# Proceedings of the
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## 38° Congresso Brasileiro de Espeleologia

## VOLUME VI / VII

Session 16 (part I): Subterranean Biology

# Proceeding of the 19th International Congress of Speleology

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# FOREWORD

The proceedings of the 19th International Congress of Speleology, held in Belo Horizonte (Brazil) from July 20 to 27, 2025, comprise seven volumes that gather over 400 scientific papers (extended abstracts) distributed across sixteen thematic sessions. These works were evaluated by an extensive group of reviewers, each guided by a Brazilian and a foreign coordinator, aiming to ensure their collective relevance, proper presentation, and compliance with the UIS Code of Ethics. The layout of the articles was prepared by the authors themselves and is their sole responsibility; however, the editorial team made an extra effort to maintain a minimum standard in their presentation.

These proceedings represent the work of more than a hundred individuals—scientists, technicians, and explorers who voluntarily dedicated thousands of hours to their production. To all of them, our heartfelt thanks. We hope you enjoy it and that the science compiled herein broadens the technical and academic horizons of karst and cave science.

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### Session 16 - Part I: Subterranean Biology

## Session 16 - Part I

# SUBTERRANEAN BIOLOGY

# First records of arthropods in Cueva de la Curva, Jalpan de Serra, Querétaro, Mexico

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## Abstract

The persistence of cave bats in fragmented landscapes depends on their ability to withstand disturbances. In this study, we assessed the impact of rock blasting and heavy machinery on bat activity in a mining area, using PIT tags for continuous monitoring. Our results indicate that climatic factors and distance to rock blasting play a key role in regulating bat activity. Based on our findings, we provide valuable insights for cave management in mining regions, emphasizing the need to prioritize microclimate maintenance to mitigate local species loss.

## Resumen

Se presentan los primeros registros faunísticos de artrópodos de la Cueva de la Curva, Querétaro, México. Los grupos encontrados pertenecen a 12 taxa distintos, siendo los ácaros (Acari) los más abundantes. Pese a estar cercana a una comunidad, se detectó una buena riqueza de organismos. Con estos primeros registros se contribuye al conocimiento biospeleológico del país y de las cavidades de la región de la Sierra Gorda de Querétaro.

## 1. Introduction

The Northeast of the state of Querétaro, Mexico, belong to the Province of the Sierra Madre Oriental, and their landscapes are highlands with rocks marine sedimentary, with a relief mostly Karst and with elements of forest vegetation temperate and tropical (JONES & SERRANO 2016). The Sierra Gorda Biosphere Reserve is located in this province, in an alignment of rocks limestone and steep mountain ranges, with drainage mostly underground; it has a development of vertical type caves, which capture water and take it to the media underground (LAZCANO 2017). These factors as well the geological and tectonic factors have had great influence on the development of the caves. In addition, it is part of the five provinces of the Transition Zone Mexican derived from a complexity physiographic that it has an overlapping affinities Nearctic and Neotropical, highlighting not only one mixture of taxa different from other regions, if not for their particular evolution *in situ* (MORRONE 2019).

Mexico has a rare and equally rare cave fauna (REDDELL 1981; ZAGMAJSTER et al. 2018); but lack a lot by study at level taxonomic and evolutionary (LAZCANO 2017). Mexico is diverse in cavities in all territory, and the Sierra Gorda is a privileged region, both in number as diversity of cavities, but it is unknown to Mexicans, including the majority of inhabitants of the states of Querétaro and Guanajuato, that comprise the area of Sierra Gorda (LAZCANO 2017).

This work contributes to the knowledge of the diversity of Mexican cave fauna and particularly in the caves of the Sierra Gorda Queretana.

## 2. Materials and methods

The “Cueva de la Cuerva” (Fig. 1) is located in the town of San Juan de los Durán, Jalpan de Serra, close to the Ecotourism center “Rincón de Ojo de Agua”, in the Sierra Gorda Biosphere Reserve. The locality belongs to Sierra Alta Escarpada (Karst Huasteco subprovince). The vegetation in the area is montane cloud forest. This is small cave, but its importance is essential since it inhabit by bats, and also has been use in ecotourism (https://ciudadypoder.mx/lo-mas-reciente/cueva-de-la-curva-un-escondite-entre-estalagmitas/).

The cave is below the level of the ground and consists of a main hall that you can be inside standing up, and three chambers. There is a slight slope towards the hall which continues horizontally. Into the cave on the left side are the entrance to the chambers; the first chamber is connected by a narrow corridor approximately 1.5 m wide and 3 h high, that constitute a dark area and you cand standing up inside. In this chamber there are significant amounts of guano and differences in environmental humidity are detected. The entrance to the second chamber is connected directly to the hall, with a 6 m wide and 5 m deep approximately. This is a dark area but there are few amounts of guano. The entrance of the third chamber is located at the back of the hall, is narrow and is raised approximately 1 m from the ground. This chamber was not explored in order to no disturb a group of bats that were there. The hall has a dolina, which allows the light entrance at certain times during the day and input of a significant amount of organic matter as litter.

They were carried out manuals samplings of arthropods as specimens were found along the Cave tour, from the entrance to the deepest area. These samples were placed directly into jars with 95% alcohol and were taken to the Laboratorio de Ecología de Artrópodos en Ambientes Extremos, UMDI-J, Facultad de Ciencias, UNAM, to be separated, counted, and identified.

In the shadow area, two leaf litter samples of 95 cm2 were taken and carried to the laboratory to be processed through Berlese funnels for 6 days, the first three at environmental temperature and the other 3 with a heat source; arthropods extracted were fixed in 95% alcohol and were separated and review under the microscope stereoscopic.

In the deep zone of darkness of the first chamber, two guano samples also of 95 cm$^2$ were taken and were processed in the same way as leaf litter.

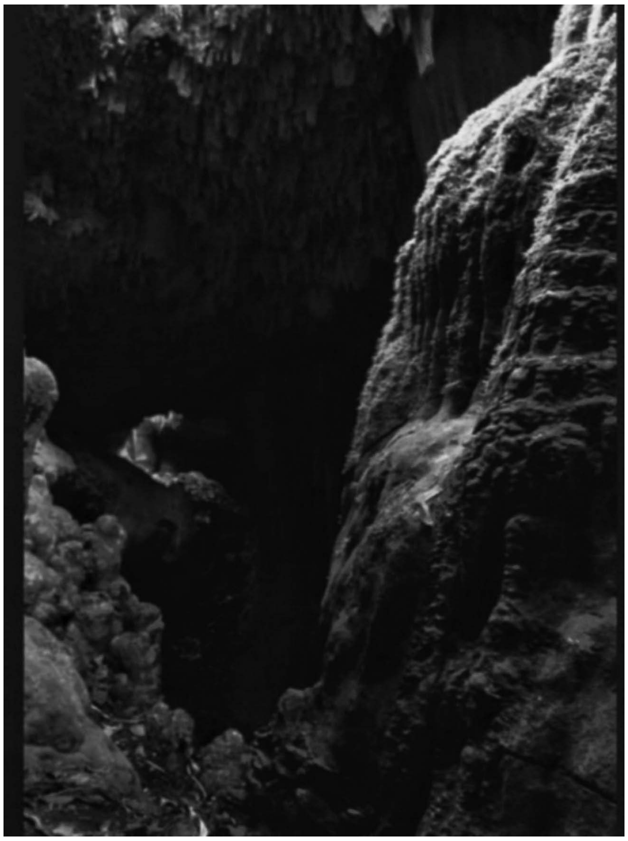

***Figure 1:*** *Entrance area of the Cueva de la Curva, San Juan de los Durán, Jalpan de Serra, Querétaro, Mexico.*

## 3. Results

A total of 11 taxa were recorded, belong to 9 orders (*Opiliones, Araneae, Diptera, Coleoptera, Orthoptera, Hemiptera, Isopoda, Psocoptera* and *Pseudoscorpionida*), as well Classes *Diplopoda* and *Collembola*, and the subclass *Acari*. The groups obtained in the different biotopes (leaf litter guano, walls by the manual sampling), vary in composition (Fig. 2). The manual sampling allowed collect individuals of groups more visible like the orthopterans (Fig. 3) and see also particular behaviors. The orthopterans were found from the entrance to the deep area of the cave. The samples of leaf litter presented the largest diversity (Fig. 2), since 9 taxa were recorded, with mites being the most abundant, followed by springtails. Finally, the sampling on the walls and the floor allows obtain other groups such as harvestmen and beetles.

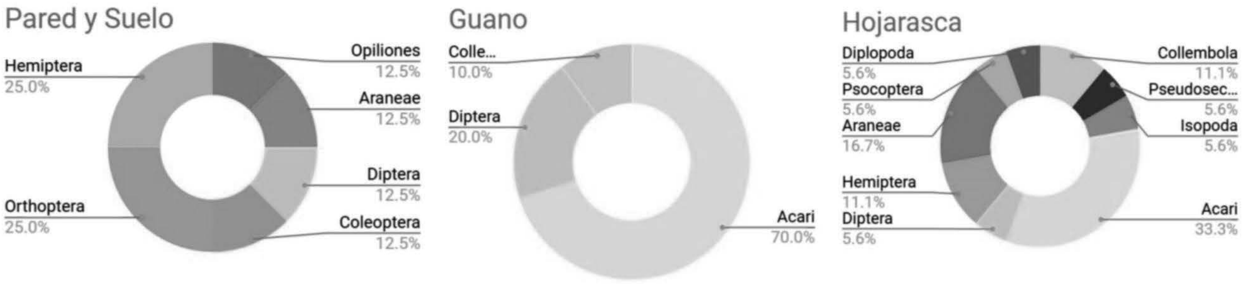

***Figure 2:*** *Arthropods present in each biotope in the Curve Cave, San Juan de los Durán, Jalpan de Serra, Querétaro, Mexico.*

## 4. Discussion

This is the first record of fauna for the Cueva de la Curva, nevertheless the cave is visited by the population and tourism. Our results are a contribution that adds to the knowledge of the fauna of this regio. The cave is located within a montane cloud forest, which usually contains species very diverse and particular (JONES et al. 2008). It is known that the caves are affected by the surrounding environment, where the dynamics and ecology of each cave is strongly supported by the vegetation type and responsible for the local conditions of the cave. In addition, the presence of bats in the cave contributed to the resources in the cave, promoting a complex community dynamic and increase the diversity of arthropod community. The presence of a skylight in the ceiling of the cave as well It increases the niches as it allows for a contribution of material organic considerable extension in the transition zone.

The collections of the three biotopes allowed throw data general communities inside the cave. In the samples through manual sampling, obtain specimens of groups of arthropods visibly large and allowing us notice behaviors, for example the orthopterans were observed walking or resting from the walls of the entrance, but also in the transition zone and deep zone of total darkness, in the deep zone they were observed near of guano on the wall and on the floor.

This occurred also with the harvestmen but with greater incidence in the dark areas and deep areas of the cave, mainly on the walls.

In the case of guano, the groups detritivores, such as collembola, acari and Diptera larvae They were the only ones presents; the first two are from the groups further common in caves, particularly in guano (FERREIRA & MARTINS 1999; PALACIOS-VARGAS 2011).

***Figure 3:** Orthoptera collected 2 m from the cave entrance, inside a crack on the wall.*

## 5. Conclusion

The few studies biospeleological studies in the area, due is difficult carry out comparisons between invertebrate communities of the Sierra Gorda, and their particular taxon complexity distinctive of this province geographic. However, the groups here collected allow have information about this particular cave and contributes to the knowledge of biodiversity. It is crucial to focus on the study of cave communities, especially on the preservation of bats as they form the trophic base for the structure of many cave communities as arthropods.

## Acknowledgements

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# Hymenoptera (Insecta: Hymenoptera) associated with cave environments in Mexico

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## Abstract

The study of Hymenoptera in caves has been largely unknown in Mexico. There are no specific faunistic works on their taxonomy and the usual behavior of these organisms within cave environments has not been elucidated. The frequent presence of these organisms in cave environments could probably be hiding something more than just being rare visitors. This work is another contribution to the taxonomic study, biospeleological data and diversity of Hymenoptera in cave environments in Mexico.

## Resumen

En México, el estudio de himenópteros en cuevas ha sido en gran parte desconocido. No hay trabajos faunísticos específicos para su taxonomía y no se ha dilucidado el comportamiento habitual de estos organismos dentro de los ambientes cavernícolas. Probablemente la asiduidad de estos organismos hacia los ambientes de cuevas podría estar escondiendo en realidad algo más que solo ser visitantes poco comunes. Este trabajo es una aportación más hacia el estudio taxonómico, datos bioespeleológicos y diversidad de himenópteros en ambientes de cuevas en México

## 1. Introduction

Mexico, along with other countries, contains one of the most diverse cave faunas of any region in the World (REDDELL 1981, ZAGMAJSTER et al. 2018). That diversity can be mainly explained by the existence of large cave systems in a wide variety of habitats and with different origins (REDDELL 1981). This great species richness and ecosystem diversity, as well as a long history of constant changes by geological and climatological events, place the Mexican territory in such particular biogeographic patterns, show a notable complexity in flora and fauna (MORRONE 2019). This is observed also in the case of the Mexican cave fauna, which includes species from temperate groups but with fauna mainly related to the tropics (REDDELL 1982). Cave dwellers are subject to the characteristics of the cave habitat, and in the case of Hymenoptera, it has been found that they have become part of the underground food web (PAPE 2016, 2024). Although they are one of the most diverse orders of insects (FORBES et al. 2018), the presence of Hymenoptera in caves has been very poorly recorded, since only between 200 and 250 species of Hymenoptera associated with caves are known (MOLDOVAN 2004). Within them, of the 38 known families, ants (Formicidae) are the most frequently found associated with caves, even with one species considered a troglobitic (ROCIN & DEHARVENG 2003, ROMERO 2009). Hymenoptera in caves perform various functions, such as guano cleaners, foragers (DATTILO et al., 2012), as common visitors and predators of endemic cave animals (PAPE, 2016). Many groups can nest at the entrances (Fig. 1), and in the case of bees and wasps, they are frequent in the walls (MALDOVAN, 2004). However, the role that they play in hypogeal environments has not been clearly established, but there is evidence that they are important ecological elements of cave ecosystems, and their study allows obtaining important information on natural history, distribution, and biogeography (VAS & KUTASI 2016).

In this work, the Hymenoptera material in two of the most important collections of cave fauna in Mexico was reviewed, in order to summarize the information available on this group in Mexican caves.

## 2. Materials and methods

A review and identification of the exclusive material from biospeleological collections on cave fauna was carried out. The material comes from the collections of the Anita Hoffmann Acarological Collection Laboratory, which is now associated with the National Acarological Collection (CNAC-UNAM) and the Laboratory of Ecology and Systematics of Microarthropods (LESM), of the Faculty of Sciences of the UNAM. As well as from some collections carried out in the Laboratory of Ecology of Arthropods in Extreme Environments, of the Multidisciplinary Teaching and Research Unit (UMDI-J, FC) Juriquilla campus of the UNAM.

Those samples that contained Hymenoptera were selected, and the specimens obtained were examined under a stereoscopic microscope with an attached camera. The range of dates of collection of the material covered from 1979 to 2019. This material is the result of field work by researchers and students from different projects. A single manual collection was also carried out in the Cueva de la Curva, located near of San Juan de los Durán, Jalpan de Serra, which is part of the Sierra Gorda Biosphere Reserve, in the Querétaro state. The individuals were placed in 95% ethyl alcohol for later assembly and identification.

Identification was carried out with the help of specialized dichotomous keys, for example, for ant genera MACKAY & MACKAY (1989) was used, while for the rest of the Hymenoptera GAULD & BOLTON (1988) and FERNÁNDEZ & SHARKEY (2006) were used.

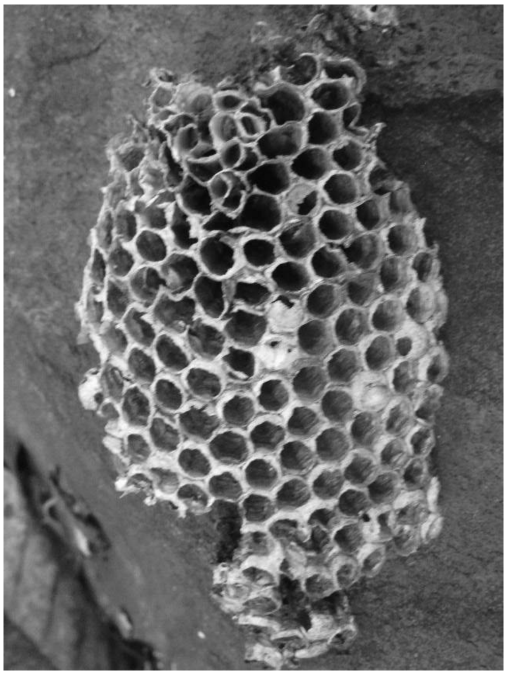

***Figure 1:*** *Nest of wasp in the entrance of a cave in State of Morelos, Mexico.*

## 3. Results

From the material in the collections, a total of 33 samples containing Hymenoptera were obtained, which come from 25 caves distributed in 10 states of the country (Fig. 2).

A total of 614 individuals were obtained, represented in 24 subfamilies belong to 18 families. The family with the greatest distribution and best represented in the caves corresponds to the ants (Formicidae), with a total of 565 individuals, compared to the 39 individuals of the wasp group. The ants found belong to 9 subfamilies, while the wasps are represented by 14 subfamilies. From the bee group, there are only 10 individuals, from the Apoidea superfamily with 4 families: Apidae, Sphecidae, Crabronidae and Halictidae (Fig. 3).

The ants of the subfamily Myrmicinae were the ones with the greatest distribution, since they were present in 19 caves in 9 states, followed by Formicinae present in 16 of the caves in 9 states and Ponerinae present in 7 of the caves in 5 states; of the subfamilies Amblyoponinae, Dolichoderinae and Proceratiinae, only one specimen of each was obtained.

As for the group of wasps, they were only present in 11 of the 25 caves studied and the group of bees in only 3 caves, Guerrero, Querétaro and Veracruz.

The caves where the Hymenoptera were recorded were: Gruta de Xtacumbilxunaán (Campeche); in Guerrero: Gruta de Aguacachil or Resumidero de Zacatecolotla, Gruta de La Joya or Resumidero de La Joya and Grutas de Juxtlahuaca; Cueva del Toro (Morelos); for the state of Puebla, Cueva de Tasolalpan Sur, Cueva de Chignahuapan and Cueva de Cohuatichan or Resumidero de Cohuatichan; for the state of Querétaro, Cueva de los Riscos, Cueva del Judío and Cueva de la Curva; for the state of San Luis Potosí, Cueva de las Cotorras and Sóntano de las Huahuas; for the state of Tabasco, Cueva de Coconá, Cueva del Barro and Cueva de las Sardinas or Cueva de Villaluz; for the state of Veracruz, Sótano del Hoyo and Cueva del Rey del Oro; for the state of Yucatán, Cueva Siete Aguas, Cueva del Rancho Sambulá, Gruta de Tzabnáh, Cenote Xkekén and Cueva de Calcehtok, and finally for the state of Oaxaca, Cueva Cheve.

***Figure 2:*** *Map of the states with studied caves with Hymenoptera.*

## 4. Discussion

The study of Hymenoptera in caves has been poor developed, so there is a great lack of knowledge of the group in these environments. There are no specific faunal studies in our country, and few has been elucidated about the relationship to cave environments and their classification. For this reason, biological inventories are necessary to obtain an overview of the basic knowledge of a place or a group of organisms (TOLEDO, 1994). Since the scientific identification of any organism is essential in the basis of any biological work (JONES & MARTINES- LUQUE, 2020), identifying cave hymenopterans in Mexico is of great relevance.

Despite the development of speleology, it is estimated that 90% of caves have not been visited for biological studies (CASTAÑO-MENESES et al. 2005). Most of the studies are carried out on other groups of arthropods in general. There are no specific works on faunal studies for Hymenoptera in caves and their relationship with these environments, nevertheless the importance of this order in all the terrestrial ecosystems. For this reason, it is essential remark the relevance of the review of materials kept in the collections that provide new data to this group and the species present in the caves. Even more, the development of specific studies on Hymenoptera and their ecology, which allows a better representation of the group in these ecosystems. Providing data that clarify their presence in these ecosystems. In addition, they are important and regular components of underground systems. As the ants that regulate guano assemblages in many caves in various regions, their habits may also promote the transition of nutrients between the outside and the inside of the cave, foraging outside the cave and incorporating organic matter into it. They have even been found in shallow horizontal zones and feeding on endemic cave animals (PAPE, 2016, WILSON, 1962). However, none of these guano species exhibit a cave-related adaptation in their external morphology (RONCIN & DEHARVENG, 2003), and only one or two species have been suggested as true cave dwellers. Also, the assigned ecological group must be based on their behavior and the level of association with these habitats and does not necessarily depend on the presence of troglomorphy since they do not always exhibit an easily apparent adaptive morphology (PAPE, 2016).

Therefore, the importance of the taxonomic review of this group associated with cave environments is essential, since the taxonomy and ecology within caves are poorly known to allow a greater generalization of their classification, especially in the case of bee wasps, since they are organisms found in transition zones between cave environments, the ground and the outside. This work provides important faunal data to understand the diversity of extensive areas with particular biogeographic characteristics.

| Subfamilies | States |
|---|---|
| Apinae | Querétaro |
| Amblyoponinae | San Luis Potosí |
| Anomaloninae | Querétaro |
| Diapriinae | Guerrero, San Luis Potosí |
| Dolichoderinae | Veracruz |
| Dorylinae | Guerrero, Querétaro, Veracruz |
| Doryctinae | Guerrero |
| Ectatomminae | Guerrero, Veracruz |
| Eumeninae | Querétaro |
| Formicinae | Guerrero, Morelos, Puebla, Querétaro, San Luis Potosí, Tabasco, Veracruz., Yucatán. |
| Mesochorinae | Puebla, Querétaro |
| Mesoninae | Puebla |
| Microgastrinae | Yucatán |
| Myrmicinae | Campeche, Guerrero, Morelos, Puebla, Querétaro, San Luis Potosí, Tabasco, Veracruz, Yucatán. |
| Neorhacodinae | Guerrero, Querétaro |
| Opiinae | Guerrero |
| Ponerinae | Guerrero, Puebla, Querétaro, San Luis Potosí, Tabasco, Yucatán |
| Polistinae | Oaxaca |
| Pristocerinae | San Luis Potosí |
| Pseudomyrmicinae | Guerrero, Querétaro, San Luis Potosí |
| Proceratiinae | Yucatán |
| Scelioninae | Guerrero, Puebla, Querétaro |
| Sphaeropthalminae | Veracruz |

***Figure 3:*** *Subfamilies of Hymenoptera present in the caves of 10 states of the country.*

## 5. Conclusion

This work is an important added value for the probable recording of new species present in cave environments. It also provides intrinsic value by coming from the review of collection specimens that have remained under protection for a long time, due to the lack of experts interested in addressing the subject.

The review of protected specimens can give us an overview of the natural faunal history of caves in Mexico. And provide interesting faunal data from the past of the caves, since several of these caves are disturbed because they are tourist spaces. In addition, it is necessary to consider a broader extension of the methods of collecting Hymenoptera in these ecosystems that will give us more success in capturing, mainly wasps and bees. As well as the realization of particular and specific studies of Hymenoptera that can better clarify their ecological role within cave ecosystems.

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# Do we still need to collect cave species to evaluate the effects of environmental predictors?

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## Resumo

A utilização de espécies em estudos de monitoramento é um grande desafio devido às incertezas taxonômicas e biogeográficas, além do impacto que as coletas sistemáticas podem ter em populações frágeis de espécies cavernícolas. Neste cenário, investigamos o uso de categorias taxonômicas superiores, como famílias, para monitorar a biodiversidade subterrânea e suas relações com preditores ambientais. Com base em dados de comunidades de invertebrados subterrâneos em cavernas da região sudeste do Pará, Brasil, o estudo testou três hipóteses sobre a correlação entre a riqueza e composição de espécies e famílias, assim como a resposta dessas categorias a preditores ambientais. Os resultados indicaram uma correlação significativa entre a riqueza e composição de espécies e famílias, com a riqueza de famílias apresentando um modelo mais parcimonioso em comparação com as espécies. Além disso, a composição de famílias foi mais sensível aos preditores ambientais do que as espécies. Este estudo sugere que o uso de famílias como proxy para monitoramento contínuo da biodiversidade pode ser uma ferramenta eficaz e menos invasiva, permitindo a análise de grandes volumes de dados sem a necessidade de coleta intensiva.

## Abstract

The use of species in monitoring studies is a great challenge due to taxonomic and biogeographic uncertainties, in addition to the impact that systematic collections can have on fragile populations of cave species. In this scenario, we investigate the use of higher taxonomic categories, such as families, for monitoring subterranean biodiversity and their relations with environmental predictors. Based on data from subterranean invertebrate communities in caves of the southeastern Pará region, Brazil, the study tested three hypotheses regarding the correlation between species and family richness and composition, as well as their response to environmental predictors. The results indicated a significant correlation between the species and family richness and composition, with family richness presenting a more parsimonious model compared to species. Furthermore, family composition was more sensitive to environmental predictors than species. This study suggests that using families as a proxy for continuous biodiversity monitoring can be an effective and less invasive tool, enabling the analysis of large volumes of data without the need for intensive collection.

## 1. Introduction

One of the main concerns of ecologists is to find relationships between environmental predictors and response variables. In general, the collection of environmental data allows the construction of robust databases with well-defined scales. For example, if we have georeferenced information on the exact locations of the samples, we can obtain environmental data from large online databases, such as climate (FICK & HIJMANS 2017) or land use (CARDOSO et al. 2021). If we want to obtain these data independently on finer scales, we can use automated tools or collection processes, such as data loggers, which can collect a wide variety of variables, such as temperature and humidity (MAMMOLA & ISAIA 2018).

On the other hand, the collection of ecological data on species is fraught with taxonomic and biogeographic uncertainties, leading to the construction of imprecise databases that are constantly updated, consequently altering the results of previous studies (CARDOSO et al. 2011). Within the scope of cave fauna studies, there is an additional challenge of the fragility of subterranean ecosystems, where species have relictual distribution patterns and are under strong anthropogenic pressure (MAMMOLA et al. 2019). In this sense, how can we assess the effect of environmental predictors on subterranean communities given the significant limitations in taxonomic knowledge and species distribution? Furthermore, how can we make decisions while avoiding biases in results that may arise from these issues?

One way to address these taxonomic inaccuracies is by using higher and relatively well-resolved taxonomic categories (HEINO & SOININEN 2007) or Operational Taxonomic Units (OTUs) (FERNANDES et al. 2016). These approaches have shown relative success in representing predictor effects when compared to species-level data (ZOU et al. 2020), making them a viable alternative for studies that do not require more complex interpretations based on finer taxonomic categories.

From this perspective, is the use of higher taxonomic categories effective in representing the effect of predictors on cave communities? To answer this question, this study aims to assess whether family-level data can serve as a proxy for the effects of environmental predictors in continuous monitoring studies. Three hypotheses will be tested: (i) Family richness and composition data are correlated with species-level data; (ii) Species and family richness exhibit similar patterns in response to environmental predictors; (iii) Species and family composition exhibit similar patterns in response to environmental predictors.

## 2. Material and methods

We constructed two databases of the subterranean invertebrate community from 24 ferruginous caves in Serra Leste, southeastern Pará, Brazil. These caves are in a transitional area between the Mato Grosso tropical dry forests and the Xingu-Tocantins-Araguaia moist forests ecoregions (DINERSTEIN et al. 2017). The 24 caves were sampled in four field campaigns during the wet and dry seasons of 2022 and 2023, totaling 96 samples.

Invertebrate sampling was conducted through active searching across all accessible sections of the caves, focusing on microhabitats such as organic deposits, fissures, and the undersides of rocks and boulders, where the likelihood of finding organisms is higher (WYNNE et al. 2018). Some specimens of each species were collected using forceps, brushes, and entomological nets, preserved in 100% alcohol, and taken to the laboratory for identification with the assistance of taxonomists. Other observed specimens were recorded and mapped onto the topographic maps of the caves (FERREIRA 2004).

During each sampling event, we collected data on environmental predictors, including the area and diversity of organic resources found in the caves, as well as landscape structure data. The area (in square meters) of organic resources (guano, leaf litter, plant material, and roots) was recorded simultaneously with faunal sampling using dedicated software for digital scanning. Additionally, satellite images corresponding to the faunal sampling period were classified into the following land cover categories: canga (ironstone outcrops), hydric features, forest, mining, and pasture. The area (in hectares) of these categories was then measured within a 250 m buffer surrounding the caves. Finally, the distances (in meters) from the caves to mining areas (including pits and infrastructure) and water bodies were calculated.

To test Hypothesis 1, after verifying that the data did not follow a normal distribution, we applied Kendall's non-parametric test (chosen over Spearman's due to tied ranks, function cor.test from the stats package) (HOLLANDER et al. 2013) to assess whether species and family richness were correlated. Subsequently, we constructed matrices using the Jaccard index (function beta, BAT package) and performed a Mantel test to evaluate the correlation between species and family composition data (function mantel, vegan package) (BORCARD et al. 2018).

For Hypothesis 2, we built generalized linear mixed models (GLMMs) with a negative binomial distribution (function glmer.nb, lme4 package) to test whether species and family richness were influenced by any of the predictors, setting caves as a random predictor (since the same 24 caves were sampled across four different campaigns) (BATES et al., 2014). In summary, model construction followed these steps: predictor scaling due to different magnitudes (function scale, base package), selection of predictors with correlation coefficients below 0.7 (function chart. correlation, performanceanalytics package), sequential exclusion of predictors until all had a variance inflation factor (VIF) < 3 (function vif, car package), and stepwise removal of non-significant predictors to obtain the most parsimonious models (JOHNSON & OMLAND, 2004; ZUUR & IENO 2016; ZUUR et al. 2010). Logical interpretation of the models was expressed using the report function (report package).

For Hypothesis 3, we performed a permutational multivariate analysis of variance (PERMANOVA) (function adonis2, vegan package) to test whether species and family composition were influenced by any predictors (BORCARD et al. 2018). This step followed the same procedures as the composition matrix construction and predictor selection described previously. Finally, a principal coordinates analysis (PCoA) using the Jaccard index (function capscale, vegan package) was conducted to obtain multidimensional scaling (MDS1 and MDS2) axis values, which were used to visualize spatial patterns of species and family communities in relation to the significant predictors identified in PERMANOVA (BORCARD et al. 2018). All analyses were conducted in R software (R CORE TEAM 2025).

## 3. Results

A statistically significant, strong positive correlation (COHEN 1988) was found between species richness and family richness (tau = 0.807, z = 11.434, $p < 0.001$). This indicates that sites with higher species diversity also tend to have higher family diversity. The correlation between the species and family composition matrices was also significant and of large magnitude (r = 0.771, p = 0.001), suggesting that community structure at the family level reflects the structure at the species level (Figure 1 - a and b).

Considering the GLMM results (Table 1, Figure 1 - c and d), the predictor resource richness had a positive and statistically significant effect on both species' richness (z = 2.189, p = 0.028) and family richness (z = 2.277, p = 0.022). The positive z-value indicates that as resource richness increases, species and family richness also increase, with a slightly stronger relationship observed for family richness.

Additionally, the Akaike Information Criterion (AIC) of the GLMMs showed that the family richness model (AIC = 700.500) is preferable to the species richness model (AIC = 783.900), with a difference of ΔAIC = 83.400, indicating that the family-level model provides a more parsimonious fit (JOHNSON & OMLAND 2004).

For species composition, the predictors distance to hydric features, distance to mining, hydric features, forest, mining, pasture, resource richness, guano, and leaf litter had significant effects ($p < 0.05$) (Table 2). The predictor distance to hydric features explained the largest proportion of variance ($R^2 = 0.026$).

For family composition, the same predictors (except leaf litter) were also significant. Distance to hydric features again explained the largest proportion of variance, with a higher value ($R^2 = 0.033$). Overall, families were more sensitive to environmental predictors than species, with higher $R^2$ values for the predictors.

The ordination of family composition displayed a spatial structure with larger vectors for the significant environmental predictors, indicating stronger correlations between these predictors and variations in the subterranean community (BORCARD et al. 2018). Additionally, the PCoA axes explained a greater proportion of variance compared to the species composition data (Figure 1 - e and f).

| Response variable | Predictor | Estimate | SE | z value | p | AIC |
|---|---|---|---|---|---|---|
| Species richness | Intercept | 4.076 | 0.057 | 70.389 | <0.001 | 783.900 |
| | Resource richness | 0.055 | 0.025 | 2.189 | 0.028 | |
| Family richness | Intercept | 3.733 | 0.048 | 77.189 | <0.001 | 700.500 |
| | Resource richness | 0.051 | 0.022 | 2.277 | 0.022 | |

***Table 1:*** *Summary of the GLMMs explaining variations in species and family richness in the target caves monitored. For each predictor variable, the estimate, standard error, coefficient value (z), significance level (p), and Akaike Information Criterion (AIC) are provided.*

| Response variable | Predictor | Df | SS | R² | F | p |
|---|---|---|---|---|---|---|
| Species composition | Distance to hydric features | 1 | 0.893 | 0.026 | 2.686 | 0.001 |
| | Distance to mining | 1 | 0.598 | 0.017 | 1.799 | 0.001 |
| | Hydric features* | 1 | 0.553 | 0.016 | 1.663 | 0.001 |
| | Forest* | 1 | 0.596 | 0.017 | 1.794 | 0.001 |
| | Mining* | 1 | 0.554 | 0.016 | 1.667 | 0.001 |
| | Pasture* | 1 | 0.816 | 0.024 | 2.455 | 0.001 |
| | Resource richness | 1 | 0.506 | 0.015 | 1.522 | 0.005 |
| | Guano | 1 | 0.653 | 0.019 | 1.965 | 0.001 |
| | Leaf litter | 1 | 0.426 | 0.012 | 1.281 | 0.028 |
| | Residual | 86 | 28.589 | 0.836 | - | - |
| Family composition | Distance to hydric features | 1 | 0.793 | 0.033 | 3.480 | 0.001 |
| | Distance to mining | 1 | 0.460 | 0.019 | 2.018 | 0.002 |
| | Hydric features* | 1 | 0.436 | 0.018 | 1.914 | 0.001 |
| | Forest* | 1 | 0.493 | 0.021 | 2.162 | 0.001 |
| | Mining* | 1 | 0.372 | 0.016 | 1.632 | 0.009 |
| | Pasture* | 1 | 0.613 | 0.026 | 2.690 | 0.001 |
| | Resource richness | 1 | 0.378 | 0.016 | 1.657 | 0.007 |
| | Guano | 1 | 0.496 | 0.021 | 2.176 | 0.001 |
| | Residual | 87 | 19.829 | 0.830 | - | - |

***Table 2:*** *Summary of the PERMANOVA analysis, presenting the best models explaining the dissimilarity of species and family communities in the target caves monitored. For each predictor variable, the degrees of freedom relative to the total (Df), sum of squares (SS), adjusted coefficient of determination (R²), pseudo-F statistic (F), and significance level (p) are provided. (*) Area in 250-meter radius buffers.*

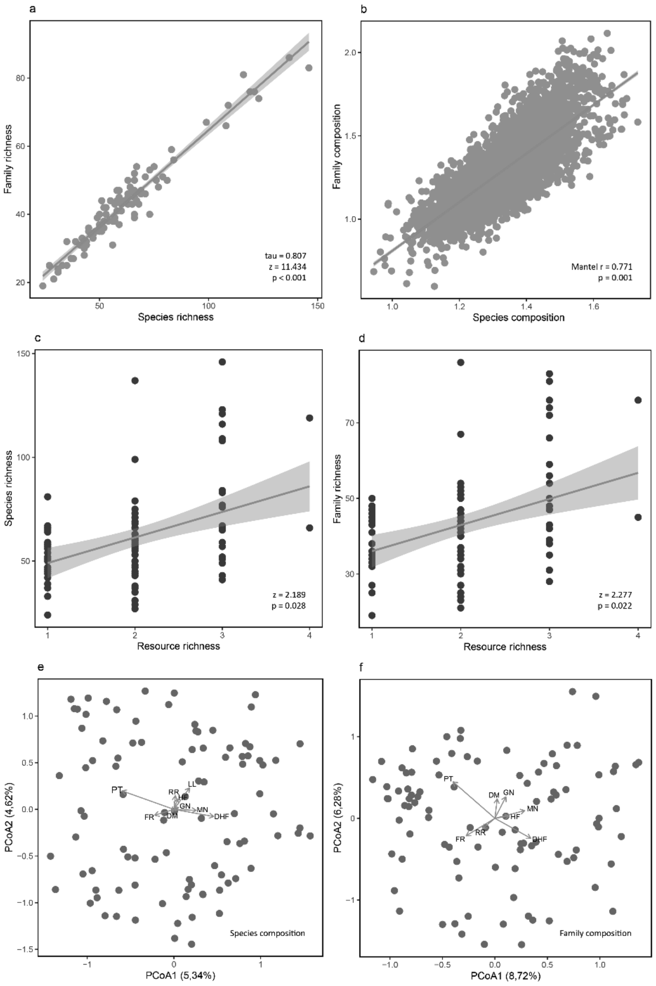

***Figure 1:*** *Graphical representations of significant relationships obtained from the analyses. (A) Relationship between species and family richness based on Kendall's correlation; (B) Relationship between species and family composition based on the Mantel test; (C and D) Relationship between species and family richness, respectively, and resource richness according to the GLMM; (E and F) PCoA of species and family composition, respectively, indicating the vectors of significant predictors. In the scatter plots, the central line represents the linear relationship between variables, with the gray area indicating the 95% confidence interval. The coefficient value (z) and significance level (p) are also provided. In the PCoA, the abbreviations are: DHF – Distance to hydric features, DT – Distance to mining, HF – Hydric features, FR – Forest, MN – Mining, PT – Pasture, RR – Resource richness, GN – Guano, LL – Leaf litter.*

## 4. Discussion

This study highlights the potential of higher taxonomic categories as a tool for long-term monitoring of subterranean biodiversity. The strong relationship observed between species richness and family richness reinforces the possibility of inferring ecological patterns from broader taxonomic levels, allowing for the assessment of subterranean community dynamics in response to environmental predictors. This approach encourages the adoption of less invasive methodologies, enabling the systematic monitoring of subterranean communities without the need for frequent collections that might compromise the integrity of the analyzed populations. Our results further suggest that family richness and composition can serve as a robust proxy for estimating the influence of environmental predictors in long-term monitoring, opening new perspectives for biodiversity studies in the context of increasing demand for rapid and accurate information on ecosystem conservation status.

Statistically, what underlies the greater explanatory power of analyses with the family database? The data exhibits lower variability and are more homogeneous, thus resulting in models with better fit (MONTGOMERY et al. 2021). Ecologically, families group species that share diagnostic characteristics and relatively more specialized niches, leading to greater data variability among species (BOLNICK et al. 2007). However, analyzing species increases identification complexity, something more evident in the case of cave invertebrates due to their small body size and the inability to visualize diagnostic characters in situ, which can only be seen with optical instrumentation (ZAGMAJSTER et al. 2010). Nowadays, methods are used to collect representatives of each species and map all their equivalents in cave maps, allowing for the collection of richness and relative abundance data (WYNNE et al. 2019). But is this method suitable for monitoring studies that require continuous collections over long periods?

One of the main challenges in collecting ecological data is the accumulation of biological material that, after identification, is no longer used. This problem is particularly relevant in subterranean ecology studies, where specimen collection can be invasive and negatively impact sensitive ecosystems (MAMMOLA et al. 2019). Monitoring aimed at verifying the influence of environmental predictors over time, using collection and mapping methodology, generates a huge volume of biological material. This material is deposited in collections, coming from the same caves that are periodically collected, storing the same species in alcohol that will accumulate in jars. Luckily, some groups may be reviewed by a taxonomist. Additionally, unless the field team is experienced, after collecting a specimen of a species a few millimeters in size, visually assuming that all similar specimens are the same species is risky. This visual refinement of the other specimens observed with this method may lead to identification errors and data bias, as incorrect geographical location can distort the interpretation of distribution patterns and diversity (CARDOSO et al. 2011).

However, using higher taxonomic categories or OTUs also has limitations, being unsuitable for studies with other objectives. For example, studies aimed at checking geographic distribution, ecological interactions, or functional diversity will be more effective with species-level data (GIBERT & DEHARVENG 2002). Also, depending on the country's legislation, the definition of caves prioritized for conservation may be based on species identity, as is the case in Brazil (BRAZIL 2008; MMA 2017). In these cases, even if there is an impact on fauna resulting from specimen inventories, it is necessary, unlike systemic collections, such as those in monitoring studies.

The use of taxonomic categories that can be identified in situ opens the perspective of using automated digital tools, such as machine learning algorithms and taxonomic databases, which can accelerate the analysis of large data volumes without the need for additional specimen collection (VALAN et al. 2019). For example, we developed a tool for subterranean fauna monitoring that allows real-time identification of fauna at least up to the family level, and it can be adapted to regional databases. We applied this tool in the same 24 caves from this study during two additional monitoring campaigns in 2024. We were able to identify 100% of 122,395 subterranean fauna specimens found in these caves during the two campaigns, at least up to the family level. Except for a few Hemiptera and Acari specimens that required confirmation, all others were identified in real time in the field, without the need for collection.

## 5. Conclusion

The use of higher taxonomic categories proves effective for long-term subterranean biodiversity monitoring, demonstrating that family richness can serve as a robust proxy to infer ecological patterns and the influence of environmental predictors. However, it is important to consider the limitations of this methodology for studies that require more detailed taxonomic resolution, such as those focused on geographic distribution and ecological interactions between species. The proposed approach allows for less invasive monitoring, reducing the need for frequent collections and minimizing negative impacts on ecosystems. Additionally, the use of automated digital tools for field identification can accelerate data analysis, making the process more efficient.

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# Speleological recovery: results of faunal recovery in caves located in ferruginous substrate of The Iron Quadrangle, Minas Gerais, Brazil

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## Abstract

Mining activities often require cave destruction, which can lead to irreversible impacts on subterranean ecosystems. Brazilian law mandates the recovery, transportation, and preservation of geological and biological elements from these caves to mitigate biodiversity loss. Subterranean species, often highly specialized and habitat-restricted, are particularly vulnerable to disruption, with some potentially being unknown to science. This study details the faunal recovery conducted in 10 iron caves in the Tico-Tico Mine, Minas Gerais, Brazil, between September 2020 and July 2023. Three sample methods were used: manual plotting (detailed microhabitat searches), exhaustive manual collection (comprehensive scanning), and the Winkler extractor (litter sampling). These methods recorded ecological conditions such as light, humidity, and available trophic resources. Recoveryd invertebrates were preserved for laboratory identification, while vertebrates were documented and guided out of the caves. The entrances were then sealed to prevent re-entry. A total of 979 organisms from 184 morphospecies were recoveryd, including arthropods, bats, frogs, and mollusks. Exhaustive collection captured three times more individuals and 3.5 times more species than the Winkler extractor, though overlap between methods was minimal. No recoveryd species exhibited troglomorphism or were threatened, but three species new to science were found. Combining methods is essential to maximize biodiversity recovery efforts.

## 1. Introduction

The exploration of mineral resources may require the destruction of caves, which are legally protected by Brazilian law (BRASIL 2022, FERREIRA et al. 2022). Rescuing fauna from caves is a critical aspect of environmental conservation, especially in contexts where human activities such as mining or infrastructure development threaten these fragile ecosystems (IBAMA 2017, BRASIL 2022). Caves are home to unique and often endemic species that are highly sensitive to disturbances (CULVER & PIPAN 2019, FRAGA et al. 2023). Rescuing subterranean fauna in mining areas is essential, as these species often have restricted habitats and specialized adaptations, making them particularly vulnerable to habitat disruption (CULVER & PIPAN 2019). Some species may even be unknown to science. Without intervention, such activities can lead to irreversible biodiversity losses.

The destruction of natural subterranean cavities involves the recovery, transportation, and destination to scientific collections of geological and biological elements due to the irreversible negative impact (IBAMA 2017, BRASIL 2022). The methodology for cave fauna recovery begins with a thorough preliminary assessment. Surveys are conducted to identify resident species, map the cave system, and evaluate habitat conditions (IBAMA 2017). Based on these findings, a site-specific recovery protocol is developed, focusing on non-invasive capture techniques to minimize stress and habitat disturbance. Trained personnel use specialized tools to safely capture and document the fauna, which are then relocated to ecologically similar habitats free from existing threats. Post-relocation monitoring is crucial to ensure the survival and adaptation of the recoveryd species (IBAMA 2017).

Incorporating cave fauna recovery into environmental licensing processes is essential to mitigate these impacts and align development projects with conservation goals (IBAMA 2017). This approach ensures compliance withconservation laws and helps balance economic growth with environmental stewardship. Despite the importance of these efforts, cave fauna recovery presents challenges, such as accessing remote cave systems and addressing the specific needs of subterranean species, many of which are not well studied (CULVER & PIPAN 2019). Careful planning, expert collaboration, and ongoing monitoring are essential to overcome these difficulties. By integrating recovery operations into licensing processes, we can better protect the biodiversity of these unique habitats while enabling sustainable development. In this study, we present the results of the faunal recovery conducted in iron caves before cave destruction in Minas Gerais, Brasil.

## 2. Materials and methods

The 10 caves sampled were in the Tico-Tico Mine area, municipality of Brumadinho, Minas Gerais, southeastern Brazil. Three active and passive capture techniques were applied for the biospeleological recovery: manual collection by plotting specimens.

The manual collection by plotting specimens consists of a detailed search in different microhabitats, with the aim of determining the composition of the fauna in the caves. The species have their abundance estimated by counting the organisms in the field, with their capture location noted on a sketch of the cave (FERREIRA 2004). There is no time limit or sampling area, so the collection is completed when the collector judges that all the specimens present have been sampled and counted. Manual collection by exhaustion involves a detailed scan of the entire cavity area, regardless of the type of substrate and without any limitations on collection time. During sampling, all individuals sighted will be captured, avoiding prior morphological classification (Figure 1; OLIVEIRA 2014). Collection using the Winkler extractor consists of taking samples from the litter in caves in order to inventory the fauna present in this type of substrate. The samples were shaken using sieves before insertion into the extractors to dislodge the fauna and remove excess material. After sieving, the remainder was transferred to a coarse-mesh fabric bag and washed with a mini-Winkler extract (Fisher 1999) for a period of 72 hours. The material filtered by the extractor was fixed in 70% alcohol (Figure 2; FISHER 1999).

The fauna recovery was carried out once per cave between September 2020 and July 2023. The biospeleological recovery methodology included ecological characterization, photographic recording, collection, and analysis of the recoveryd fauna. Lighting conditions, substrate humidity, and available trophic resources were recorded. The collected invertebrate organisms were fixed in 70% alcohol and transported to the laboratory for identification. The recorded taxa were checked for their threatened status using national and international red lists. The collected material was destined for institutional scientific collections. When present, vertebrates were counted, identified, and chased away. All cave entrances were closed with shade cloth to prevent vertebrates from entering. We evaluated species richness, abundance and compared sampling methods (exhaustive collection and Winkler extractor) using statistical tests (Kruskal-Wallis) performed in the PAST 3.0 software (HAMMER 2012).

The recorded taxa were verified for their threat status using the current official lists: List of Threatened Species of the State of Minas Gerais (COPAM 2010), Ministry of the Environment Ordinance (MMA 2022), IUCN Red List of Threatened Species (IUCN 2020). The collected material was sent to the following depository institutions: Biology Department of the Federal University of Lavras (*Annelida*, *Entognatha*, *Insecta*, *Malacostraca*, *Maxilipoda*, *Mollusca*, *Nematoda*, *Onychophora* and *Platyhelminthes*), Soil Fauna Reference Collection – Collembola Systematics and Conservation Laboratory of the State University of Paraíba (Collembola) and Arachnids and Myriapods Collection of the Special Laboratory of Zoological Collections of the Butantan Institute (IB-SP) (*Arachnida* and *Myriapoda*). The speleological recovery methodology was based on the Speleological Recovery Plan (BIOESPELEO 2019) (Figure 3).

***Figure 1:*** *Collection by Winkler extractor in cave SAZ-0039.*

***Figure 2:*** *Collection by Winkler extractor in cave SAZ-0039.*

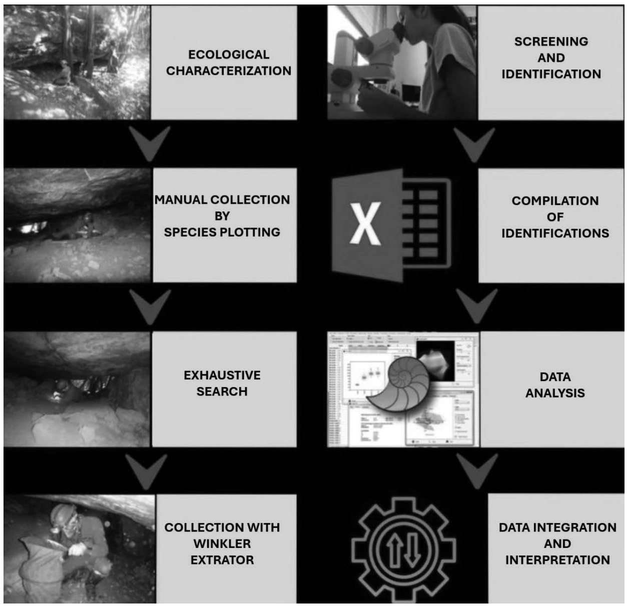


***Figure 3:*** *Schematic summary of the speleological recovery methodology and data analysis.*

## 3. Results

We recoveryd 979 organisms representing 184 (morpho)species. Three phyla were recorded: Arthropoda (N = 942, S = 172), Chordata (Chiroptera (N = 31, S =7), Anura (N = 3, S = 2) and Mollusca (N = 3, S = 3) (Table 1). Average abundance and richness per cave were 97.9 (±69.6 sd) individuals and 31.8 (±13.5 sd) (Figure 4). *Atta* sp.1, *Solenopsis* sp.1 (*Hymenoptera: Formicidae*), *Oribatida* sp.1 (*Arachnida: Sarcoptiformes*), *Theridiidae* sp.1 (*Arachnida: Araneae*), and *Zelurus* sp.1 (*Insecta*: *Hemiptera*) were the most abundant morphospecies, representing 23.2% of total abundance. *Arthropoda* represented 96.2% (N = 942) of all organisms recoveryd form caves. Insects (mostly *Hymenoptera*, N = 218) represented 50.3% (N = 492) of all individuals recoveryd, followed by *Arachnida* (31.2%, N = 305; mostly *Araneae*, N = 149). We also encountered Chordata (31 records of chiropterans (Figure 5), three records of anurans) and *Mollusca* (three records of gastropoda).

We sampled three times more individuals with exhaustive collection (N = 726, average±sd = 72.6±51.2) than Winkler extractors (N = 235, 23.5±25.5) (Table 2). Similarly, exhaustive collection sampled 3.5 times more (morpho)species (25.3±10.3) than Winkler extractors (7.2±5.6). Shared (morpho)species between both methods were very low (range: 0–3, 1.3±1.0) (Table 2). During the recovery events, we found no troglomorphic or threatened species. We recoveryd seven specimens of *Mesabolivar* sp.nov. (*Araneae*) new to science from three caves.

Caves varied in light conditions, from fully lit to low light, but never completely aphotic. Different organic substrates were observed, including animal and plant debris, which were distributed sparsely or in patches. Anthropogenic materials (e.g., paper, bottles) were also found in one cave. Previous studies on the relevance of caves have shown troglomorphic species and species new to science. However, no species recoveryd exhibited troglomorphism or was listed as threatened. New species to science were recoveryd from three caves.

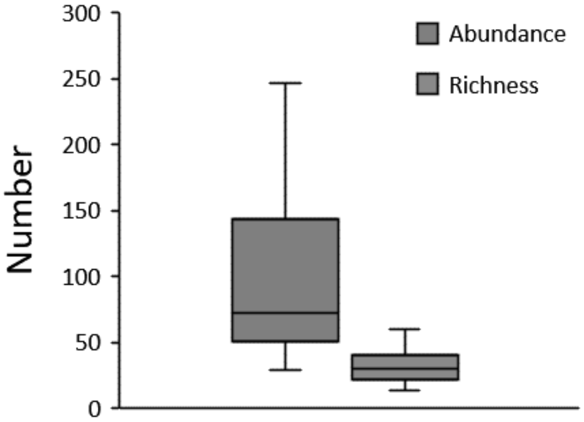
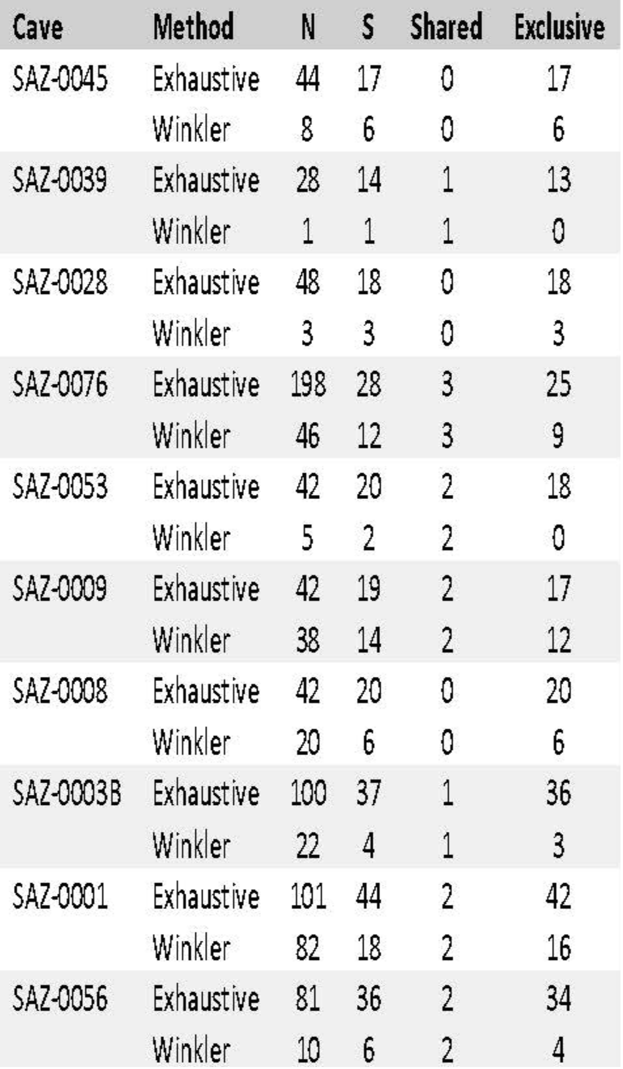


*Figure 4: Distribution of number of individuals (abundance) and species (richness) of organisms recoveryd from caves.*

| **Phylum** | **Class** | **Order** | **Total** |
|---|---|---|---|
| Arthropoda | Arachnida | Araneae | 149 |
| | | Ixodida | 1 |
| | | Mesostigmata | 32 |
| | | Opiliones | 10 |
| | | Palpigradi | 2 |
| | | Pseudoscorpiones | 27 |
| | | Sarcoptiformes | 58 |
| | | Trombidiformes | 26 |
| | Chilopoda | Geophilomorpha | 1 |
| | | Other | 2 |
| | Collembola | Entomobryomorpha | 93 |
| | | Neelipleona | 3 |
| | | Symphypleona | 23 |
| | Diplopoda | Julida | 3 |
| | | Polydesmida | 4 |
| | | Spirostreptida | 7 |
| | Diplura | Diplura | 1 |
| | Insecta | Blattodea | 35 |
| | | Coleoptera | 42 |
| | | Diptera | 14 |
| | | Hemiptera | 62 |
| | | Hymenoptera | 218 |
| | | Lepidoptera | 18 |
| | | Neuroptera | 3 |
| | | Orthoptera | 46 |
| | | Psocodea | 50 |
| | | Siphonaptera | 2 |
| | | Zygentoma | 2 |
| | Malacostraca | Isopoda | 6 |
| | Symphyla | | 2 |
| Chordata | Amphibia | Anura | 3 |
| | Mammalia | Chiroptera | 31 |
| Mollusca | Gastropoda | Ellobiida | 3 |
| **Morphospecies** | | | **184** |
| **Abundance** | | | **979** |

***Table 1:*** *List of of organisms recoveryd from caves.*



| Cave | Method | N | S | Shared | Exclusive |
|---|---|---|---|---|---|
| SAZ-0045 | Exhaustive | 44 | 17 | 0 | 17 |
| | Winkler | 8 | 6 | 0 | 6 |
| SAZ-0039 | Exhaustive | 28 | 14 | 1 | 13 |
| | Winkler | 1 | 1 | 1 | 0 |
| SAZ-0028 | Exhaustive | 48 | 18 | 0 | 18 |
| | Winkler | 3 | 3 | 0 | 3 |
| SAZ-0076 | Exhaustive | 198 | 28 | 3 | 25 |
| | Winkler | 46 | 12 | 3 | 9 |
| SAZ-0053 | Exhaustive | 42 | 20 | 2 | 18 |
| | Winkler | 5 | 2 | 2 | 0 |
| SAZ-0009 | Exhaustive | 42 | 19 | 2 | 17 |
| | Winkler | 38 | 14 | 2 | 12 |
| SAZ-0008 | Exhaustive | 42 | 20 | 0 | 20 |
| | Winkler | 20 | 6 | 0 | 6 |
| SAZ-0003B | Exhaustive | 100 | 37 | 1 | 36 |
| | Winkler | 22 | 4 | 1 | 3 |
| SAZ-0001 | Exhaustive | 101 | 44 | 2 | 42 |
| | Winkler | 82 | 18 | 2 | 16 |
| SAZ-0056 | Exhaustive | 81 | 36 | 2 | 34 |
| | Winkler | 10 | 6 | 2 | 4 |

***Table 2:*** *Number of individuals (N), species (S), shared and exclusive species of organisms recoveryd from caves for exhaustive collection and Winkler extractors.*

***Figure 5:*** *Some species of vertebrate organisms recoveryd from caves.*

## 4. Discussion

Among the species recorded during the biospeleological recovery, no troglobitic (cave-obligate) or troglomorphic (organisms with morphological adaptations to subterranean environments) species were identified. Likewise, no species listed as threatened on national or international red lists were detected. However, the discovery of individuals belonging to a new spider species underscores the ecological significance of the studied caves as reservoirs of potentially unknown biodiversity. This finding highlights the need for continued efforts to document subterranean fauna, particularly in biodiverse yet understudied regions such as iron caves in Brazil.

The results revealed notable differences in the efficacy of the two collection methods applied (FISHER 1999, OLIVEIRA 2014). Exhaustive searches yielded significantly greater species richness and individual abundance compared to the Winkler extractor. This method involves a detailed visual inspection of all accessible microhabitats, allowing researchers to target organisms across a wide range of ecological niches. Conversely, the Winkler extractor, which focuses on leaf litter and sediment-dwelling species, contributed fewer records but captured unique taxa not detected through exhaustive searches. These findings suggest that the two methods are complementary rather than redundant, each contributing to a more comprehensive understanding of cave biodiversity.

This study underscores the necessity of employing diverse and integrative collection techniques to maximize capture efforts during biospeleological recoverys. Reliance on a single methodology could lead to an incomplete assessment of cave ecosystems, particularly given the ecological heterogeneity of subterranean environments. The complementary nature of exhaustive searches and the Winkler extractor supports their simultaneous use, enabling researchers to effectively sample both generalist and microhabitat-specific species.

From a conservation perspective, these findings emphasize the importance of biodiversity assessments in caves slated for destruction due to mining or other anthropogenic activities. The absence of troglobitic and threatened species in this study does not diminish the ecological value of these caves, as the presence of a new spider species demonstrates their role as critical habitats for unique and potentially endemic fauna. Future studies should continue refining collection techniques and exploring innovative methods to enhance the detection and conservation of subterranean biodiversity.

## 5. Conclusion

Among the recorded species during the biospeleological recovery, no troglobitic, troglomorphic, or threatened species were identified. However, the discovery of individuals belonging to a new spider species highlights the potential for caves to harbor previously unknown biodiversity. The findings underscore the importance of thorough and diversified collection methods during cave fauna recoverys.

The results showed that the exhaustive search method yielded significantly higher species richness and individual abundance compared to the Winkler extractor. Exhaustive searches involve detailed scanning and capturing of organisms across various microhabitats within the cave, which allows for the identification of a broader range of species. In contrast, the Winkler extractor, while useful for sampling litter-dwelling organisms, captured fewer species and individuals. These differences highlight the need for complementary approaches, as each method targets distinct ecological niches and organism types.

To maximize the effectiveness of biospeleological recoverys, employing multiple collection techniques is crucial. A combination of exhaustive searches and specialized methods like the Winkler extractor ensures comprehensive sampling of cave biodiversity. This integrative approach is vital not only for identifying the full spectrum of species present but also for documenting ecological conditions that inform future conservation strategies.

## Acknowledgments

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# Habitando folhas e cavernas: troglomorfismos clássicos são aplicáveis aos estafilinídeos amazônicos?

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## Resumo

Investigamos a diversidade de Staphylinidae em geossistemas ferruginosos da Amazônia, buscando compreender como o ambiente determina o padrão morfológico das espécies em ambiente hipógeo. Realizamos a pesquisa, realizada na Floresta Nacional de Carajás (Pará, Brasil), onde amostramos diferentes estratos do solo. Observamos que 35,65% das espécies edáficas têm características adaptadas ao ambiente subterrâneo, como redução ou ausência de olhos e despigmentação, que poderiam ser confundidos com troglomorfismos. A análise indicou que maior cobertura de dossel está associada a organismos menores, e a robustez aumenta em estratos mais profundos do solo. O estudo contribui para a compreensão da morfologia da fauna subterrânea, indicando que o alongamento de apêndices pode ser o carcter que melhor diferencia organismos edáficos dos troglóbios.

## Abstract

We investigate the Staphylinidae diversity in Amazon ferruginous geosystems, aiming to understand how the environment drives the morphological pattern of species in hypogean environments. The research was conducted in the Carajás National Forest (Pará, Brazil), where we sampled different soil strata. We observed that 35.65% of the edaphic species have characteristics adapted to the subterranean environment, such as reduction or absence of eyes and depigmentation, which could be mistaken for troglomorphisms. The analysis indicated that higher canopy cover is associated with smaller organisms, and robustness increases in deeper soil strata. The study contributes to understanding the morphology of subterranean fauna, suggesting that appendage elongation may be the characteristic that best differentiates edaphic organisms from troglobites.

## 1. Introduction

Besouros da família Staphylinidae são caracterizados, em sua maioria, pelo corpo alongado e élitros curtos, expondo parte do abdômen. São organizados em uma das famílias mais diversas do mundo, sendo que no Brasil são quase 3.000 espécies/subespécies descritas (ASENJO et al. 2019). Muitas espécies são capazes de ocupar diferentes habitats de acordo com o seu estágio de vida ou alterações temporais das condições ambientais. Outras possuem hábitos de vida mais específicos, sendo que em alguns casos são altamente adaptados à vida em cavernas (ex. ASENJO et al. 2017, ASENJO et al. 2018).

Para acessar a fauna especializada ao ambiente subterrâneo é necessário o emprego de técnicas específicas de amostragem (ANDÚJAR e GREBENNIKOV 2021). Métodos adequados conferem maior acurácia ao estudo de padrões ecológicos da comunidade. O ambiente subterrâneo pode ser separado em diferentes compartimentos que formam um contínuo no qual as espécies são capazes de transitar (GIACHINO e VAILATI 2010). O compartimento mais superficial corresponde à área edáfica – solo profundo, em sequência encontra-se o Meio Subterrâneo Superficial (MSS) e por fim o domínio subterrâneo mais profundo, onde estão as cavernas (MAMMOLA et al. 2016). Características comum a esses ambientes incluem a ausência de luz, havendo uma estabilização das condições microclimáticas à medida que se distancia da superfície (MAMMOLA et al. 2016).

Os ambientes hipógeos, incluindo as cavernas, exercem forte seleção direcional resultando em alterações morfológicas previsíveis em função de convergências adaptativas (TRONTELJ et al. 2012). As espécies de besouros especializadas ao ambiente hipógeo geralmente são anoftálmicas, despigmentadas e sem asas. Portanto, essas características não permitem a categorização das espécies entre os diferentes compartimentos. Por outro lado, existe um gradiente de especialização da fauna à medida que se distancia da superfície, espécies de besouros tendem a apresentar alongamento do corpo e de apêndices locomotores (CHEN et al. 2021), o que não é observado nas espécies edáficas. Com isso, a compreensão dos mecanismos que atuam na seleção de características morfológicas auxilia na categorização ecológico-evolutiva das espécies. Essa categorização tange aspectos fundamentais à conservação de espécies no Brasil, uma vez que a legislação brasileira define critérios de preservação de acordo com a categorização das espécies em cavernas (JAFFÉ et al. 2016). Dessa forma é crucial o desenvolvimento de técnicas adequadas que auxiliem no diagnóstico das espécies de acordo com o habitat preferencial (ANDÚJAR e GREBENNIKOV 2021).

Nesta perspectiva, partindo da hipótese que muitas características consideradas troglomórficas são compartilhadas por espécies edáficas e troglóbias, e que características do habitat são responsáveis por determinar os atributos das espécies, o presente estudo visa identificar troglomorfismos clássicos em coleópteros edáficos (Staphylinidae) em geossistemas ferruginosos amazônicos, além de investigar se há um padrão de distribuição dessa fauna pelos diferentes estratos do solo. De forma específica, objetiva-se: i) Realizar um estudo detalhado em ambiente edáfico associado à densa floresta tropical amazônica; ii) listar características consideradas troglomórficas na fauna edáfica; iii) determinar quais parâmetros ambientais, incluindo microclimáticos, profundidade de serrapilheira ou de cobertura de dossel, são responsáveis por selecionar atributos morfológicos relacionados ao ambiente hipógeo.

## 2. Materiais e Métodos

**Área de estudos**

A região de amostragem de fauna edáfica se encontra em solo da floresta Amazônica, situadas na Floresta Nacional de Carajás (FLONA Carajás) no sudeste do Pará, Brasil, que abrange 400 mil hectares (MOTA et al. 2015). A região é caracterizada por paisagens tipicamente conhecidas como canga, com predomínio de savanas rupestres/arbustivas (SANTOS e VARAJÃO 2004, JACOBI et al. 2007). Entretanto, nas vertentes inclinadas há solos mais profundos, possibilitando o estabelecimento de florestas ombrófilas (MOTA et al. 2015). Nessas regiões de florestas concentram-se a área de coleta do presente estudo que compreenderam dois sítios amostrais (Fig. 1).

Nas áreas selecionadas, os indivíduos foram amostrados ao longo de três transectos paralelos, equidistantes em 60m (Fig. 1). Cada transecto é composto por três quadrantes, considerados as unidades amostrais, que indicam o ponto central do quadrante amostral (9m de lado) (Fig. 1, adaptação de OLIVEIRA et al. 2019). Cada quadrante foi subdividido por grades latitudinais e longitudinais de 3 m. Em cada quadrante, os espécimes foram amostrados usando três métodos diferentes, amostras de solo (AS), amostras de serrapilheira (APL) e amostras de feno (HBT).

**Amostragem em campo**

Para a amostragem do extrato mais superficial, selecionamos os locais para coleta de 4L de serrapilheira. Peneiramos o material coletado em sifter cilíndrico. Em seguida inserimos em Funis de Berlese-Tullgren para extração da fauna por uma semana.

Acessamos o extrato médio por meio de armadilhas de feno umedecido. Dispomos o feno em envelopes de 25x25cm em tela metálica (malha de 2cm). Nos quadrantes, instalamos cinco armadilhas (30-40cm de profundidade), por 8 semanas. Em seguida, inserimos as amostras em Funis de Berlese-Tullgren para extração da fauna por uma semana.

A amostragem de fauna em solo mais profundo seguiu os procedimentos detalhados em ANDUJAR e GREBENNIKOV (2021). Escolhemos os locais de solos úmidos e bem arejados. Após a remoção das camadas superficiais de serrapilheira e solo, acessamos o solo entre 30 e 40 cm de profundidade. Após a retirada do material grosso, realizamos o processo de flotação em que o material em suspensão é coletado com uma peneira de malha fina. Em laboratório colocamos o material em extratores específicos. São duas bandejas sobrepostas, a inferior contendo água, a superior sem fundo é separada da inferior por duas malhas (uma de 1 x 1 cm e outra de 2 x 2mm), onde é colocado o material resultante da flotação. Lâmpadas incandescentes de 40w são posicionadas sobre os coletores durante 7 ciclos de 48 horas.

**Identificação e atributos funcionais**

Após a triagem e identificação ao menor nível taxonômico possível com a designação de morfótipos, depositamos vouchers de cada espécie na Coleção de Invertebrados Subterrâneos da Universidade Federal de Lavras (ISLA-UFLA) e também no Museu Paraense Emílio Goeldi.

Para cada morfótipo elencamos a presença ou ausência de características troglomórficas, compreendendo a anoftalmia, coloração pálida, alongamento de antenas e alongamento de estruturas locomotoras. Consideramos as particularidades dos táxons para a atribuição dos troglomorfismos.

Para abordagem dos atributos funcionais das espécies, selecionamos as subfamílias Pselaphinae e Osoriinae, uma vez que estas subfamílias possuem dentre seus representantes, espécies que apresentam adaptações morfológicas ao ambiente edáfico (ANDUJAR e GREBENNIKOV 2021). Seguindo a metodologia proposta por PEREZ-SANCHEZ et al. (2021) medimos os atributos morfológicos diretamente nos indivíduos coletados (comprimento do corpo, comprimento da antena e tamanho do olho, sendo corrigidas pelo comprimento total do corpo), calculamos dois outros atributos (robustez e razão élitro) com base em uma combinação de outras medidas morfológicas (Fig. 2 e Fig. 3).

**Análise dos atributos funcionais**

Por fim, para avaliar a resposta dos atributos funcionais das espécies de Osoriinae e Pselaphinae, amostradas no presente estudo, às condições ambientais realizamos análises de co-inércia RLQ (DOLÉDEC et al. 1996). Vale destacar que dentre as variáveis ambientais foi incluído os três métodos de coleta distintos como um proxy da profundidade do local de amostragem. Além dessas incluímos dados microclimáticos, profundidade de serrapilheira e cobertura de dossel. Os atributos funcionais utilizados estão destacados a seguir (Fig. 3). Essas análises fazem uso de três tabelas, sendo que na primeira delas são inseridas variáveis ambientais (R), na segunda insere-se a distribuição de abundância das espécies nas amostras (L) e na terceira, os atributos funcionais de cada espécie (Q) (DRAY e LEGENDRE 2008, DRAY et al. 2014).

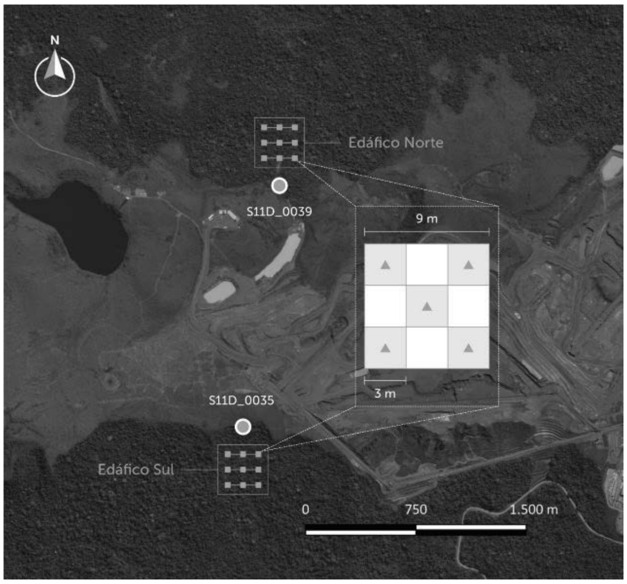


***Figura 1:*** *Líquen crostoso presente em um cacto e vegetação associada as cavidades estudadas.*

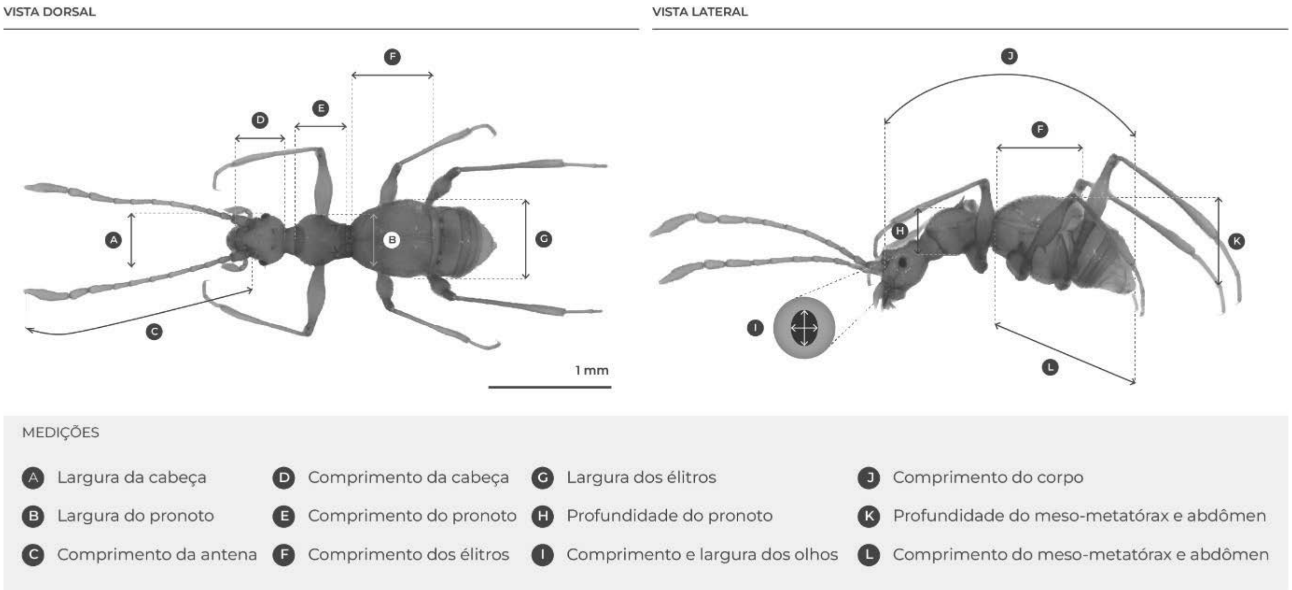
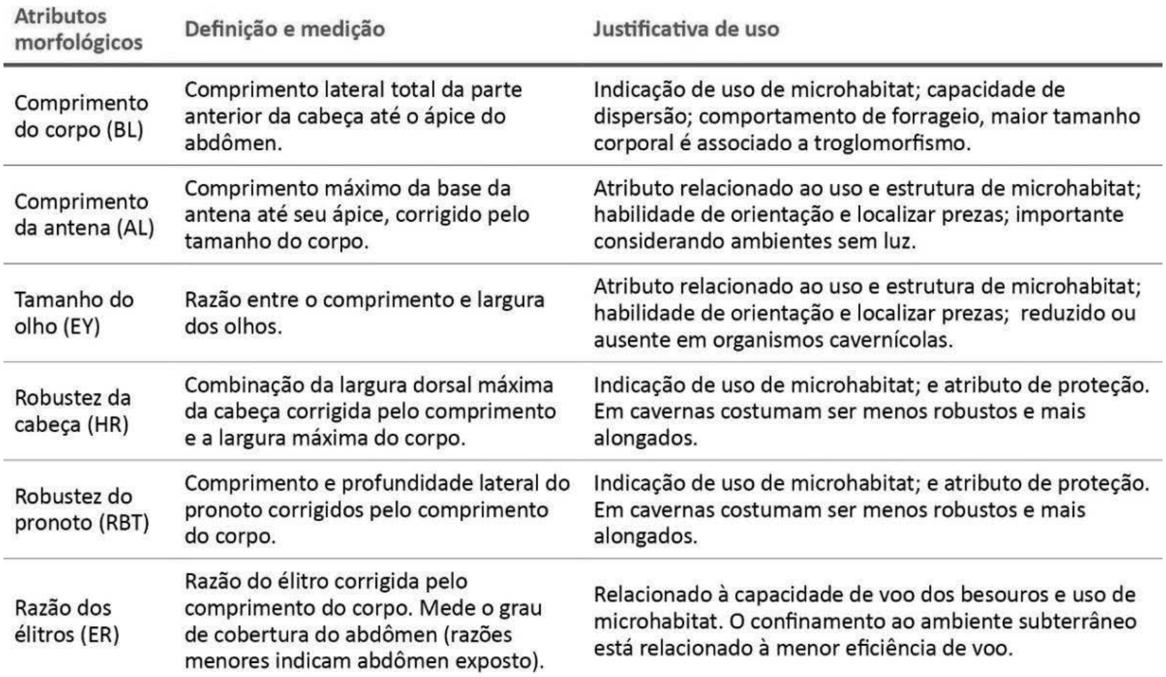


***Figura 2:*** *Representação esquemática dos atributos funcionais inferidos com base em medidas morfométricas. Largura máxima da cabeça e largura, profundidade e comprimento do pronoto foram utilizados para calcular a robustez. Comprimento dos élitros, comprimento do abdômen e meso-metatórax foram utilizados para calcular a razão dos élitros. Imagem: Oxarthrius (Oxarthrius) inexpectatus Asenjo et al. 2018.*



| Atributos morfológicos | Definição e medição | Justificativa de uso |
|---|---|---|
| Comprimento do corpo (BL) | Comprimento lateral total da parte anterior da cabeça até o ápice do abdômen. | Indicação de uso de microhabitat; capacidade de dispersão; comportamento de forrageio, maior tamanho corporal é associado a troglomorfismo. |
| Comprimento da antena (AL) | Comprimento máximo da base da antena até seu ápice, corrigido pelo tamanho do corpo. | Atributo relacionado ao uso e estrutura de microhabitat; habilidade de orientação e localizar prezas; importante considerando ambientes sem luz. |
| Tamanho do olho (EY) | Razão entre o comprimento e largura dos olhos. | Atributo relacionado ao uso e estrutura de microhabitat; habilidade de orientação e localizar prezas; reduzido ou ausente em organismos cavernícolas. |
| Robustez da cabeça (HR) | Combinação da largura dorsal máxima da cabeça corrigida pelo comprimento e a largura máxima do corpo. | Indicação de uso de microhabitat; e atributo de proteção. Em cavernas costumam ser menos robustos e mais alongados. |
| Robustez do pronoto (RBT) | Comprimento e profundidade lateral do pronoto corrigidos pelo comprimento do corpo. | Indicação de uso de microhabitat; e atributo de proteção. Em cavernas costumam ser menos robustos e mais alongados. |
| Razão dos élitros (ER) | Razão do élitro corrigida pelo comprimento do corpo. Mede o grau de cobertura do abdômen (razões menores indicam abdômen exposto). | Relacionado à capacidade de voo dos besouros e uso de microhabitat. O confinamento ao ambiente subterrâneo está relacionado à menor eficiência de voo. |

***Figura 3:*** *Definição e justificativa de uso (com referências) dos atributos funcionais utilizados em Staphylinidae. Modificado de Pérez-Sánchez et al. (2021).*

## 3. Resultados

**Descrição da fauna**

Foram amostrados 273 indivíduos pertencentes à família Staphylinidae, os quais correspondem a 115 morfoespécies distintas distribuídas em 11 subfamílias: Aleocharinae (86 indivíduos), Euasthetinae (2 indivíduos), Leptotyphlinae (9 indivíduos), Osoriinae (19 indivíduos), Oxytelinae (10 indivíduos), Paederinae (6 indivíduos), Pselaphinae (66 indivíduos), Scaphidiinae (3 indivíduos), Scydmaeninae (55 indivíduos), Staphylininae (15 indivíduos) e Tachyporinae (2 indivíduos). A assembleia de Staphylinidae exibiu frequência de distribuição de poucas espécies mais amplamente distribuídas, sendo que a maioria foi considerada rara, sendo contabilizados somente um ou dois indivíduos (Figura 2.1). Por outro lado, uma única espécie, *Paratorchus* sp.1 (Osoriinae) foi representada por 37 indivíduos.

**Troglomorfismos**

Das 115 morfoespécies identificadas na área de estudo, 41 (35,65%) apresentaram algumas características atribuídas a troglomorfismos. Dentre esses atributos, o mais frequente foi a ausência ou redução de estruturas oculares, sendo observado em 23 das morfoespécies. A despigmentação foi observada em 13 morfótipos, já o alongamento de apêndices locomotores ou sensoriais (antenas) foi observado em 5 morfótipos apenas (Fig. 4). A morfoespécie que apresentou o maior número de atributos relacionados ao ambiente subterrâneo foi Metopiellus sp., apesar de a espécie possuir pigmentação pronunciada, apresenta todos os demais atributos analisados.

**Atributos funcionais**

A partir da análise da relação entre os atributos funcionais morfológicos, espécies de Osoriinae e de Pselaphinae e as características da paisagem, foi possível verificar alguns padrões. A correlação do segundo eixo gerado pelo modelo a partir da análise de RLQ é baixa (0,107) (Fig. 5). A variação dos parâmetros ambientais é bem preservada nos dois primeiros eixos (94,28%). Já em relação aos atributos funcionais, foi preservada cerca de 67,85% da variação dos dados nos dois primeiros eixos.

A partir das correlações apresentadas foi possível observar que a maior cobertura de dossel determina a ocorrência de organismos de menor tamanho corporal. Os olhos e a razão dos élitros são menores em locais com maior cobertura por serra pilheira. Os organismos são mais robustos nos estratos mais profundos. Para o comprimento das antenas, as relações com o ambiente foram pouco expressivas, explicando menos de 12% da variação observada (Fig. 6).

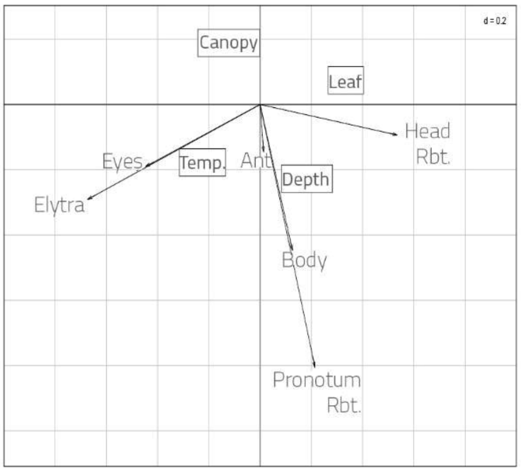


*Figura 5:* *Coeficientes dos atributos morfológicos e das variáveis ambientais representando os resultados da análise de RLQ.*

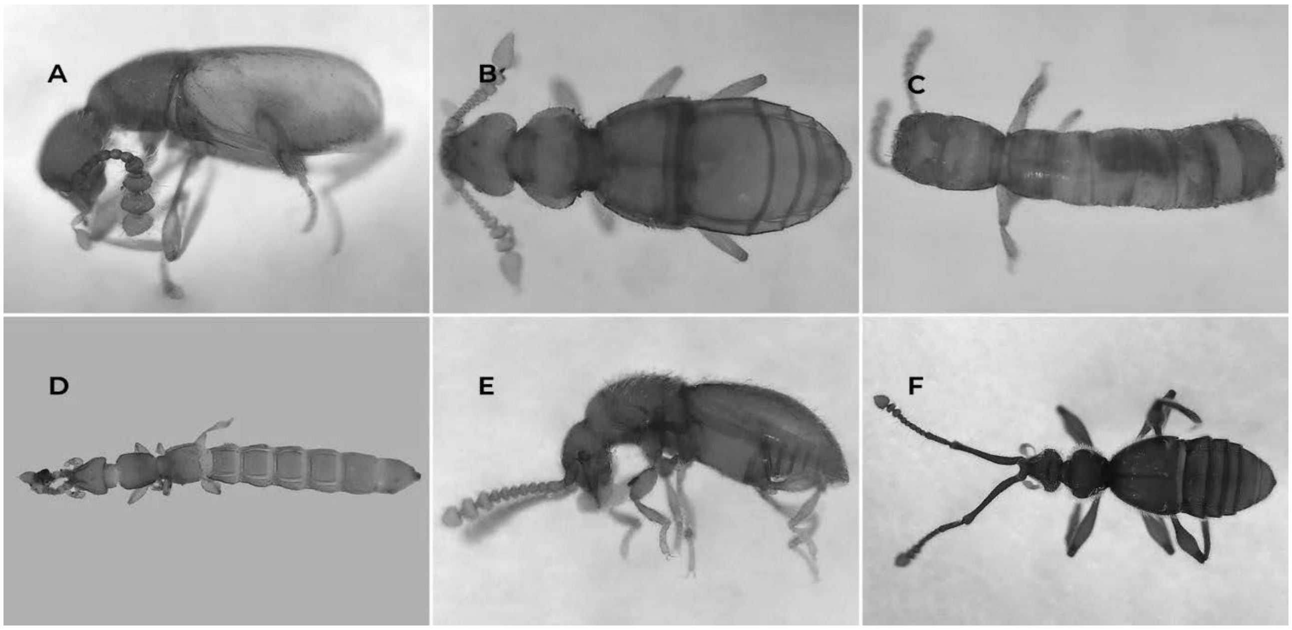
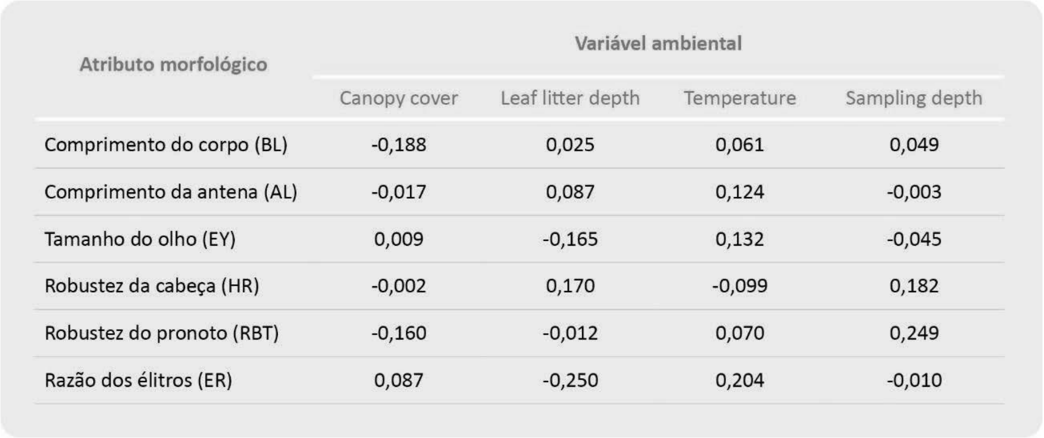


*Figura 5:* *Características consideradas troglomórficas em organismos edáficos.*



| Atributo morfológico | Variável ambiental | | | |
|---|---|---|---|---|
| | Canopy cover | Leaf litter depth | Temperature | Sampling depth |
| Comprimento do corpo (BL) | -0,188 | 0,025 | 0,061 | 0,049 |
| Comprimento da antena (AL) | -0,017 | 0,087 | 0,124 | -0,003 |
| Tamanho do olho (EY) | 0,009 | -0,165 | 0,132 | -0,045 |
| Robustez da cabeça (HR) | -0,002 | 0,170 | -0,099 | 0,182 |
| Robustez do pronoto (RBT) | -0,160 | -0,012 | 0,070 | 0,249 |
| Razão dos élitros (ER) | 0,087 | -0,250 | 0,204 | -0,010 |

*Figura 6:* *Correlação entre os atributos morfológicos de Osoriinae e Pselaphinae com as variáveis ambientais.*

## 4. Discussão

O presente estudo, permitiu a amostragem de alta riqueza de espécies da família Staphylinidae edáficas na região (115 espécies), revelando o rico padrão de diversidade existente no solo de reservas de floresta Amazônica. Os atributos morfológicos das espécies também refletem tal diversidade, nos quais todos os troglomorfismos analisados foram observados nos exemplares edáficos, sendo que o alongamento de apêndices foi o menos frequente. Por fim, indicamos que a maior cobertura de dossel determina a ocorrência de organismos de menor tamanho corporal. Os olhos e a razão dos élitros são menores em locais com maior cobertura por serra pilheira. Os organismos são mais robustos nos estratos mais profundos

Os Staphylinidae se destacam frente à outras famílias de coleópteros na colonização de ambientes edáficos. Espécies edáficas costumam apresentar características morfológicas que incluem o corpo pequeno e alongado, ausência ou redução de asas e estruturas oculares, atributos que são comuns a alguns grupos de Staphylinidae (COIFFAIT 1979, DAJOZ 1980). Dessa forma, a alta diversidade de Staphylinidae no solo se deve a afinidade pelo ambiente, como ocorre com muitos representantes de Pselaphinae e a maioria dos Osoriinae conhecidos (ANDÚJAR e GREBENNIKOV 2021). As características do ambiente e a morfologia da fauna edáfica contribuem para um padrão de alto endemismo de muitas das espécies de Coleoptera encontradas em solo (BAEHR 2014, GIACHINO et al. 2021), as quais podem eventualmente acessar o ambiente cavernícola.

Ademais, o padrão morfológico de distribuição dos estafilinídeos frente as características do habitat indicam que o ambiente edáfico funciona como um filtro ambiental. Besouros de corpo longo e de coloração mais escura se associam a florestas densas (VANDEWALLE et al. 2010). Tais padrões morfológicos foram pouco frequentes nos exemplares amostrados. De forma contrária, encontramos forte associação entre a maior robustez do pronoto com a profundidade de amostragem, atributo relacionado à proteção do indivíduo (FOUNTAIN-JONES et al. 2015).

Muitas espécies apresentaram morfologia convergente com a encontrada em espécies cavernícolas, o que dificulta classificações do status ecológico-evolutivo das espécies. Por outro lado, o atributo que confere maior distinção entre os grupos é a baixa frequência de apêndices (antenas e pernas) nos organismos edáficos.

## 5. Conclusão

Estudos como este representam avanços para a conservação da biodiversidade de espécies. Alguns dos táxons encontrados no presente estudo compreendem espécies novas, carecendo uma descrição formal de sua morfologia, a partir da qual é possível começar a se traçar estratégias à conservação. Além disso, a morfologia dos besouros edáficos dificulta a definição do status ecológico da fauna subterrânea em seus extratos distintos. Considerando-se a importância na definição precisa desse status, entender padrões de diversidade de distribuição das espécies é fundamental para a elaboração de estratégias de conservação.

## Agradecimentos

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# River bank influencing spatial distribution of terrestrial cave invertebrates

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## Abstract

O curso d’água presente em uma caverna pode alterar a estrutura da comunidade de invertebrados encontrada tanto nas proximidades quanto em áreas mais distantes do rio. Para testar essa hipótese, foram realizadas coletas em áreas na margem do curso d’água e em regiões mais secas da caverna, utilizando setores e quadrantes para delimitar as unidades amostrais. Além dos invertebrados coletados, também foram analisadas características ambientais. A análise revelou uma diferença significativa na composição de invertebrados entre as áreas próximas e distantes do curso d’água. Portanto, pode-se concluir que a presença de um corpo d’água em uma caverna influencia significativamente a variedade de nichos ecológicos e a composição de espécies no interior da caverna.

## Abstract

The watercourse present in a cave can alter the structure of the invertebrate community found both near and far from the river. To test this hypothesis, collections were made at areas on the edge of the watercourse and in drier areas of a cave, using sectors and quadrants to delimit the sample units. In addition to the collected invertebrates, environmental characteristics were also analyzed. The analysis revealed a significant difference between the composition of invertebrates in areas close to and far from the watercourse. Therefore, it can be concluded that a body of water in a cave significantly influences the variety of ecological niches and species composition inside a cave.

## 1. Introduction

Subterranean ecosystems serve as “natural laboratories” due to their structural simplicity and lower biodiversity than surface environments (POULSON & WHITE, 1969; MAMMOLA, 2019). Their semi-isolation from the surface environment results in unique characteristics, such as the complete absence of light in deeper areas, preventing the development of photoautotrophic organisms along with consistently high humidity and stable temperatures throughout the year (BADINO, 2010). Due to these conditions, these ecosystems are typically oligotrophic (energy-limited), relying primarily on organic matter from external sources, though exceptions exist (ISKALI & ZHANG, 2015).

Research on subterranean environments expands our understanding of these ecosystems and provides valuable insights into evolutionary and ecological processes in more complex systems (POULSON & WHITE, 1969; MAMMOLA, 2019). Factors such as species dispersal limitations, climatic conditions, and habitat heterogeneity play crucial roles in shaping beta diversity (species turnover and nestedness) and influence the structure of cave-dwelling invertebrate communities (CALDERÓN-PATRÓN et al., 2016; SOUZA-SILVA et al., 2021; REIS-VENÂNCIO et al., 2022; 2024). However, these relationships and the variables associated with observed patterns depend directly on the scale of analysis (MAMMOLA et al., 2019).

To better understand these patterns and interactions, it is essential to apply methodologies at multiple scales, capturing the diverse responses of subterranean fauna with greater precision (MAMMOLA et al., 2019, 2020; SOUZA-SILVA et al., 2021; REIS-VENÂNCIO, 2024). Research on organismal response scales is fundamental for developing more effective conservation strategies.

Several factors influencing Neotropical cave communities are already well-documented, including landscape structure (PELLEGRINI et al., 2016), cave linear development (SIMÕES et al., 2015), seasonal variations (BENTO et al., 2016), lithology (SOUZA-SILVA et al., 2011), environmental heterogeneity (SOUZA-SILVA et al., 2021; FURTADO-OLIVEIRA et al., 2022; REIS-VENÂNCIO et al., 2022; 2024), and the presence of water (SIMÕES et al., 2015). Water bodies, in particular, play a crucial role in the dispersal and distribution of various groups, as flooding events can cause disturbances that alter community composition (PACIOGLU et al., 2019). Additionally, underground river banks serve as vital vectors for organic matter transport, supplying essential resources that resident species can exploit (SOUZA-SILVA et al., 2012). Given these dynamics, further exploration of the specific effects of river banks on cave community structure is necessary and promising. Besides that, a water body can provide surrounding air and floor moisture inside subterranean habitats and affect faunal distribution (SIMÕES et al., 2015).

In this context, the present study aims to investigate the influence of a river bank on the composition and richness of terrestrial invertebrates in a limestone cave. We also seek to understand how floor habitat characteristics surrounding a river bank vary spatially.

## 2. Materials and methods

Sampling was carried out during a single collection event during the dry season of 2021 at the Gruta da Lapa Cave, located in Niquelândia, northeastern Goiás, Brazil. The region's climate is classified as Aw (tropical with a dry winter from March to October) (ALVARES et al., 2013) and is part of the Cerrado biome. The cave features an active autogenic drainage system that flows out of it.

Terrestrial invertebrates were recorded through visual searches and active manual collection using forceps and brushes (FERREIRA, 2004; WYNNE et al., 2019). Sampling was conducted at both micro and mesoscales within predefined units. At the mesoscale, sampling sectors measured 30 m$^2$ (3 m × 10 m) and were positioned at ground level. Three 1 m$^2$ quadrants were placed equidistantly, representing the microscale sampling level. Four sectors (12 quadrants) were established in areas adjacent to the cave's river bank, while two sectors (6 quadrants) were placed in drier regions farther from the water.

Collected specimens were stored in labeled containers filled with a 70% ethanol solution to ensure proper preservation. All specimens were deposited in the Subterranean Invertebrate Collection of Lavras (ISLA), affiliated with the Center for Subterranean Biology Studies at the Federal University of Lavras (biologiasubterranea.com.br). In the laboratory, specimens were sorted using optical stereomicroscopes and identified to the lowest possible taxonomic level with identification keys. They were then categorized into morphotypes (OLIVER & BEATTLE, 1966a, b).

For floor substrate characterization and quantification at the mesoscale, each sector was divided into ten 3 m$^2$ sections, where the percentage composition of different substrates and available resources was estimated following the methodology of SOUZA-SILVA et al. (2021). We calculated each substrate type's total proportions and arithmetic mean per sector.

The floor substrate (e.g., guano, animal and plant detritus, fine sediment, gravel, boulders, speleothems and, bedrock) characterization and measurement were performed at the microscale using digital photographs taken in the field before sampling. In the laboratory, substrate class percentages were calculated using the ImageJ software.

For general data analysis, total invertebrate richness was determined by summing the morphospecies within the sample units. Invertebrate abundance was assessed as the total count of individuals collected.

To quantify substrate, shelter, and resource diversity within each sampling unit, we applied the Shannon Diversity Index (H') (CARDOSO et al., 2022; PELLEGRINI et al., 2016; SOUZA-SILVA et al., 2021; FURTADO-OLIVEIRA et al., 2022). Substrate diversity was calculated by applying the index to all substrate classes (e.g., guano, leaf litter, branches, boulders, cobblestones, gravel, and silt). Resource diversity was assessed by considering only organic substrates on the cave floor (e.g., guano, leaf litter, and branches). Shelter diversity was calculated based on the proportions of medium and small boulders, coarse and fine gravel, and cobblestones.

### 2.1. Data Analysis

The analysis was performed only using quadrats as the sectors didn`t present sufficient numbers for statistical analysis. However, sectors were used to account for the richness of the cave fauna.

Generalized linear models (GLMs) (ZUUR, 2009) were constructed to assess the influence of habitat structure parameters (substrate, resource, and shelter diversities) on invertebrate richness at the microscale, comparing areas near the river bank to drier cave sections. The model was built assuming a Poisson distribution. Model fit was visually evaluated and assessed using the 'simulateResiduals' function from the DHARMa package (HARTIG, 2022). Additionally, model overdispersion was tested. We then tested differences in mean invertebrate richness between the two areas using the Mann-Whitney U test.

To compare faunal similarity between the two communities (river-bank-adjacent vs. dry regions), we conducted a Permutational Multivariate Analysis of Variance (PERMANOVA) based on a Bray-Curtis similarity matrix, which was first transformed using the Hellinger method. Additionally, the Similarity Percentages (SIMPER) analysis was performed to identify the species contributing to the dissimilarities between groups, also based on the Bray-Curtis matrix.

Finally, beta diversity and its partitioning between the two communities were calculated using the 'beta' function from the BAT package (CARDOSO et al., 2015). All statistical analyses were conducted in R (R CORE TEAM, 2023).

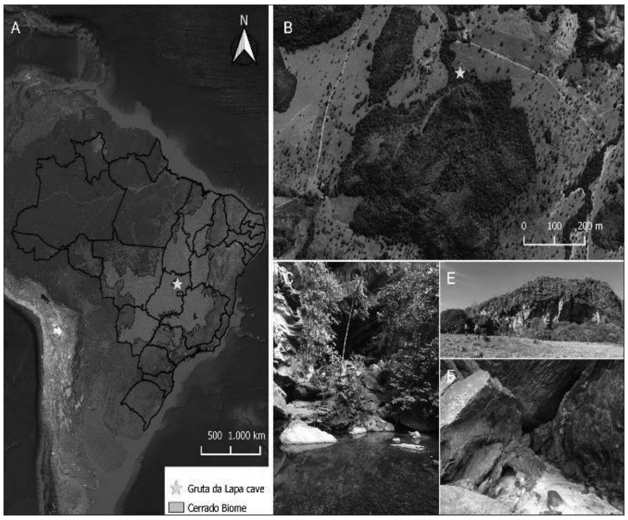

***Figure 1:*** *Location of the cave in Goiás state (A) and the landscape features (B and E). Cave entrance (E) and water course (C).*

## 3. Results

51 morphospecies of invertebrates were sampled within 16 orders (Figure 2). The average species richness was 2.25 species in areas adjacent to the river and 3 species in areas distant from the river. Araneae, Diptera, Coleoptera and, Hemiptera were the richest orders, respectively (Figure 2). In quadrants near from the river bank, 256 individuals could be counted, and 152 in the quadrants located in the dry portion of the cave.

The Mann-Whitney test showed no difference in average richness between areas close to and far from the river bank (W = 46.5; $p_{value} = 0.1669$).

A significant difference was observed in the composition of the invertebrate fauna in quadrants near and far from the river bank ($R^2 = 0.12$; F = 2.20; $p_{value} = 0.02$). SIMPER revealed that Phalangopsidae sp1 occupying the riverbanks and Scytodidae sp, found in the drier areas, contributed most to the dissimilarity from wet to dry areas. None of the tested predictors showed a significant relationship with the richness of terrestrial invertebrate species.

The beta diversity between quadrants close to and far from the river bank was $\beta_{total} = 0.78$, with species turnover having the most significant influence ($\beta_{repl} = 0.45$). At the same time, the nestedness component was lower ($\beta_{repl} = 0.33$).

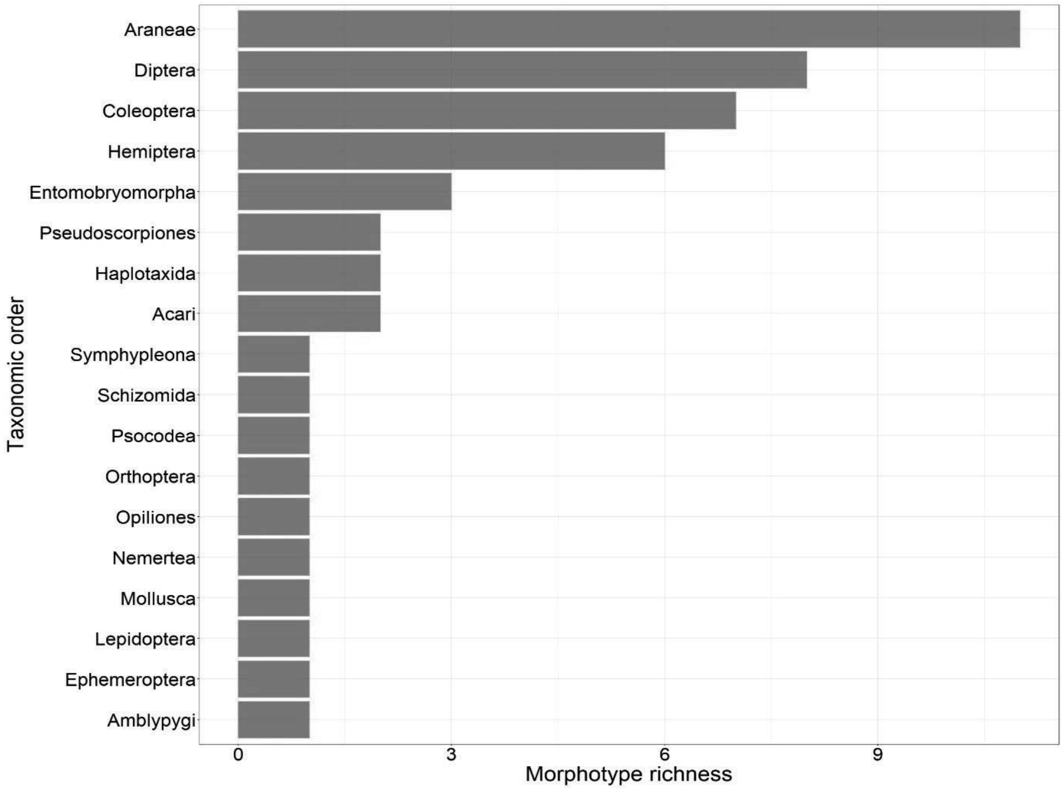

***Figure 2:*** *Richness of morphospecies by taxonomic order recorded from Gruta da Lapa Cave, Niquelândia, northeastern Goiás, Brazil.*

## 4. Discussion

The study revealed that the presence of a watercourse significantly influences the composition and diversity of terrestrial cave invertebrates. Specifically, the presence of a waterbody contributes to increased moisture levels in both the air and on the cave floor, creating an ideal environment for species that thrive in humid conditions. This elevated humidity supports the species' survival and carries organic matter within the cave (WEINSTEIN, 1994; SOUZA-SILVA et al., 2012). Furthermore, the study highlights that other critical environmental parameters, such as temperature fluctuations and humidity levels, play a vital role in shaping the distribution patterns of terrestrial invertebrate communities within caves. Variations in these conditions can lead to marked differences in species richness and composition, thereby emphasizing the importance of watercourses as ecological drivers in subterranean environments (SOUZA-SILVA et al., 2021). However, the change in moisture between the wettest and driest portions of the cave does not occur continuously but abruptly, which may explain the high turnover of species observed in the sampled units.

On the other hand, certain species of cave-dwelling invertebrates have been observed seeking refuge in microhabitats characterized by slightly lower humidity levels. This behavior can occur near the cave entrance and deeper within the cave, influenced by various environmental factors such as temperature and moisture gradients and strategic considerations related to food availability. Our research has indicated that invertebrates, some taxa like Phalangopsidae, Carabidae, Entomobryoidae, Chironomidae, Veliidae, and Psyllipsocidae typically thrive in more humid areas, where moisture supports their physiological needs and enhances their foraging opportunities. In contrast, other groups, including Lepidoptera larvae, Scytodidae, Filistatidae, and Nesticidae, tend to inhabit drier cave areas. This distinction in habitat preference highlights the diverse adaptations of cave invertebrates to their unique ecological niches, shaped by the interplay of humidity and resource availability (Figure 3). Overall, the presence of water bodies within a cave is considered an element that tends to increase invertebrate richness, not only by providing more moisture but also as a via of organic resources, which are transported into subterranean environments through rivers, floods (SOUZA-SILVA et al., 2012). Therefore, more humid environments are expected to have greater species richness (SIMÕES et al., 2022). However, we must consider that the origin of the cave stream is autogenic, meaning that the direction it flows is from the interior to the exterior, causing it to have passed through various physical barriers that filter the organic matter it carries, preventing coarse organic compounds while allowing finer or dissolved substances, which may not be useful for many invertebrates, potentially directly affecting the richness of the environment (SIMON et al., 2007).

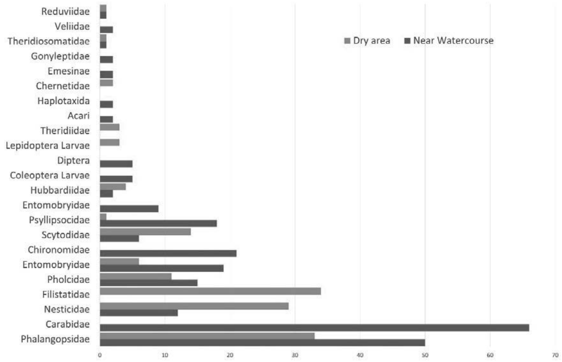

***Figure 3:*** *Distribution of the most abundant morphotypes concerning the cave's dry and wet area.*

## 5. Conclusion

The presence of a watercourse in a cave environment is crucial for assessing biodiversity, once the stream within the cave affects the composition of invertebrate species. Additionally, the humidity gradient in the various sections of the cave supports the presence of different species. Therefore, it can be concluded that a body of water in a cave significantly influences the variety of ecological niches and species richness.

## Acknowledgments

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# Anemophilous Mycobiota in Bat Caves of Northeast Brazil

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## Resumo

Os fungos são organismos bem adaptados às condições das cavernas, e frequentemente são isolados nesses habitats, principalmente em cavernas que possuem uma grande comunidade de morcegos, como as bat caves. A principal forma de disseminação desses fungos é pelo ar, sendo então chamados de fungos anemófilos. Este trabalho teve o objetivo de identificar os fungos do ar nas bat caves Boqueirão de Lavras no Ceará, e Urubu no Rio Grande do Norte, Brasil. Foram isolados, ao total, 42 fungos que após a identificação foram agrupados em 10 gêneros e 17 espécies pertencentes ao filo Ascomycota, os gêneros *Aspergillus* e *Cladosporium* foram os mais abundantes. As cavernas estudadas apresentaram uma notável riqueza de fungos em suspensão no ar do seu ambiente, e todos os fungos encontrados já foram relatados em outras cavernas no Brasil e no mundo. Portanto, este estudo colaborou com a estimativa global de fungos presentes em cavernas, além de fornecer dados micológicos para a futura elaboração dos seus respectivos planos de manejo.

## Abstract

Fungi are well adapted organisms to cave conditions and are frequently isolated in these habitats, especially in caves that host large bat communities, known as bat caves. The primary means of fungal dissemination is through the air, thus they are referred to as anemophilous fungi. This study aimed to identify airborne fungi in the bat caves Boqueirão de Lavras in Ceará, and Urubu in Rio Grande do Norte, Brazil. A total of 42 fungi were isolated, which, after identification, were grouped into 10 genera and 17 species belonging to the phylum Ascomycota, with *Aspergillus* and *Cladosporium* being the most abundant genera. The studied caves exhibited a notable richness of fungi suspended in the air of their environment, and all the fungi found have been previously reported in other caves in Brazil and worldwide. Therefore, this study contributed to the global estimation of fungi present in caves, as well as provided mycological data for the future development of their respective management plans.

## 1. Introdução

*Bat caves* são cavernas que abrigam grandes comunidades de morcegos, são utilizadas por eles como locais para acasalamento, criação de filhotes, digestão de alimentos, entre outras atividades do seu metabolismo (PIMENTEL & BERNARD, 2024). Os morcegos presentes nessas cavernas influenciam na disponibilidade de nutrientes para outros organismos, devido à deposição do guano, que serve como fonte alimentar para a biota cavernícola, incluindo os fungos (REIS et al. 2023). Os fungos são organismos amplamente distribuídos em todo planeta, e sua presença em cavernas foi documentada pela primeira vez em 1741, (DOBAT, 1967). Desde então, estudos têm mostrado que os ambientes subterrâneos detém uma diversidade fúngica altamente difundida em todos os seus substratos (VANDERWOLF et al. 2013). A Caatinga é uma das mais importantes florestas tropicais brasileiras, cobre a maioria dos estados da região Nordeste do país e possui uma diversidade de fauna e flora bem estabelecida (BEZERRA et al. 2017). Diversas cavernas e *bat caves* estão inseridas nesse ecossistema, e algumas já possuem registros da micobiota presente em seus substratos inclusive com a descrição de novas espécies (CUNHA et al. 2020; PEREIRA et al. 2021; CARVALHO et al. 2022; ALVES et al. 2022; LIMA et al. 2024). Os fungos produzem esporos a partir do seu ciclo reprodutivo que serve como forma de disseminação da espécie, esses esporos podem permanecer em suspensão ou ser transportados por correntes de ar, o que os caracteriza como fungos anemófilos. Quando inalados, esses esporos podem causar alergias ou infecções fúngicas, especialmente em indivíduos imunocomprometidos. Nas cavernas, a manifestação patológica mais conhecida é a Histoplasmose, que ocorre pela inalação dos esporos do fungo *Histoplasma capsulatum* (VICENTINI et al. 2012). O estudo micológico em cavernas torna-se necessário para a segurança biológica de visitantes e pesquisadores, sendo um tópico exigido na elaboração do plano de manejo de uma caverna (CECAV, 2008). Além disso, o registro da micobiota cavernícola tem finalidade de assegurar a conservação desses ambientes, visto que os fungos anemófilos fazem parte desse ecossistema sensível, contribuindo diretamente com a sobrevivência de diversas espécies. Além disso, as cavernas podem abrigar espécies ainda desconhecidas ou novas ocorrências fúngicas, que podem ser perdidas com os impactos ambientais e antrópicos. Nesse contexto, o presente estudo teve como objetivo isolar e identificar fungos presentes no ar de duas *bat caves* do Nordeste do Brasil, fornecendo dados para o conhecimento da micobiota cavernícola e a garantia de visitações mais seguras.

## 2. Materiais e Métodos

Foram selecionadas duas cavernas para a realização deste estudo: Caverna 1 – Boqueirão de Lavras (6°42'45.05''S, 38°57'28.10''O), localizada no município de Lavras da Mangabeira, estado do Ceará; e Caverna 2 – Urubu (05°34'22.8''S, 37°39'08.8''O) localizada no município de Felipe Guerra, estado do Rio Grande do Norte (Figura 1). Em ambas as cavernas, foram definidos três pontos de coleta: entrada, meio e fim, abrangendo toda a extensão das cavernas.

*Figura 1: A - Entrada da caverna Boqueirão de Lavras; B - Ambiente interno da caverna Urubu.*

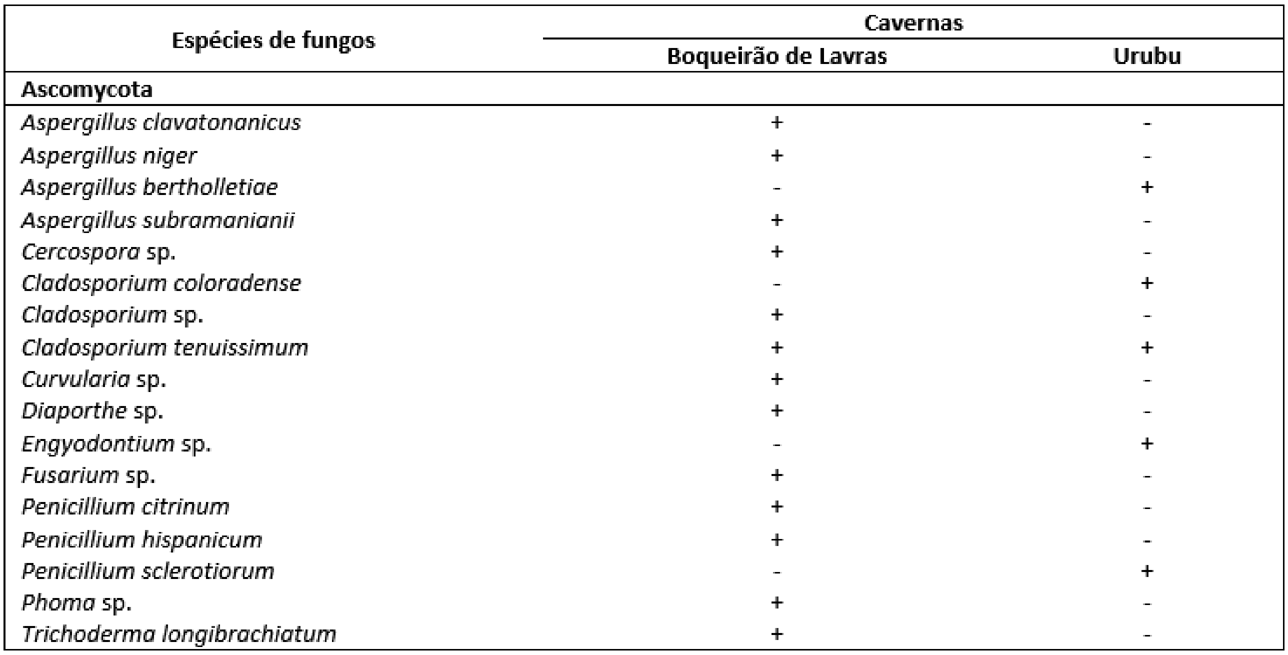
A coleta dos fungos anemófilos foi realizada utilizando a metodologia de sedimentação passiva em meio de cultura contida em placas de Petri (gravity settling culture plate method). Em cada ponto, foram abertas três placas de 90 mm contendo Ágar Sabouraud acrescido de cloranfenicol (0,1 mg/L). As placas foram posicionadas equidistantes umas das outras, a 1 metro do chão, em suportes de PVC, por 20 minutos (CUNHA et al. 2020). Após a exposição, as placas foram fechadas, identificadas, embaladas e levadas ao laboratório, onde foram incubadas a 25 °C por até 14 dias no escuro. A cada dia, as placas eram verificadas para observação do crescimento fúngico. O número de Unidades Formadoras de Colônia (UFC) por placa foi utilizado para estimar a abundância fúngica. As colônias que apresentavam morfologias distintas foram sub cultivadas em Ágar Sabouraud acrescido com cloranfenicol (0,1 mg/L) para restringir o crescimento de bactérias e incubadas nas mesmas condições anteriores. Após a purificação, as colônias fúngicas foram preservadas em uma solução de glicerol a 30%, juntamente com solução salina, e armazenadas na coleção de trabalho do Laboratório de Taxonomia e Biotecnologia Utilizando Fungos.

Para a identificação dos fungos foi realizada a extração do DNA com as instruções do Kit de extração de DNA genômico da Promega (Wizard Genomic DNA Purification Kit). Após a extração, regiões específicas do DNA foram amplificadas através da Reação em Cadeia da Polimerase (PCR) utilizando primers como ITS1 e ITS4 para ITS rDNA, Bt2a e Bt2b para tubulina (TUB2), CMD5 e CMD6 para calmodulina (CAL), EF-728F e EF-986R, 983 e 2218 para fator de alongamento da tradução (TEF1) e RPB2-5f2/RPB2-7cR para subunidade da RNA polimerase II (RPB2), a depender dos gêneros conforme a indicação da literatura, e as programações do termociclador conforme BEZERRA et al. (2017). Os produtos de PCR foram analisados através de eletroforese em gel de agarose a 1%, purificados com as enzimas EXO+SAP (Alkaline Phosphatase/ Exonuclease l) (Celco, Brasil) conforme as orientações do fabricante e encaminhados para sequenciamento na Plataforma Multiusuária de Sequenciamento de DNA do Centro de Biociências da UFPE, utilizando os mesmos primers da amplificação por PCR.

Para a análise filogenética, as sequências de DNA foram editadas e comparadas com outras sequências do banco de dados do GenBank/ NCBI. As análises filogenéticas foram realizadas utilizando as sequências obtidas neste estudo juntamente com as sequências de referência obtidas na base de dados GenBank, seguindo os tratamentos taxonômicos de cada gênero. As sequências foram alinhadas com as obtidas neste estudo usando a ferramenta online MAFFT v.7, e os alinhamentos foram editados manualmente no MEGA v.7. As relações filogenéticas foram analisadas por meio de uma árvore de verossimilhança (ML), utilizando o programa RAxML-HPC BlackBox (8.2.12) (STAMATAKIS, 2014) na plataforma CIPRES Science Gateway (MILLER et al. 2010), utilizando configurações pré estabelecidas. As árvores geradas foram visualizadas no software FigTree (RAMBAUT, 2009) e utilizadas para a determinação das espécies dos isolados (dados não mostrados).

## 3. Resultados

A abundância de fungos anemófilos nas cavernas estudadas foi determinada pelo número total de Unidades Formadoras de Colônias (UFC) em cada ponto de coleta. Ao todo, foram contabilizadas 787 UFC nas duas cavernas, sendo a caverna Boqueirão de Lavras a que apresentou maior número de UFC (435) em comparação à caverna Urubu. Após as análises, foram identificados 26 fungos na caverna Boqueirão de Lavras e 16 fungos na caverna Urubu, totalizando 42 isolados, todos pertencentes ao filo Ascomycota. Os isolados foram agrupados em 10 gêneros: *Aspergillus*, *Cercospora*, *Cladosporium*, *Curvularia*, *Diaporthe*, *Engyodontium*, *Fusarium*, *Penicillium*, *Phoma* e *Trichoderma*. Os gêneros Aspergillus e Cladosporium foram os mais frequentes, com quatro espécies cada, seguidos por *Penicillium* com três espécies. Os demais gêneros foram representados por uma espécie cada. No total, foram obtidas 17 espécies diferentes, refletindo a riqueza de fungos anemófilos das cavernas estudadas (Figura 1).



| **Espécies de fungos** | **Cavernas** | |
|---|---|---|
| | **Boqueirão de Lavras** | **Urubu** |
| **Ascomycota** | | |
| *Aspergillus clavatonanicus* | + | - |
| *Aspergillus niger* | + | - |
| *Aspergillus bertholletiae* | - | + |
| *Aspergillus subramanianii* | + | - |
| *Cercospora* sp. | + | - |
| *Cladosporium coloradense* | - | + |
| *Cladosporium* sp. | + | - |
| *Cladosporium tenuissimum* | + | + |
| *Curvularia* sp. | + | - |
| *Diaporthe* sp. | + | - |
| *Engyodontium* sp. | - | + |
| *Fusarium* sp. | + | - |
| *Penicillium citrinum* | + | - |
| *Penicillium hispanicum* | + | - |
| *Penicillium sclerotiorum* | - | + |
| *Phoma* sp. | + | - |
| *Trichoderma longibrachiatum* | + | - |

***Figura 1:*** *Riqueza de espécies fúngicas no ar das cavernas Boqueirão de Lavras/CE e Urubu/RN [+ Presença; - Ausência].*

## 4. Discussão

Este estudo realizou um levantamento de fungos anemófilos em duas bat caves do Nordeste brasileiro. A avaliação da riqueza fúngica em ambientes cavernícolas é essencial, tanto para garantir a segurança biológica do turismo quanto para colaborar com a estimativa global de fungos. Observou-se alta abundância de fungos no ar das cavernas, com 435 UFC na caverna Boqueirão de Lavras e 352 UFC na caverna Urubu, resultados consistentes com estudos anteriores em outras cavernas do Nordeste CUNHA et al. (2020) registraram 673 UFC de uma bat cave no Parque Nacional do Catimbau/PE, e ALVES et al. (2022) obtiveram 526 UFC em uma no Parque Nacional da Furna Feia/RN, evidenciando a alta abundância de fungos no ar em cavernas e bat caves do Nordeste. As espécies de fungos identificadas neste estudo pertencem ao filo Ascomycota, corroborando com outros estudos em cavernas, que indicam esse filo como o mais representativo nos isolamentos fúngicos (VANDERWOLF et al. 2013). Os gêneros *Aspergillus* e *Cladosporium* foram os mais frequentes, juntamente com o *Penicillium*, resultados também observados em cavernas do Nordeste (CUNHA et al. 2020; ALVES et al. 2022; LIMA et al. 2024). A presença dominante desses gêneros pode ser justificada pela grande produção de esporos e a rápida disseminação desses propágulos pelo ar (BIAGIOLI et al. 2023). Os demais gêneros encontrados neste estudo já foram registrados anteriormente em cavernas no Brasil e no mundo (VANDERWOLF et al. 2013). A composição fúngica nas cavernas pode ser influenciada pelo ambiente externo, principalmente na zona de entrada, onde há maior comunicação com o exterior (SÁNCHEZ-MORAL et al. 2021). De forma geral, os estudos sobre os fungos em cavernas tratam esses organismos como originados do ambiente externo (ZHANG et al. 2018). Os fungos podem ser introduzidos na caverna por animais, correntes de ar, água, folhas e até mesmo os visitantes e pesquisadores, que transportam propágulos fúngicos que se incorporam à biota fúngica local (ZHELYAZKOVA et al. 2020). Os morcegos também atuam como agentes de dispersão fúngica, e um estudo realizado na Polônia mostrou que a quantidade de fungos foi maior quando havia uma alta concentração de morcegos na caverna, sugerindo que esses animais são determinantes na dispersão de fungos anemófilos em locais de hibernação (KOKUREWICZ et al. 2016).

## 5. Conclusão

As *bat caves* Boqueirão de Lavras e Urubu apresentaram uma notável riqueza de fungos anemófilos. A maioria das espécies encontradas no ar dessas cavernas já foi registrada em outras cavernas do Brasil e do mundo. Embora a amostragem tenha sido limitada a um único período de coleta, os resultados indicaram uma taxa significativa de fungos de espécies distintas, sugerindo que as *bat caves* abrigam uma diversidade de fungos em suspensão no ar. Alguns isolados requerem análises filogenéticas adicionais, com foco em regiões específicas do DNA, para confirmação das espécies e uma avaliação mais precisa da riqueza fúngica. Outros estudos estão em andamento nas cavernas, envolvendo a análise de substratos como guano/sedimento e corpo de morcegos, com o objetivo de entender melhor a comunidade fúngica local. Este estudo contribui para estimativas nacionais e globais de fungos, fornece dados micológicos para futuros planos de manejo das cavernas estudadas e, por consequência, reforça a necessidade de conservação das cavernas, especialmente da região da Caatinga.

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# Adaptability and subterranean plasticity of *Bokermannohyla martinsi* (Anura: Hylidae).

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## Abstract

*Bokermannohyla martinsi* utilizes iron caves as shelter, especially during the dry season (autumn and winter), when external climatic conditions become adverse. During the rainy season, they seek external habitats for reproduction. In locations with suitable environmental conditions, the species exhibits continuous activity and reproduction throughout the year. Here, we investigate the ecological plasticity of *B. martinsi* in caves with perennial water bodies, examining whether it is active (including reproductive activity) year-round in these caves. We also list the environmental factors that, along with morphological, physiological, and behavioral adaptations, facilitate the colonization of caves by the species. *Bokermannohyla martinsi* is endemic to the Iron Quadrangle (Quadrilátero Ferrífero) region (Minas Gerais, Brazil), has a saxicolous habit, and occurs restrictively in rocky streams and creeks at high altitudes. The loss of quality and extent of its habitat is the primary threat faced by this vulnerable species. This study reinforces the importance of caves as habitats for *B. martinsi*, particularly due to mining, highlighting the need for the conservation of caves in Iron Quadrangle as well as the adjacent areas.

## 1. Introduction

Most amphibians exhibit an aquatic larval phase and a terrestrial adult phase (WELLS, 2007). Adult anurans, in addition to pulmonary respiration, also breathe through the skin (WELLS, 2007). Thus, maintenance of skin moisture is important for adequate gas exchange (WEBER, 2004; WELLS, 2007). Consequently, they are highly dependent on humid environments, being found in areas of high humidity or near water sites (WELLS, 2007). As ectothermic animals, the body temperature of anurans varies with ambient temperature, making them susceptible to changes in temperature and humidity in the environment (WEBER, 2004; WELLS, 2007). Most caves exhibit high humidity and mild temperatures (WEBER, 2004), and are also characterized by environmental stability, with little daily and annual variation in temperature and humidity (FERREIRA, 2005; ANDRADE et al., 2021). Thus, various species of anurans utilize natural cavities to shelter from unfavorable environmental conditions in the surrounding epigean environment, avoiding adverse temperatures and desiccation (WEBER, 2004).

Many anurans use caves to temporarily shelter from climatic adversities (WEBER, 2004). Species that return to the surface to complete their life cycle, such as for feeding or reproduction, can be classified as trogloxenes (SKET, 2008). However, when environmental resources are available, caves are also used as foraging and breeding sites by some species of anurans (WEBER, 2004), allowing them to complete their entire life cycle within the caves. In this case, some species may maintain permanent subterranean populations, being classified as eutroglophiles (SKET, 2008).

In this context, a study conducted in Serra do Gandarela National Park (SGNP) showed that *Bokermannohyla martinsi* utilizes iron caves year-round as shelter (ANDRADE et al., 2021). Increased usage was recorded during the dry and cold period (autumn and winter), when external climatic conditions become adverse (ANDRADE et al., 2021). In the upper part of the SGNP, where these caves are located, most streams are temporary and remain mostly dry during the dry season (ANDRADE et al., 2021). Thus, during this period, the species takes advantage of caves for shelter, as these have high humidity and are more environmentally stable (ANDRADE et al., 2021). Most iron caves in SGNP do not contain watercourses inside due to their altimetric positioning in the landscape, above 1,500 meters, being close to the top of the relief (PILÓ et al., 2015). During the rainy season, when temporary streams have water, most individuals leave the caves to reproduce in these streams (ANDRADE et al., 2021).

Ecological plasticity is the ability of organisms to respond differently to various environmental stimuli even with the same genetic background (NOVOPLANSKY, 2002). *Bokermannohyla martin*si is a territorial species that is normally active year-round in mountainous streams (MAGALHÃES et al., 2018). In the Santuário do Caraça Private Natural Heritage Reserve, another site within its distribution, *B. martinsi* tadpoles have been recorded throughout the year (AFONSO & ETEROVICK, 2007), indicating that the species has continuous reproduction in locations with suitable environmental conditions. At the Santuário do Caraça and the Itacolomi State Park, both tadpoles and adults of *B. martinsi* have been recorded in quartzitic caves with perennial water bodies (ANDRADE et al., 2021; 2023). Therefore, we aim to investigate the ecological plasticity of *Bokermannohyla martinsi* regarding the use of caves under different environmental conditions. Our hypothesis is that the species exhibits activity, including reproductive activity, throughout the year in caves with perennial water bodies. We also discuss whether morphological, physiological, and behavioral adaptations influence cave occupancy by the species.

## 2. Materials and Methods

We visited twenty caves distributed across two conservation units within the Iron Quadrangle (IQ), namely: (1) Serra do Gandarela National Park (SGNP) (20°06'03"S, 43°39'50"W), located in the central-northeastern region of the IQ; and (2) Itacolomi State Park (ISP) (20°26'32"S, 43°27'46"W), located in the southeastern region of the IQ (Fig. 1). The IQ is a geologically distinct area located in central-southeastern region of the state of Minas Gerais, Brazil, comprising iron-rich and quartzitic mountains and covering an area of approximately 7,000 km² (AZEVEDO et al. 2012).

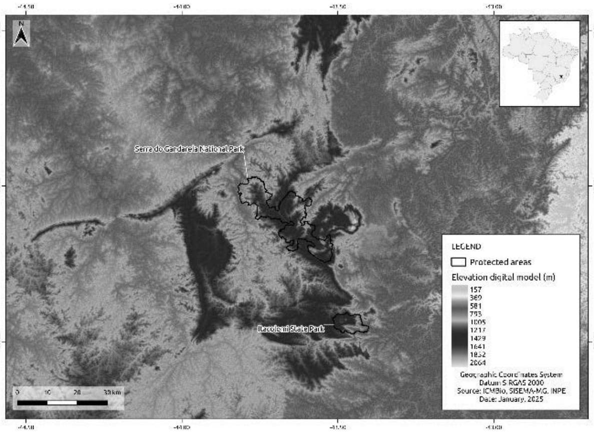

***Figure 1:** Altimetric map showing the region of the Iron Quadrangle and the conservation units studied: Serra do Gandarela National Park (SGNP) and Itacolomi State Park (ISP). Author: Tiago Castro Silva.*

The area studied in the ISP is characterized by being drained by groundwater that flows between blocks, within caves, at the bottom of dolines, and in canyons (Fig. 2A and 2B) (GLÖCKNER, 1981). In contrast, the iron caves investigated in SGNP do not exhibit watercourses within them due to their altimetric positioning in landscape, as previously reported (Fig. 2C and 2D).

Each cave was visited four times between October 2022 and September 2023, keeping a minimum interval of two months between data collections. The sampling was undertaken within each of four three-month periods that we considered as early wet season (Spring: October–December), late wet season (Summer: January–March), early dry season (Autumn: April–June), and late dry season (Winter: July–September).

The search for anurans (adults and tadpoles) and reproductive activities (e.g., vocalization, amplexus, spawning) was carried out during daylight hours by two researchers, using flashlights, through active searching without time constraints, based on vocalization and visual observation. It is important to note that we did not employ any form of marking for individual identification of specimens. Thus, a specimen may have been recorded in more than one season.

We recorded the activity level of each adult anuran at the time of its location as (1) inactive: resting with eyes closed, unresponsive to the flashlight, body on the surface and limbs curled, with hands and feet beneath the body (sensu ANDRADE et al., 2021); (2) active: awake with limbs extended and responsive to the flashlight.

The light incidence at the location of the adult was classified into three categories: (1) photic: near the entrance, with direct sunlight incidence at some point during the day and the presence of photosynthesizing organisms; (2) dysphotic (twilight): with indirect light incidence (without direct sunlight); and (3) aphotic: without light incidence (adapted from SOARES et al., 2013). We classified the substrates of the microhabitat where the adults were found as: (1) rock, (2) soil, and (3) sand. To investigate the lithology of the caves, we utilized the National Register of Speleological Information (Canie) from the National Centre for Research and Conservation of Caves (ICMBio/Cecav) and bibliographic surveys, with subsequent validation of the information during field activities. The bodies of water present within the caves were classified as perennial or intermittent, as well as lentic or lotic.

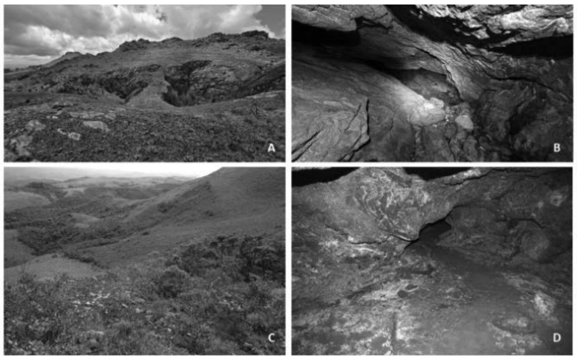

***Figure 2:** (A) External environment in the Itacolomi State Park (ISP), showing the sinkholes where caves and underground watercourses are located; (B) Quartzite cave in the ISP, showing a lotic water body; (C) External environment in Serra do Gandarela National Park (SGNP), showing the ferruginous rock field (canga); (D) Ferruginous cave in SGNP. Author: Maurício Andrade.*

## 3. Results

We obtained 419 records (111 records of adults and 308 in the larval stage – tadpoles) in 16 (80%) of the 20 sampled caves, comprising nine caves in the ISP and seven in the SGNP. From these records, 75 (67.5%) were obtained in the SGPN and 36 (32.5%) in the ISP. Adults were observed in all seasons within the caves of both conservation units. The seasons with the highest number of adult records were autumn in the SGNP (n=24) and spring and summer in the ISP, both with ten records. Conversely, the seasons with the lowest number of adult records were summer in SGNP (n=15) and autumn and winter in ISP (n=8) (Fig. 3 and 4A). In caves of the ISP, seven adults were recorded vocalizing in spring, three in summer, one in autumn, and one in winter. We did not record any individual vocalizing in caves of the SGNP.

Tadpoles were recorded only in caves of the ISP and in all seasons of the year. Winter and summer were the seasons with the highest number of tadpole records (n=94 and n=93, respectively), while spring had the lowest number of records (n=43) (Fig. 3, 4B, and 4C). The tadpoles exhibited different sizes, suggesting multiple breeding events. No amplexus, spawning, or recently metamorphosed froglets were recorded within the caves.

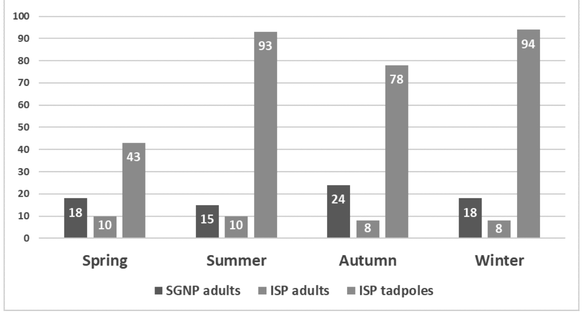

***Figure 3:** Number of adults and tadpoles of Bokermannohyla martinsi recorded per season in caves of Serra do Gandarela National Park (SGNP) and Itacolomi State Park (ISP).*

Most adults (n=100; 90%) were found in the dysphotic zone of the caves. Ten individuals (9%) were found in the aphotic zone, and only one individual (1%) was recorded in the photic zone. In SGNP, 97% of the adults were found in the dysphotic zone and 3% in the aphotic zone. In ISP, 75% of adults were recorded in the dysphotic zone, 22% in aphotic zone, and 3% in photic zone (Fig. 5).

In SGNP, 98.5% of individuals were recorded on rocks and 1.5% on soil. In ISP, 69.5% were recorded on rocks, 8.5% on sand, and 2.5% on soil. In this conservation unit, we recorded seven (19.5%) individuals only acoustically. It was not possible to visualize the substrates due to unfeasible access to the places where they were positioned (Fig. 6).

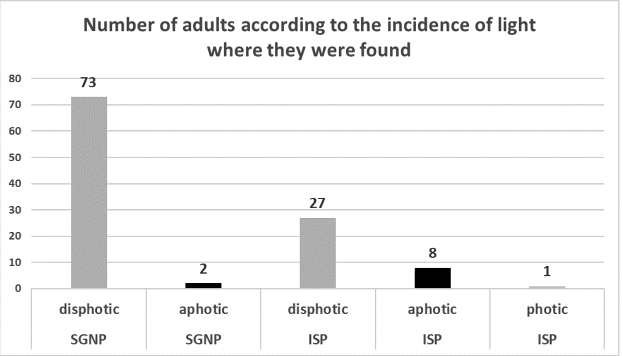

***Figure 5:*** *Number of adults of Bokermannohyla martinsi found in caves of Serra do Gandarela National Park (SGNP) and Itacolimi State Park (ISP) according to the incidence of light in the location where they were found.*

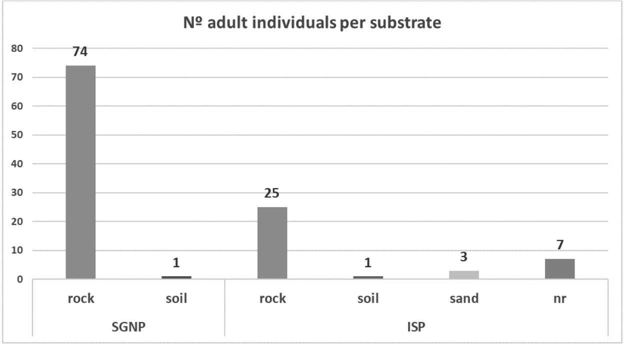

***Figure 6:*** *Number of adults of Bokermannohyla martinsi found in caves of Serra do Gandarela National Park (SGNP) and Itacolimi State Park (ISP) according to substrate used. Legend: not recorded (nr).*

In SGNP, the majority of adult B. martinsi were inactive during both the rainy (85%) and dry (88%) seasons. In contrast, at ISP, most adults were active during the rainy period (65%) and exhibited both active and inactive states during the dry period (Fig. 7).

Regarding the lithologies of the surveyed caves, all caves at the ISP are composed of quartzitic rocks. At this location, we recorded bodies of water in eight caves, all classified as perennial and lotic. At the ISP, nearly all individuals were recorded in caves with watercourses, with only one individual recorded in a cave without any water body. In SGNP, half of the sampled caves are quartzitic, while the other half are composed of ferruginous rocks. At this site, we recorded individuals in four ferruginous caves and three quartzitic caves. However, in quartzitic caves, we recorded only five individuals (6.5%). Thus, ferruginous caves accounted for 93.5% of the adult records in SGNP. Water bodies were recorded in two quartzitic caves in SGNP, classified as intermittent and lentic. Four individuals of B. martinsi were recorded in these caves.

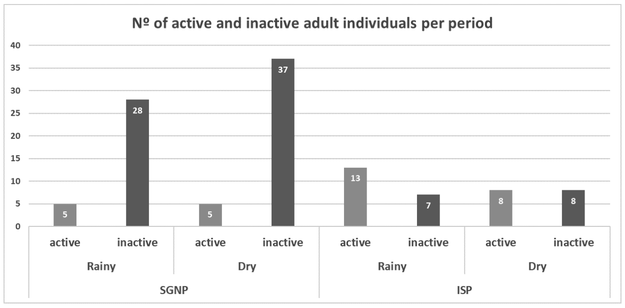

***Figure 7:*** *Activity level of adults of Bokermannohyla martinsi, by dry and rainy season, found in caves of Serra do Gandarela National Park (SGNP) and Itacolimi State Park (ISP).*

## 4. Discussion

*Bokermannohyla martinsi* exhibited continuous use (throughout all seasons) of the ferruginous caves of the SGNP and the quartzitic caves of the ISP, either with or without water bodies. As recorded by ANDRADE et al. (2021), we also observed that the species utilized the ferruginous caves more during the dry period. Therefore, these caves represent a potential refuge for the species when the streams are dry and external climatic conditions become adverse (ANDRADE et al., 2021). The reduced use of these caves during the rainy season is likely related to the more favorable climatic conditions in the epigean environment and the search for streams for reproduction (ANDRADE et al., 2021). During this period, temporary streams retain water, allowing the reproduction and development of tadpoles (ANDRADE et al., 2021).

To avoid water loss during the day, nocturnal anurans typically rest in an "inactive" posture, thereby reducing the exposed surface area (e.g., ETEROVICK et al., 2020). Although we sampled during the day, adults varied in activity patterns, being predominantly inactive throughout the year in the SGPN, whereas in the ISP they were predominantly active in the rainy season and either active or inactive in the dry season. The record of activity in the ISP may be related to the availability of breeding habitats (with evidence of reproduction in these habitats, i.e., records of tadpoles in the bodies of water within the caves).

Fidelity to breeding, feeding, and estivating sites is a common characteristic among anurans (SINSCH, 1990). ANDRADE et al. (2021) demonstrated the fidelity of specimens of *B. martinsi* to the ferruginous caves of SGNP. Due to this fidelity and the territoriality of the species, we believe that specimens likely use the quartzitic caves of the ISP throughout the year.

The reproduction and feeding of anurans in caves are significantly influenced by the presence of water (SPERANDEI et al., 2024). In quartzitic caves of the ISP, which contain perennial and lotic water bodies, vocalizing adults and tadpoles have been recorded in all seasons. In these caves we observed a greater abundance of *B. martinsi* during the rainy period, as also recorded in epigean environments (AFONSO & ETEROVICK, 2007). Six caves surveyed in ISP have watercourses that are not connected upstream with other external water bodies. Thus, the hydromorphology of these caves prevents the transport of tadpoles from outside. In these caves, although we did not record any spawn or couples in amplexus, we did observe tadpoles, females, and calling males, indicating the occurrence of reproduction. Therefore, we can conclude that the species exhibits ecological plasticity, remaining active and reproducing throughout the year in caves that provide the required environmental conditions.

The degree of association of species with the cave environment is intrinsically linked to the combination of morphological, physiological and behavioral adaptations (HOWARTH & MOLDOVAN, 2018). The saxicolous habit, the presence of digital discs, interdigital membranes, and nocturnal activity pattern are eco-physiological characteristics found in some anuran species that utilize caves (BISWAS, 2014) and are also present in *B. martinsi*.

In our study, the species showed a preference for the disphotic zone of the caves, followed by the aphotic zone, as also observed by ANDRADE et al. (2021). Most troglophilic and trogloxenic species, including anurans, are found in twilight (disphotic) zones of caves, characterized by high humidity and mild temperatures, favorable microclimatic conditions (OSEEN & WASSERSUG, 2002; WEBER, 2004; LUNGHI et al., 2018). In these areas, which have lower visibility, anurans are also better protected from predators that rely on visual orientation (OSEEN & WASSERSUG, 2002).

Regarding the microhabitat, most recorded adults were found on rocky substrates, as observed by LIMA et al. (2013) in epigean environment. Records of males vocalizing in rock crevices in streams demonstrate their adaptation and strong association with the rocky environment (SILVEIRA et al., 2019). The presence of rocky substrates in both epigean and hypogean microhabitats, combined with the saxicolous and nocturnal habits of the species, are factors that enable its use of caves and adaptability to the cave environment.

Anurans exhibit a sedentary habit, with migratory behavior limited by the demands of water balance and thermoregulation (SINSCH, 1990). To date, studies conducted in the SGNP have characterized *B. martinsi* as a trogloxene species (ANDRADE et al., 2021). However, given its sedentary habit and a favorable environment for reproduction, thermoregulation, and maintenance of water balance, it is possible that some individuals may complete their entire life cycle in caves and could be classified as eutroglophilic, what remains to be tested.

Although foraging in caves has been reported for anurans (LURÍA-MANZANO & RAMÍREZ-BAUTISTA, 2017), there is currently no data demonstrating that the species feeds on cave-dwelling invertebrates. Nevertheless, it is highly likely that it does so due to the availability of prey in this environment, as noted by FERREIRA (2005).

*Bokermannohyla martinsi* is an endemic species of the Iron Quadrangle (IQ), with an estimated range of occurrence of 3,816 km² (BASTOS et al., 2023), occurring restrictively in rocky streams at high altitudes (SILVEIRA et al., 2019). Thus, given the species' limited range and restricted habitat preferences, caves constitute an environment that the species has colonized, thereby increasing its niche within its small distribution area.

In addition to being endemic, *B. martinsi* is classified as "Vulnerable" (VU) due to its limited range of occurrence (< 20,000 km²) and the ongoing decline in the extent and quality of its habitat (IUCN, 2023). The primary threat to the species is mining, which is responsible for the continuous decline in the quality and extent of its habitat (BASTOS et al., 2023). The IQ is one of the world's major iron mining areas (PINHEIRO et al., 2014), posing an increasing threat to the caves and the species' habitat. Furthermore, wildfires, urban expansion, and agricultural development in the region also contribute to the degradation and fragmentation of its habitat (IUCN, 2023). The species does not adapt well to disturbed environments, and some subpopulations are no longer found, possibly being locally extinct (BASTOS et al., 2023). Therefore, this study corroborates the importance of conserving the areas where the species occurs as a whole, including the caves.

*Bokermannohyla martinsi*, like other anurans, is negatively affected by global warming, which is causing droughts and rising temperatures in its distribution range (IUCN, 2023). However, habitat loss is the most urgent threat faced by this species, affecting approximately 70% of its distribution area (IUCN, 2023), less than 30% of which is protected by conservation units (IUCN, 2023). Thus, we strongly recommend the establishment of fully protected conservation units in IQ to preserve caves, breeding habitats, and populations of this sedentary species. Monitoring populations is also essential for the ongoing assessment of anthropogenic impacts on the species and its risk of extinction.

## 5. Conclusion

Our study provides new insights into the ecology of *Bokermannohyla martinsi* and contributes to the understanding of the use and adaptation of anurans to caves, highlighting the importance of this environment in their life history and generating support for the planning and implementation of conservation actions that take into account caves and their inhabitants.

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# Caves as key habitats for the acoustic detection of brazilian threatened bat species

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## Abstract

*Natalus macrourus* and *Furipterus horrens* are aerial insectivorous bat species considered threatened in Brazil, mainly due to habitat loss and cave exploitation. Both species produce high-frequency calls (over 120 kHz), making them challenging for passive acoustic monitoring, as high call frequency dissipates easily through atmospheric attenuation affecting the detection. We conducted an acoustic experiment testing two different microphones in seven caves of Serra dos Carajás, and we conducted a literature review of acoustic surveys in the species distribution areas. Our results suggest that AudioMoths detects fewer calls of the species than SongMeter recorders in caves. Even though AudioMoth had less detection in caves, it is greater than in open ecosystems, where we found few studies that detected *F. horrens* and one that detected a similar species – *N. tumidirostris*. We reinforce that caves can be considered key habitats for acoustic detection of these species, and bat surveys made in open environments should include passive acoustic monitoring in caves if the area is documented to have caves. Choosing an effective microphone is essential to ensure the presence of these threatened bat species.

## 1. Introduction

Traditional methods (mist nets, harp-trap, and active search) are the commonly used techniques in bat cave monitoring programs, but they are limited by logistics, time, and money (KUNZ et al. 2009). The use of autonomous recorders for the cave bat monitoring has increased over the last decades and it has the advantages of a non-invasive method, with the possibility to record bats over long periods (REVILLA-MARTÍN et al. 2020). Bat cave acoustic monitoring from temperate zones is already established including sampling protocols (REVILLA-MARTÍN et al. 2020). This is not the case for tropical regions that are megadiverse, and where acoustic monitoring is an ongoing challenge limited by a matching diversity in echolocation calls (e.g., YOH et al. 2020) and budget constraints.

Many factors can influence bat acoustic detection in highly diverse tropical caves including microphone quality, sampling rates, filtering configurations, and sampling designs, among others (KUNBERGER et al. 2023). However, one of the main challenges in acoustic ultrasound monitoring is the detection of high-frequency calls. High-frequency calls can attenuate more in the air, thus compromising detection distances (GRIFFIN 1971). This factor introduces bias in species detection, particularly when comparing recordings from different microphone quality (KUNBERGER et al. 2023). Microphone quality is commonly measured by signal-to-noise ratio (SNR) which is in Decibel value that is logarithm of ratio of a standard signal's power to the noise power of the microphone created by its self-noise (DARRAS et al. 2019).

The Amazon harbors one of Earth´s highest diversity of bats, with a minimum of 144 species occurring within the territory pertaining to Brazil, as recently compiled (TAVARES et al. 2024). Eight of nine Neotropical families of bats are species of aerial foragers with specialized echolocation calls, allowing bat researchers to identify species using standard acoustic call parameters (ARIAS-AGUILAR et al. 2018). Particularly, two species (*Furipterus horrens* and *Natalus macrourus*) of these aerial foragers emit high-frequency calls over 120 kHz and can be found in large populations on caves in the Eastern Amazon, in the "Serra dos Carajás" region (TAVARES et al. 2012). *Furipterus horrens* and *N. macrourus* exhibit conspicuous behaviour in caves, where their presence commonly is associated with the abundance of crevices and domes (BARROS & BERNARD 2023), making them difficult to capture with hand nets. They easily detect mist nets and harp traps positioned at cave entrances, further complicating traditional survey methods. The combination of their cave-dwelling behavior and high-frequency calls makes these species particularly challenging to monitor. Both species are classified as "Vulnerable" on the Brazilian Red List of Endangered Species (Portaria MMA Nº 148 2022), highlighting the need for effective monitoring protocols and acoustic detection strategies. The caves of Carajás provide an ideal setting to test different microphone types and configurations for improving the detection of these elusive bat species.

In this context, we compared the number of bat passes of *F. horrens* and *N. macrourus* between two of the most used ultrasound recorders (AudioMoth and SongMeter Bat) in caves with different entrance sizes. With a comprehensive literature review of *F. horrens* and *N. macrourus* acoustic recordings conducted in the distribution area of the species, we investigate the variation of the recording number of *F. horrens* and *N. macrourus* between different recorder types in non-cave areas compared with our study.

## 2. Materials and methods

We conducted our study in the Carajás National Forest (FLONA Carajás). Located within Serra dos Carajás in southeastern Amazonia, Brazil (-6.06 (lat), -50.04 (long)), it comprises a unique mosaic of iron-rich subterranean systems with savanna-like “canga” vegetation and Amazonian rainforests (PILÓ et al. 2015). The caves were distributed in two isolated plateaus of Serra dos Carajás that are known to house caves largely populated with several bat species (TAVARES et al. 2012). We installed pairs of AudioMoths 1.1.0 with onboard microphones (OPEN ACOUSTIC DEVICES, UK) and SM4Bats with SMM-U1 microphones (Wildlife Acoustics, USA) at seven caves between August 2022 and September 2024.

AudioMoth 1.1.0 comes with Knowles MEMS microphones and SM4Bats with SMM-U1 Knowles FG. Both microphones can record up to 192 kHz, and are omnidirectional. Recorders were deployed side by side in the cave walls, approximately fifty centimeters from the main entrance of each cave. The microphones of SM4Bats were always installed facing the interior of the caves and the AudioMoths had the microphones always installed at the top of the equipment. We used entrance height as a proxy for entrance size. The two recorders were programmed to register bat activity in real-time with a 384 kHz sample rate. Microphone gain is the adjustment of the input sensitivity, which amplifies or reduces the strength of recorded sounds, and to test this in call detection of high-frequency species, we made two experiments: in one experiment, both recorders were set in medium gain, and in another, both recorders were configured in high gain. The AudioMoths were programmed to record for five seconds followed by 25 seconds of sleeping, and the SM4Bats were programmed to record for 15 seconds after they were triggered. For our analyses, we only considered the activity captured by the SM4Bats at the same intervals that AudioMoths were programmed to be recording (for example 18:00:00, 18:00:30, 18:01:00). Bat activity was recorded between 17:30 and 06:00 in both recorders. We retrieved a total of 73 recording nights, and 39 nights for AudioMoth and 34 nights for SM4, totaling 4080 recording minutes. We identified the bat calls of *F. horrens* and *N. macrourus* manually using the Kaleidoscope Software (WILDLIFE ACOUSTICS, Inc., USA), and classified them as bat passes when detecting at least two recognizable search-phase calls per species. We used our local library containing calls from tent-released and hand-released bats as references for the identification (Fig. 1). All analyses were conducted in R Studio (RStudio Team, 2023). We used a generalized linear mixed models (GLMM) with the package “glmmTMB” to compare the number of bat passes for each species with the type of recorder (SM4 and AudioMoth), gain setting (medium and high), cave entrance size and interaction between gain and cave entrance.

For the literature review, we searched the Web of Knowledge and Google Scholar for papers and preprints that used bat acoustic inventory or monitoring. We only selected studies made in the distribution of *F. horrens* and *N. macrourus* based WILSON & MITTERMEIER (2019). We selected only studies that the acoustic recorders were programmed to capture frequencies over than 120 kHz, covering the search-phase calls of the species. Indeed, we included only studies that provided a complete list of sonotypes and bat species. A total of 14 papers were included, and in some of these we considered only the detection of *F. horrens* due they are not located in the range distribution of *N. macrourus*. Indeed, as N. macrourus has lower acoustic detection in the studies, we included a survey made in Colombia with a recording of a sister species *Natalus tumidirostris*, that emits calls with a frequency of maximum energy of 122 kHz similar to *N. macrourus* (BARATAUD et al. 2013).

## 3. Results

We found echolocation calls of *F. horrens* in all studied caves and *N. macrourus* echolocation calls in six of seven caves of FLONA Carajás. The acoustic characteristics of the bat species follows partially the parameters described by LÓPEZ-BAUCELLS et al. (2019), they described that *Natalus macrourus* have modulated frequency with final quasi-constant frequency calls with frequency of maximum energy (FME) above then 110 kHz. In our acoustic monitoring, we observed the same basic call form, but the FME was mostly above than 120 kHz (Fig. 1). While the acoustic parameters described by LÓPEZ-BAUCELLS et al. (2019) for *F. horrens* matches totally: extremely frequency modulated calls with FME between 130 and 170 kHz (Fig. 1).

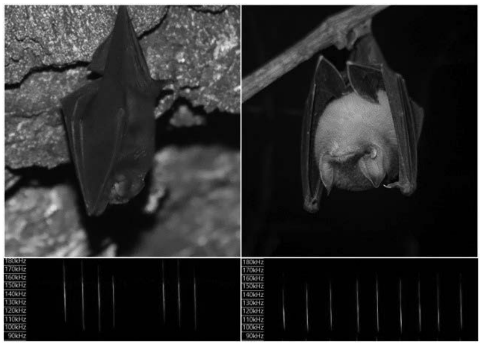

***Figure 1:*** *Thumbless Bat (Furipterus horrens) picture and echolocation calls in the left and Brazilian Funnel-Eared Bat Natalus macrourus picture and echolocation calls on the right. (Photos by Giulliana Appel and Thayse C. Benathar)*

We recorded 892 bat passes in total, considering both recorder types. *Furipterus horrens* had 456 bat passes with higher activity recorded in N1_0168 and S11C_0153 (Fig. 1), corresponding to almost 65% of all recorded bat passes. While, *N. macrourus* had 436 bat passes with higher activity in N4WS_0015 and S11C_0153 representing 86% of the bat passes (Fig. 2). SongMeter SM4 detected 873 bat passes in all studied caves (except for S11B_0094) and AudioMoths recorded only 19 bat passes distributed in three caves (S11B_0094, S11C_0153, and N1_0168) (Fig. 2).

Notably, SongMeters detected significantly more activity than Audiomoth for both high-frequency species (Fig. 3A and B). Entrance size did not affect the activity of the species (Fig. 2A and B), and the gain configuration did not influence the recording bat passes on *F. horrens* activity (Fig. 3A). The gain effect was not analyzed for *N. macrourus* due to an excess of zero values, as no bat species of this species were recorded in the three caves where the gain was set to high on AudioMoths and SongMeters.

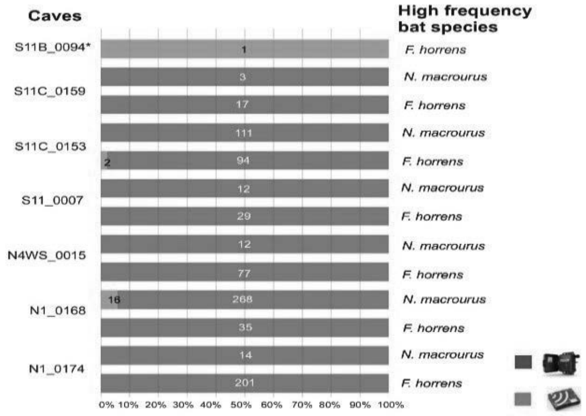

***Figure 2:*** *Raw numbers and percentages relative to total activity per bat species recorded considering the two types of recorders (blue are the SongMeters and orange the AudioMoths) in each studied cave of FLONA Carajás.*

We found a variation in the recorder types used in acoustic inventories through the distribution of the two high-frequency species, included different models of SongMeter (SM3Bat+, SM3 Bat, SM4 Bat+ and SM4 Bat FS), AudioMoth, Batcorder (EcoObs, Inc., Germany) and D1000x Bat Detector (Petterson Elektronic, Inc, Sweden) (Fig. 4). More than half of the studies used SongMeter models (57.1%), followed by AudioMoth (28.5%) and Batcorder and D1000X were both used in only one study (Fig. 4). The predominant mode of acoustic survey was passive and except for one study, the surveys were made in open environments (Fig. 4). Studies that used AudioMoth and Batcorder has not detected the high-frequency species (Fig. 4). The active acoustic survey conducted using D1000X Bat detector recorded a sonotype that included calls from both species. Although it is not possible to confirm which species is, the results demonstrate that this detector can record frequencies above 120 kHz. The Thumbless bat (*F. horrens*) was recorded in only three studies all located in the Amazon biome (Fig. 4). The Brazilian Funnell-Eared Bat (*N. macrourus*) was not detected in any acoustic survey even in localities where are registers of caves (Fig. 4). On the other hand, its close relative *Natalus tumidirostris* was recorded in a study conducted in Colombia using SM4 Bat FS, also in the Amazon, in an area with cave (Fig. 4).

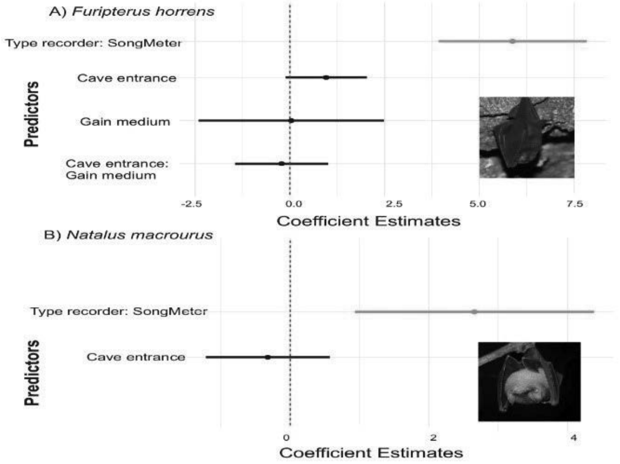

***Figure 3:*** *Effects of recorder type, cave entrance size, gain configuration and their interaction with entrance on activity of F. horrens (A) and N. macrourus (B) in seven caves of FLONA Carajás. Effect estimated are based on the fixed effects, characterized by its mean (dot) and credible intervals (95% CI, lines). Red estimates indicate significant positive effects and black estimates non-significant effects.*

The *F. horrens* activity, relative to the minute's effort in the studies that detected the species, changed from a minimum of 0.0002 to a maximum of 0.002 recordings per minute showing that even using SM2Bat+ the activity recording is low (Fig. 4). For *N. tumidirostris*, the activity relative was calculated based in at least one register as the authors did not mention the exact number of bat passes of this species (0.00009, Fig. 4). The sonotype *Furipterus/Natalus* was the highest activity relative found in the studies (Fig. 4) with 0.009 recordings per minute's effort. The highest activity relative found in the review was 11 times lower than our study for both *N. macrourus* and F. horrens (0.1 recordings per minute's effort, Fig. 4).

| N | Species | Recording per min effort | Method | Recorder model | Where? | Biome | Cave presence in the region? |
|---|---|---|---|---|---|---|---|
| 1 | *F. horrens*<br>*N. macrourus* | 0.0025<br>0 | PA | SM2Bat+ | Open space | AM | N |
| 2 | *F. horrens*<br>*N. macrourus* | 0.0009<br>0 | PA | SM2Bat+ | Open space | AM | N |
| 3 | *F. horrens* | 0 | PA | SM3 Bat | Open space | AM | N |
| 4 | *F. horrens*<br>*N. macrourus* | 0.0002<br>0 | PA | SM2Bat+ | Open space | AM | N |
| 5 | *Furipterus /Natalus* | 0.0099 | AC | D1000x Bat Detector | Open space | AT | N |
| 6 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | Audiomoth | Open space | CA | Y |
| 7 | *F. horrens* | 0 | PA | Audiomoth | Open space | CE | Y |
| 8 | *F. horrens* | 0 | PA | SM2Bat+ and SM4BAT | Open space | CE | Y |
| 9 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | Audiomoth | Open space | AM | N |
| 10 | *F. horrens*<br>*N. tumidirostris* | 0<br>0.00009* | PA | SM4BAT FS | Open space | AM | Y |
| 11 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | SM2Bat+ | Open space | AT | N |
| 12 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | Batcorder | Open space | AT | N |
| 13 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | SM2Bat+ | Open space | CA, CE | Y |
| 14 | *F. horrens*<br>*N. macrourus* | 0<br>0 | PA | Audiomoth | Open space and cave | AM | Y |
| our study | *F. horrens* | 0.1110<br>0.0006 | PA | SM4Bat+<br>Audiomoth | Inside cave | AM | Y |
|  | *N. macrourus* | 0.1000<br>0.003 |  | SM4Bat+<br>Audiomoth |  |  |  |

***Figure 4:*** *Information of the papers found in the literature review. Studies: (1) TORRENT et al. (2018); (2) APPEL et al. (2022); (3) GOMES et al. (2020) ; (4) LÓPEZ-BAUCELLS et al. (2021) ; (5) HEER et al. (2015) ; (6) MERAMO et al. (2022) ; (7) SILVA et al. (2023) ;(8) FALCÃO et al. (2024) ; (9) CARVALHO et al. (2023) ; (10) DÍAZ-B et al. (2023) ; (11) FALCÃO et al. (2021) ; (12) GREGORIN et al. (2022) ; (13) PEREIRA et al. (2022); (14) GOMES & BERNARD (2024). Method : PA = Passive acoustic monitoring/ AC = Active acoustic monitoring. Biome: AM = Amazon; AT = Atlantic Forest; CA = Caatinga; CE = Cerrado/ Savanna. Cave presence: Y = Yes; N = No. * This study not mentioned the exact number of recordings so we estimated based in one recording.*

## 4. Discussion

Our study revealed significant variation in the acoustic detection of high-frequency bat species depending on the type of recorder and the locations of the recordings based on experiments and literature review. The detection of bat calls by autonomous recorders is known to vary according to the biology of the species sampled, such as their foraging behavior, also according to the position of the microphone in relation to the bat recorded, variation in call parameters (e.g., frequency of maximum energy, duration, call structure), as well as extrinsic environmental features such as weather conditions and vegetation/obstacles clutter (REVILLA-MARTÍN et al. 2020). We found that bat passes for F. horrens and N. macrourus were substantially higher with Songmeter recorders compared to AudioMoths. This result followed the prediction that microphones with higher signal-to-noise ratio are more sensitive in record high or low frequency calls (ADAMS et al. 2019). AudioMoth 1.1.0 microphones have 44 dBA of signal-to-noise ratio (1 kHz re Pa), whereas SMM-U1 (microphones of SM4Bat) has 80 dBA (1 kHz re Pa) (OPEN ACOUSTIC DEVICES, 2024; WILDLIFE ACOUSTICS 2024). Our results highlight a compromise between these sources of variability and demonstrate limitations of uttermost importance when the study goals include inventorying and/or monitoring the high-frequency bat species using subterranean ecosystems.

The Thumbless Bat and the Brazilian Funnel-Eared Bat are considered vulnerable in Brazil (Portaria MMA N°148, 2022). The use of low signal-to-noise ratio microphones to record and monitor these species in the Amazonian caves is a matter of great concern because the acoustic monitoring results may reveal false negative detections or less activity than is. Following the crescent wave of inclusion of acoustic data in general Biodiversity surveys (APPEL et al. 2022) there has been an increase in requests made by regulatory agencies for the use of acoustic recording as a complementary method in bat inventories related to Environmental Impact Assessments (EIA) through Brazilian Federal and state laws. These demands have been widely expanded for inventories and monitoring of caves classified as "maximum relevance" according to the Federal Decree Nº6.640 of the Brazilian Environmental Law (November 7, 2008). As we reinforce here the differences in acoustic species detection inside the caves must be considered in the exigences of environmental agencies.

Our study did not find an effect of different microphone gain settings and cave entrance sizes on *F. horrens* and *N. macrourus* calls detection. However, our findings may are limited by the dataset and the low number of replicates. The cave entrances of the studied caves varied slightly, with a minimum of 0.71 meters and maximum of 2.1 meters and an average of 1.15 meters. Serra dos Carajás (Carajás'Ridges) hosts over 1,500 iron caves distributed across the plateaus (PILÓ et al. 2015) and future studies should explore a wider range of cave entrance sizes to determine whether they influence the acoustic detection of high-frequency species. Large cave entrance may the microphone does not efficiently record these high-frequency calls as these bats will not be close enough, even for sensitive microphones. We observed a little tendency for smaller cave entrances combined with medium gain setting to result in fewer detections of *F. horrens* calls. Although, we tested high-gain settings in only three caves over three recording nights, so it is necessary additional sampling nights to ensure statistically reliable comparisons of microphone gain settings, in different recorder types.

Our literature review of studies across the species' distribution confirmed that SongMeter models had a higher detection rate per minute effort of *F. horrens* compared to AudioMoths and Batcorder. However, we could not confirm this pattern for *N. macrourus*, as we found one survey that recorded *N. tumidirostris*, a related species. This detection occurred in an open ecosystem with caves in the area, suggesting that caves likely increased the possibility of recording *Natalus*, as this genus is considered primarily cave-dwelling (BARROS & BERNARD 2023). None of the studies that recorded *F. horrens* calls had presence of caves (TORRENT et al. 2019; LÓPEZ-BAUCELLS et al. 2021; APPEL et al. 2022) and they did not record any *N. macrourus* calls, despite the extensive sampling effort in the area (LÓPEZ-BAUCELLS et al. 2021).

Using SongMeter recorders, we recorded eleven times higher detections of *F. horrens* and *N. macrourus* calls per minute effort inside the caves than the detections of the studies made in open ecosystems. Caves can be key habitats for ensuring the acoustic presence of these high-frequency species, and the use of sensitive microphones is essential for accurately detecting these species. Bat acoustic surveys made in preserved open environments should include passive or active acoustic monitoring in caves if the area is documented to have caves, increasing the probability of detection these elusive species.

It is important to mention that regardless of the microphone employed, all microphones provide an approximate snapshot of the calls that occurred in the environment (KUNBERGER et al. 2023). As hotspots of cave-bat diversity are primarily concentrated in tropical, developing, and low-income countries, and with fewer resources for monitoring cave-bat population dynamics, we suggest using AudioMoths to record the medium-frequency call species and combining it with other methods such as harp traps, mist nets, and active searching inside the caves. Another alternative to recording is the active acoustic search using USB ultrasonic microphones, such as Echo Meter Touch (WILDLIFE ACOUSTICS, Inc., USA) and Dodotronic microphones. In our surveys inside the Carajás caves, we usually used the Echo Meter Touch 2 PRO microphone coupled with a smartphone to register *Furipterus horrens* presence. In fact, a study by DARRAS et al. (2019) recommends designing connections for independent and high-performance microphones that properly adapt to the ranges of frequencies.

## 5. Conclusion

The growing integration of acoustic data in biodiversity assessments and cave conservation policies in Brazil, underscores the need for standardized and effective monitoring protocols, especially for threatened species. SongMeter recorders consistently outperformed AudioMoths in detecting *F. horrens* and *N. macrourus*, as shown by our comparison experiment in the caves and by the literature review related to acoustic monitoring across the species' distribution area. The recording detection per minute effort of *F. horrens* and *N. macrourus* is substantially higher when made in the caves compared to open environments across the Brazilian Biomes (Fig. 3), so we emphasize that caves are key habitats to ensure the species' presence in the area. Ultimately, our study showed that the choice of acoustic monitoring equipment must align with the study objectives and target species. To improve bat conservation efforts, particularly in tropical regions where financial resources for monitoring are often scarce, we recommend combining multiple survey techniques to maximize species detection and ensure accurate assessments of bat populations in caves and surrounding habitat.

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# Aumento da biodiversidade de invertebrados após a remoção de material particulado em cavidades subterrâneas naturais

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## Resumo

O processo de extração de calcário do ambiente natural pode gerar diversos tipos de impactos, incluindo a liberação de material particulado no ar em quantidades superiores às originalmente observadas. Esse material, pode se depositar em cavernas e alterar o ecossistema hipógeo. Neste estudo, apresentamos os resultados obtidos da resposta da comunidade de invertebrados cavernícolas após a remoção de material particulado acumulado em seis cavernas localizadas no município de Matozinhos, Minas Gerais. Os dados observados uma semana após a remoção do MP mostram que houve um aumento na riqueza de espécies, na diversidade e na equitatividade da comunidade de invertebrados, em comparação ao período anterior à limpeza. Contudo, o número total de indivíduos apresentou uma redução no período pós-limpeza. A composição de espécies, por outro lado, manteve-se inalterada entre os períodos pré e pós-limpeza. Dessa forma, os resultados indicam que as atividades de remoção de material particulado podem proporcionar benefícios à fauna cavernícola, ao menos em curto prazo.

## Abstract

(Increase of invertebrate biodiversity after the removal of particulate matter from caves). Limestone extraction can generate several types of impacts, including the emission of large amounts of particulate matter (PM) into the air. This PM can deposit inside caves and change the subterranean ecosystem. In this study, we investigate the response of subterranean invertebrate communities after the removal of particulate matter from six caves located at Matozinhos municipality, Minas Gerais state. Our results shows that one week after the removal of PM there was an increase in invertebrate's species richness, diversity and evenness when compared to the period before cleaning. However, total number of individuals decreased after cleaning. On the other hand, species composition did not change after PM removal. In this way, our results indicate that PM removal can improve subterranean fauna, at least, in short term.

## 1. Introdução

A restauração ecológica tem como objetivo iniciar ou acelerar a recuperação de ecossistemas que foram degradados, danificados ou destruídos (SER, 2004). A sua aplicação tem sido, na maioria das vezes, eficaz em ecossistemas epígeos, melhorando as condições ambientais em relação ao estado degradado (REY BENAYAS et al., 2009; SUDING, 2011). Por exemplo, vários estudos têm relatado um aumento nos parâmetros de biodiversidade, serviços ecossistêmicos e até mesmo socioeconômicos após a restauração (e.g. REY BENAYAS et al., 2009 ; CROUZEILLES et al., 2016).

A restauração ecológica tem sido cada vez mais aplicada nos ecossistemas epígeos (ARONSON & ALEXANDER, 2013). E, apesar da sua rápida consolidação e crescimento científico (SUDING, 2011), a escassez de informações é uma realidade (MONTOYA et al., 2012). Essa carência de informações é ainda maior quando se trata da restauração de ecossistemas subterrâneos. A restauração de cavidades naturais subterrâneas é relativamente nova e sua prática não tem sido tão comum como nos demais ecossistemas. A maioria das publicações sobre o assunto são internacionais e baseadas em estudos de caso.

Os projetos de restauração em cavernas têm sido implementados em diversos países, principalmente nos Estados Unidos, usando uma variedade de técnicas para reparar os danos diretos e indiretos causados pela ação humana (HILDRETH-WERKER & WERKER, 2006). O foco principal destes projetos tem sido a recuperação de cavernas turísticas, a fim de resgatar a sua estética, proteger seus valores histórico-culturais e/ou reduzir os impactos sobre a fauna subterrânea (HILDRETH-WERKER & WERKER, 2006). Dentre as principais práticas de restauração executadas em cavidades naturais subterrâneas no mundo estão: remoção de pichações, recuperação de espeleotemas, remoção de resíduos sólidos, remoção de organismos fotossintetizantes, que se desenvolvem devido a introdução de luz artificial, remoção de poeira e restos orgânicos advindos de atividades de uso público, remoção de preenchimentos artificiais e remoção de pegadas e marcas sobre feições frágeis (HILDRETH-WERKER & WERKER, 2006). Embora seja uma prática ainda pouco difundida no Brasil, alguns projetos de restauração de cavernas já foram realizados, sendo quase todos eles em cavernas turísticas (ALT & MOURA, 2023).

Atualmente, a restauração de cavernas tem tomado novos rumos no Brasil, e vem sendo aplicada em ambientes subterrâneos vedados ao uso público, a fim de mitigar os impactos causados pelas atividades humanas (e.g. atividades de mineração) (SOUZA et al., 2022). Nestes casos, o objetivo não é tão voltado para a melhoria da beleza cênica, mas sim uma tentativa de deixar estes sistemas o mais similar possível a sua condição natural original (e.g. melhorar as condições ambientais do ecossistema como um todo) (SOUZA et al., 2022). Alguns projetos de licenciamento ambiental têm, por exemplo, focado na limpeza de material particulado (e.g. poeira) oriundo de empreendimentos minerários, e que se acumulou nas cavidades ao longo dos anos. Apesar de não existirem estudos que comprovem os efeitos negativos do material particulado sobre o ecossistema cavernícola, acredita-se que este pode causar alguns impactos ambientais devido a introdução de poluentes como metais pesados e óxidos. Como impactos podemos citar : impactos negativos sob a fauna, microclima, espeleogênese e mudanças químicas e estruturais em espeleotemas e superfícies rochosas. Sendo assim, a remoção do material particulado pode representar uma importante ferramenta para reverter os danos de ecossistemas hipógeos (ALT & MOURA, 2020). E, avanços nesta área do conhecimento são essenciais a fim de tornar a restauração uma prática comum nos esforços de conservação de ambientes subterrâneos.

Apesar da redução dos impactos sobre a fauna estar entre os objetivos dos projetos de restauração em cavernas do mundo todo, até o momento, apenas dois estudos avaliaram se estas práticas são realmente eficazes para a recuperação da fauna (SOUZA et al., 2002; MANENTI et al. 2019). Nesse sentido, ainda faltam pesquisas abrangentes que investiguem a eficácia da restauração em cavidades subterrâneas. Conhecer o sucesso da restauração é extremamente importante, pois traz informações sobre os benefícios desta estratégia, assim como de suas limitações.

Sendo assim, o objetivo do presente estudo é avaliar a resposta da comunidade de invertebrados cavernícolas após a limpeza de material particulado em seis cavernas inseridas em rochas calcárias. O material particulado foi sendo depositado nestas cavidades ao longo de décadas em decorrência das atividades de mineração. Especificamente pretende-se avaliar : 1) houve um aumento na riqueza de espécies, número de indivíduos, diversidade de espécies e equitatividade após a limpeza das cavidades ? 2) houve mudanças na composição de espécies após a intervenção da limpeza ?

## 2. Materiais e Métodos

**Área de estudo**

A área do estudo está localizada no município de Matozinhos, na porção norte da região Metropolitana de Belo Horizonte, Minas Gerais. As cavidades alvo do projeto de restauração estão inseridas na área do empreendimento minerário da Mineração Belocal Ltda.

**Cavernas alvo da restauração e seus impactos**

Estudos espeleológicos implementados pela empresa Spelayon Consultoria Ambiental detectaram a presença de material particulado em cavidades localizadas na área do empreendimento. Sendo assim, foi proposto um Plano Executivo de Recuperação Ambiental, com a finalidade de implementar medidas de mitigação de impactos, assim como técnicas de recuperação ambiental (e.g. limpeza do material particulado), com posterior monitoramento, para avaliar a eficácia das estratégias de recuperação utilizadas. O objetivo deste plano de recuperação é promover uma melhoria das condições ambientais dos ecossistemas cavernícolas (e.g. fauna, recursos tróficos e ambiente físico) através, principalmente, da limpeza do material particulado. Nesse sentido, foram selecionadas seis cavidades piloto (BM-36: UTM E 595271/ UTM N 7840083; BM-50: 595220/ 7840266; BM-104: 595032/7840324; BM-108: 595015/ 7840405; BM-116: 595017/ 7840488; BM-120: 595325/ 7840458), a fim de testar a eficácia das técnicas de limpeza.

**Treinamento téorico-prático da equipe**

A equipe técnica responsável pela limpeza das cavernas, constituída por quatro espeleólogos com formação nas áreas de engenharia ambiental, geologia, biologia e arqueologia, passou por um treinamento prévio teórico-prático sobre as técnicas de limpeza de material particulado que tem sido aplicadas no mundo inteiro. Este treinamento foi ministrado pelos pesquisadores eslovenos Dra. Rosana Cerkevnik e Sr. Borut Petric, gestores do Parque Cavernas de Škocjan (Eslovênia).

**Diagnóstico, planejamento e inventário**

Antes da aplicação das técnicas de limpeza, as cavernas foram setorizadas no mapa topográfico com o objetivo de facilitar a logística e organização do processo. Nesse sentido, foi realizado um mapeamento e diagnóstico de seus impactos ambientais, para organizar e facilitar a tomada de decisões e planejamento das ações de restauração, embasando as atividades de limpeza. Nesta etapa foram também identificadas as áreas de maior fragilidade dos sistemas (superfícies de baixa, média e alta fragilidade, assim como recursos orgânicos/organismos fotossintetizantes recobertos por material particulado), que exigiram cuidados específicos.

Uma semana antes das cavernas passarem pela intervenção da limpeza foi realizada amostragens da comunidade de invertebrados cavernícolas e um registro fotográfico inicial com a finalidade de documentar a situação das cavernas antes do processo de recuperação ambiental. Tanto a amostragem de fauna como os registros fotográficos continuarão sendo realizados para avaliar a eficácia dos esforços de restauração.

**Limpeza das cavidades**

A limpeza das cavernas foi feita seguindo as diretrizes propostas por HILDRETH-WERKER & WERKER (2006). Já que todo o processo de limpeza deve ser pensado de forma a não ocasionar mais distúrbios para a estrutura do ambiente e fauna, antes de começar as atividades de limpeza, foram sinalizadas nas cavidades elementos frágeis a ação mecânica (e.g. espeleotemas delicados, objetos com potencial valor histórico, materiais orgânicos), com o objetivo de proteger o patrimônio espeleológico e evitar danos ao ambiente. Sempre que possível (quando o tamanho da cavidade permitiu), foram estabelecidas trilhas para o deslocamento da equipe de campo com fitas de sinalização, com a finalidade de minimizar o pisoteamento.

Antes de qualquer ponto da cavidade ser limpo, foi efetuada a busca ativa de organismos da fauna subterrânea pelo bioespelólogo. Isso foi feito, pois invertebrados podem ser encontrados na camada de sujidades acumulada ou em substratos naturais existentes nestes locais. Os organismos encontrados foram afugentados ou translocados para outro setor em que a limpeza não estivesse acontecendo. A translocação ocorreu de preferência para setores que já haviam sido limpos. O manuseio dos invertebrados se deu através da utilização de pinças, pincéis ou sugadores entomológicos, e a translocação dos exemplares realizada com a acomodação dos espécimes em recipientes plásticos, com tampa, de forma temporária, enquanto eram levados de um setor a outro. Ressalta-se que locais em que não foi possível realizar a remoção prévia da fauna, não passaram pelo processo de limpeza. Ressalta-se que locais em que não foi possível realizar a remoção prévia da fauna, não passaram pelo processo de limpeza.

A limpeza das cavidades foi realizada utilizando uma combinação de estratégias, e a escolha destas estratégias foi feita de acordo com a realidade de cada setor das cavernas. Um dos métodos utilizados foi a limpeza mecânica a seco, através de uma escova com cerdas macias de poliéster, e uma pá para coleta do material particulado. A limpeza a seco foi feita também em locais com espeleotemas e superfícies mais frágeis, utilizando, nestes casos, pincéis de ponta fina e cerdas macias. Em locais onde existia alta concentração de material particulado foi realizada a limpeza por aspiração. Nesse caso, a limpeza a seco, com uso de escovas e pincéis, não é recomendada e nem eficaz, pois causa a mobilização do material particulado, deixando-o em suspensão na cavidade. A limpeza por aspiração foi realizada através de aspiradores de mão convencionais. O material particulado removido tanto pela limpeza a seco, como pelo aspirador, foi condicionado em sacos plásticos e/ou bombonas e descartado em locais apropriados para este tipo de resíduo. A limpeza mecânica úmida foi realizada sempre que os pesquisadores julgaram que a limpeza a seco não gerou resultados satisfatórios. Ela foi efetuada com a utilização de pulverizadores manuais contendo água local, extraída através de poços artesianos, e que não passa por etapas químicas de tratamento, como adição de floculantes ou cloro. Para auxiliar nesse processo, a escova de cerdas macias também foi utilizada. Para evitar que a água atingisse outros locais da cavidade, foi utilizada uma barreira de contenção (e.g. auxílio de tecido ou esponja ou lonas que sirva para barrar o escoamento da água. Em todos os casos foi feita a coleta do efluente em baldes para destinação final. A limpeza das 06 (seis) cavidades aconteceu entre maio/2022 à novembro de 2022.

### Amostragem da comunidade de invertebrados

A comunidade de invertebrados foi amostrada uma semana antes (pré-limpeza) e uma semana depois (pós-limpeza) da intervenção de limpeza acontecer. Em cada cavidade foi realizada a coleta de toda a comunidade de invertebrados utilizando método de coleta ativa (WYNNE et al., 2019). Os procedimentos de coleta foram feitos seguindo a setorização das cavidades.

### Análises estatísticas

As análises foram feitas sob duas perspectivas. Na primeira perspectiva o objetivo foi avaliar os padrões gerais de resposta das comunidades biológicas após a limpeza das cavidades (usando os dados de todas as cavidades em conjunto). Assim, as cavidades entraram na análise como unidade amostral. Na segunda perspectiva, a análise foi feita separadamente por cavidade, sempre que possível (quando a cavidade apresentava pelo menos três setores). Neste caso os setores das cavidades entraram como unidade amostral. Só não foi possível realizar as análises separadamente para as cavernas BM-108 e BM-116.

Para avaliar se a riqueza média de espécies, número médio de indivíduos, diversidade média de espécies e equitatividade média mudou após a limpeza das cavidades foi feita uma análise de modelos lineares generalizados mistos (GLMMs). A variável explicativa foi o tempo de amostragem (antes da limpeza e após a limpeza). As cavidades ou os setores das cavidades foram incluídos como variáveis aleatórias para controlar a dependência temporal e espacial do experimento.

Para avaliar se existem diferenças na composição de espécies de invertebrados coletados antes e após a limpeza das cavidades foram realizadas análises de NMDS e de PERMANOVA. A matriz de composição de espécies foi transformada em uma matriz triangular de similaridade utilizando o índice de Jaccard (presença e ausência das espécies). Todas as análises foram realizadas no software R (R DEVELOPMENT CORE TEAM, 2025).

## 3. Resultados

A limpeza das seis cavidades piloto resultou na remoção de cerca de 645 kg de material particulado, sendo 77 kg da BM-36, 379 kg da BM-50, 15 kg da BM-104, 8 kg da BM-108e 147 kg da BM-116 e 17 kg da BM-120.

Todos os parâmetros das comunidades biológicas avaliados mudaram após a limpeza das cavidades (Figura 3). A riqueza média de espécies, diversidade e equitatividade aumentaram após a limpeza das cavernas. Diferentemente, o número médio de indivíduos sofreu uma diminuição significativa após a limpeza (Figura 1).

Ao avaliar as cavernas separadamente, foi verificado que a BM-36 apresentou os mesmos resultados citados anteriormente. Na cavidade BM-50 foi observado apenas a diminuição do número de indivíduos após a limpeza. A cavidade BM-116 exibiu um aumento da diversidade e equitatividade e diminuição do número de indíviduos. E, a cavidade BM-120 apresentou um aumento da riqueza pós-limpeza.

A composição de espécies baseada na presença e ausência foi considerada estatisticamente semelhante pré e pós limpeza das cavidades. O gráfico de NMDS confirma esse resultado já que os pontos se encontram dispersos no espaço e não agrupados pelas categorias antes e após limpeza. Além disso, os intervalos de confiança das categorias se sobrepõem (Figura 2 ; Figura 3). Nesse caso, a variável cavidade foi considerada mais importante, já que pontos relacionados a uma mesma cavidade se encontram mais próximos um do outro em relação a pontos de cavidades diferentes. Ao avaliar as diferenças na composição de espécies de cada cavidade separadamente, foi verificado que apenas na BM-36 houve mudanças significativas na composição entre um período e outro (Figura 3).

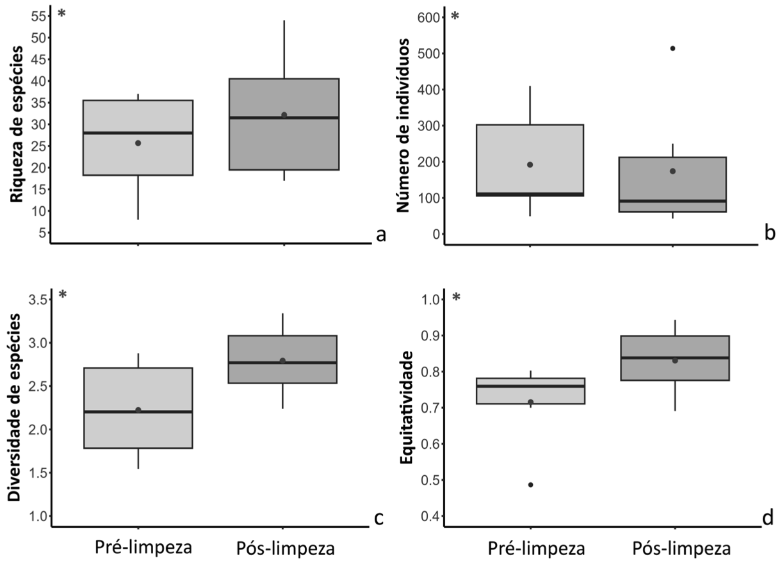


***Figura 1:*** *Riqueza média (a), número médio de indivíduos (b), diversidade média de espécies (c) e equitatividade média (d) registradas nas seis cavidades alvo da recuperação no período pré-limpeza e pós-limpeza. O círculo verde no gráfico de boxplot representa média e a linha contínua a mediana das variáveis. O asterisco (*) indica diferença significativa entre as categorias (p < 0.05).*

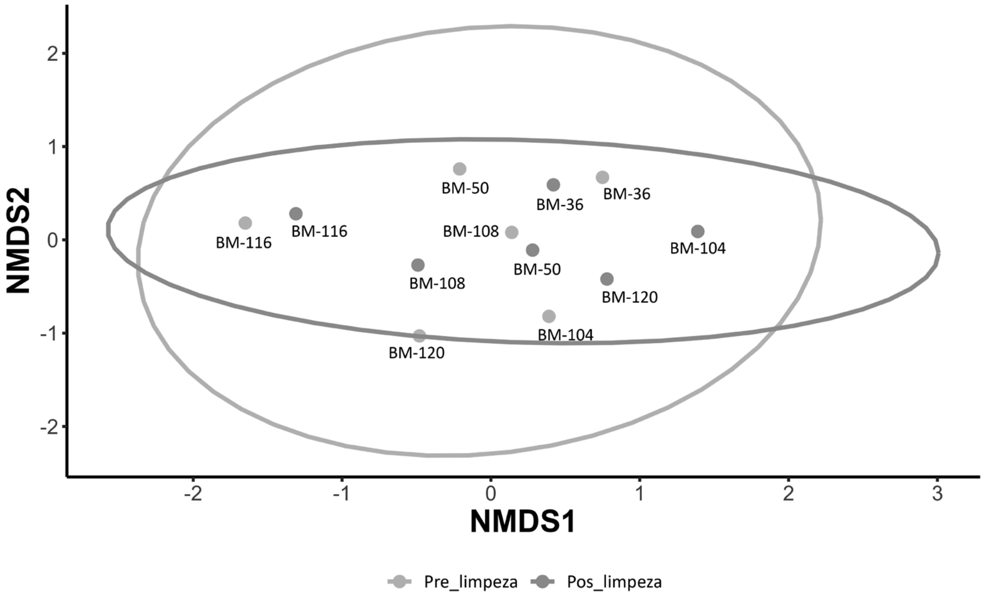
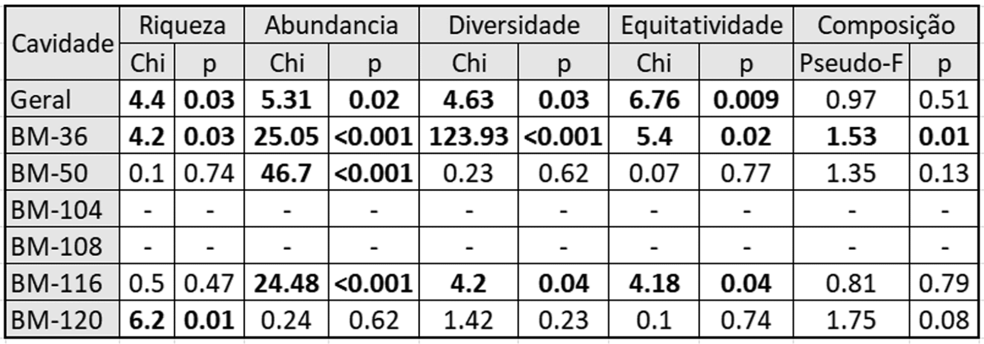


***Figura 2:** Análise de NMDS da composição de espécies baseada na similaridade de Jaccard para comparar as cavidades pré e pós limpeza. Cada círculo representa uma cavidade alvo da recuperação.*



| Cavidade | Riqueza | | Abundancia | | Diversidade | | Equitatividade | | Composição | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Chi | p | Chi | p | Chi | p | Chi | p | Pseudo-F | p |
| Geral | **4.4** | **0.03** | **5.31** | **0.02** | **4.63** | **0.03** | **6.76** | **0.009** | 0.97 | 0.51 |
| BM-36 | **4.2** | **0.03** | **25.05** | **<0.001** | **123.93** | **<0.001** | **5.4** | **0.02** | **1.53** | **0.01** |
| BM-50 | 0.1 | 0.74 | **46.7** | **<0.001** | 0.23 | 0.62 | 0.07 | 0.77 | 1.35 | 0.13 |
| BM-104 | - | - | - | - | - | - | - | - | - | - |
| BM-108 | - | - | - | - | - | - | - | - | - | - |
| BM-116 | 0.5 | 0.47 | **24.48** | **<0.001** | **4.2** | **0.04** | **4.18** | **0.04** | 0.81 | 0.79 |
| BM-120 | **6.2** | **0.01** | 0.24 | 0.62 | 1.42 | 0.23 | 0.1 | 0.74 | 1.75 | 0.08 |

***Figura 3:** Resultados das análises de GLM e PERMANOVA para a comparação da riqueza, abundância diversidade, equitatividade e composição de espécies entre o período pré e pós-limpeza.*

## 4. Discussão

Os resultados aqui apresentados mostram a resposta de curto prazo (uma semana) das comunidades de invertebrados após a remoção do acúmulo de material nas cavidades. Ao contrário do esperado, essa remoção não causou um efeito negativo imediato sobre a fauna cavernícola. Mesmo que sejam implementados todos os tipos de cuidados, a limpeza das cavidades envolve uma série de intervenções que podem ter impactos negativos sobre a fauna (e.g. pisoteamento, a limpeza *sensu stricto*, abrasão de substratos, transposição dos invertebrados). Essa hipótese foi criada com base em vários estudos que mostram o impacto do turismo na biodiversidade subterrânea (e.g. PELLEGRINI & FERREIRA, 2016; PIANO et al., 2022). Assim, acreditava-se que poderia existir um impacto negativo imediato logo após a limpeza, e subsequentemente as comunidades iriam recuperar ao longo do tempo.

No entanto, os resultados se mostram muito promissores, até mesmo por se tratar de um curto prazo após a recuperação. A maioria dos parâmetros das comunidades avaliados aumentaram após a limpeza. Apenas o número de indivíduos diminuiu. Além disso, a composição de espécies permaneceu a mesma entre os períodos considerados, possivelmente por se tratar de um intervalo de tempo curto entre as amostragens. Só foi encontrada mudanças na composição na cavidade BM-36. Esta é uma das cavernas que possui maior área de contato com o meio externo, o que pode explicar a mudança significativa na identidade das espécies entre os períodos.

Algumas cavidades apresentaram um aumento significativo na riqueza e/ou diversidade de espécies ou uma tendência ao aumento destas variáveis. Ou seja, após a intervenção, a entrada de espécies no sistema foi maior que a saída de espécies. Além disso, o número de indivíduos se tornou mais equitativo entre as espécies presentes, aumentando assim os valores de diversidade.

No geral, a equitatividade também aumentou depois da limpeza. De acordo com HILLEBRAND et al. (2008) a alteração de ecossistemas é capaz de afetar fortemente a equitatividade das comunidades, sendo esta métrica muito importante para entender a resposta dos organismos a impactos de origem antrópica. Isso acontece porque muitas espécies se beneficiam na presença de impactos, aumentando em abundância e dominando numericamente as comunidades, o que gera uma baixa equitatividade. Esta baixa equitatividade pode influenciar a interação das espécies, os processos ecológicos e a resistência e resiliência das comunidades. Antes da limpeza, na maioria das cavidades a equitatividade era baixa, havendo a dominância de uma ou poucas espécies em relação às outras, o que pode indicar a presença de comunidades biológicas mais alteradas neste período. Após a limpeza, houve a diminuição da abundância de várias espécies que eram dominantes, o explica a diminuição do número de indivíduoss após a limpeza, tornando as comunidades mais equitativas (abundância mais homogeneamente distribuída entre as espécies). Essa maior equitatividade pode ter auxiliado a aumentar a riqueza de espécies dos sistemas após a limpeza, pois comunidades mais equitativas tendem a abrir espaço para a entrada de novas espécies por aumentar a possibilidade de coexistência (HILLEBRAND et al., 2008).

Existem duas hipóteses capazes de explicar esse aumento da biodiversidade logo após a remoção do material particulado das cavidades: 1) ausência do material particulado; 2) resposta da fauna a distúrbios (e.g. limpeza). Alguns estudos já mostraram, que distúrbios de intensidade intermediária podem maximizar a biodiversidade. Apesar da alta vulnerabilidade dos organismos subterrâneos, distúrbios em ecossistemas cavernícolas não necessariamente resultam na diminuição da biodiversidade. Tudo irá depender da duração, intensidade e cobertura espacial deste distúrbio (FAILLE et al., 2014). O distúrbio aqui aplicado (e.g. remoção do material particulado) foi de certa forma positivo.

A restauração de ecossistemas cavernícolas é um tema novo no Brasil e são poucos os estudos científicos que avaliaram o sucesso destas estratégias. Mais escassos ainda são as investigações sobre os impactos da recuperação de cavernas sobre a fauna subterrânea. Até o momento temos o trabalho de Souza et al. (2022), que avaliaram a resposta das comunidades cavernícolas à remoção de material particulado em quatro cavidades. Sua conclusão foi que não existiam fundamentos que possibilitassem atestar se a atividade de limpeza trouxe benefícios ou interferências negativas às comunidades subterrâneas. Desta forma, nosso estudo é o primeiro a alegar que as atividades de limpeza das cavidades podem ser benéficas para a fauna, pelo menos a curto prazo.

## 5. Conclusão

Os resultados aqui apresentados mostram, que a remoção de material particulado pode ser uma estratégia importante na restauração de cavidades, não ocasionado impactos negativos imediatos para a fauna cavernícola.

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# Beyond geographic boundaries: *Thiothrix*-crustacean symbioses across sulfidic ecosystems

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## Abstract

Symbiotic relationships between sulfur-oxidizing bacteria of the genus *Thiothrix* and various crustacean hosts have been documented in sulfide-rich environments, notably in the Movile Cave and the Mangalia region. This study aims to explore these associations beyond these well-studied sites, focusing on the presence of *Thiothrix* in amphipods, copepods, ostracods, and isopods from other sulfidic habitats. Using Fluorescence In Situ Hybridization (FISH) and Scanning Electron Microscopy (SEM), we provide clear visual support of *Thiothrix* filaments colonizing the exoskeletons of crustaceans. Our findings suggest that the symbiotic relationship between *Thiothrix* and crustaceans is more widespread than previously thought and may play a significant ecological role in various ecosystems characterized by high hydrogen sulfide concentrations.

## Résumé

Les relations symbiotiques entre les bactéries sulfure-oxydantes du genre *Thiothrix* et divers crustacés hôtes ont été documentées dans des environnements riches en sulfure, notamment dans la grotte de Movile et la région de Mangalia. Cette étude vise à explorer ces associations au-delà de ces sites bien étudiés, en se concentrant sur la présence de *Thiothrix* chez les amphipodes, copépodes, ostracodes et isopodes dans d'autres habitats sulfurés. En utilisant l'hybridation in situ en fluorescence (FISH) et la microscopie électronique à balayage (SEM), nous apportons un appui visuel clair de la colonisation des exosquelettes de crustacés par des filaments de *Thiothrix*. Nos résultats suggèrent que la relation symbiotique entre *Thiothrix* et les crustacés sont plus répandues qu'on ne le pensait auparavant, et qu'elles pourraient jouer un rôle écologique significatif dans divers écosystèmes particulièrement riche en hydrogène sulfuré.

## Resumen

As relações simbióticas entre bactérias oxidantes de enxofre do gênero *Thiothrix* e diversos hospedeiros crustáceos foram documentadas em ambientes ricos em sulfeto, notadamente na Caverna de Movile e na região de Mangalia. Este estudo busca explorar tais associações além desses locais amplamente estudados, focando na presença de *Thiothrix* em anfípodes, copépodes, ostrácodes e isópodes em outros habitats sulfidizados. Utilizando Hibridação Fluorescente in situ (FISH) e Microscopia Eletrônica de Varredura (SEM), fornecemos evidências visuais claras da colonização dos exoesqueletos dos crustáceos por filamentos de *Thiothrix*. Nossos resultados sugerem que a relação simbiótica entre *Thiothrix* e crustáceos é mais difundida do que se reconhecia anteriormente e pode desempenhar um papel ecológico significativo em diversos ecossistemas caracterizados por altas concentrações de sulfeto de hidrogênio.

## 1. Introduction

Extreme environments characterized by high concentrations of hydrogen sulfide ($H_2S$), such as sulfidic caves and hydrothermal vents, often harbor specialized symbiotic associations. Many invertebrates inhabiting these ecosystems have evolved physiological adaptations that facilitate survival in such chemically challenging conditions, frequently involving microbial partners that contribute to host metabolism and detoxification processes (Anderson et al., 1987; Wilmot & Vetter, 1990; Cavanaugh et al., 2006; Dubilier et al., 2008; Tokuda et al., 2008; Petersen et al., 2010; Pakes et al., 2014; Sun et al., 2022). While hydrothermal vents provide a well-documented setting for studying these associations, sulfidic caves offer distinct advantages for symbiosis research, including logistical accessibility, stable environmental conditions, and the potential for repeated sampling. Both ecosystems share key characteristics, including a reliance on chemosynthetic primary production, yet caves permit more detailed investigations into host-microbe interactions.

Symbiotic interactions between microorganisms and invertebrates play a crucial role in the functioning of sulfidic ecosystems. The genus *Thiothrix* comprises filamentous, sulfur-oxidizing bacteria commonly found in these environments, where they frequently associate with various aquatic invertebrates. Among these, amphipods represent a particularly well-documented group of hosts. *Thiothrix*-amphipod relationships has been recorded across several geographically distinct sulfidic habitats in Europe. However, while caves have been known since ancient times, and among sulfurous caves, Grotta del Fiume (part of the Frasassi complex) and the Frasassi Cave were discovered in 1948 and 1953, respectively, by the Italian Alpine Club (CAI) of Jesi and Fabriano, they remain largely unexplored and poorly studied. In contrast, black smokers were discovered in 1978 (Jannash, 1985) and have already been extensively studied, despite being significantly more difficult to access.

The first documented case of *Thiothrix*-crustacean associations in fresh water cave was in the Frasassi cave system (Italy), where *Thiothrix* filaments were observed on the amphipod *Niphargus* ictus (Dattagupta et al., 2009). Subsequent studies expanded these findings to *Niphargus frassasianus* and *Niphargus montanarius* within the same cave system (Bauermeister et al., 2012), suggesting that such relationships might be more common than initially recognized. In marine environments, *Thiothrix* bacteria have been identified as epibionts on the amphipod Urothoe poseidonis collected from coastal sediments near Wimereux, France (Gillan & Dubilier, 2004), highlighting their potential for broader ecological distribution.

Further research in Romania has revealed that *Thiothrix* symbioses are not limited to amphipods. In the Movile Cave and surrounding sulfidic groundwater systems of Mangalia, *Thiothrix* bacteria have been identified on multiple microcrustaceans, including copepods, ostracods, and isopods (Chauveau et al., 2024). These findings suggest that *Thiothrix* has a broader host range than previously assumed, adapting to different crustacean species in diverse sulfidic habitats. Despite these observations, the prevalence and ecological significance of *Thiothrix*-crustacean associations in other sulfidic environments remain underexplored.

The functional significance of *Thiothrix* symbiosis with amphipods and other crustaceans remains an open question, with competing hypotheses regarding its ecological role. The detoxification hypothesis suggests that sulfur-oxidizing bacteria may reduce local $H_2S$ concentrations, thereby mitigating toxicity for their hosts. However, an experimental study testing the effects of antibiotic-induced bacterial depletion in *Niphargus* failed to demonstrate a survival advantage linked to the presence of these bacteria (Bauermeister et al., 2013). Alternatively, the nutritional hypothesis proposes that *Thiothrix* may supply their hosts with organic carbon derived from sulfide oxidation, similar to known nutritional mutualisms in deep-sea vent crustaceans (Ponsard et al., 2013). To date, this hypothesis has not been tested in *Niphargus*, leaving open the question of whether these bacterial symbionts play a direct metabolic role in host physiology. Given that crustaceans inhabiting sulfidic environments face dual constraints of sulfide toxicity and oxygen limitation, *Thiothrix* symbiosis may confer selective advantages, yet the precise nature of these benefits remains unresolved. Moreover, the specificity of this association raises broader questions: why have similar relationships not been documented in other crustaceans?Is this symbiosis limited to temperate marine and freshwater ecosystems, or does it extend to other environments? or beyond the Palearctic biogeographic region?

Although genomic data from symbiotic *Thiothrix* strains are currently unavailable, insights from related free-living and non-symbiotic *Thiothrix* genomes suggest potential metabolic adaptations relevant to life in sulfidic environments. These include genes associated with sulfur oxidation, oxidative stress resistance, and biofilm formation (Lapidus et al., 2011; Ravin et al., 2021; Ravin et al., 2023). Comparative studies of Thiothrix-host interactions across different ecological settings highlight recurrent features of this symbiosis. Preliminary observations in Movile Cave suggest that *Thiothrix* may also colonize other crustaceans, such as ostracods and copepods (unpublished data). Across diverse aquatic and subterranean environments, *Thiothrix* consistently associate with specific anatomical structures on their hosts, such as setae and appendages near the gills (Gillan & Dubilier, 2004; Dattagupta et al., 2009; Bauermeister et al., 2012; Flot et al., 2014). These consistent attachment sites may provide optimal conditions for bacterial sulfide oxidation and/or nutrient exchange, reinforcing the functional significance of this symbiosis in extreme environments.

Despite extensive documentation of *Thiothrix*-crustacean associations, the underlying molecular mechanisms governing these relationships remain poorly understood. Hypotheses suggest that *Thiothrix* could provide detoxification benefits by oxidizing hydrogen sulfide, thereby reducing its toxicity to the host, or alternatively contribute to the host's nutrition through the production of organic compounds via chemosynthesis. However, empirical evidence supporting these hypotheses remains limited, emphasizing the need for further research to elucidate the nature of these associations.

## 2. Materials and methods

**Sample Collection**

A total of twelve sampling missions were conducted across multiple sulfidic environments worldwide. These included marine sediments in Wimereux and the paleontological cave of La Rigotte in France, the Movile Cave and the Black Sea in Romania, Melissotrypa Cave, Lake Vouliagmeni, Kaiafa Cave, Sulfur Beach, and the marine hydrothermal springs on the island of Milos in Greece, the Frasassi Cave and Vulcano Island shallow-water black smokers in Italy, the Godarville Tunnel in Belgium, and Lake Yu in Japan. These locations share the common characteristic of being sulfur-rich environments capable of sheltering crustaceans within their ecosystems.

To sample these sites effectively, various techniques were employed depending on accessibility and habitat conditions. Sampling methods included SCUBA diving and speleology-based collection, utilizing specialized tools such as Pasteur pipettes and plankton nets. Specimens were immediately preserved in 4% paraformaldehyde for subsequent molecular and morphological analyses.

**Molecular approach :**

A *Thiothrix*-specific 16S rRNA primer (Flot et al., 2014) was used in combination with metabarcoding techniques to analyze more than 400 individual specimens. The presence of *Thiothrix* was assessed on various anatomical regions, including setae and appendages, allowing for a comprehensive phylogenetic reconstruction of *Thiothrix*-crustacean associations. This analysis revealed previously unrecognized clusters within the *Thiothrix* genus, suggesting that their distribution may be more geographically constrained than host-specific.

**Imaging Approaches :**

Based on these findings, Fluorescence In Situ Hybridization (FISH) was performed to further localize *Thiothrix* on crustacean exoskeletons. Hybridized samples were washed to remove unbound probes, mounted on slides, and examined under epifluorescence microscopy to confirm bacterial colonization patterns.

To complement these molecular analyses, scanning electron microscopy (SEM) was employed to investigate the spatial organization of *Thiothrix* strains when co-existing on the same host. Crustacean specimens were dehydrated through a graded ethanol series, critical-point dried, and sputter-coated with gold. SEM imaging provided high-resolution visualization of filamentous bacteria, enabling quantification of their relative abundance across different host species. Furthermore, this approach elucidated variations in bacterial colonization patterns under distinct environmental conditions, shedding light on the complexity of *Thiothrix*-crustacean interactions.

## 3. Results and discussion

Our expanded 16S rRNA phylogenetic analysis provides new insights into *Thiothrix*-crustacean associations across different sulfidic environments, revealing both previously described and novel lineages. Compared to earlier studies, our dataset includes a broader taxonomic and geographic sampling, allowing us to refine the phylogeny of *Thiothrix* symbionts. The analysis suggests the presence of new OTUs within *Thiothrix*, distinct from those previously reported. However, the true diversity within this genus remains insufficiently characterized due to limited available data. These novel lineages highlight an overlooked diversity of *Thiothrix* in symbiotic associations, reinforcing the idea that multiple distinct groups may exist but are yet to be fully described.

*Thiothrix* has been detected on *Asellus* isopods from the sulfidic environment of the Godarville Tunnel in Belgium. These observations extend the known host range of *Thiothrix* beyond previously studied amphipods and copepods, confirming their presence on isopods in subterranean sulfidic waters.

In addition to molecular data, scanning electron microscopy (SEM) images from Movile copepod's provide the first evidence of *Thiothrix* colonization on Eucyclops specimens that he collected in Movile Cave.

The bacterial filaments are primarily localized along setae and intersegmental joints all over the lower part of the body and fewer on the upper part, areas likely to provide optimal conditions for microbial attachment and growth. The filamentous morphology and rosette-like structures observed in SEM are characteristic of *Thiothrix*.

Although these preliminary findings strongly suggest a widespread presence of *Thiothrix* in sulfidic habitats beyond Mangalia, further confirmation using Fluorescence In Situ Hybridization (FISH) is still required to unambiguously identify these filaments as *Thiothrix*.

## 4. Conclusion

Our study provides new insights into the distribution and potential ecological role of *Thiothrix*-crustacean symbioses beyond the well-documented Movile and Mangalia regions. The expanded phylogenetic framework and high-resolution imaging presented here suggest that *Thiothrix* associations are not restricted to specific host taxa or geographic locations but instead occur across a wide range of sulfidic environments.

The discovery of *Thiothrix* filaments on crustaceans from diverse habitats, including marine sediments, brakish water lake and sulfidic caves, highlights the resilience and adaptability of these bacteria. Moreover, the presence of previously unknown OTUs suggests that *Thiothrix* diversity is still underestimated and likely shaped by local environmental factors rather than strict host specificity.

While FISH and SEM have provided clear morphological evidence of *Thiothrix* colonization, future studies should focus on functional analyses to elucidate the metabolic contributions of these bacteria to their hosts and inversely. Stable isotope probing and transcriptomic analyses could help clarify whether *Thiothrix* provides detoxification or nutritional benefits to crustaceans in these extreme habitats.

Overall, our findings suggest that *Thiothrix*-crustacean symbioses are more widespread and ecologically significant than previously assumed. By integrating molecular and imaging approaches, this study lays the groundwork for further investigations into the role of sulfur-oxidizing bacteria in shaping microbial-invertebrate interactions in sulfidic ecosystems.

## Acknowledgments

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# Artropofauna de la cueva turística “Cueva de Nerja” (Málaga, España)

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## Abstract

**Arthropofauna in the tourist cave “Cueva de Nerja” (Málaga, Spain)**

Nerja Cave (Málaga, Spain) has been declared as Cultural Interest Site, with a category of Archaeological area. It has been sampled from July 2017 to July 2019 to evaluate the variation of the entomofauna. Non-invasive baited pitfall traps with respect to the archaeological sediment have been specifically designed for this study. 60 sampling points were placed, 20 traps in the Tourist Galleries (GT), 16 in the High Galleries (GA) and 24 in the New Galleries (GN). 11786 specimens of arthropods have been captured. It has identified 73 taxa. Specimen captures show differences between the three areas of the cave. Thus, GT reaches 74.97% of the collected fauna, GA 11.20% and GN 13.82%. GT possess an abundance of arthropods between five and almost seven times more than the other galleries. GA shows the least fauna and less diversity. Geostatistical analysis has been carried out to combine the presence of entomofauna and the temporal variation of the number of captures in each trap. It shows the existence of three site with maximum variation (two in GT and one in GN). Some species, such as the cave cricket, phorid dipterans and acari, suggest areas of connection with the outside in the GNs.

## Resumen

La Cueva de Nerja (Málaga, España) está declarada Bien de Interés Cultural, con categoría de Zona Arqueológico. Se muestreó desde julio de 2017 hasta julio de 2019 para evaluar su entomofauna. Se han diseñado unas trampas de caída cebadas no invasivas respecto al sedimento arqueológico. Se situaron 60 puntos de muestreo, 20 trampas en las Galerías Turísticas (GT), 16 en las Galerías Altas (GA) y 24 en las Galerías Nuevas (GN). Se han capturado 11786 ejemplares de artrópodos y se han identificado 73 taxones. Tanto el número de capturas, como la diversidad de los grupos taxonómicos, evidencian diferencias entre las tres zonas de la cueva. Así, GT abarca el 74,97% de la fauna colectada, con 8821 individuos; GA el 11,20%, con 1324 ejemplares y GN el 13,82%, con 1631 ejemplares. Las GT poseen una abundancia de artrópodos entre cinco y casi siete veces más que las otras galerías. El análisis geoestadístico realizado evidencia la existencia de tres puntos de máxima variación (dos en GT y uno en GN). Por otro lado, la tipología de algunas especies, como los dípteros fóridos, los ácaros y un grillo cavernícola, sugieren zonas de conexión con el exterior en las GN.

## 1. Introducción

La Cueva de Nerja (Málaga, España) es una de las cuevas turísticas de España que recibe mayor número de visitantes al año, el cual supera el medio millón de personas (DEL ROSAL et al., 2009). Esta cavidad tiene la particularidad de poseer asentamientos prehistóricos desde hace más de 35000 años (MEDINA-ALCAIDE et al., 2023). Debido a ello, la Cueva de Nerja está declarada Bien de Interés Cultural, con categoría de Zona Arqueológica (BOJA, 2006).

La cavidad en conjunto tiene 4823 metros de desarrollo horizontal, contando la longitud de todas sus galerías, con una superficie de 35484 m2. La Cueva de Nerja se divide en tres tramos bien diferenciados sobre la planta topográfica. Un tramo inicial que constituyen las Galerías Turísticas (GT), segundo tramo las Galerías Altas (GA) y tercer tramo las Galerías Nuevas (GN). De ellas, tan sólo es visitable la zona correspondiente a GT. Situando la cota ±0 en la entrada, la GT desciende hasta -30,6 metros, siendo la punto más alto en GN a +67,27 metros. Las GT se iluminan con luz artificial desde primera hora de la mañana hasta el cierre de la cueva. El resto de la cavidad, GA y GN, son visitadas únicamente por investigadores; y aunque disponen de cierta iluminación, su uso es muy puntual a lo largo del año. Para el conocimiento de las variaciones abióticas de la cavidad, existen una serie de sensores en diferentes puntos que permiten registrar las condiciones ambientales y evaluar su variación en el tiempo (LIÑÁN et al, 2018).

Los primeros datos sobre la fauna de esta cavidad datan de inicios de los años 60, cuando se citaron tres especies de coleópteros y se describió *Platyderus speleus* (COBOS, 1961). Posteriormente, se realizan algunas prospecciones esporádicas, con la cita de 8 taxones, algunos de los cuales fueron incorrectamente identificados (WALLACE, 1985). El primer estudio sistemático de la entomofauna en la cavidad se desarrolla en el bienio 2000-2001 (DEL ROSAL et al., 2009) con un censo total de 26 especies, de las cuales se describieron dos endemismos: el pseudoescorpión *Chthonius nerjaensis* y el dipluro *Plusiocampa baetica*. Además, se proporcionaron mapas con la distribución de estas especies a lo largo de la cueva, tanto en la zona turística como en las GA y parte de las GN. Por tanto, este estudio representa una buena base para evaluar, veinte años después, cuál ha sido la dinámica de la fauna de esta cavidad y su posible relación con las visitas y el cambio climático. Por ello en el proyecto desarrollado en 2017-2025 (con un paréntesis de tres años durante la Pandemia de la Covid) se plantearon los siguientes objetivos. Primero, realizar un inventario actual de la artropodofauna presente en la cavidad, su variación estacional en cuanto a la composición y fenología de las especies y asociar la distribución a factores bióticos (recursos tróficos) y abióticos mediante un estudio geoestadístico espacio-temporal. Segundo, establecer los niveles de vulnerabilidad de las especies y de la cueva en relación con el uso turístico, junto con posibles medidas de protección de la fauna y conservación del patrimonio.

## 2. Material y métodos

Como ya se ha indicado, la declaración de la Cueva de Nerja como de Sitio Arqueológico, conlleva una serie de restricciones en cuanto la alteración del sustrato de toda la cavidad y los atrayentes empleados en las trampas. Por ello fue necesario diseñar unas trampas de caída no invasivas que hemos denominado “tipo túmulo” utilizando propilenglicol como conservante y sobrasada como cebo (CASTILLO et al., 2022). Se han dispuesto un total de 60 trampas repartidas por toda la cavidad, un tercio en la zona visitable (GT) y dos tercios en la zona no turística distanciadas unas de otras un mínimo de diez metros (20 en GT, 16 en GA y 24 en GN); excluyéndose las áreas de grandes caos de bloques de las GA y GN (Fig. 1). Todas las muestras se han procesado en el laboratorio de Entomología de la Universidad de Almería separándose los diferentes grupos taxonómicos y conservándose en alcohol a 70° para su posterior identificación taxonómica.

Para el análisis espacial de la población de artrópodos y su variación temporal se han empleado técnicas geoestadísticas de interpolación espacial mediante kriging (GRINGARTEN & DEUTSCH, 2001).

La desviación típica en el número de capturas en cada punto de muestreo se ha utilizado como estadístico para delimitar las zonas de la cueva en función del mayor o menor grado de variación temporal de las poblaciones de entomofauna. Un análisis inicial evidenció una clara tendencia decreciente en el número de capturas desde la entrada turística, por lo que también se consideró una deriva lineal en la construcción del variograma que define la correlación espacial en el número de capturas.

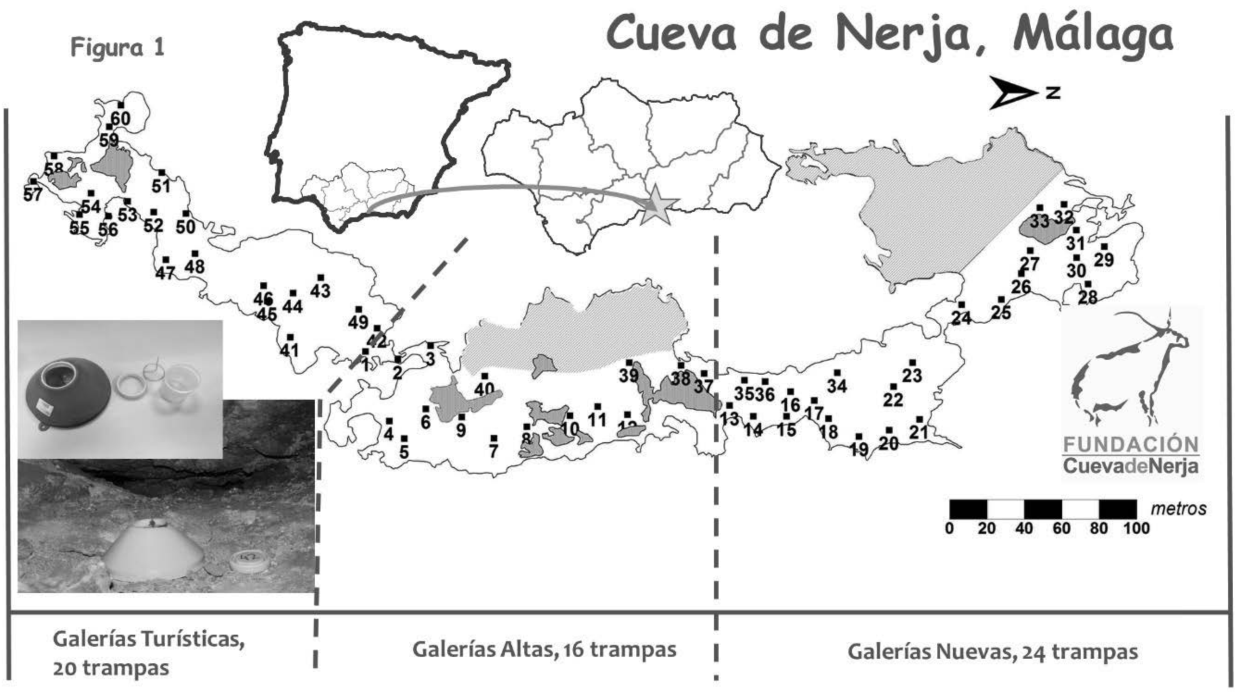


*Figura 1:* *Localización de la Cueva de Nerja, “trampa-túmulo”, y ubicación de las trampas. Escala en metros.*

## 3. Resultados

Se han capturado 11786 ejemplares de artrópodos agrupados en 73 taxones. Ello eleva el número de taxones registrado en la actualidad a 95, duplicando los 45 registrados en estudios precedentes. En la Figura 2 se ha representado la distribución espacial de capturas en la cavidad y capturas acumuladas por trampa. La incidencia de fauna a lo largo de la cueva presenta diferencias muy marcadas. Las GT arrojan un total de 8859 individuos, lo que representa casi el 75% de las capturas. Mientras que en las GA se han capturado 1352 ejemplares y 1639 en la GN, lo que representa aproximadamente el 11% y el 14% respectivamente. Con relación a las categorías taxonómicas presentes en cada una de las galerías, se produce el mismo efecto, con porcentajes de 44, 17 y 14, respectivamente, para cada una de las galerías. Las GT son, con diferencia, las que presentan mayores registros de fauna, tanto cuantitativa como cualitativamente, siendo las otras dos zonas de la cavidad más similares. Este hecho ya se puso en evidencia en los estudios anteriores (DEL ROSAL et al., 2009), donde se apuntaba como origen a la gran disponibilidad de materia orgánica disponible a causa de las visitas. No obstante, existe otro factor determinante que condiciona directamente esta circunstancia, precisamente la conexión con el exterior. El análisis de las especies que aparecen en cada galería proporciona una información muy relevante sobre las condiciones ambientales particulares en éstas y confiere especial significación a la distribución y localización de determinados taxones. El 54% de los ejemplares capturados en las GT corresponden al psocóptero *Psyllipsocus ramburii* Selys-Longchamps, 1872 que constituye el 24% en GA y tan sólo el 1,16% en GN. La segunda especie en importancia en GT es Petaloptila *malacitana* Barranco, 2010, donde representa algo más del 20%, descendiendo al casi el 6% en GA y tan sólo el 0,24% en GN. Se trata de un grillo troglobio endémico de cavidades malagueñas y que puede ser empleada como bioindicadora; su presencia en esta cavidad va a ser objeto de un estudio específico. El tercer grupo en importancia en estas galerías son los ácaros con 674 ejemplares, (de los que se han identificado 25 especies, fundamentalmente oribátidos, seguidos por los mesostigmátidos), que representan casi el 14% de las capturas. Estos arácnidos disminuyen su presencia en las otras dos zonas de la cavidad. Tanto en GA, como en GN, el grupo taxonómico más abundante son los colémbolos, que representan más del 50% de la fauna en ambas zonas, seguido del psocóptero antes mencionado en GA y de los dípteros fóridos en GN. Este último grupo, también es un bioindicador de conexiones con el exterior. Los troglobios endémicos de la Cueva de Nerja como el dipluro *Plusiocampa baetica* Sendra, 2004 y el pseudoescorpión *Chthonius nerjensis* Carabajal, García y Rodríguez 2001 parecen distribuirse de forma opuesta en la cavidad. Así el primero aparece a lo largo de toda ella, si bien el mayor número de capturas se produce en GN, las galerías más profundas donde llega a representar casi el 20% de las capturas. Por el contrario, el pseudoescorpión solo se ha capturado en GT y con una representación de tan sólo 10 ejemplares (0,11%).

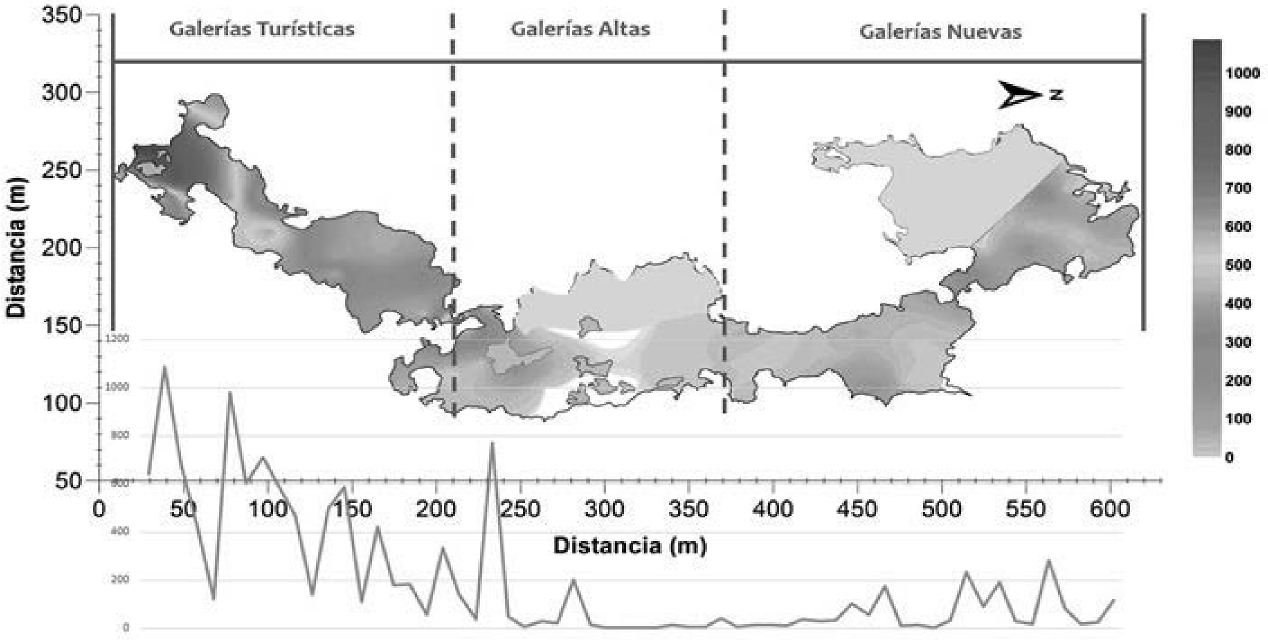


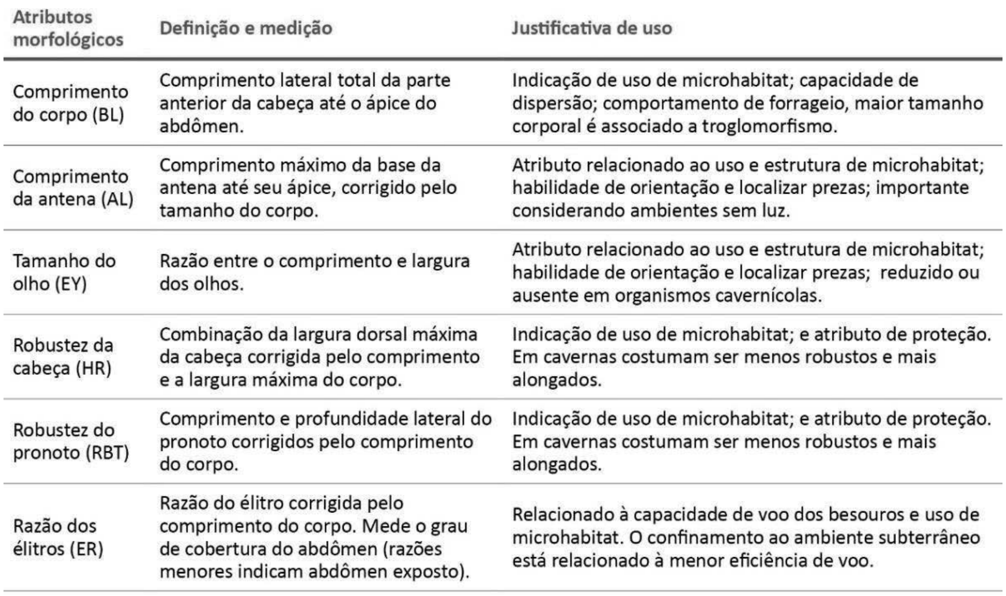
***Figura 2:*** *Distribución espacial de capturas en la cavidad y capturas acumuladas por trampa.*

## 4. Discusión

En el conjunto de la cavidad, se han detectado tres lugares que corresponden a los de máxima variación y que coinciden con algunos de los sitios de máxima abundancia. La zona con la máxima variación corresponde a la entrada a la cueva y las GT, esto produce un gran flujo de fauna fundamentalmente con conesiones al exterior, tanto por la zona de entrada como por varios sumideros que existen en las zonas superiores de las GT. El segundo lugar con mayor variación temporal se localiza en la zona de acceso de las GT a GA. El tercer punto de mayor variación se localiza al fondo de las GN, donde el valor de la variación de ejemplares es de 60-80 individuos. Estos tres lugares señalados de máxima variación deben indicar puntos de flujo de fauna. Precisamente estos lugares de máxima variación poblacional coinciden con zonas de flujo de corrientes en la cavidad (LIÑÁN et al., 2020). Las especies que ocasionan estas dinámicas locales son diferentes según la zona, en general son *Psyllipsocus ramburii* y *Petaloptila malacitana* en los dos primeros casos y el colémbolo *Lepidocyrtus flexicollis* en el tercero. Estas tres especies presentan un comportamiento troglófilo en la cavidad.

De las espcies de artrópodos citados, cabe especial mención para los que se pueden considerar bioindicadores. Por un lado, los que su presencia indican una conexión con el exterior, entre los que podemos mencionar los dípteros fóridos, la gran mayoría de los ácaros detectados y un caso particular que representa el grillo cavernícola *Petaloptila malacitana*.

Por lo general, la fauna de las cuevas es escasa salvo cuando existe aporte de nutrientes. Así, las cavidades que poseen colonias de murciélagos, presentan zonas de enorme abundancia siempre que estas colonias se mantengan en el tiempo. En la Cueva de Nerja no existen actualmente grandes aglomeraciones de estos mamíferos, aunque hay restos de acumulación de guano antiguo en algunos lugares de la cavidad, pero que no presentan acumulación profusa de fauna, probablemente debido a la perdida de nutrientes con el paso del tiempo.



| Atributos morfológicos | Definição e medição | Justificativa de uso |
|---|---|---|
| Comprimento do corpo (BL) | Comprimento lateral total da parte anterior da cabeça até o ápice do abdômen. | Indicação de uso de microhabitat; capacidade de dispersão; comportamento de forrageio, maior tamanho corporal é associado a troglomorfismo. |
| Comprimento da antena (AL) | Comprimento máximo da base da antena até seu ápice, corrigido pelo tamanho do corpo. | Atributo relacionado ao uso e estrutura de microhabitat; habilidade de orientação e localizar prezas; importante considerando ambientes sem luz. |
| Tamanho do olho (EY) | Razão entre o comprimento e largura dos olhos. | Atributo relacionado ao uso e estrutura de microhabitat; habilidade de orientação e localizar prezas; reduzido ou ausente em organismos cavernícolas. |
| Robustez da cabeça (HR) | Combinação da largura dorsal máxima da cabeça corrigida pelo comprimento e a largura máxima do corpo. | Indicação de uso de microhabitat; e atributo de proteção. Em cavernas costumam ser menos robustos e mais alongados. |
| Robustez do pronoto (RBT) | Comprimento e profundidade lateral do pronoto corrigidos pelo comprimento do corpo. | Indicação de uso de microhabitat; e atributo de proteção. Em cavernas costumam ser menos robustos e mais alongados. |
| Razão dos élitros (ER) | Razão do élitro corrigida pelo comprimento do corpo. Mede o grau de cobertura do abdômen (razões menores indicam abdômen exposto). | Relacionado à capacidade de voo dos besouros e uso de microhabitat. O confinamento ao ambiente subterrâneo está relacionado à menor eficiência de voo. |

***Figura 3:*** *Número de especies por grupos taxonómico presente en la cavidad. End: endemismo ; TX: trogloxeno ; TF: troglófilo ; TB: troglobio. *endemismo encontrado en otras localidades.*

## 5. Conclusión

El presente estudio ha elevado considerablemente el número de taxones censados en la cavidad, duplicando el número de especies registadas a principios del mismo. Además ha permitido el descubrimiento de una nueva especie de araña cuya descripción taxonómica está en prensa para su publicación.

El estudio ha evidenciado la influencia de la repercusión que representa la exposición de las salas turísticas al público. Su afectación se realiza de dos modos, el incremento de materia orgánica que introducen los visitantes (de forma voluntaria o involuntaria) y la iluminación de las Galerías Turísticas. Ello se ha puesto de manifiesto en el incremento de las poblaciones de psocópteros por la proliferación de algas sobre las calizas. La Fundación de la Cueva de Nerja está realizando un gran trabajo de investigación para la contención de esta proliferación, tanto para preservar la integridad de las formaciones como las pinturas rupestres que existen sobre ellas.

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# Assessment of the impacts of management and sampling strategy on the hematological profiles of *Furipterus horrens*

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## Abstract

Assessing hematological indices is crucial for evaluating animals' health since these measures reflect nutritional status, disease presence, and environmental responses. However, interpreting such parameters in wild species is challenging due to the stress imposed by capture and handling. This study examined the hematological and biochemical profiles of *Furipterus horrens* to evaluate how intrinsic factors (sex, age, and reproductive stage) and methodological variables (sampling strategy, handling time, and seasonal variations) affect their physiological condition. Sampling was conducted at the UC Flona de Carajás, where bats were captured in two periods—pre-foraging and post-foraging—allowing comparisons between fasting and fed states. After capture, biometric data and blood samples were collected to determine hemoglobin and glucose levels. The results revealed that reproductive females exhibit significantly higher body weight, while plasma glucose levels are elevated in bats captured after foraging and decline with prolonged handling time. These outcomes underscore the importance of standardizing collection protocols and minimizing handling time to reduce stress-induced alterations. Such measures ensure the accuracy of physiological data and support effective conservation strategies for the species.

## 1. Introduction

Assessing hematological parameters is fundamental for evaluating the physiological state and overall health of animals in both wild and captive environments. These measures not only indicate nutritional status and disease presence but also reveal environmental changes such as shifts in habitat quality (Crooks 2003; Dutton 2003; McLaughlin 2007). A range of factors—including sex, age, reproductive status, environmental conditions, geographical location, disturbances, stress, and food availability—can influence these profiles (Paksuz, 2022).

A major challenge in field research is distinguishing the natural variation in immune and hematological functions from alterations induced by capture and handling stress, particularly since blood is typically collected after capture, introducing a delay (Romero and Romero 2002; Buehler *et al.* 2008; Davis *et al.* 2008). Stressors, such as physical restraint, can change immune cell counts within 20 minutes (Dhabhar and McEwen 1997; McLaren *et al.* 2003) and modify erythrocyte parameters within an hour due to plasma volume shifts (Fisher and Crook 1962; Fletcher and Boonstra 2006; Teague *et al.* 2007). Johnstone *et al.* (2012) stress the need to clarify how and for how long stress impacts measures of hypothalamic-pituitary-adrenal axis activation, and whether these remain interpretable over varying time intervals. In free-ranging bats, the effect of short-term capture stress on innate immune function is still uncertain, highlighting the importance of rapid processing.

This study tests the hypotheses that: (1) sex, age class, and reproductive stage modulate key hematological parameters; (2) these parameters differ between pre-foraging and post-foraging states; (3) seasonal variation influences them; and (4) capture and handling time affect hematological metrics. We also characterize the hematological profiles and body conditions of the free-living bat *Furipterus horrens*, aiming to identify an optimal sampling strategy that minimizes stress during blood collection.

## 2. Methods and Materials

### Experiments and Bat Capture

Bats were captured using harp traps placed at the entrances of eight cavities in the UC Flona de Carajás (Figures 1 and 2). Shade nets were installed to funnel the bats toward the traps. Captures occurred at two periods—pre-foraging (17:30–18:00) and post-foraging (04:30–05:00)—to compare fasting and fed conditions. All individuals appeared healthy, and traps were inspected every 10 minutes to prevent prolonged restraint. Each bat was placed in an individual cotton bag until biometric data (capture time, sex, weight, age class according to Brunet-Rossinni & Wilkinson, 2009, and reproductive status per Racey, 2009) and blood samples were obtained. The elapsed time from initial disturbance to sampling was recorded; afterward, bats were hydrated and released near the capture site. All procedures followed the American Society of Mammalogists protocols (Sikes, 2016) and were approved by SISBio (permit 83170-1).

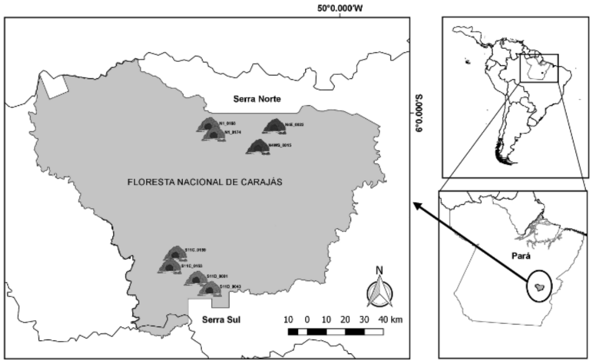

***Figure 1:*** *Map of the UC Carajás National Forest (FLONA), indicating the location of the caves studied.*

**Blood Sampling**

For sampling, bats were gently held in dorsal recumbency with one wing extended while shielded from light by a cotton bag. The sampling site was aseptically prepared with 70% alcohol, and blood was drawn by puncturing the superior propatagial or uropatagial vein with a 6 mm × 0.25 mm needle. Blood collected in a 0.5 mL syringe was used to measure hemoglobin concentration (HB, g/dL) with a HemoVet analyser (ECO Diagnóstica, Minas Gerais, Brazil) and glucose concentration (GL, mg/dL) with an ECOCheck Meter (TaiDoc Technology Corporation, New Taipei City, Taiwan).

**Statistical Analysis**

We employed maximum likelihood-based Linear Models (LMs) and Generalised Linear Mixed Models (GLMMs; Zuur *et al.*, 2009), treating physiological variables (body condition-BC, HB, and GL) as responses while experimental treatments, feeding cycles, and processing time served as fixed factors; caves and feeding cycles were included as random factors. After exploratory analysis to determine data distributions, models were fitted in R using the "glmmTMB" package (Brooks *et al.*, 2023). Model quality was verified with the "DHARMa" package (Hartig, 2022) and effect plots generated with "jtools" (Longo, 2022). Critical breakpoints in the relationship between physiological measures and processing time were identified via piecewise regression using the "segmented" package (Muggeo, 2003).

## 3. Results

The three variables, HB, GL, and BC, were evaluated to establish reference values for *F. horrens*. These values allow for the inference of possible correlations between sexes, different ages, and reproductive stages. We captured 79 individuals of *F. horrens* in eight cavities within the UC FLONA of Carajás. Of the total sampled individuals, 33 were females and 48 were males. Not all individuals provided data for all the studied variables, so individuals with missing data were filtered out for each specific subsequent analysis.

Among the physiological variables studied, only weight (body mass - g) varied according to sex (Kruskal H: 18.13, df = 1, p = 0.006), with females being heavier than males (Figure 2A). Regarding age class, 59 individuals were adults, 12 were sub-adults, and 10 were juveniles, and in this case, none of the physiological variables studied varied significantly between these classes. Concerning reproductive stage, 47 of the sampled individuals were non-reproductive and 34 were reproductive. In this case, both weight (body mass – g; Kruskal H: 15.54, df = 1, p = 0.008) responded to the reproductive stage of the animal (Figure 2B).

We obtained significant results when we evaluated the interaction between sex and reproductive stage influencing weight (t = -2.993, df = 77, p = 0.003; Figure 2C), where reproductive females are on average significantly heavier than non-reproductive females and males in general. Regarding body condition, reproductive females present, on average, significantly lower Scaled Mass ratios (t = 2.083, df = 76, p = 0.04; Figure 2D). Thus, we demonstrated the importance for future analytical steps of considering reproductive females separately when modelling body condition.

We evaluate the effect of sampling bats at the start of their foraging activity, when they are emerging from their roosts while fasting (n=54), and sampling them at the end of their foraging period when they are returning to their roosts well-fed (n=94). Among all the physiological variables tested, only glucose concentration responded significantly (F = 33.32, d.f. = 75, p = 0.00006), showing higher values in individuals sampled at the end of their foraging period (Figure 2E). Furthermore, we did not find an effect of sex related to the reproductive period.

Glucose concentration (F = 12.18, d.f. = 57, p = 0.001) decreased significantly as the time between capture and biometric processing of individuals increased. We identified critical points of change that suggest time limits for processing individuals and collecting hematologic variables (Figure 2F). We analyzed the influence of seasonality, dry season (72) and rainy season (76) on hematological parameters. We found a positive correlation between weight (Z=-2.43, p=0.0152; Figure 2G) and hemoglobin concentration (Z=-2.42, p=0.0152; Figure 2H), showing that breeding bats tend, on average, to have greater weight and higher hemoglobin concentration during the dry season.

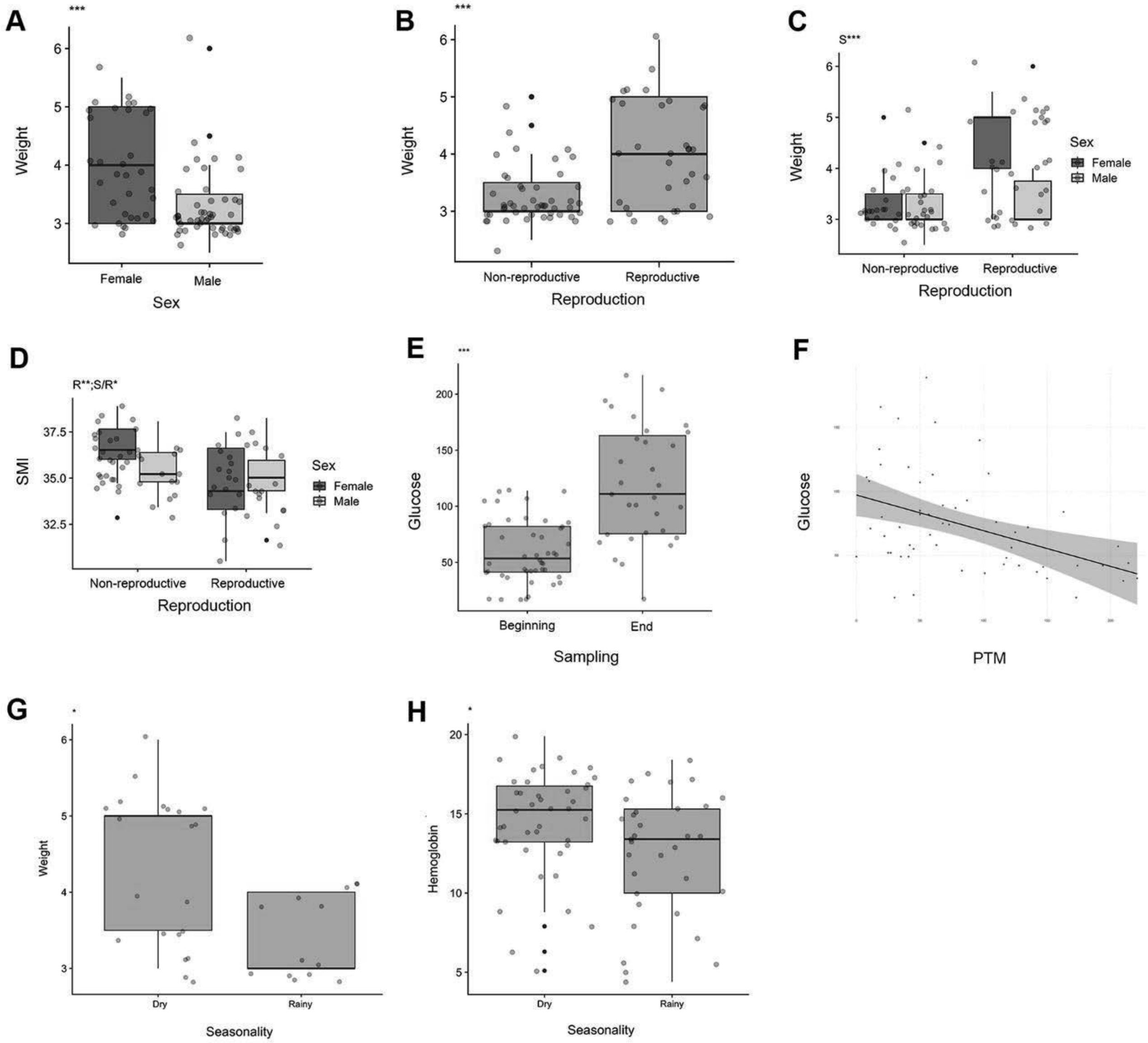

***Figure 2:*** *Effect of interaction between A) weight and Sex; B) weight and Reproductive stage; C) Sex and Reproductive stage influencing weight; D) sex and Reproductive stage influencing scaled mass; E) period of bat capture on the estimated glucose concentration (mg/dL); F) time animals were restrained until they were processed for data collection (TPM in minutes) on glucose; G) weight and seasonality; H) Hemoglobin concentration (mg/dL) and seasonality in F. horrens individuals.*

## 4. Discussion

In this study, we provide baseline information on the physiological state of *F. horrens* inhabiting the UC FLONA of Carajás, in the southeast of the Brazilian Amazon. We show that weight varies according to sex and reproductive stage. In this regard, we emphasize that intrinsic factors, weight and reproductive stage, should be considered in the interpretation of hematological and biochemical parameters in *F. horrens*. Additionally, we demonstrate that these parameters, along with hemoglobin and glucose, vary between different sampling strategies, seasonality, and handling time in the field. Below, we discuss these findings from a methodological perspective and conclude with a summary of the practical implications of these findings for field studies in the conservation physiology of bats.

When evaluating the effect of sampling bats at the beginning of their foraging activity (fasting) and at the end of the foraging period (fed), in physiological variables analyzed, we observed significant variation only in plasma glucose levels, with higher values in individuals sampled at the end of their foraging period (Figure X). The average glucose levels were twice as high between the start (3.30 ± 0.51 mmol/L) and the end of their foraging activities (6.64 ± 0.51 mmol/L).

The plasma glucose levels in fed insectivorous bats (Peng *et al.* 2016) are similar to those reported for other species of frugivorous and sanguivorous bats (Freitas *et al.*, 2005; Pinheiro *et al.*, 2006) and other mammals also fed on protein-rich diets (Kettelhut *et al.*, 1980; Opazo *et al.*, 2004). However, our findings indicate lower concentrations in *F. horrens* (3.30 mmol/L) compared to other insectivorous species, such as *Myotis pilosus* and *Scotophilus heathi* with 4.05 mmol/L (Freitas *et al.*, 2010; Mang *et al.*, 2016). Additionally, our results differ from the evidence reported by Freitas *et al.* (2010) regarding the effect of sex on blood glucose in insectivorous bats, where females are more susceptible to fasting periods, suggesting variable energy metabolism due to the costs and investments in reproduction. We did not observe any effect of sex on plasma glucose in *F. horrens*.

We suggest that the best sampling strategy, aiming to minimize the stress caused by handling individuals and collecting physiological samples, would be to sample the bats at the end of the foraging period (from 04:30 onwards), as plasma glucose levels, in both males and females, are higher and within the expected range for insectivorous bats. This represents an alternative approach to minimizing undesirable deaths resulting from the handling of a sensitive species such as *F. horrens*.

Physical restraint during the collection of bat samples can alter certain hematological parameters (Heard *et al.* 1998); therefore, the sooner blood is collected after capture, the less significant the alterations will be (Strobel *et al.* 2015). Considering this, we tested possible correlations between the duration of the procedure and hematological parameters during the handling of bats, including the use of an on-site analyzer to measure blood samples immediately after collection. We documented that handling time significantly influences glucose, and these levels progressively decrease.

The elevation of blood glucose levels, resulting from the conversion of lipids into glycogen by glucocorticoids, is often used as an indicator of stress axis activation in bats and other vertebrates (Widmaier and Kunz, 1993; Wingfield *et al.* 1998; Malisch *et al.* 2018, Boonstra *et al.* 2001, Edwards *et al.* 2022). This process is believed to be part of the adaptive stress response, as the mobilized energy can be directed toward escape behaviors or other mechanisms essential for survival (Sapolsky *et al.* 2000). However, the observed reduction in glucose concentration diverges from expectations, as an increase in glycaemic levels following manipulation, characterizing stress-induced hyperglycemia, was anticipated. Therefore, further research involving different species is necessary to investigate glucose levels at varying time intervals to enhance our understanding of the glycemic response to capture, handling, and restraint.

Blood profiles can be influenced by various external factors such as seasonality (Sealander, 1964; Korine *et al.*, 1999), time of day (Wesrhuyzen, 1978; Minematsu *et al.*, 1995), exposure to cold (Horton, 1981; Lochmiller *et al.*, 1985), and the availability and quality of food (Hellgren *et al.*, 1988; Wyk *et al.*, 1993). In bats, the maintenance of blood parameters is significantly conditioned by physiological aspects and the seasonal variations of the life cycle (Riedesel, 1977; Hecht *et al.*, 2015).

Hemoglobin levels in *F. horrens* (4.4-19.9 g/dL) fall within the ranges reported in previous studies on African (Korine *et al.*, 1999) and Neotropical bat species (Valdivie and Tamsitt, 1970), aligning more closely with those of other insectivorous species (Jurgens *et al.*, 1981; Arevalo *et al.*, 1987; Wołk and Bogdanowicz, 1987; Albayrak *et al.*, 2016; Rashid *et al.*, 2016; Bandouchova *et al.*, 2020; Paksuz, 2022).

Tavares *et al.* (2023) emphasize that spatial use by *F. horrens* in the same locality as our study is strongly related to seasonality, with greater movements observed during this period. However, the authors note that this behaviour contradicts expectations, as *F. horrens* is one of the smallest Neotropical bat species. Due to their small body size, it was anticipated that they would exhibit energetic constraints, as documented for other bats (Fenton, 1997) and mammals (Lindstedt *et al.*, 1986; Kelt and Vuren, 1999). We believe that, during the dry season, *F. horrens* likely adjust its hemoglobin levels and weight to higher proportions to forage over greater distances in search of food and water, a behavior consistent with the movement patterns reported by Tavares *et al.* (2023).

## 5. Conclusions

Our findings demonstrate that hematological parameters in *F. horrens* are significantly influenced by intrinsic factors such as sex and reproductive stage, with important implications for animal welfare during sampling. To ensure reliable comparisons both within and among species, it is essential to adopt standardized, reproducible sampling protocols with minimal handling time. This study offers specific recommendations regarding optimal timing and duration for collecting hematological samples, balancing data precision and animal welfare. However, as these guidelines are based on a single bat species from the UC FLONA de Carajás, further research involving multiple species and habitats is needed to fully elucidate how life history, reproductive status, age, and seasonal factors influence hematological variability.

## Acknowledgements

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# Bridging gaps in bat-flower interactions: literature review and case study in iron-ore caves

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## Abstract

In the Carajás FLONA caves, large populations of nectar-eating bats make these sites strategic for the study of bat-flower interactions (BFI), essential for conservation actions. We conducted a systematic review to identify gaps in knowledge about BFI and evaluated efficient methods, highlighting direct observation (40%), combination with pollen assessment (30%) and the exclusive use of the latter (30%). Pollen assessment was more effective, recording four times more interactions than direct observation. We developed an innovative protocol for collecting pollen directly from the bodies of bats, resulting in 124 DNA extractions with concentrations between 0.6 and 6.87 ng/µL. We used primers for amplification of the ITS region, with products between 500 and 550 bp, including a positive control with Ipomoea sp. pollen. The initial protocol proved to be viable for studies using metabarcoding, presenting great potential to advance the identification of bat-pollinated plants in poorly studied regions and in the Carajás FLONA. Aiming to create the possibility of a reference database to identify plant pollen in Carajás. These advances strengthen the understanding of the IMF and promote conservation in these regions

## 1. Introduction

Bats are crucial for ecosystem services, suppressing pests consuming disease vectors like malaria mosquitoes (KEMP *et al.* 2019), and aiding seed dispersal and pollination (KUNZ 2003). Bat pollination, although less common than bird or insect pollination, supports many important plants (Fleming *et al.* 2008), with nectarivorous bats enhancing plant reproductive success by dispersing pollen and maintaining genetic diversity (KUNZ *et al.* 2011).

The Carajás National Forest (FLONA) hosts savanna-like vegetation called “cangas” or ferruginous fields with the world’s highest richness of natural subterranean caves tied to iron ore deposits (PILÓ *et al.* 2015), providing critical shelters for bats (FUREY & RACEY 2016). Large bat colonies contribute organic resources, like guano, to cave fauna (PIMENTEL *et al.* 2022).

Traditional methods for documenting bat-flower interactions involve collecting pollen from bat fur and directly observing bat-flower interactions, which are time-consuming and labor-intensive. Pollen identification requires expert analysis, but environmental DNA and metabarcoding can offer faster and higher-resolution analyses (HARPER *et al.* 2019). In FLONA, plant-invertebrate interaction studies are advancing, but nectar-eating bats remain underexplored, highlighting a knowledge gap. Bat-flower interactions (BFI) can be considered an Eltonian deficit in biodiversity knowledge, and these data deficits hinder biodiversity assessments and conservation (Hortal *et al.*, 2015). This study aims to address this gap by developing protocols for bat capture and pollen sampling.

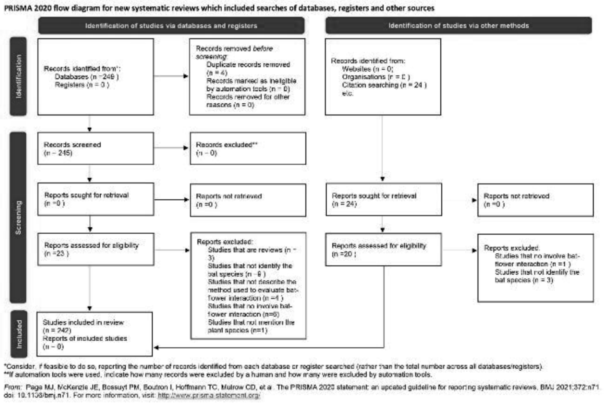
We propose to update the state of bat pollination research and outline a protocol for studying bat-plant interactions in FLONA using metabarcoding tools. We will assess knowledge gaps, identify global techniques for bat-flower interactions, compare capture, and pollen collection methods through a literature review of bat-flower studies.

## 2. Materials and methods

**Literature review and statistical analysis**

A systematic search was conducted on Web of Knowledge, Google Scholar, Scielo, and Scopus for peer-reviewed publications, excluding theses and dissertations. Search terms included ‘bats’ OR ‘Chiroptera’ AND ‘pollination’ OR ‘interaction’ OR ‘bat-flower interaction,’ with equivalents in

Spanish and Portuguese to encompass nectarivorous bat studies. The review covered publications from 1965 to September 2022. Inclusion criteria required publications to: (i) be peer-reviewed; (ii) describe at least one bat-flower interaction; (iii) detail investigation methods; (iv) provide species names of plants and bats; and (v) exclude reviews. The PRISMA flow diagram (PAGE *et al.* 2021) outlined the review process (Figure 1).



*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).
**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/

***Figure 1:*** *The PRISMA diagram illustrates the systematic review of published studies investigating the interactions between flowers and bats. ** Publications that were found by automatic tools.*

Key extracted data included: (1) geographic and temporal context; (2) publication details; (3) habitat type; (4) study design (empirical/experimental); (5) evidence type; (6) bat response level; (7) interaction evaluation methods (observation, pollen collection); (8) pollen collection site (fur, feces, digestive tract); (9) observation techniques; (10) pollen identification methods; (11) plant and bat taxa; and (12) number of interactions.

The interaction network was made considering only the studies made in caves. We used "ggraph" package (Thomas2024) for the interaction network figure 3.To test whether methods influenced the number of interactions, Generalized Linear Models (GLM) were performed. Three analyses compared: (1) observation, pollen evaluation, and their combination (145 studies); (2) pollen samples from fur, feces, and their combination (77 studies); and (3) observation methods (video monitoring, focal observation, or both; 95 studies). Analyses included only undisturbed habitats and caves. Residual dispersion was assessed with DHARMa, applying a negative binomial family in the MASS package. All analyses were conducted in R Studio (R STUDIO 2022).

**Protocol for studying bat-flower interactions in Carajás**

To capture bats in the caves, we use a harp trap that is installed at the entrance to the cavity, late at night (4:30 am), to capture the bats after their feeding period. All the captured bat individuals were released after the sample collection.

Strict methodological rigor is essential to prevent contamination during metabarcoding analysis. All procedures used disposable gloves, and replaced as needed to minimize contamination risks. Two types of samples, feces, and pollen, were collected from each captured bat. For feces, bats were kept in cloth bags for at least one hour to allow defecation. Fecal samples were collected using sterile cotton swabs and stored in 1.5 mL microtubes with absolute ethanol. Pollen samples were collected with moistened swabs (ultrapure water), passed over various body parts (e.g., wings, abdomen, head, mouth, ears, and face), even when pollen was not visible (Figure 3). Multiple swabs were used per bat, and pollen collection was limited to nectarivorous and frugivorous bats. Samples were stored at -20 ºC and later transferred to -80 ºC at the Instituto Tecnológico Vale. Metadata was recorded for each sampling site, including cavity number, capture time, bat activity (entering/exiting), biometric data, sex, and age. Metadata was organized in a digital spreadsheet linked to individual and sample identifiers.

The extraction process began with workspace asepsis, performed in a laminar flow hood cleaned with 2% sodium hypochlorite and 70% ethanol, followed by UV sterilization. Negative controls (water instead of DNA) were included for each extraction round, following the same protocols as DNA samples. DNA extraction used the DNeasy PowerSoil Kit (QIAGEN) for faecal samples and the DNeasy Plant Mini Kit (QIAGEN) for pollen samples, as per manufacturers' protocols, with final elution in 30 µL. DNA quantification was performed using a Qubit® 3.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit. NGS sequencing (Ion Torrent and Illumina MiSeq) targeted the ITS region using primers ITS2F and ITS3R (CHIOU *et al.*, 2007), synthesized by IDT. The ITS region was chosen for its variability between species and extensive representation in plant databases.

PCR amplification was conducted in triplicate for each sample, with negative (water) and positive (DNA from Ipomoea sp. pollen) controls. Reaction mixtures (12.5 µL) included buffer, MgCl2, dNTPs, TBT, DMSO, primers, Taq polymerase, DNA, and water. Thermocycling conditions included initial denaturation (94°C, 3 min), 35 cycles of denaturation (94°C, 1 min), annealing (55.5°C, 1 min), and extension (72°C, 1 min), with a final extension at 72°C for 10 min. Amplicons were assessed via 1% agarose gel electrophoresis and purified with SPRI beads (Agencourt AMPure XP) at a 0.8X ratio.

## 3. Results

**Literature review**

The final dataset comprised 242 studies, selected from an initial total of 269 publications, after the exclusion of 27 articles that did not meet the inclusion criteria (Figure 1). These studies were published in 99 journals, 11 of which accounted for half of the publications, with an emphasis on tropical ecology, botany, and mammal biology. The publications, spanning the period from 1957 to 2022, showed a significant increase after the year 2000.

Most studies were conducted in South America (92), North America (57), Asia (39), and Central America (33), with limited contributions from Oceania (15) and Africa (11). The research covered 43 countries, predominantly Brazil (63), Mexico (50), and Costa Rica (23), while half of the countries had only one study.

Preserved habitats, such as primary forests and undisturbed deserts, accounted for most research (153), whereas 65 studies examined altered environments, including urban areas, plantations, and regenerating forests. Caves were underrepresented, with only 23 studies, primarily in the Neotropics (16), while the Oriental (4), Nearctic (2), and Australasian (1) regions were under-sampled.

Empirical studies dominated (139), followed by mixed empirical-experimental approaches (90), while purely experimental studies were rare (5). Observation was the primary method (170), either alone (96) or combined with pollen analysis (74). Pollen analysis alone was used in 70 studies, while methods like acoustic monitoring and telemetry were rare (2 each).

Pollen collection focused mainly on bat fur (25), followed by combined fur and fecal samples (19) and fecal samples alone (16). When collected from fur, gelatine (30) was the most used medium, followed by jelly (18) and swabs (10), though 29 studies did not specify the method. Conventional microscopy dominated pollen identification (101 studies), whereas scanning electron microscopy (1) and metabarcoding (3) were rare.

Studies using pollen analysis recorded more bat-flower interactions than those relying solely on observation. Combining fecal sample analysis with pollen collection from fur further increased interaction records (Figure 2). However, focal observation and video monitoring did not significantly affect the number of recorded interactions.

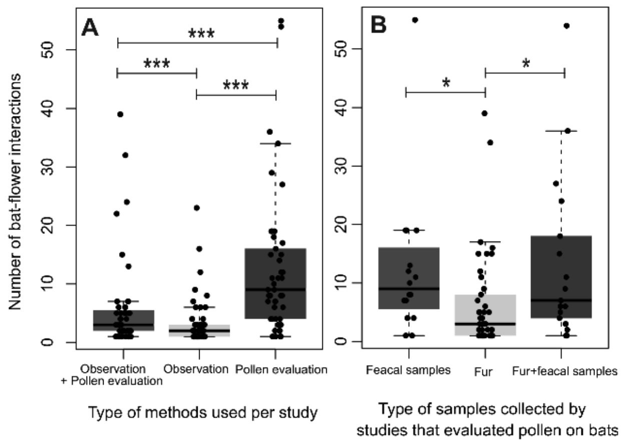


***Figure 2:** A) Number of bat-flower interactions related to the type of method or combination of methods used in each study in our systematic review. B) Number of bat-flower interactions related to the type of samples or combinations.*

Total of 116 bat species and 18 taxonomic groups were identified visiting flowers, with 2,048 recorded interactions. The top 21 species accounted for 70.1% of these interactions (Table 1). *Glossophaga soricina* led with 311 visits (15% of total interactions) and was the most cited (82 studies – 33%). *Eonycteris* spelaea followed (8% of interactions – 169 visits) but was studied less frequently than *Leptonycteris curasoae* (30 studies), *Anoura geoffroyi* (23), A. *caudifer* (21), and *Phyllostomus discolor* (21).

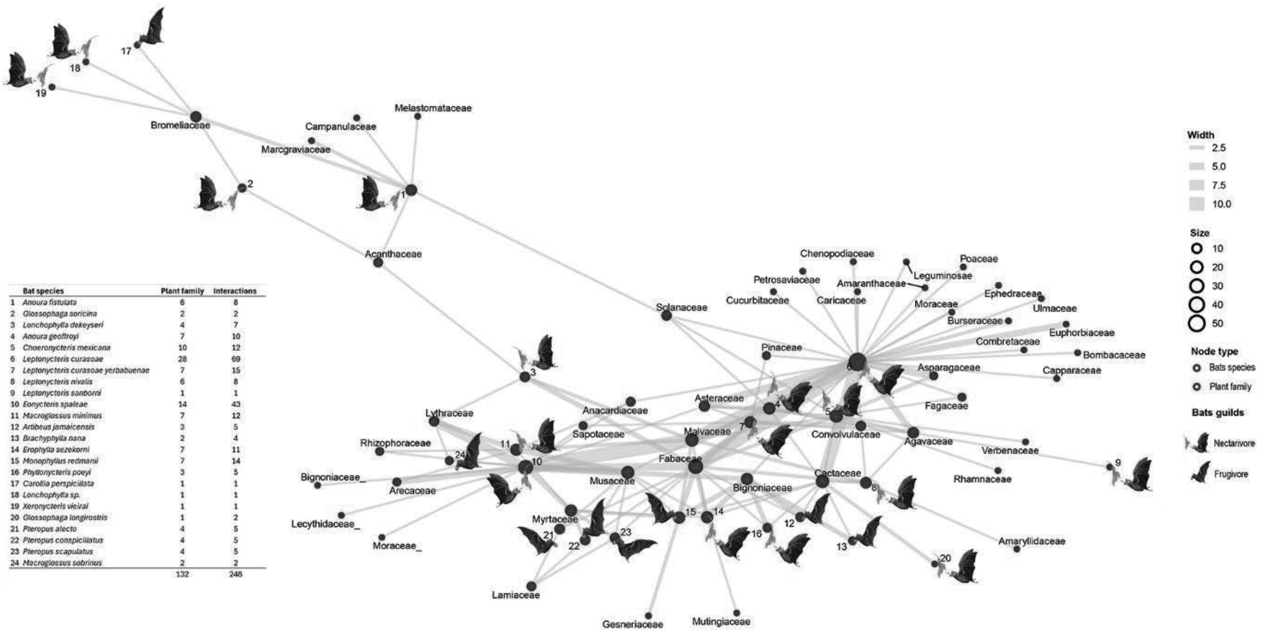
Among cave-dwelling species, 20 visited flowers (Figure 3), with *Choeronycteris mexicana* recording the highest visits (56), followed by *Glossophaga soricina* (28). Nectarivorous bats from the Lonchophyllinae subfamily were underrepresented, with only four studies: *Lonchophylla dekeyseri* (2), *L. mordax* (1), and *Xeronycteris vieirai* (1).

Interactions with 113 plant families were recorded. Thirteen families, interacting with nine bat subfamilies, accounted for 66% of all interactions (1,369). Glossophaginae interacted with all 13 families, with 65% of interactions involving Fabaceae, Malvaceae, Cactaceae, and Bignoniaceae. Lonchophyllinae lacked records for Agavaceae but showed strong interactions with Fabaceae, Malvaceae, and Cactaceae, particularly the latter. Stenodermatinae interacted with nearly all 13 families.

Macroglossinae had the most interactions with Musaceae, contributing 30% of its total. Pteropodinae and Cynopterinae had the fewest, each interacting with only four families, with Myrtaceae as the main one for Pteropodinae. Carollinae interacted mostly with Fabaceae and Malvaceae (70%), while Phyllostominae favored Fabaceae (60%). Rousettinae interacted with seven of the 13 families, with 60% of records involving Fabaceae, Malvaceae, and Bignoniaceae.

| Bat species | Subfamily | Family | Nº of flower interactions | Nº studies |
|---|---|---|---|---|
| *Glossophaga soricina* | Glossophaginae | Phyllostomidae | 311 | 82 |
| *Eonycteris spaleae* | Rousetiinae | Pteropodidae | 169 | 18 |
| *Leptonycteris curasoae* | Glossophaginae | Phyllostomidae | 117 | 30 |
| *Anoura caudifer* | Glossophaginae | Phyllostomidae | 78 | 21 |
| *Anoura geoffroyi* | Glossophaginae | Phyllostomidae | 74 | 23 |
| *Artibeus lituratus* | Stenodermatinae | Phyllostomidae | 70 | 6 |
| *Sturnira lilium* | Stenodermatinae | Phyllostomidae | 66 | 10 |
| *Artibeus jamaicensis* | Stenodermatinae | Phyllostomidae | 57 | 13 |
| *Phyllostomus discolor* | Phyllostominae | Phyllostomidae | 53 | 21 |
| *Choeronycteris mexicana* | Glossophaginae | Phyllostomidae | 49 | 16 |
| *Lonchophylla mordax* | Lonchophyllinae | Phyllostomidae | 45 | 6 |
| *Glossophaga commissarisi* | Glossophaginae | Phyllostomidae | 42 | 12 |
| *Monophyllus redmanii* | Glossophaginae | Phyllostomidae | 39 | 10 |
| *Carollia perspicillata* | Carollinae | Phyllostomidae | 36 | 16 |
| *Cynopterus brachyotis* | Cynopterinae | Pteropodidae | 35 | 8 |
| *Xeronycteris vieirai* | Lonchophyllinae | Phyllostomidae | 34 | 3 |
| *Artibeus fimbriatus* | Stenodermatinae | Phyllostomidae | 33 | 1 |
| *Mystacina tuberculata* | - | Mystacinidae | 33 | 3 |
| *Macroglossus minimus* | Macroglossinae | Pteropodidae | 32 | 8 |
| *Syconycteris australis* | - | Pteropodidae | 32 | 6 |
| *Musonycteris harrisoni* | Glossophaginae | Phyllostomidae | 31 | 5 |
| | | **Total of interactions** | **1436** | |

***Table 1:** Bat species recorded visiting flowers in our literature review, and the number of studies per bat species. Only the bat species with at least 30 interactions with flowers were described in this table.*



| | Bat species | Plant family | Interactions |
|---|---|---|---|
| 1 | *Anoura fistulata* | 6 | 8 |
| 2 | *Glossophaga soricina* | 2 | 2 |
| 3 | *Lonchophylla dekeyseri* | 4 | 7 |
| 4 | *Anoura geoffroyi* | 7 | 10 |
| 5 | *Choeronycteris mexicana* | 10 | 12 |
| 6 | *Leptonycteris curasoae* | 28 | 69 |
| 7 | *Leptonycteris curasoae yerbabuenae* | 7 | 15 |
| 8 | *Leptonycteris nivalis* | 6 | 8 |
| 9 | *Leptonycteris sanborni* | 1 | 1 |
| 10 | *Eonycteris spaleae* | 14 | 43 |
| 11 | *Macroglossus minimus* | 7 | 12 |
| 12 | *Artibeus jamaicensis* | 3 | 5 |
| 13 | *Brachyphylla nana* | 2 | 4 |
| 14 | *Erophylla sezekorni* | 7 | 11 |
| 15 | *Monophyllus redmanii* | 7 | 14 |
| 16 | *Phyllonycteris poeyi* | 3 | 5 |
| 17 | *Carollia perspicillata* | 1 | 1 |
| 18 | *Lonchophylla sp.* | 1 | 1 |
| 19 | *Xeronycteris vieirai* | 1 | 1 |
| 20 | *Glossophaga longirostris* | 1 | 2 |
| 21 | *Pteropus alecto* | 4 | 5 |
| 22 | *Pteropus conspicillatus* | 4 | 5 |
| 23 | *Pteropus scapulatus* | 4 | 5 |
| 24 | *Macroglossus sobrinus* | 2 | 2 |
| | | 132 | 248 |

***Figure 3:** Interaction network of plant families visited by cave bats and which use caves in a complementary way. Table shows the numbers that each bat species corresponds to in the interaction network, number of plant families and number of interactions.*

**Protocol for studying bat-flower interactions in Carajás**

A total of 187 pollen samples and 61 fecal samples from different species of nectar-eating bats were collected in eight caves of FLONA Carajás. We first tested the effectiveness of the DNA extraction kits for each type of sample, pollen and feces. The DNeasy PowerSoil kit was more efficient for feces samples and the DNeasy Plant Mini Kit for pollen samples.

Based on positive DNA extractions for each type of sample, we tested the amplification efficiency of the primers (ITS2F and ITS3R) through the temperature gradient test. The best primer annealing temperature for pollen samples was 55.5°C and for fecal samples it was 56.5°C. We subsequently established an amplification protocol for both samples. During the tests, we observed that the amplification signals from feces samples were stronger than from pollen samples (Figure 4); this pattern is related to the amount of DNA between the samples. DNA was extracted from 124 pollen samples, with DNA concentrations ranging from 0 to 6.87 ng/µL. Of the extracted samples, we performed PCR on 47, with positive amplifications for 16 samples. The amplification of the ITS region was between 500 and 550 bp for all samples, as well as for positive control (flower test 01; Figure 4).

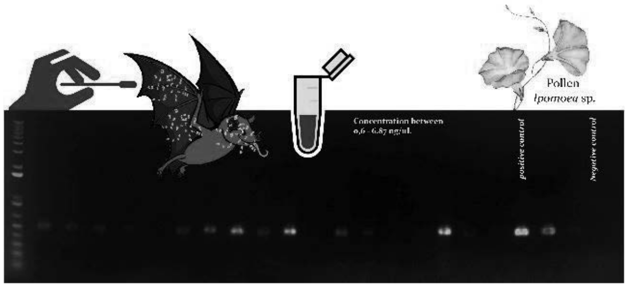


***Figure 4:*** *Agarose gel 1% of pollen samples collected directly from the bodies of bats. The bands represent the amplifications of the ITS marker.*

## 4. Discussion

Our comprehensive literature review identified 242 studies on bat-flower interactions (IBF). From this analysis, we identified knowledge gaps and assessed the most used and effective methods for describing these interactions. Based on these findings, we propose an initial protocol for applying DNA metabarcoding to pollen samples collected from the fur and feces of pollinating bats in the Carajás National Forest (FLONA).

Most studies on bat pollination (72%) have been conducted in the Neotropical region, mainly in Brazil and Mexico. Other under-sampled regions include Australasia (6%), the Afrotropical region (4%), the Palearctic (2%), and the Nearctic (1%). Our results indicate that records are limited to local interactions between specific bat and plant species, generally restricted to one or two study areas. Notably, the northern Neotropics have a high number of data-deficient bat species, while the Oriental region (15%) has the highest density of threatened species (FRICK *et al.* 2020), highlighting the need for further studies with broader geographical coverage.

Tropical and dry forests, deserts, savannas, and coastal areas were the primary environments investigated (78%). The diversity of these ecosystems forms a mosaic where bat-plant interactions play a key role in maintaining tropical biodiversity (NOR ZALIPAH *et al.*, 2016; AZIZ *et al.* 2017; STEWART & DUDASH, 2017). However, increasing landscape modification due to human activities alters plant and bat species composition, impacting mutualistic relationships (MELDRUM *et al.* 2024). Land use changes are among the main factors driving Neotropical biodiversity loss (VOIGT & KINGSTON 2016). Understanding plant-pollinator interactions is crucial for assessing community structure and ecosystem stability in the face of environmental changes (MONTOYA, PIMM & SOLÉ, 2006; KAISER-BUNBURY & BLÜTHGEN, 2015; ZAMORA-GUTIERREZ *et al.*, 2021).

Globally, caves are important refuges for bats, with specific characteristics influencing their use (FUREY & RACEY 2016). The IUCN estimates that 49% of bat species use caves (TANALGO *et al.* 2022). However, studies on BFI involving cave-dwelling bats are scarce (11%) and do not cover critical regions such as the Afrotropical and Indomalayan regions, where several species face severe threats (FRICK *et al.* 2020). These gaps are particularly evident for nectarivorous bats of the subfamily Lonchophyllinae. We identified 20 pollinating species, of which eight substantially depend on caves. Among these, *Leptonycteris nivalis* and *Lonchophylla dekeyseri* are listed as threatened, *Choeronycteris mexicana* and *Leptonycteris yerbabuenae* as near-threatened, *Leptonycteris curasoae* as vulnerable, and *Xeronycteris vieirai* as data deficient. The Brazilian Cerrado, the world's most biodiverse savanna, harbors one of Brazil's few threatened nectarivorous bat species, *L. dekeyseri* (Phyllostomidae, Lonchophyllinae), with limited data on its dietary specialization and foraging patterns (AGUIAR *et al.* 2006). Another relevant hotspot is the Caatinga, the largest dry forest in the Neotropics, home to the little-studied X. vieirai.

BFI has predominantly been described through morphological identification of pollen grains collected from bat fur or feces via microscopy. However, the lack of distinctive morphological traits and the scarcity of reference collections hinder plant species identification. Environmental DNA (eDNA)-based techniques can overcome these limitations but remain underutilised, representing only 1% of studies. eDNA has shown potential for plant species identification from fecal samples (AZIZ *et al.*, 2017;) and bat fur samples (SWIFT *et al.* 2018).

Bats are the most relevant mammalian pollinators responsible for pollinating at least 528 species of angiosperms across 67 families. They play a crucial role in ecosystems, agricultural production, and food security, contributing to the reproduction of ecologically and economically important tropical plants (FLEMING *et al.* 2009). The Neotropical subfamilies Glossophaginae and Lonchophyllinae account for 869 and 143 interactions, respectively. Highly specialized for nectar consumption (DATZMANN 2010), these subfamilies pollinate 549 plant species across 191 genera and 62 families, showing generalist behavior regarding chiropterophilous plants (MUCHHALA *et al.* 2024). Meanwhile, pteropodid bats, which are frugivorous and nectarivorous, recorded 451 interactions and pollinated 168 species in 100 genera and 41 families. The most relevant botanical families for bats include Malvaceae, Musaceae, Fabaceae, and Cactaceae (PIECHOWSKI *et al.* 2010). Despite the importance of this ecosystem service, bat pollination remains poorly understood and requires further research, particularly on endemic nectarivores of Lonchophyllinae.

To advance this research, we optimized a protocol for collecting and amplifying ITS2 and 3 markers for sequencing via metabarcoding. Standardized collection was essential for obtaining adequate DNA, resulting in a high amplification success rate for both pollen and fecal samples. Although methodological challenges exist in plant DNA amplification (FAZEKAS *et al.* 2008), our results show that appropriate adjustments can improve this method's efficiency.

## 5. Conclusion

Our review highlights the importance of bat pollination and the knowledge gaps surrounding this interaction, bringing a new perspective on filling the Eltonian gap. The use of eDNA has great potential to advance the identification of pollinated plants, particularly in understudied regions such as caves. The optimized DNA metabarcoding protocol proved to be effective in analyzing pollen and feces in bats, representing a promising tool to improve the understanding of these interactions. Future studies should prioritize broader geographic coverage and the use of innovative techniques to fill the existing gaps and strengthen the conservation of bats and the plants that depend on them.

## Acknowledgments

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# Diversidade temporal das comunidades de morcegos cavernícolas

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## Abstract

Understanding the factors that structure bat communities across space and time is essential for community ecology. This study investigated the temporal beta diversity of bats in caves located in Conceição do Mato Dentro, Minas Gerais, Brazil, between 2018 and 2024. Ninety-one caves distributed across three geomorphological units were analyzed, with semiannual sampling to evaluate community composition in relation to climatic and seasonal variables. Thirteen species from the family Phyllostomidae were identified, with variations in composition across locations and time periods. Temporal beta diversity (TBI) indicated that the Serra da Ferrugem locality exhibited greater dynamism, with species gains and losses influenced by climatic variables such as precipitation, temperature, and humidity. Mixed linear models showed a positive relationship between species gains and continuous climatic variables, but no such relationship was observed for species losses or TBI.The results indicate that local conditions and climatic variations play crucial roles in the dynamics of bat communities, highlighting the importance of environmental heterogeneity and resource availability. This study advances the understanding of the interactions between abiotic and biological factors that shape bat assemblages in karst ecosystems.

## 1. Introdução

Uma das questões centrais em ecologia de comunidades é compreender como as espécies estão distribuídas no espaço e no tempo, e a maneira pela qual estes agrupamentos podem ser influenciados pelo ambiente abiótico e pelas interações entre as populaçoes de espécies (LEIBOLD *et al.*, 2004; BEGON *et al.*, 2006; VELLEND, 2010). No contexto espacial, WHITTAKER (1972) introduziu o conceito de diversidade beta para descrever variações na composição da comunidade entre locais em uma região, complementando as definições de diversidade alfa, que representa a diversidade local, e diversidade gama, que se refere à diversidade regional (WHITTAKER, 1960; MITTELBACH & MCGILL, 2019). A diversidade beta é uma métrica essencial para investigar as mudanças na composição de espécies em diferentes escalas espaciais e temporais., podendo ser particionada em processos de substituição de espécies (*turnover*) e ganho ou perda de espécies (*nestedness*) (BASELGA, 2010; LEGENDRE & GAUTHIER, 2014; SHIMADZU *et al.*, 2015; SOCOLAR *et al.*, 2016). Esses processos são especialmente relevantes para entender as respostas das comunidades às variações ambientais e às interações ecológicas, sobretudo em ecossistemas sujeitos a sazonalidade ou impactos antropogênicos (LEGENDRE, 2019). Morcegos desempenham um papel ecológico crucial devido às várias funções que exercem nos ecossistemas, como polinização, dispersão de sementes e controle de populaçoes de insetos (KUNZ *et al.*, 2011). Muitas espécies utilizam cavernas como abrigo, local de alimentação e reprodução, o que torna esses ambientes essenciais para a sobrevivência das espécies (GNASPINI & TRAJANO, 2000). Devido à variedade de estratégias alimentares dos morcegos neotropicais, que incluem frugivoria, nectarivoria e insetivoria (PATTERSON *et al.*, 2003), a composição dessas assembleias é frequentemente influenciada por variações sazonais (MELLO, 2009; STEVENS & AMARILLA-STEVENS, 2012, STEVENS, 2013; BOBROWIEC *et al.*, 2014; ORTÊNCIO-FILHO et al., 2014). Estudos indicam que a diversidade beta está relacionada à heterogeneidade ambiental, mostrando que os morcegos respondem diretamente à estrutura do habitat e aos gradientes ambientais (LÓPEZ-GÓNZALEZ, 2004, STRADA-VILLEGAS *et al.*, 2012; LÓPEZ-GÓNZALEZ *et al.*, 2015, VARZINCZAK *et al.*, 2018). No entanto, pouco se sabe sobre a influência temporal e sazonal desses fatores na composição das comunidades. Essa lacuna é particularmente relevante, pois as condições ambientais no entorno de cavernas podem variar significativamente entre as estações climáticas, influenciando diretamente a dinâmica e a composição das comunidades de morcegos. Assim, o presente estudo tem como objetivo avaliar se os padrões de diversidade temporal das comunidades de morcegos em cavernas varia entre estações climáticas e entre os diferentes locais de estudo. Ao integrar métricas de diversidade e fatores climáticos, espera-se avançar na compreensão das forças que estruturam as comunidades de morcegos ao longo do espaço e do tempo.

## 2. Materiais e Métodos

**Local de estudo**

O estudo foi realizado em área protegidas da Anglo American Minério de Ferro Brasil S/A no município de Conceição do Mato Dentro, Minas Gerais, Brasil (Fig.1). A área de estudo está localizada na Serra do Espinhaço Meridional. O clima regional é classificado como subtropical úmido, com temperatura média anual de 20,6 °C e a precipitação média anual é de 1.424,4 mm. O período chuvoso ocorre entre outubro e março. Já o período seco se estende entre abril e setembro (INMET, 2024). Foram amostradas 91 cavernas em três Unidades Geomorfológicas (UG): 10 cavernas na UG Serra da Ferrugem, 41 na UG Serra de Santo Antônio e 40 na UG Serra do Cangueiro. As amostragens ocorreram semestralmente entre abril de 2018 e maio de 2024, totalizando 13 campanhas. Cada caverna foi visitada uma vez por campanha, com incursões diurnas e captura de morcegos utilizando puçás. As identificações seguiram as chaves e descrições de GARDNER (2008) e DÍAZ *et al.* (2016), com taxonomia baseada em GARBINO *et al.* (2024). Exemplares foram coletados para formar uma coleção de referência, depositados na Coleção de Mamíferos do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG).

**Variáveis climáticas**

Dados históricos mensais de temperatura, precipitação e umidade relativa do ar (2018 a 2024) foram obtidos da estação convencional de Conceição do Mato Dentro (83589), operada pelo INMET.

**Partição da diversidade beta temporal.**

A diversidade beta temporal foi analisada utilizando o *Temporal Beta-diversity Index* (TBI). Também utilizamos a dissimilaridade de Jaccard para dados de incidência para calcular as dissimilaridades entre pares de amostragens. Os dados de todos os sítios amostrais de cada localidade foram somados para representar uma amostra por localidade e então ser avaliada temporalmente. Para tal, nós comparamos a variação na composição ao longo das campanhas amostral em relação à primeira amostral (T1 × T2, T1 × T3, ... T1 × T13).

**Modelos estatísticos**

Modelos Lineares Generalizados (GLMs) foram utilizados para testar diferenças nos padrões temporais da composição de morcegos (variáveis resposta: TBI, ganhos e perdas de espécies) em relação à localidade, estação e interação entre os dois fatores (variáveis preditoras). Modelos Lineares Generalizados de Efeitos Mistos (GLMMs) foram usados para testar a relação entre os padrões temporais da composição de morcegos (variáveis resposta: TBI, ganhos e perdas de espécies) e variáveis climáticas (precipitação, temperatura média e umidade relativa do ar), considerando a identidade das localidades como efeito aleatório nos modelos, a fim de verificar uma relação global independentemente de cada localidade.

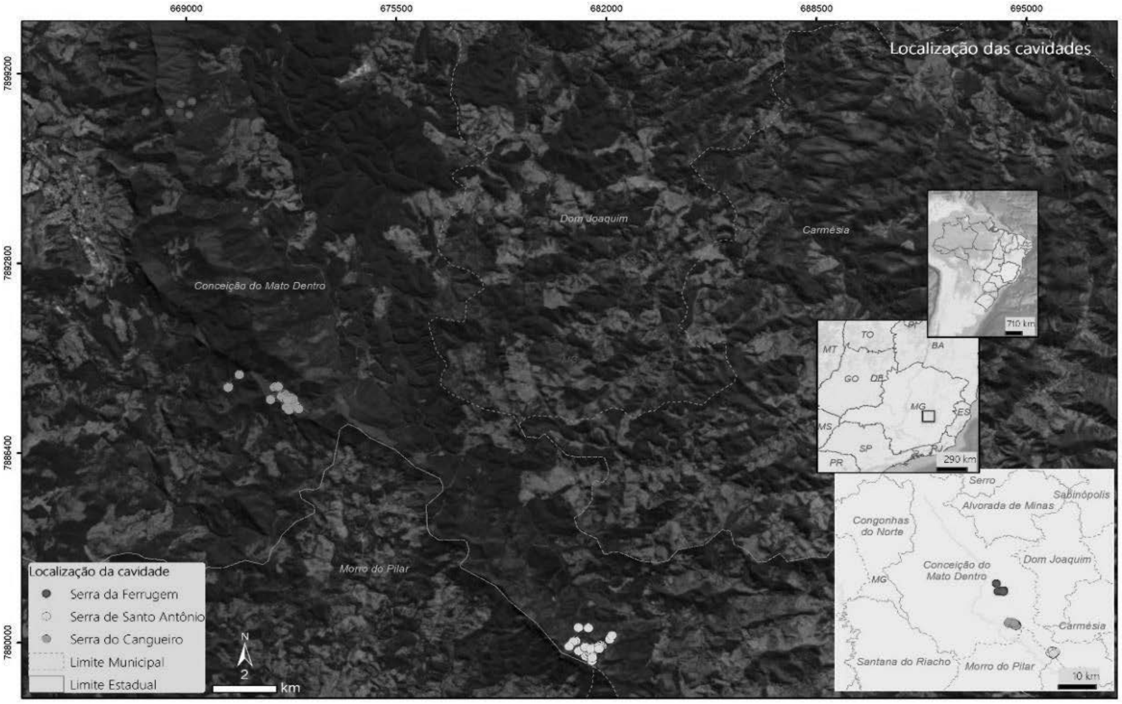

***Figure 1:*** *Location map of caves in the three study areas.*

## 3. Resultados

Foram realizados 501 registros e identificadas 13 espécies de morcegos, todas da família Phyllostomidae: 157 registros (31,3%) e nove espécies (69,2%) na Serra da Ferrugem (SFE), 198 registros (39,5%) e 11 espécies (84,6%) na Serra do Cangueiro (SCA) e 146 registros (29,1%) e 10 espécies (76,9%) na Serra de Santo Antônio (SSA). As espécies com maiores números de registros foram: *Glossophaga soricina* (31,3%), *Mimon bennettii* (17,8%), *Carollia perspicillata* (Carollinae; 8,8%), *Chrotopterus auritus* (8,4%) e *Micronycteris microtis* (8,2%), que representaram juntas 74,5% do total de registros.

A partição da diversidade beta temporal mostrou que a localidade SFE teve as maiores variações de TBI ao longo das amostragens (comparações de cada amostragem em relação à primeira) (Fig.2). De forma interessante, nesta localidade os valores de TBI foram os mesmos (0,25) entre T1 × T2, T1 × T6 e T1 × T11, coincidindo com períodos de chuva e mais representados por ganhos do que perdas de espécies. Os maiores valores de TBI foram (0,83) ocorreram nas coletas 5 e 9, dominados por ganhos de espécies. Em geral, tanto ganhos como perdas foram importantes para guiar a variação temporal na composição de morcegos na localidade SFE. Na localidade SCA, foram observados valores elevados de TBI (0,67), seguidos por um declínio até a 5ª coleta e certa estabilidade (~0,30) até a 9ª coleta, representados tanto por perdas como ganhos de espécies.

A 10ª coleta apresentou o maio valor de TBI (0,80), representado exclusivamente por perda de espécies, seguidos por acentuado declínio das coletas seguintes (Fig. 2). Na localidade SSA, os valores de TBI foram similares nas primeiras amostragens (0,40), totalmente representados por perda de espécies. Após um leve ganho de espécies, as perdas fizeram que o valor de TBI voltasse ao inicial (7ª amostragem). Perdas pronunciadas de espécies dominaram na fase final de amostragem (amostragens 8, 10, 12 e 13), as quais fizeram que o valor de TBI retornasse a um patamar próximo ao valor inicial (0,5) (Fig.2).

Os GLMs revelaram que os valores de TBI não diferiram entre localidades, estações e a interação entre esses dois fatores (Fig. 3). Ganho e perda de espécies ao longo do tempo diferiram entre localidades, mas não entre estações. A localidade SFE apresentou maiores ganhos de espécies em comparação com as demais localidades, enquanto a localidade SSA teve maiores perdas de espécies do que SCA apenas, independentemente da estação (Fig.3). Os GLMMs não detectaram relação entre as variáveis climáticas e os valores de TBI e perda de espécies (Fig.4). Por outro lado, os valores de ganho de espécies estiveram positivamente relacionados com as três variáveis climáticas (Fig.4).

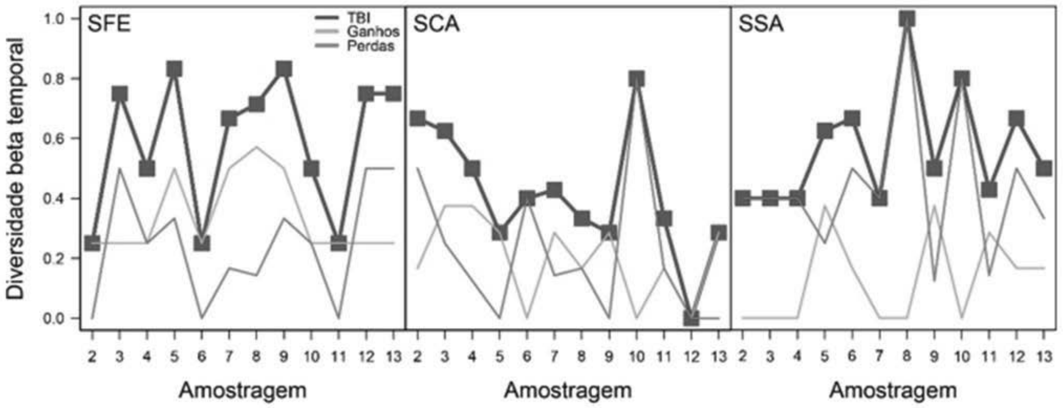

***Figure 2:*** *Temporal beta diversity patterns and components of species gains and losses; samples were compared in relation to the first collection campaign, throughout the 13 samplings (April 2018 to May 2024) of bat assemblages collected in caves from three locations in the municipality of Conceição de Mato Dentro, Minas Gerais, Brazil. SFE = Serra da Ferrugem; SCA = Serra do Cangueiro; SSA = Serra de Santo Antônio.*

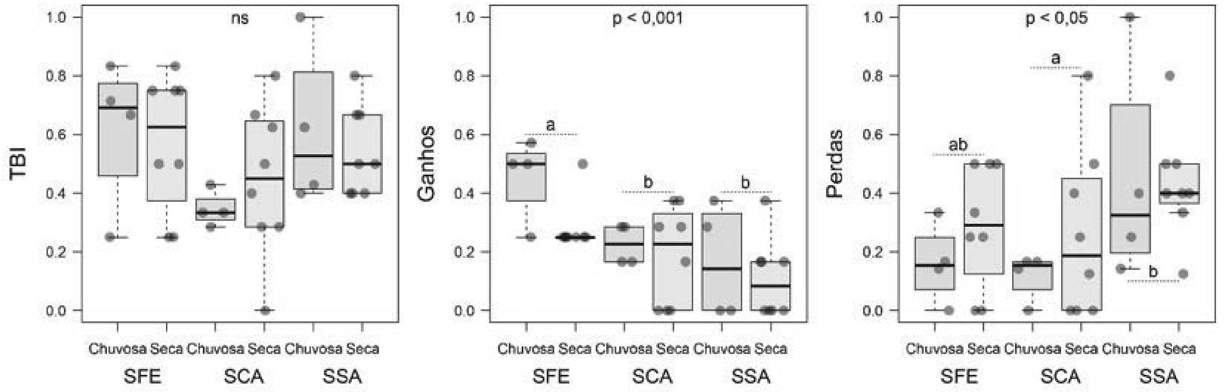

***Figure 3:*** *Distribution of TBI values, gain and loss of bat species in relation to location and sampling season. Bat species were collected during 13 campaigns (April 2018 to May 2024) in caves from three locations in the municipality of Conceição de Mato Dentro, Minas Gerais, Brazil. SFE = Serra da Ferrugem Natural Monument; SCA = Serra do Cangueiro; SSA = Serra de Santo Antônio. Observed values are represented by circles. 'ns' means non-significant statistical model. Different letters indicate statistical differences in the contrast analysis.*

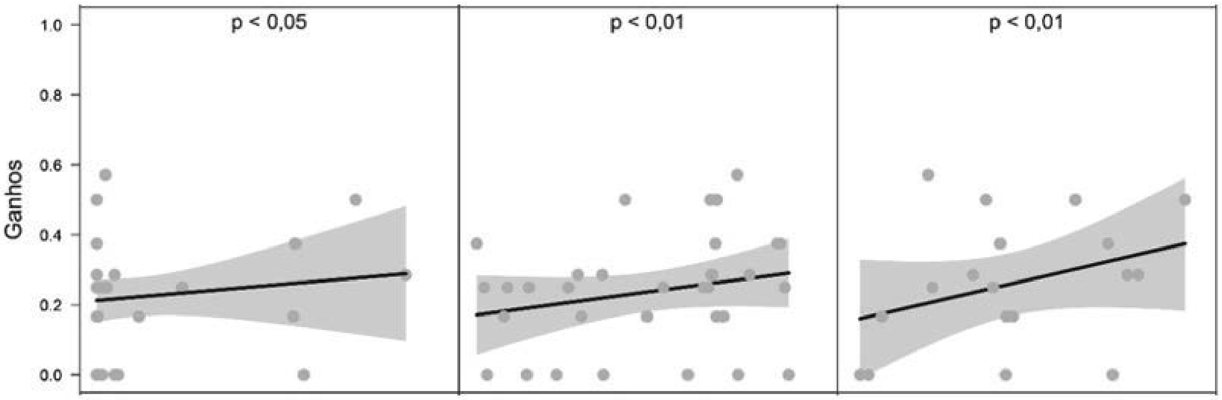

***Figure 4:*** *Relationship between TBI, gain and loss of bat species and the climatic variables of precipitation, average temperature and relative humidity. The bat species were collected during 13 campaigns (April 2018 to May 2024) in caves of three locations in the municipality of Conceição de Mato Dentro, Minas Gerais, Brazil. SFE = Serra da Ferrugem; SCA = Serra do Cangueiro; SSA = Serra de Santo Antônio. Observed values are represented by circles. 'ns' means non-significant statistical model. The polygon next to the model line represents the 95% confidence interval.*

## 4. Discussão

Todas as espécies registradas pertencem à família Phyllostomidae. No Brasil, esta é a família mais diversa com 95 espécies, o que corresponde a mais da metade dos morcegos conhecidos no país (GARBINO *et al.*, 2024).

Os índices de diversidade beta temporal (TBI) revelaram diferentes dinâmicas na composição das comunidades de morcegos nas três localidades, refletindo variações em ganhos e perdas de espécies ao longo do tempo. As maiores flutuações nos valores de TBI em SFE, compostas por ganhos e perdas moderadas, destacam uma alta variabilidade na composição da comunidade nesse local. Os períodos de maior TBI coincidiram com eventos dominados por ganhos de espécies, especialmente nas estações chuvosas. Em contraste, na localidade SSA a dinâmica da composição da comunidade foi marcada por perdas de espécies, independente da estação climática. Já em SCA, a dinâmica nos valores de TBI foi mais estável. A importância de fatores sazonais direcionando os padrões de atividades dos morcegos neotropicais foram demonstrados em outros estudos (BOBROWIEC *et al.*, 2014; ORTÊNCIO-FILHO *et al.*, 2014). Além dos fatores sazonais, outros determinantes ambientais específicos de cada localidade podem estar relacionados às diferenças observadas nos padrões de TBI. Esses resultados podem indicar um dinamismo das comunidades de morcegos em cavernas, com alterações frequentes ao longo do tempo, moldadas por diferentes fatores como a estrutura do habitat (ESTRADA-VILLEGAS *et al.*, 2012; LÓPEZ-GONZÁLEZ *et al.*, 2015, LOPÉZ-BAUCELLS *et al.*, 2022) ou a disponibilidade de alimentos associada a fenologia da flora local (MELLO, 2009). Os modelos lineares generalizados demonstraram que os ganhos e perdas de espécies apresentaram diferenças marcantes entre as localidades. Especificamente, a localidade SFE registrou maiores ganhos de espécies, enquanto em SSA houve maiores perdas. A ausência de diferenças sazonais indica que os ganhos e perdas de espécies não são modulados pela estação climática. É possível que condições ambientais locais ou características específicas dessas áreas, como maior heterogeneidade de recursos, favoreçam ou limitam a permanência e colonização de espécies de morcegos em cavernas. Embora os modelos lineares generalizados não tenham indicado influência das estações climáticas, o modelos lineares generalizados de efeitos mistos apresentaram uma relação positiva significativa ao considerar as variáveis climáticas contínuas (temperatura, umidade e precipitação), refletindo uma diferença na capacidade explicativa entre as variáveis categóricas (seca e chuva) e contínuas. A análise dos GLMMs revelou uma relação significativa entre as variáveis climáticas e o ganho de espécies ao longo do tempo, mas não com a o TBI (índice de diversidade beta temporal) ou perda de espécies. Especificamente, o ganho de espécies apresentou uma correlação positiva com precipitação, temperatura média e umidade relativa do ar, indicando que mudanças nas condições climáticas, como aumento da temperatura e umidade, podem influenciar positivamente a colonização ou aparecimento de novas espécies de morcegos. De acordo com HEITHAUS *et al.*, (1975), nas regiões tropicais, a quantidade de morcegos frugívoros tende a ser mais elevada durante os períodos caracterizados por calor e alta umidade. Essa maior ocorrência pode ser atribuída à maior disponibilidade de recursos alimentares, conforme apontado FLEMING (1986). Estudos realizados por MELLO (2009) e ORTÊNCIO-FILHO *et al.*, (2010) indicaram que as variações sazonais na temperatura exercem influência na estruturação das comunidades de morcegos, possivelmente relacionados a alterações nos padrões de uso de sítios de alimentação, decorrentes da redução na disponibilidade de recursos tróficos no local.

## 5. Conclusão

O estudo demonstrou que as comunidades de morcegos que utilizam cavernas apresentam alta variabilidade temporal em suas composições, influenciadas principalmente por fatores climáticos e características locais da paisagem. A diversidade beta temporal destacou diferenças entre localidades. Além disso, os ganhos de espécies mostraram-se positivamente relacionados à precipitação, temperatura e umidade, reforçando o papel do clima na dinâmica dessas comunidades, além de destacar a importância de integrar métricas de diversidade com fatores ambientais para a compreensão e conservação das espécies de morcegos e do ecossistema cavernícola.

## Agradecimentos

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# Behaviors in light and darkness: a review of amblypygid behavior in caves and surface habitats

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## Abstract

Research on the behavior of cave-dwelling amblypygi remains notably scarce. A bibliographic review conducted through the Web of Science database focused on studies of amblypygi behavior in both epigean and hypogean habitats. The results revealed a significant disparity between the two, with far more research available on epigean amblypygi. The review also categorized the types of behaviors studied, with navigation and reproductive behaviors being the most commonly examined. The Phrynidae family was the most frequently observed, particularly in epigean habitats. While studies on amblypygi behavior have gained increased attention over the years, a clear gap remains in understanding the behavioral biology of these animals, particularly in cave environments. Cave-dwelling specimens are notably underrepresented in research, leading to a limited understanding of their behavior compared to their epigean counterparts.

## Résumé

La recherche sur le comportement des amblypyges cavernicoles reste remarquablement rare. Une revue bibliographique effectuée à travers la base de données Web of Science s'est concentrée sur les études portant sur le comportement des amblypyges dans les habitats épigés et hypogés. Les résultats ont révélé une disparité significative entre les deux, avec une documentation beaucoup plus abondante sur les amblypyges épigés. La revue a également classé les types de comportements étudiés, les comportements de navigation et de reproduction étant les plus fréquemment examinés. La famille des Phrynidae a été la plus souvent observée, en particulier dans les habitats épigés. Bien que les études sur le comportement des amblypyges aient gagné en visibilité au fil des ans, il existe encore une lacune claire dans la compréhension de leur biologie comportementale, en particulier dans les environnements souterrains. Les spécimens cavernicoles sont nettement sous-représentés dans la recherche, ce qui limite notre compréhension de leur comportement par rapport à leurs homologues épigés.

## 1. Introduction

Caves are among the most unique ecosystems on Earth (MAMMOLA *et al.*, 2022), characterized by complete darkness and a tendency toward oligotrophy (CULVER & PIPAN, 2009). These extreme conditions impose significant selective pressures on the physiological and behavioral traits of organisms inhabiting subterranean environments (CULVER & PIPAN, 2009), with behavioral adaptations often being the first to emerge when species encounter such novel conditions (MAYR, 1963).

One group of arachnids capable to thrive in these challenging environments is the order Amblypygi. Many species of Amblypygi have adapted to and become abundant in cave systems. Their antenniform legs, which allow them to navigate and perceive their surroundings without relying on vision, facilitate a range of unique behaviors (WEYGOLDT, 2000).

Despite their importance in understanding the behavior of cave-dwelling animals, research focusing on subterranean amblypygids remains limited. To address this gap, we conducted a bibliographic review to examine behavioral differences between surface-dwelling and cave-dwelling amblypygids.

## 2. Materials and methods

We conducted a bibliographic review of studies focusing on , utilizing the Web of Science database as our primary source.

The search was guided by two primary keywords: "*Amblypygi behavior*" and "*whip-spider behavior*". These terms were first searched independently and then in combination with the term "cave" to refine the results.

We selected studies that addressed any behavioral aspects of amblypygids, thoroughly analyzing each work to ensure its relevance and alignment with our research objectives.

The data extracted from the selected studies were organized into a table, categorized by title, study methodology, type of behavior, species analyzed, and habitat type (epigean or hypogean).

All graphical representations of the data were generated using R software, version 4.2.3.

## 3. Results

A search conducted in the Web of Science database identified 62 studies relevant to the keywords and objectives of our bibliographic review.

The behaviors analyzed in these studies were categorized based on their frequency, with the most commonly studied being navigation (26%), reproductive behavior (24%), and agonistic/predatory behavior (11%).

The habitat types of the analyzed animals were also recorded, revealing that 83% of the studies focused on epigean habitats, while only 17% investigated hypogean habitats, as reported in the reviewed articles.

The species mentioned in the articles were linked to the study modality (in situ or ex situ), showing a higher prevalence of ex situ studies, with species from the Phrynidae family being the most frequently studied (Figure 1). The relationships between the identified categories are illustrated in Figure 2.

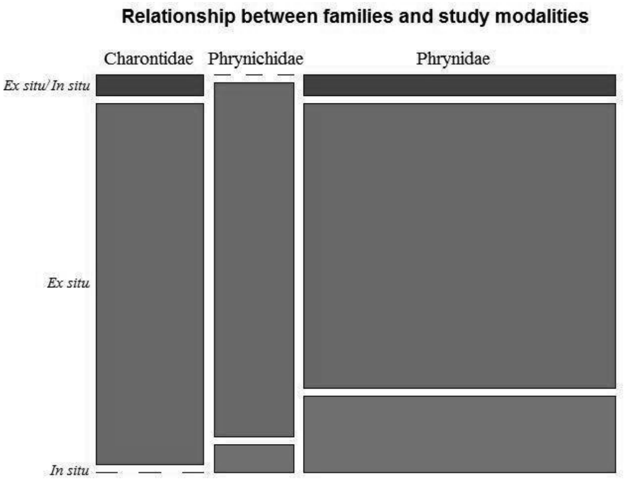

***Figure 1:*** *A mosaic chart displaying the proportionate distribution of study modalities across the families of amblypygi, providing a visual representation of the relative sizes and relationships within the dataset.*

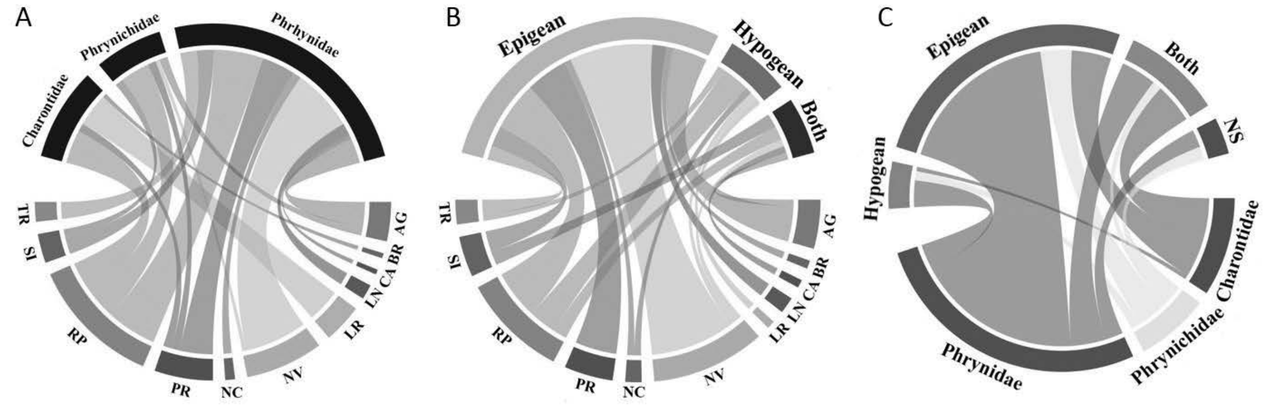

***Figure 2:*** *Chord diagrams that illustrates the interrelationships between (A) the type of behaviors, at the bottom, and the families, at the top; (B) the type of behaviors, at the bottom, and the habitats, at the top; (C) the families, at the bottom, and the habitats, at the top. AG = Agonistic; BR= behavioral repertory; CA = Cannibalism; LN = Learning; LR = Locomotor rhythm; NV = Navigation; NC = Necrophagy; NS = not specified; PR = Predation; RP = Reproductive; SI = Social interactions; TR = Territorialism.*

## 4. Discussion

According to Chapin & Hebets (2016), significant foundational studies on the behavioral biology of amblypygids have been developed over recent decades, providing an essential basis for further research. While amblypygid behavior has been the subject of numerous studies, the authors highlight its substantial untapped research potential.

Based on the data collected, certain aspects of amblypygid behavior, particularly navigation and reproduction, are relatively well-studied. These two behaviors are likely more frequently examined due to the feasibility of conducting controlled ex situ experiments. However, a noticeable gap emerges when focusing on cave-dwelling amblypygids, with only 17% of studies targeting subterranean species. Chapin (2015) addressed this gap by comparing behavioral traits such as hunting, aggression, and vigilance between epigean and hypogean specimens.

Such studies are crucial for understanding the behavioral differences between surface and cave-dwelling species, emphasizing the need for further research into the unique behavioral adaptations of amblypygids to subterranean environments. This emerging field has the potential to shed light on the ecological roles of amblypygids within cave ecosystems.

The results of this review also reveal a notable diversity of families, genera, and species analyzed in the reviewed studies. The Phrynidae family stands out as the most prominently studied group, particularly in epigean habitats. This underscores the need for increased focus on less-studied species and families, especially those inhabiting caves, to deepen our understanding of these important predators and their ecological significance in subterranean environments.

## 5. Conclusion

It can be concluded that while research on amblypygi behavior has garnered increasing attention over the years, a significant gap persists between their behavioral biology and our current understanding. Cave-dwelling specimens remain notably underrepresented in studies compared to their epigean counterparts, leading to a limited comprehension of their behaviors. Among the behavioral aspects investigated, navigation and reproduction are the most frequently studied, with the Phrynidae family being the most commonly represented in the reviewed literature.

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# Utilizar ou morar: o que a quiropterofauna das cavernas de Natalândia-MG nos sugere sobre uso sazonal de abrigos e sua relevância?

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## Abstract

In the present study we carried out a survey of bats present in four caves recently cataloged by the Speleological Group of the University of Brasília in Natalândia/MG. A total of 82 individuals of 11 species were captured. The most abundant species, Artibeus planirostris, is not commonly found in caves and the fact that most individuals are in the reproductive stage suggests the use of the cavities as maternity or reproduction roosts. Sampling in other seasons can reveal whether roost use is continuous or seasonal, which underscores the importance of temporal sampling to detect species that do not use caves all year round. The three species of vampire bats in the region were also sampled. The conservation of the karst area and the environment around the cavities is of fundamental importance for the preservation of these species of bats and of all the individuals that depend on the energy introduced into the cave by these individuals.

## Résumé

Dans la présente étude, nous avons réalisé une étude des chauves-souris présentes dans quatre grottes récemment cataloguées par le Groupe Spéléologique de l'Université de Brasilia à Natalândia/MG. 82 individus de 11 espèces ont été capturés. L'espèce la plus abondante, Artibeus planirostris, ne se trouve pas couramment dans les grottes et le fait que la plupart des individus soient en phase de reproduction suggère l'utilisation des cavités comme maternités ou sites de reproduction. L'échantillonnage à d'autres saisons peut révéler si l'utilisation des abris est continue ou saisonnière, ce qui souligne l'importance de l'échantillonnage temporel pour détecter les espèces qui n'utilisent pas les grottes toute l'année. Les trois espèces de chauves-souris vampires de la région ont également été échantillonnées. La conservation de la zone karstique et de l'environnement autour des cavités est d'une importance fondamentale pour la préservation de ces espèces de chauves-souris et de tous les individus qui dépendent de l'énergie introduite dans la grotte par ces individus.

## Resumen

En el presente estudio, realizamos un censo de murciélagos presentes en cuatro cuevas recientemente catalogadas por el Grupo Espeleológico de la Universidad de Brasilia en Natalândia/MG. Se capturaron 82 individuos de 11 especies. La especie más abundante, Artibeus planirostris, no se encuentra comúnmente en cuevas y el hecho de que la mayoría de los individuos se encuentren en etapa reproductiva sugiere el uso de cavidades como salas de maternidad o lugares de reproducción. El muestreo en otras estaciones puede revelar si el uso de refugio es continuo o estacional, lo que resalta la importancia del muestreo temporal para detectar especies que no utilizan cuevas durante todo el año. También se muestrearon las tres especies de murciélagos vampiros de la región. La conservación de la zona kárstica y del entorno circundante a las cavidades es de fundamental importancia para la preservación de estas especies de murciélagos y de todos los individuos que dependen de la energía introducida por estos en la cueva.

## 1. Introdução

As cavidades naturais são importantes abrigos para morcegos, os vertebrados com maior sucesso na utilização desses habitats (FUREY & RACEY, 2016). Além de moradia, esses ambientes conferem proteção contra predadores e rigor climático, além de garantir o contato social intraespecífico (TRAJANO, 1984). Os invertebrados troglóbios se mantêm utilizando o guano de morcegos (GNASPINI & TRAJANO, 2000; SALGADO *et al.*, 2014), que tem relevante papel no aporte energético desse tipo de ecossistema (FERREIRA *et al.*, 2007).

As cavernas são abrigos de diversas espécies de morcegos que contribuem com serviços ecossistêmicos que impactam a economia humana, como a dispersão de sementes, a polinização e supressão de pragas agrícolas (KUNZ *et al.* 2011; MEDELLIN *et al.* 2017; AGUIAR *et al.*, 2021). BERNARD et al. (2012) discutem que entre os tópicos mais relevantes para elaboração de medidas para conservação de morcegos no Brasil estão o status de proteção de cavernas brasileiras e as lacunas no conhecimento da distribuição das espécies. Conhecer a fauna associada às cavernas é fundamental, pois esses levantamentos são importantes ferramentas para a tomada de decisões nos processos de licenciamento ambiental e seleção de áreas prioritárias para conservação da biodiversidade (GUIMARÃES & FERREIRA, 2015).

O município de Natalândia é uma área com alta potencialidade espeleológica (JANSEN *et al.*, 2012) e com ocorrência de registros arqueológicos (painéis, pictografias, material lítico e/ou cerâmico). A principal vegetação encontrada na área é a mata seca ou floresta estacional, associada a afloramentos carbonáticos, sendo as fitofisionomias presentes a floresta estacional semidecídua montana e a savana arborizada (IBGE, 2022), a qual ocorrem tipicamente barrigudas, aroeiras, cipós, gameleiras, angicos, ipês, aráceas, cactáceas, e também espécies urticantes ou espinhosas (EMBRAPA, 2023). Os primeiros estudos do GREGEO na região de Natalândia-MG foram realizados em 1992, com a descoberta de 11 cavidades, e desde então algumas expedições foram realizadas ao longo dos anos com o intuito de ampliar e desenvolver os estudos espeleológicos na região.

Esta é a primeira amostragem de morcegos do município de Natalândia e os nossos achados nos levaram a questionar sobre o uso das cavidades como abrigo definitivo ou temporário para reprodução, logo, o objetivo deste estudo foi conhecer os morcegos que habitam quatro cavernas de Natalândia e discutir a importância de conservação dessa área que pode servir de abrigo maternidade para a espécie *Artibeus lituratus*.

## 2. Materiais e métodos

### 2.1. Área de Estudo

A região (Figura 1) está inserida no contexto morfoestrutural das Cristas de Unaí, caracterizada por um alinhamento de serras, intercaladas por áreas rebaixadas e planaltos (XAVIER, 2007).

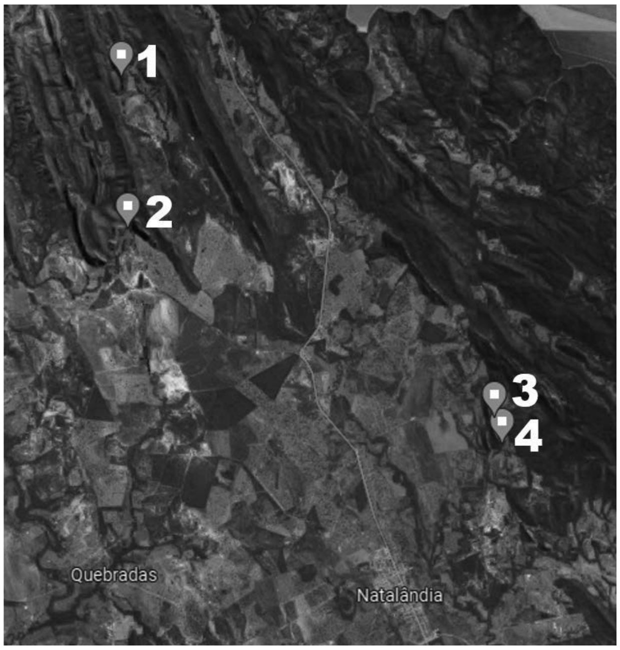


***Figura 1 :*** *Cavernas amostradas. 1 - Gruta do Ancião; 2 - Gruta Seis Bocas; 3 - Gruta Lapa da Pantera; e 4 - Gruta dos Meandros. A área do mapa compreende de 16°24’51.6”S / 46°34’00.2”W até 16°30’47.4”S / 46°27’38.9”W.*

Nestas sequências de serras e morros alinhados a aproximadamente NW-SE, são encontradas as rochas carbonáticas, atribuídas ao Grupo Paranoá; e os vales formados entre esses altos topográficos são constituídos essencialmente por rochas pelíticas do Grupo Bambuí. Ambas as áreas estão inseridas em contexto com ocorrência de carbonatos com alto potencial espeleológico do subgrupo Paraopeba e Formação Sete Lagoas (BAPTISTA *et al.*, 2015). Nos trechos com ocorrência de calcários, desenvolvem-se formas cársticas descobertas com presença de sumidouros, grutas, cavernas e dolinas, onde também foram observados sítios arqueológicos em abrigo sobre-rocha.

Na região, foram amostradas quatro cavidades: Gruta Meandros, Gruta Lapa da Pantera, Gruta Seis Bocas e Gruta do Ancião (Figura 1). Cada caverna foi amostrada uma vez entre os dias 26 e 30 de dezembro de 2022, durante a 3ª expedição do GREGEO à Natalândia-MG.

### 2.2. Captura de Morcegos

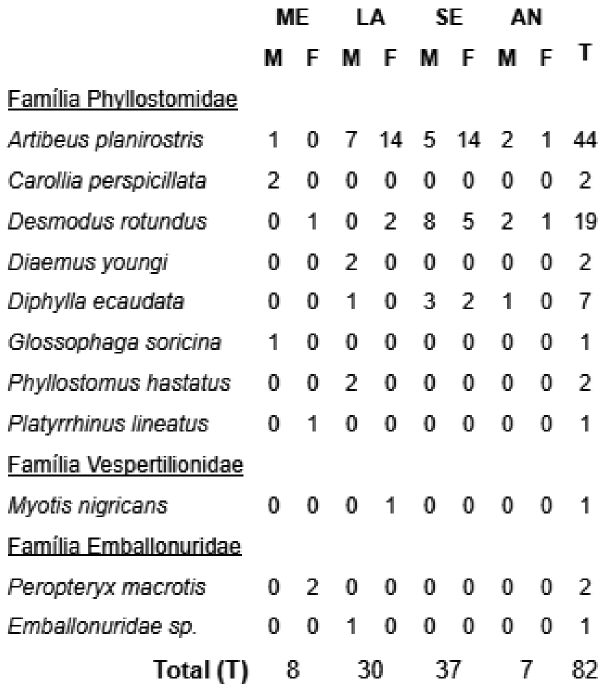
***Figura 2:*** *Rede de neblina montada na abertura da Gruta Meandros, Natalândia/MG.*

As capturas foram realizadas utilizando redes de neblina abertas na entrada das cavidades (Figura 2), obstruindo a passagem dos morcegos, de modo a maximizar as chances de captura no momento da saída do abrigo para o forrageio. As redes ficaram abertas das 17:30 às 22:30. Após a captura, os indivíduos foram acondicionados em um saco de contenção de pano até a triagem. Informações como horário de captura, peso, tamanho do antebraço, sexo, idade e estágio reprodutivo foram tomadas antes de anilhá-los e soltá-los para que pudessem forragear. A identificação dos indivíduos em campo foi auxiliada pela chave de identificação de DIAZ *et al.* (2016). Os testemunhos das espécies e indivíduos com potencial dúvida taxonômica foram coletados e tombados na Coleção de Mamíferos da Universidade de Brasília (CMUnB).

## 3. Resultados e Discussão

Durante a campanha de campo foram capturados 82 morcegos pertencentes a 11 espécies e três famílias (Figura 3). A família Phyllostomidae apresentou a maioria dos representantes (8 espécies), totalizando 95,1% de todos os indivíduos capturados. A espécie mais abundante foi *Artibeus planirostris* (53,7%), seguido de *Desmodus rotundus* (23,2%).

Em meio as espécies amostradas nesta área, observamos quase todos os hábitos alimentares presentes para morcegos: frugivoria (*A. planirostris, Carollia perspicillata e Platyrrhinus lineatus*), nectarivoria (*Glossophaga soricina*), hematofagia (*D. rotundus, Diaemus youngi e Diphylla ecaudata*), insetivoria (*Myotis nigricans e Peropteryx macrotis*) e onivoria (*Phyllostomus hastatus*). Alguns desses hábitos alimentares estão intrinsecamente associados a serviços ecossistêmicos (KUNZ *et al.*, 2011) que podem ser medidos para a região em estudos futuros, uma vez que compreende uma área com alta cobertura de agricultura e pecuária.



| | ME | | LA | | SE | | AN | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | T |
| **Família Phyllostomidae** | | | | | | | | | |
| *Artibeus planirostris* | 1 | 0 | 7 | 14 | 5 | 14 | 2 | 1 | 44 |
| *Carollia perspicillata* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Desmodus rotundus* | 0 | 1 | 0 | 2 | 8 | 5 | 2 | 1 | 19 |
| *Diaemus youngi* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Diphylla ecaudata* | 0 | 0 | 1 | 0 | 3 | 2 | 1 | 0 | 7 |
| *Glossophaga soricina* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Phyllostomus hastatus* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Platyrrhinus lineatus* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| **Família Vespertilionidae** | | | | | | | | | |
| *Myotis nigricans* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| **Família Emballonuridae** | | | | | | | | | |
| *Peropteryx macrotis* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Emballonuridae sp.* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| **Total (T)** | 8 | | 30 | | 37 | | 7 | | 82 |

*Figura 3: Lista de espécies de morcegos capturados nas cavernas de Natalândia/MG. ME= Gruta Meandros, LA=Gruta Lapa da Pantera, SE=Gruta Seis Bocas, AN= Gruta do Ancião, M= machos e F=fêmeas.*

Embora *A. planirostris* se abrigue em folhagens e não seja comumente visto em cavernas (HOLLIS, 2005; GUIMARÃES & FERREIRA, 2015), nesse estudo foi a espécie mais comum na área amostrada. O fato da maioria dos indivíduos estarem em estágio reprodutivo (Figura 4) sugere que eles estejam utilizando as cavidades para essa finalidade, como já visto no estado da Bahia (GUIMARÃES & FERREIRA, 2015; TORRES & BICHUETTE, 2019), o que reforça a importância de preservação desses ambientes cársticos que servem de abrigo maternidade para diversas outras espécies (HUTSON & MICKLEBURGH, 2001). *Artibeus planirostris* desempenha um importante papel na dispersão e indução de germinação para algumas espécies de plantas (OLIVEIRA & FREITAS LEMES, 2010). Uma amostragem na estação seca é recomendada para elucidar se essa espécie de fato se abriga nestas cavernas ou as utiliza apenas para reprodução.

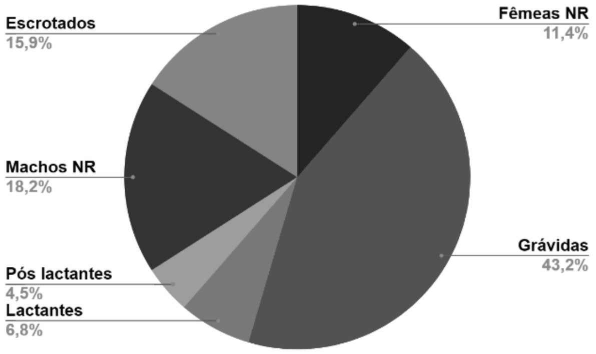


*Figura 4: Razão sexual e estado reprodutivo de Artibeus planirostris nas cavernas amostradas. NR= não-reprodutivo(a).*

Outra observação importante é o aparecimento das três únicas espécies de morcegos-vampiros do mundo, pertencentes à subfamília Desmodontinae: *Desmodus rotundus* (morcego-vampiro-comum), *Diphylla ecaudata* (morcego-vampiro-de-pernas- peludas) e *Diaemus youngi* (morcego-vampiro- de-asas-brancas). Essas espécies tendem a ser cada vez mais comuns em amostragens devido à intensa modificação do uso da terra em pastagem e criações de gado e aves (STREICKER & ALLGEIER, 2016). Até mesmo a espécie *D. youngi*, raramente encontrada em amostragens na parte sul do Brasil vem expandindo sua ocorrência (AGUIAR *et al.*, 2006; COSTA *et al.*, 2008; HOPPE *et al.*, 2019; WIIT *et al.*, 2019).

## 4. Conclusões

As cavernas de Natalândia são importantes ambientes para conservação dos morcegos, tendo em vista que são ambientes utilizados para fins reprodutivos, como verificado para a espécie *A. planirostris*. Outro aspecto que ressalta a relevância dos ambientes estudados foi o aparecimento das espécies de morcegos-vampiros que pode estar associado à intensa conversão do uso da terra para fins agropecuários. Por fim, recomendamos novas amostragens para morcegos das mesmas áreas para verificar a ocupação de *A. planirostris* na estação seca e amostragens de novas cavidades para enriquecer a lista de espécies da região, uma vez que isso pode aumentar a relevância da conservação da área.

## Agradecimentos

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# Description of a new species of *Eidmanacris* Chopard, 1956 (Orthoptera: Phalangopsidae) from a cave in northeastern Brazil

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## Abstract

Eidmanacris crickets are endemic to South America and are frequently associated with cave environments. The genus comprises 29 species, the majority of which are found in Brazilian territory. In this study, we describe a new species of Eidmanacris from a granite cave in northeastern Brazil, further reinforcing the genus association with subterranean habitats. Future inventories in unexplored karst regions may uncover additional species, expanding our understanding of the genus diversity and distribution.

## Resumo

Os grilos Eidmanacris, endêmicos da América do Sul, são frequentemente encontrados em cavernas. O gênero compreende 29 espécies, a maioria das quais ocorre em território brasileiro. Neste estudo, descrevemos uma nova espécie de Eidmanacris proveniente de uma caverna no nordeste do Brasil, reforçando ainda mais a relação do gênero com ambientes subterrâneos. Inventários futuros em regiões cársticas inexploradas podem revelar novas espécies dentro do gênero.

## 1. Introduction

With over 190 genera (CIGLIANO et al., 2025), Phalangopsidae Blanchard, 1854 (Orthoptera: Ensifera: Grylloidea) is a widely distributed cricket family found across major biogeographical regions worldwide (DESUTTER-GRANDCOLAS, 1995a; CIGLIANO et al., 2025). The majority of its genera (approximately 70%) originate from the Neotropical region, while 10% are distributed among the Ethiopian, Australasian, and Oriental regions (DESUTTER-GRANDCOLAS, 1995a). Most species are hygrophilous, thriving in humid environments such as tropical forests and caves (DESUTTER-GRANDCOLAS, 1995a). They are typically brachypterous, exhibiting reduced or absent forewings, and are characterized by elongated antennae, legs, and cerci.

The genus *Eidmanacris* Chopard, 1954 comprises 29 species and is distinguished by supra-anal plates with posterolateral projections, fore tibiae without tympana, metanotal glands with an anteromedial triangular ridge, and short forewings lacking a stridulatory file (DESUTTER-GRANDCOLAS, 1995b; DE CAMPOS et al., 2017). As these crickets are unable to produce courtship songs, they rely on secreting nutritious substances from their metanotal glands to attract potential mates (DE CAMPOS et al., 2017). The metanotal gland is considered a key taxonomic feature and is frequently used in species descriptions (DESUTTER-GRANDCOLAS, 1995b; SOUZA-DIAS et al., 2015; DE CAMPOS et al., 2017). However, three species of *Eidmanacris* lack metanotal glands: *E. endophallica* (DE MELLO, 1990), *E. fusca* Desutter-Grandcolas, 1995, and *E. tridentata* Desutter-Grandcolas, 1995 (DESUTTER-GRANDCOLAS, 1995b; DE MELLO, 2007; DE CAMPOS et al., 2017).

Species of *Eidmanacris* are frequently found in caves across southeastern and central-western Brazil. Among the 29 described species, six have been recorded in subterranean environments: *E. alboannulata* (Piza, 1960), *E. dissimilis* Desutter-Grandcolas, 1995, *E. larvaeformis* Chopard, 1938, *E. neomarmorata* De Campos, 2017, *E. scopula* De Campos, 2017, and *E. speluncae* (Mello-Leitão, 1937) (BOLFARINI et al., 2022). Notably, *E. neomarmorata*, *E. scopula*, and *E. speluncae* have caves as their type localities (CIGLIANO et al., 2025).

This study aims to describe a new species of *Eidmanacris* from a cave in northeastern Brazil, further contributing to the understanding of the genus' distribution and ecological associations with subterranean habitats.

## 2. Materials and methods

**Collection and examination:** The new species, *Eidmanacris n.* sp., was collected through active search in Toca da Raposa 2 cave (9°13'38.02"S, 35°54'47.49"W), located in the municipality of Murici, Alagoas state, Brazil. The adult male specimen was preserved in 70% ethanol and deposited in the Collection of Subterranean Invertebrates of Lavras (ISLA) at the Federal University of Lavras, Brazil.

The general morphology of the specimen was examined under a Stemi 508 (ZEISS) stereomicroscope. The phallic complex was dissected and subjected to pancreatin enzymatic digestion (ÁLVAREZ-PADILLA & HORMIGA, 2007) for 24 hours to facilitate the removal of muscle tissue and membranes. After enzymatic treatment, the phallic complex was rinsed in 70% ethanol and further analyzed under a Stemi 508 (ZEISS) stereomicroscope.

For photographic documentation, an Axio Zoom V16 (ZEISS) stereomicroscope equipped with an Axiocam 506 camera (ZEISS) was used. Images were arranged into plates to highlight key morphological features, including forewing length, posterolateral projections of the supra-anal plate, and metanotal gland structure.

The description of the phallic complex follows the terminology proposed by Desutter-Grandcolas (2003) and later refined by de Campos et al. (2017). Comparative analyses were conducted based on literature data (DESUTTER-GRANDCOLAS, 1995b; DE CAMPOS et al., 2017; CIGLIANO et al., 2025) and *Eidmanacris* specimens stored in the Collection of Subterranean Invertebrates of Lavras (ISLA).

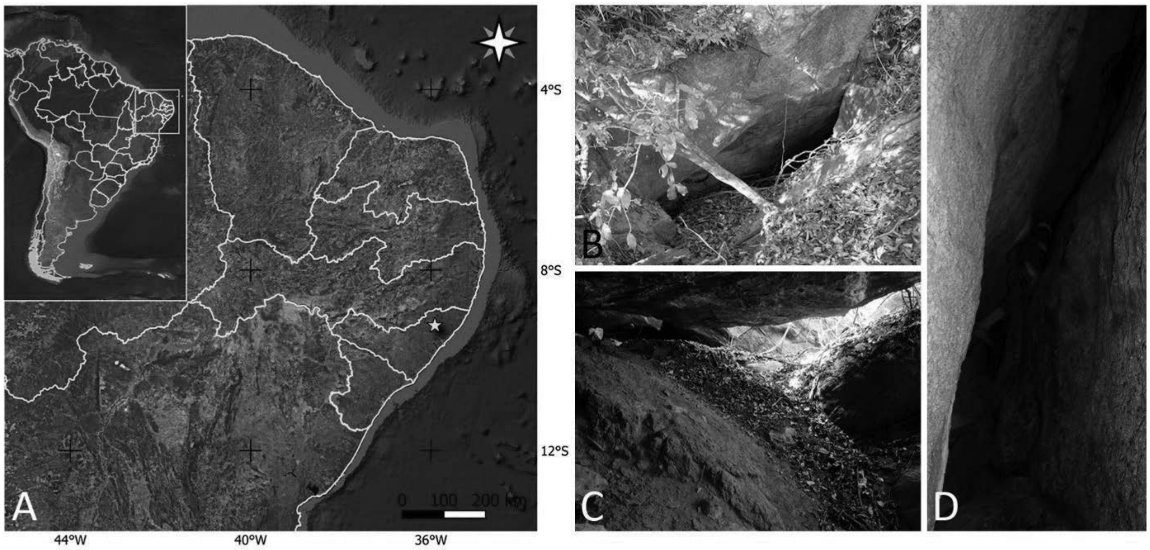

***Figure 1**: A) A) South America, northeastern region of Brazil highlighted, yellow star marks the type locality of Eidmanacris n. sp. ; B) Entrance of Toca da Raposa 2 cave ; C) First chamber of Toca da Raposa 2 cave ; D) Inner chamber of Toca da Raposa 2 cave.*

## 3. Results

**Adult male description: Head.** General color yellowish-brown; gena with an anteroventral brown spot; *fastigium* anteriorly brown; *frons* yellowish-brown, with three longitudinal brown stripes; *vertex* with a brown medial band and two light brown spots posterior to the compound eyes; *antennae* banded, with a large white portion in the middle.

**Torax**. *Pronotum* wide, brown with light brown spots; anterior margin broader than the posterior margin, lateral lobes slightly projected toward the head; *forewings* reduced, subtriangular, oriented inward, partially covering the *metanotum*, with a longitudinal white stripe, apex and inner margins white; metanotal gland present and anteriorly positioned, formed by a pair of pointed projections and an inverted-triangle ridge.

**Legs**. *Leg 1* pubescent, tympanum absent; *leg 2* pubescent, tibia serrulated, with two ventral apical spurs and one dorsolateral apical *spur*; *tibia* 3 serrulated, with eight subapical spurs (four internal and four external, the two most distal shorter than the rest) and six apical spurs (three internal and three external, external spurs shorter than the internal).

**Abdomen**. *Supra-anal plate* light yellowish-brown, covered with long setae, posterior margins ventrally brown, with two lateroposterior short projections; subgenital plate elongated, light yellowish-brown, covered with short bristles; paraprocts as long as the supra-anal plate.

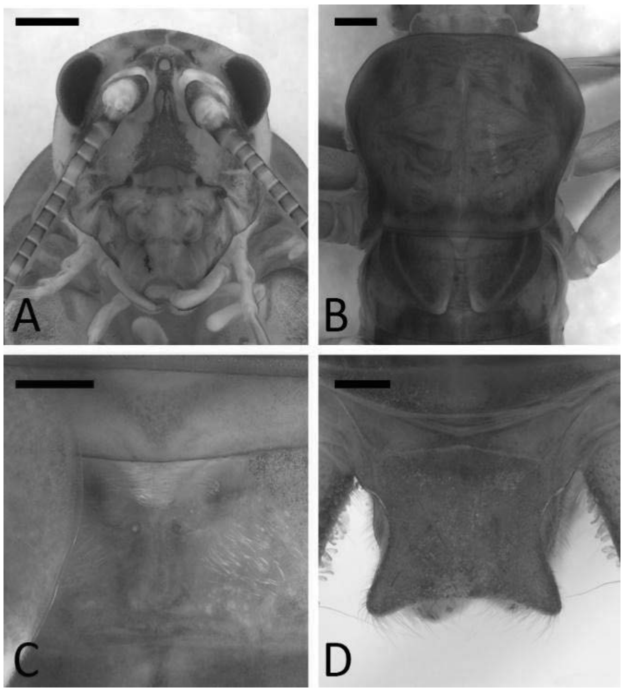

***Figure 2:** Adult male of Eidmanacris n. sp. ; A) head, frontal view ; B) Pronotum and forewings, dorsal view ; C) Metanotal gland, dorsal view ; D) Supra-anal plate, dorsal view.*

**Phallic complex.** *Pseudepiphallic base* developed, lateral portions rounded and concave; *pseudepiphallic arms* curved dorsally, apex sclerotized and slightly folded over itself, distal portion with a short pointed dorsal projection, which is directed inward; *pseudepiphallic anterior projection* subtriangular in lateral view, shorter than the *ectophallic apodeme; pseudepiphallic paramere* 1 sclerotized and concave; *pseudepiphallic paramere* 2 sclerotized, roughly T-shaped, and adjacent to the *ectophallic dorsal* projection; *ectophallic apodeme* elongated, but not as long as the *endophallus*; ectophallic arc sclerotized, inverted V-shaped; ectophallic dorsal projection broad, more sclerotized at the center, which is roughly X-shaped; *endophallic posterior portion sclerotized*, curved dorsally, surpassing the apex of pseudoepiphallic arm in length; *endophallic sclerite* concave and V-shaped; *endophallic apodeme* elongated, surpassing the ectophallic apodeme in length.

***Figure 3:*** *phallic complex ; A) Dorsal view ; B) Lateral view.*

## 4. Discussion

To date, six species of *Eidmanacris* have been recorded in caves (BOLFARINI et al., 2022). *Eidmanacris* n. sp. represents the seventh cave-dwelling species within the genus, further reinforcing its strong association with subterranean environments. The discovery of this new species highlights the importance of cave faunal inventories, as these surveys are essential for unveiling cryptic biodiversity and informing conservation efforts.

The new species described here is distinguished from its congeners by a unique combination of morphological traits, including the length of the supra-anal plate posterolateral projections, the size and shape of the forewings, and key structural features of the phallic complex, particularly the apex of the pseudepiphallic arms, the length and curvature of the ectophallic apodema, the width of the ectophallic arc, and the length of the endophallus.

Despite these distinguishing characteristics, *Eidmanacris* n. sp. shares certain morphological features with other species within the genus. Its general coloration is similar to that of *E. speluncae*, *E. melloi* De Campos, 2017, and E. minuta (de Mello, 1990), while its forewing length and shape resemble those of *E. multispinosa* Desutter-Grandcolas, 1995, *E. larvaeformis,* and *E. fusca* (DESUTTER-GRANDCOLAS, 1995b; DE CAMPOS et al., 2017). The metanotal gland structure is most similar to that of *E. speluncae*, while the supra-anal plate resembles that of *E. dissimilis* (DESUTTER-GRANDCOLAS, 1995b; DE CAMPOS et al., 2017).

The substantial geographic separation between the cave where *Eidmanacris* n. sp. was discovered and the type localities of its congeners further supports its recognition as a new species. The closest known species, *E. septentrionalis* Desutter-Grandcolas, 1995 and *E. scopula*, occur approximately 700 km and 1,200 km from the type locality of *Eidmanacris* n. sp., respectively (Cigliano et al., 2025). These species are isolated from *Eidmanacris* n. sp. by major geographical barriers, such as the São Francisco River, further corroborating its taxonomic distinctiveness.

## 5. Conclusion

The description of this new *Eidmanacris* species enhances our understanding of the distribution and diversity of Neotropical Orthoptera. *Eidmanacris* n. sp. is the seventh species of the genus recorded in caves, further reinforcing its strong association with subterranean habitats. These findings underscore the importance of caves as suitable environments for *Eidmanacris* and suggest that future surveys in unexplored regions may reveal additional species within the genus, further expanding our knowledge of its diversity and ecological adaptations.

## Acknowledgments

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# Diversity of subterranean *Aegla* (Crustacea: Decapoda: Anomura) in the Alto Ribeira karst area, southeastern Brazil

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## Abstract

*Aegla* crustaceans are freshwater anomuran decapods, endemic to temperate and subtropical watersheds of continental South America. They are frequent in caves of the Alto Ribeira karst area, São Paulo and Paraná states, a spot of high diversity of subterranean organisms, both terrestrial and aquatic. Several *Aegla* spp. form troglophilic populations (source populations both in hypogean and in epigean habitats, genetically connected by individuals commuting between them) in the Alto Ribeira, such as *Aegla strinatii*, *A. quilombola* [previously A. marginata], *A. schmitti* , *A. lachesis*, *A. lancinhas* (the only cave species from Paraná).Herein, we present an overview of the six described troglobitic species (exclusively subterranean source populations). *A. microphthalma* and *A.cavernicola*, from the Parque Estadual do Alto Ribeira – PETAR, and *A. leptochela*, from Parque Estadual Intervales – PEI, are highly troglomorphic, characterized by reduced cornea and eyestalks, slim body and chelae, elongated antennae and pereopods.; such reductions are extreme in *A. microphthalma*. Behavioral specializations are discussed. These species are Critically Endangered. *A. charon A. atropos* (Bulha d´Água region, PETAR) and *A. clotho* (Núcleo Caboclos, PETAR) are characterized by slightly reduced corneas and eyestalks. These species would be recent troglobites, considered Vulnerable.

## 1. Introduction

*Aegla* crustaceans are unique freshwater anomuran decapods, endemic to temperate and subtropical watersheds of continental South America (Schmitt, 1942) between latitudes 20° and 50°, in Brazil and in Chile, respectively (Bueno et al., 2007; Oyanedel et al., 2011). They are nocturnal opportunistic omnivores, feeding on vegetal debris, dead and living animals (Moracchioli, 2004), thus preadapted (in the sense of exaptations – Buss et al, 1998) to the subterranean life.

Several species have been recorded in caves from the Alto Ribeira karst area, São Paulo and Paraná states, since the 1970´s (e.g., Strinati, 1975; Dessen et al, 1980; Trajano,1987; Gnaspini & Trajano, 1994; Moracchioli, 1994, Bond-Buckup & Buckup, 1994; Pinto-da-Rocha,1995; Trajano & Bichuette, 2010; Fernandes et al, 2013; Maia et al., 2013; Santos et al., 2015; Bueno et al, 2016, 2017; Gallão & Bichuette, 2018; Trajano,2023; Bueno et al, 2024). These species have been classified as troglophiles (source populations both in hypogean and in epigean = surface habitats, genetically connected by individuals commuting between them –Trajano & Carvalho, 2017) or troglobites (exclusively subterranean source populations – Trajano & Carvalho, 2017). There are six described troglobitic species in Brazil: *Aegla cavernicola* Türkay, 1972, *A. leptochela* Bond-Buckup & Buckup, 1994, *A. microphthalma* Bond-Buckup & Buckup, 1994, *A. charon* Bueno & Moraes, 2017, *A. clotho* Bueno & Tavares, 2024 e *A. atropos* Bueno & Tavares, 2024 (Türkay, 1972; Bond-Buckup & Buckup ,1994; Bueno et al., 2017, 2024).

Species frequent in the Ribeira karst area, forming troglophilic populations in several caves of São Paulo and Paraná states, include *Aegla strinatii* Türkay, 1972 (Fig.1), *A. quilombola* Moraes, Tavares e Bueno, 2017 [previously *A. marginata*; see Moraes et al. 2017 for taxonomic review information] (Fig. 2), *A. schmitti* Hobbs III, 1979 (Fig. 3), *A. lachesis* Bueno & Tavares, 2024, *A. lancinhas* Bond-Buckup & Buckup, 2015 (the only cave species from Paraná state) (Moracchioli, 1994; Pinto-da-Rocha, 1995; Rocha & Bueno, 2011; Fernandes et al, 2013; Santos et al., 2015; Moraes et al., 2017; Bueno et al., 2024).

In general, troglobitic species present autapomorphies (exclusive character states) related to isolation in subterranean habitats followed by specialization to the hypogean life, named troglomorphisms. The most widespread troglomorphisms, present in taxa as diverse as planarians, arthropods and vertebrates, is the reduction or loss of visual organs and of dark pigmentation. In arthropods, elongation of appendages, as a mechanism of sensorial compensation, is also frequent. In troglobitic *Aegla* species, the most common troglomorphisms are general cuticle depigmentation, reduction of the cornea and of the eye peduncle, slimmer body shape and delicate pair of chelae, and increase in length of sensory and ambulatory appendages (antennae and pereopods) as compared to epigean congeners (Bueno et al. 2016: fig.2.3).

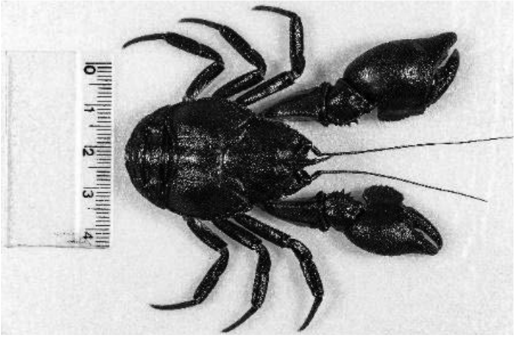

***Figure 1:*** *Aegla strinatii (Photo: Sérgio Rocha).*

**Figure 2:** *Aegla quilombola (Photo: S. Bueno).*

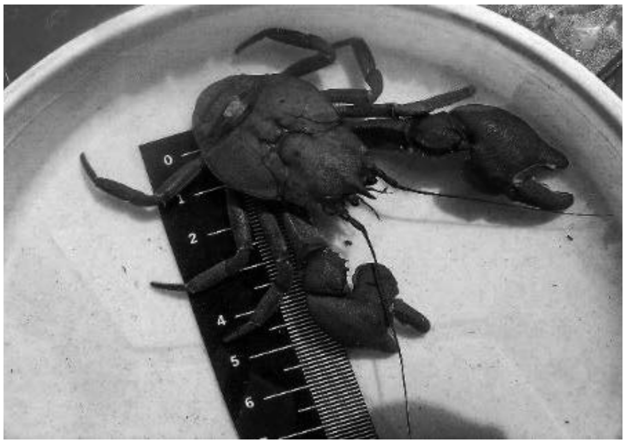

**Figure 3:** *Aegla schmitti (Photo: S. Bueno).*

## 2. Diversity of troglobitic *Aegla* species in the Alto Ribeira karst area

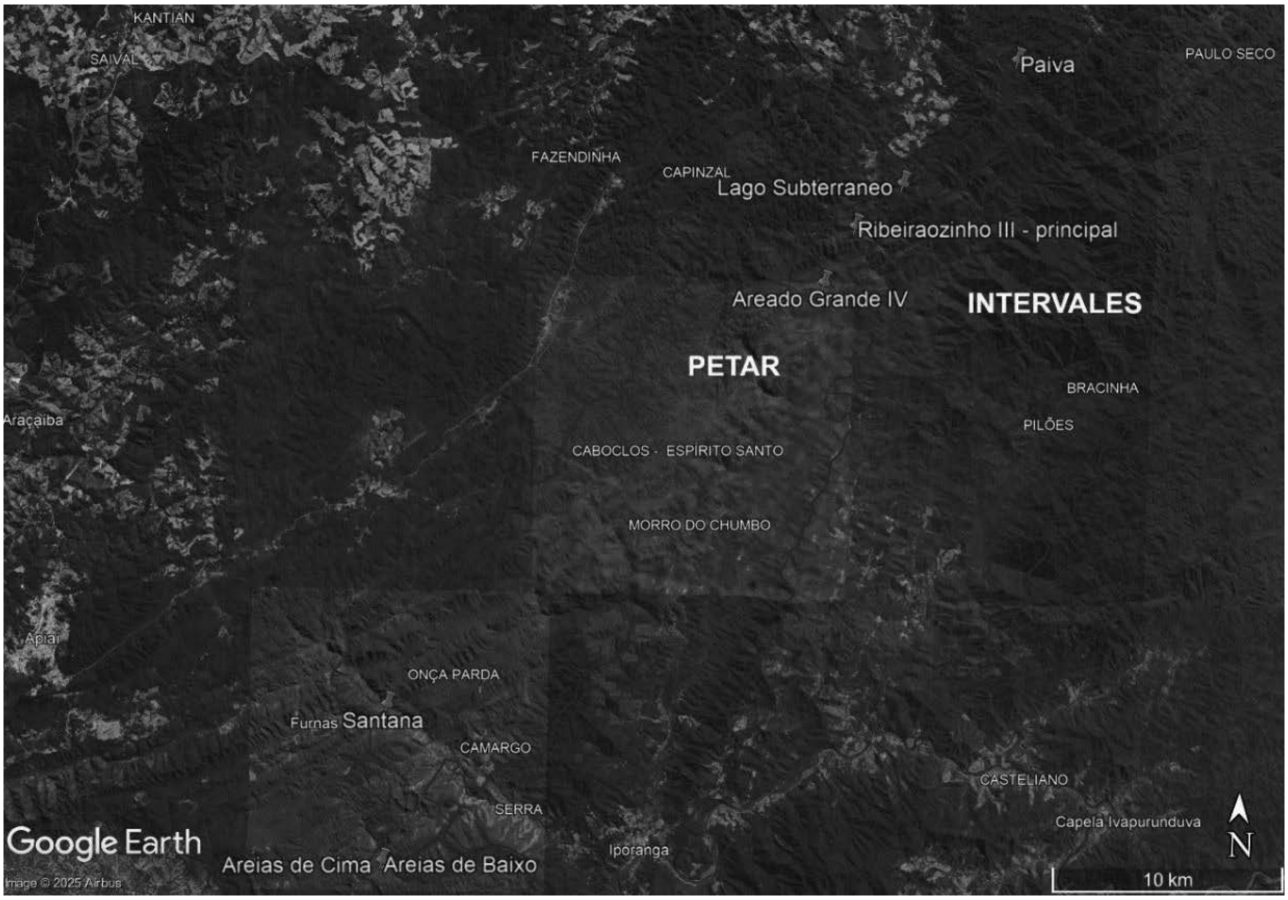

**Figure 4:** *Distribution of troglobitic Aegla in the Alto Ribeira karst area: Areias system (Aegla cavernicola) ), Santana cave (A. microphthalma), Areado Grande IV cave (A. clotho), Ribeirãozinho III cave (A. atropos), Lago Subterrâneo cave (A. charon) and Paiva cave (A. leptochela).*

The first troglobitic aeglid described was *Aegla cavernicola*, collected by P. strinati during the 1960's in the Areias system, and described by Türkay (1972). This author also described *Aegla strinatii*, a troglophilic species from Tapagem Cave (also known as Caverna do Diabo), also collected by P. Strinati.

Herein, we present a list of described troglobitic *Aegla* species, with data on locality, biology (when available) and troglomorphisms. Except for *A. charon*, these species inhabit lotic waters, being observed in rapids and slow-moving pools.

- *Aegla cavernicola* Türkay, 1972 (Fig. 5): reduced corneas and eyestalks, slim body and chelae, elongated pereopods (walking appendages). Endemic to Areias cave system, Parque Estadual Turístico do Alto Ribeira (PETAR). The highest population densities are observed in Areias de Baixo Cave (1-2 individuals.m2, in the 1990´s - Moracchioli, 1994), where blind catfish, *Pimelodella kronei*, are less frequent, but they are rare in Areias de Cima Cave, where blind catfish used to be abundant (E. Trajano, pers. obs.), indicating competition (both species are opportunistic feeders) or predation of young *Aegla* by *P. kronei.*

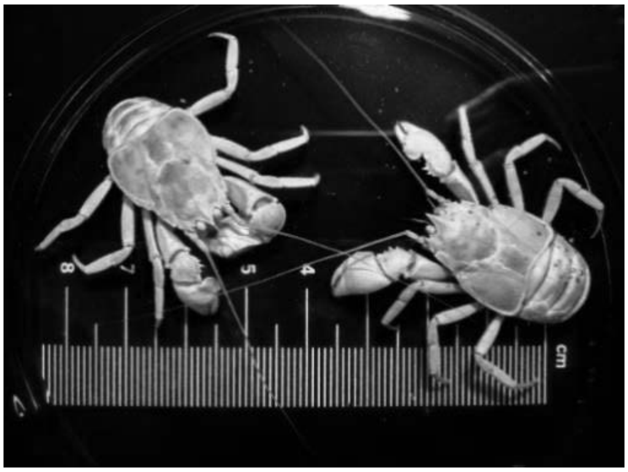

***Figure 5:*** *Aegla cavernicola (Photo: Bruno Takano).*

- *Aegla leptochela* Bond-Buckup & Buckup, 1994 (Fig. 6): reduced corneas and eyestalks, slim body and chelae, elongated antennae and pereopods, semi-pigmented in life. Endemic to Paiva Cave Fig. 12), where it is sintopic with troglophilic A. quilombola in Paiva cave, Parque Estadual Intervales (PEI). Both species used to be equally common until the 1994´s (population density = 2 ind.m2 - Moracchioli, 1994), but since then they suffered a sharp decrease in numbers (Maia et al., 2013), and nowadays they are nearly extinct in the cave (E. Trajano, pers. obs.). This is probably due to disturbance by tourists and/or introduction of carps as potential predators of young *Aegla*.

***Figure 6:*** *Aegla leptochela (depigmented in alcohol) (Photo: A.Camargo).*

- *Aegla microphthalma* Bond-Buckup & Buckup, 1994 (Fig. 7): the species that exhibits troglomorphic traits at their highest levels among troglobitic aeglids, it is characterized by having highly reduced and depigmented corneas, shortened eyestalk, depigmented white color cuticle in life, slim body and chelae, and elongated antennae and walking pereopods. It is an endemic species to Santana Cave, núcleo Betari (PETAR). Relatively common in the 1970´s, a density of 0.1 ind.m2 was estimated in the 1990´s (Moracchioli, 1994). Since then, this species was also subject to accentuated decline – only two specimens were found in 2007 (Maia et al., 2013) and the species is nearly extinct. The probable causes for this drastic decline was pollution by mining activities upstream the cave in the 1980´s, competition with Macrobrachium prawns, introduced in the Iporanga basin in the 1950´s (Maia et al., op. cit.).

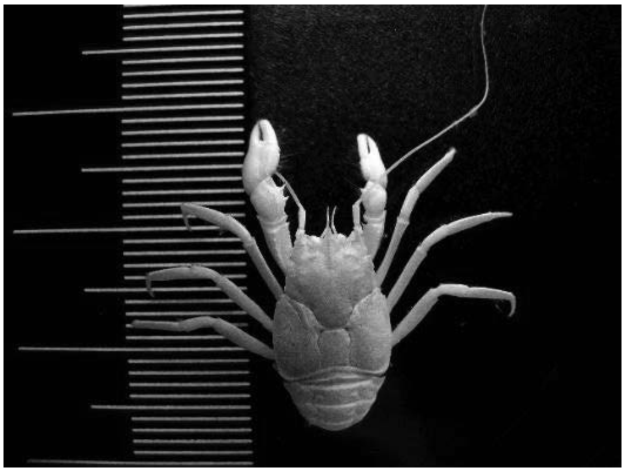

***Figure 7:*** *Aegla microphthalma (Photo: Felipe Cohen).*

Moracchioli (1994) studied the behavior of these species, comparing them with troglophilic populations and epigean species. In spite of eye reduction, A. cavernicola and A. leptochela are more photophobic than epigean Aegla spp. Cryptobiotic behavior (hiding habits) are similar in the hypogean and epigean species, except for the highly specialized A. microphthalma, that spend most of the time exposed, over the rock dens or walking around, indicating a reduction of cryptobiotic habitats, as observed for several troglobitic fishes. The troglobitic species spend less time to locate food than the epigean ones. Higher feeding efficiency may be related to the compensatory development of sensorial structures in the antennae and pereopods, which are cleaned more frequently than in epigean relatives. Reduction of intraspecific aggressiveness was observed in the troglobitic species, which may explain the reduction in chelae size.

- Aegla charon Bueno & Moraes, 2017 (Fig. 8): slightly troglomorphic, with partially reduced cornea. A remarkable autapomorphy is the presence of partially developed uniramous pleopods in adult males, whereas the absence of these appendages is the general condition for this sex in Aegla. Known from Lago Subterrâneo Cave (Fig. 11), in Bulha d´Água region, PETAR, it is the only Aegla known to inhabit lentic subterranean waters.

***Figure 8:*** *Aegla charon (Photo: A.Camargo).*

- *Aegla atropos* Bueno & Tavares, 2024 (Fig. 9): pigmented area of cornea slightly reduced. Known from Ribeirãozinho III Cave, Bulha d´Água region, PETAR.

***Figure 9:** Aegla atropos in an Aegla´s trap (Photo: A. Camargo).*

- *Aegla clotho* Bueno & Tavares 2024 (Fig. 10): cornea slightly reduced, shortened eyestalks. Known from Areado IV Cave, Núcleo Caboclos, PETAR.

***Figure 10 :** Aegla clotho (Photo: Marcelo Gonçalves).*

***Figure 11:** Lago Subterrâneo cave, habitat of Aegla charon (Photo: A.Camargo).*

***Figure 12:** Paiva cave, habitat of A. leptochela and A. quilombola (Photo: A.Camargo).*

## 3. Discussion

The Ribeira karst area is a spot of high diversity of troglobites, both aquatic and terrestrial (Trajano et al., 2016). On the scale of karst areas, paleoclimatic fluctuations may function as a primary cause of isolation and diversification of whole biotas in subterranean habitats. Based on the allopatric paleoclimatic model (Barr, 1968), Trajano (1995) predicted this high diversity in the Ribeira valley, which was a corridor of dry vegetation during glacial periods in the Pleistocene. For the aquatic fauna, the disruption of epigean drainage and differentiation in isolation from its surface ancestors during these dry periods was proposed by Trajano and Britski (1992) to explain the origin and evolution of the blind catfish, *Pimelodella kronei*.

For *Aegla* species, geological and molecular evidence indicate an independent colonization of subterranean domain by different epigean ancestors, followed by vicariance and speciation. The troglobites would be relicts of extinct epigean ancestors widely distributed in the area (Fernandes et al., 2013), whereas the present-day epigean and troglophilic populations would be the result of re-colonization of the epigean and subterranean drainages when they were reinstated during the humid warm periods.

Many troglobites, especially those living in lotic habitats, have small geographic ranges and low population densities, resulting in small population sizes. Most are highly sensitive to disturbance, as is the case of *A. microphthalma*, that did not survive in laboratory. They frequently present a K-selected life history, probably an adaptation to food scarcity. Consequently, their population turnover is very slow and their ability to recover from population losses is decreased compared to epigean taxa. Therefore, these animals generally meet quite well the criteria to receive vulnerable species status (Trajano, 2000). This would be the case of *A. charon, A. clotho* and *A. atropos*, that live in pristine areas. On the other hand, *A. microphthalma, A. cavernicola* and *A. leptochela* even though these species occur in Conservation Units (PETAR and PEI, respectively), they have suffered a drastic population decrease in the last decade and are currently considered as Critically Endangered species (Instituto Chico Mendes de Conservação da Biodiversidade, 2018).

## 4. Conclusions

Several species of the freshwater anomuran decapods, genus *Aegla*, both troglophiles and troglobites, have been recorded in caves from the Ribeira karst area, São Paulo and Paraná states, since the 1970´s.

Species with troglophilic populations include *Aegla strinatii, A. quilombola, A. schmitti , A. lachesis* and *A. lancinhas*.

Among the six described troglobitic species, *A. microphthalma* and

*A. cavernicola*, from núcleo Betari, PETAR, and *A. leptochela*, from PEI, are highly troglomorphic, characterized by reduced cornea and eyestalks, slim body and chelae, elongated antennae and pereopods.; such reductions are extreme in *A. microphthalma*. These species are critically threatened.

*A. charon, A. atropos* and *A. clotho* are characterized by slightly reduced corneas and eyestalks. They would be recent troglobites.

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# Ecology of brown spiders, *Loxosceles adelaida* (Araneae : Sicariidae) in a cave from Alto Ribeira karst area, SE Brazil

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## Abstract

The ecology of brown spiders, *Loxosceles adelaida*, was studied in Laje Branca cave, Alto Ribeira kast area, São Paulo State, SE Brazil, using mark-recapture technique. These spiders show preference to rocky substrates, concentrating near the walls, in regions with large sediment banks where nutrients carried by floods accumulate and prey such as beetles, flies, crickets and millipeds are more abundant, but not exposed in open spaces. The estimated population size was around 200-300 individuals. These are moderately sedentary spiders, with ca. ¼ of the individuals moving on average 22 m in two months. A particular type of web was recorded in the cave, named reticulated web, which differs from webs in the epigean environment by the larger area and lower density of threads, as an adaptation to habitats with low density of prey. No compelling evidence of seasonal reproduction was observed.

## 1. Introduction

More than 35 of the 81 spider-families present in the continent have subterranean representatives in South America, either as troglophiles, troglobites or trogloxenes. Among them, brown spiders, genus *Loxosceles* (Sicariidae) are widespread troglophiles in South American caves (Trajano, 2019).

*Loxosceles* spiders are distributed nearly worldwide in warmer areas, occurring in natural and anthropogenic habitats, including man-made structures. They are venomous spiders known for their bite, which produces a characteristic set of symptoms, such as pain and erythema in the bite area, that can develop into a necrotic ulcer. Although feared, brown spiders are usually not aggressive, cryptobiotic, reported as sedentary (Eickstedt, 1975). They build an irregular sheet-like web, where they capture terrestrial and flying prey.

**Figure 1:** *Brown spider, Loxosceles adelaida, female.*

Brown spiders are common in caves of South and Central America (Eickstedt, 1975; Trajano, 2019). They may be found in sediment banks, rock walls, in narrow spaces, and in corners between walls and floor, preying on millipeds, beetles (cholevids, ptylodactylids), and flies (Trajano & Gnaspini-Netto, 1991).

We investigated the ecology of *Loxosceles adelaida* Gertsch, 1967 (Figures 1 and 2) in Laje Branca Cave, Alto Ribeira karst area, SE Brazil, using the mark-recapture technique.

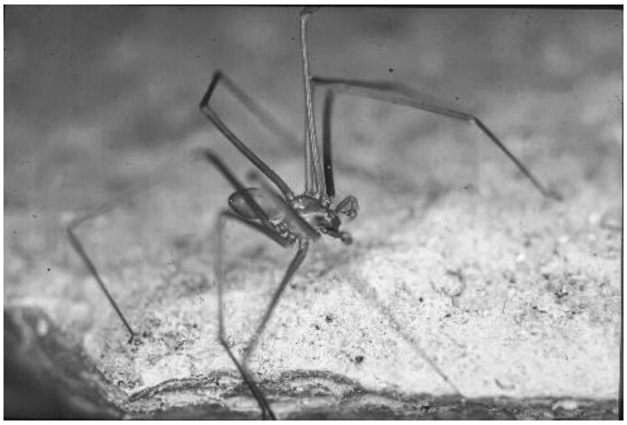


**Figure 2:** *Brown spider, Loxosceles adelaida, male.*

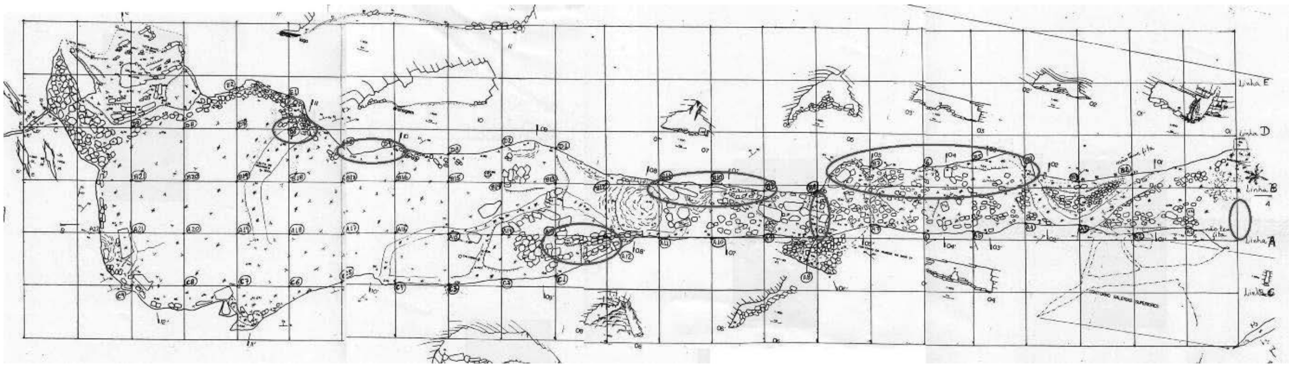


***Figura 4:*** *Laje Branca Cave map showing the quadrats and the sites where brown spiders, Loxosceles adelaida, tend to concentrate. Red circlers. = areas with more than five occurrences.*

## 3. Materials and methods

The study was carried out in 16 monthly visits from August 2001 to December 2002, totalling 39 days of fieldwork. The spiders spotted after visual inspection of potential habitats were imobilized by gently pressing them against the susbstrate with a piece of foam. Sex, life stage (adults *versus* juveniles), location in the cave quadrats, type of substrate, presence and type of web and general behavior were noted

and recorded in photographies.

Adults (males with bulbous palp, females 2.5 to 3.0 cm large) were marked with correction fluid Alta Química applied to the femurs with a thin brush. Different combinations of mark position allowed for individual marking of adults (Fig. 3). All juveniles received a single mark in the cephalothorax.

***Figure 3:*** *Marked female with ootheca on rock.*

Population sizes were estimated using the Lincoln-Petesen and the Triple Catch methods. The Triple Catch method avoids much of the inaccuracy associated with violation of equal catchability and reduces the bias inherent in non-random capture frequencies because it only requires recapturing each individual once per year.

The substrate was classified into four main categories: rock (walls, floor, boulders), between rocks and silty-sandy sediment, silty-sandy sediment, and logs and vegetal debris.

The occuppied webs were described. For a better visualization,ten of them were sprayed with wheat flour. Web areas were measured using the longest and shortest axes (Camargo, 2004). For comparison, Loxosceles webs observed in the illuminated zone of Morro Preto Cave entrance, where prey for spiders are abundant, were also measured.

Temperature and relative humidity in the collecting sites were measured. The mean temperature inside the cave was 16.4o C and relative humidity was 100%.

## 4. Results

In total, 223 adult spiders were marked, 53 (24%) males and 170 (76%) females. Thirty-six females (21,18 %) and four males (7.55%) were recaptured at least once; nine females were recaptured twice. Two hundred and thirty juveniles were marked, of which 31 were recaptured.

The monthly numbers of captures and mark recaptures of adults are in Figure 5, showing a significant variation in these proportions.

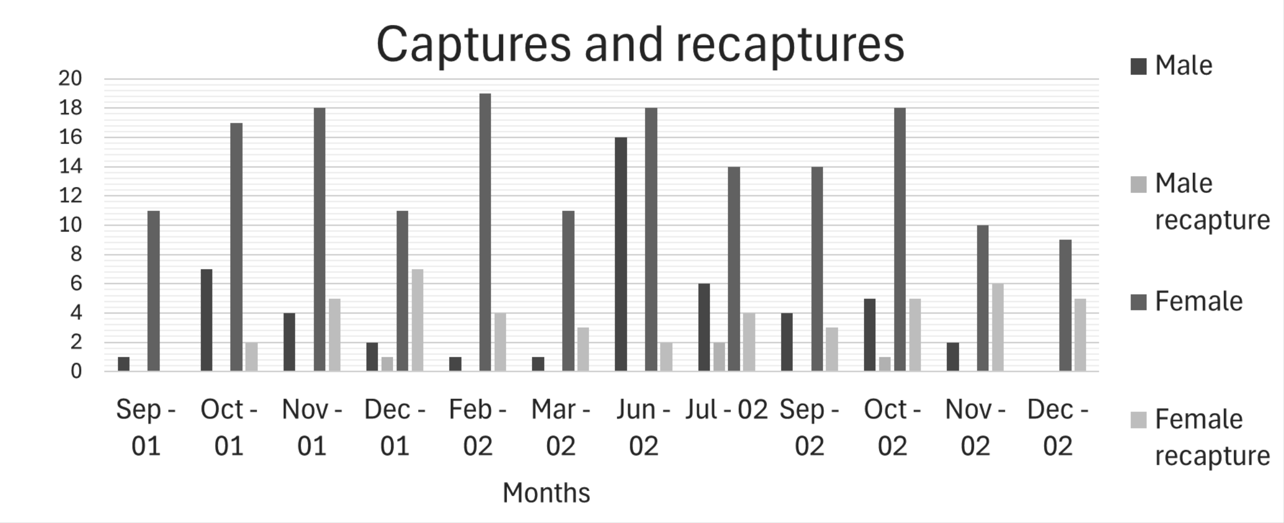


***Figure 5:*** *Monthly proportions of captures and mark recaptures of L. adelaida in the studied area in Laje Branca cave.*

The population sizes estimated by Lincoln-Petersen Index are presented in Figure 6.

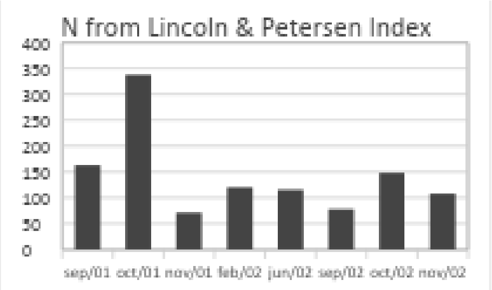


***Figure 6:*** *Population sizes of L. adelaida in the stydied area in Laje Branca cave estimated by the Lincoln-Petersen Index.*

Population sizes (number of individuals in the studied area) estimated by the Triple Catch method are: 1,150 (Sept-Oct-Nov/2001), 48 (Oct-Nov-Dec/2001), 218 (Jun-J ul-Sept/2002), 296 (Sept-Oct-Nov/2002) and 66 (Oct-Nov-Dec/2002). Extreme values (dozens by one hand, and a thousand, by the other) are probably artifacts caused by the lack of homonegeneity in the samples. We consider average values, in the order of two to three hundred individuals, a reasonable estimate for adults.

Brown tended to be concentrated near the walls, in regions with large sediment banks where nutrients carried by floods accumulate and prey were more abundant (Figure 4), but not exposed in open spaces - brown spiders were not spotted in large spaces in the middle of these banks. They seemed to prefer corners between rocky walls and boulders and the sediment.

At a distance of 300 m from the entrance, toward the distal end of the large cave conduit (to the left in Fig. 2), the spider distribution became sparser; only two captures were carried out at the distal end of the studied area.

Regarding the substrate, 67% of the spiders were captured on rock, 16% between rock and sediment, 15% on sediment, 1% on logs and vegetal debris, and 1% on other types of substrate.

Most of the recaptured individuals (30 of 40, or 75 %) were in the same site as the previous captures, including multiple recaptures. The minimum time of permanence for the spiders in the same site was one month (15 individuals or 50%), and it also having been observed two months (seven or 23,34%), three, four, and five months (six or 20%), and 10 (one or 3,34%) and 14 months (one or 3,34%) permanence. This 14 month marked spider corresponds to the highest longevity recorded for an adult female on this field study, with three recaptures. All recaptured males (four individuals) were in the same initial site, with one month (three individuals) to four months (one individual) permanences.

Eight from the ten movements were observed in spring season of 2001 (six of them) and 2002, and two others in summer and fall season. The average distance covered by spiders recorded moving was 22 m in two months, being recaptured over a new web or without one. The longest recorded distance was 60 m in two months. In one case, a female traversed 5 m in 18 hours, moving through a predominantly rocky substrate. The two more longeve female did not move. The displacements occured longitudinally inside the same strips of the plot (Fig. 4), inwards and outwards the cave distal end, evenly. One female captured with ootheca in October was recaptured after 60 days with a new ootheca 10 m far from the original mark site.

A particular type of web, called reticulate (Camargo, 2004), was identified (Figure 7), with lower density of threads covering a larger area than the observed in the epigean habitat (Table 1). The webs studied at Morro Preto cave are typical of the epigean environment, with a high density of threads, endowing an appearance of unwoven cotton.

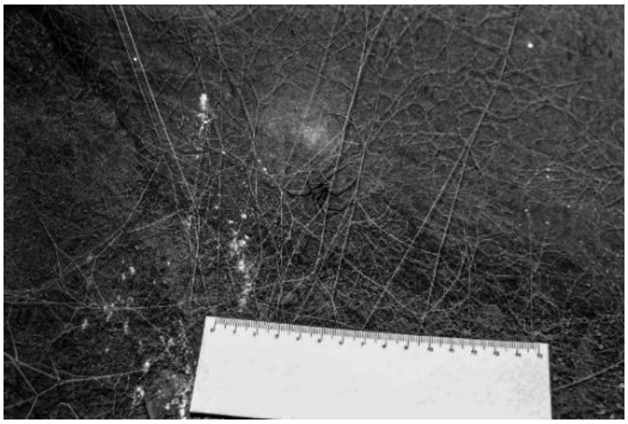


***Figure 7:*** *Reticulate web pattern observed in cave.*

Adults of both sexes and juveniles may use webs close to each other, 10-20 cm apart. In some occasions, they were observed sharing the same web. Cannibalism was not observed. Abandoned webs may be re-occupied by other individuals.

Twenty-five oothecae were found along the study (Figure 8) set on rocky substrate, most in spring of 2002. It was not observed oothecae for the two more longeve females. Dispersing spiderslings were spotted in September and December 2002.

These brown spiders were found feeding mainly on beetles (specially ptilodactylids), millipeds, crickets (Fig. 9), and flies.

***Figure 9:*** *Female L. adelaida preying on cricket.*

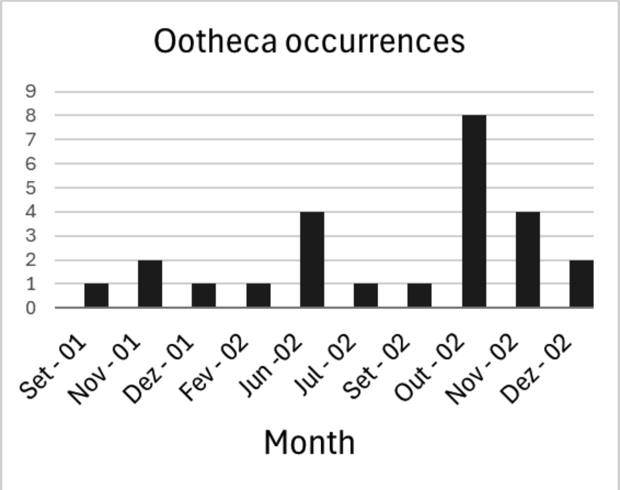
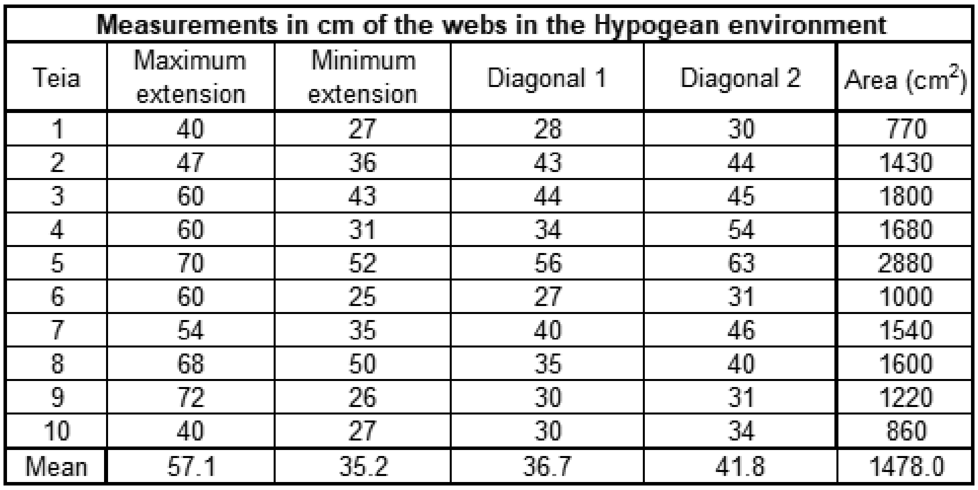
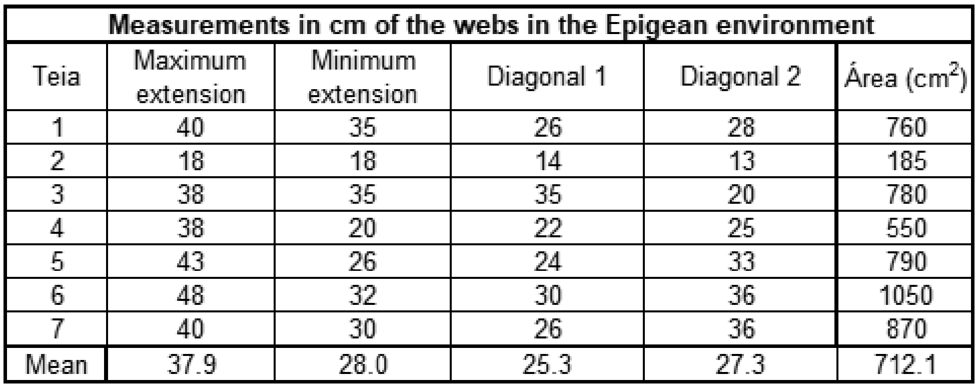


***Figure 8:*** *Monthly number of o oothecae observed in Laje Branca cave.*



| Measurements in cm of the webs in the Hypogean environment | | | | | |
|---|---|---|---|---|---|
| Teia | Maximum extension | Minimum extension | Diagonal 1 | Diagonal 2 | Area (cm$^2$) |
| 1 | 40 | 27 | 28 | 30 | 770 |
| 2 | 47 | 36 | 43 | 44 | 1430 |
| 3 | 60 | 43 | 44 | 45 | 1800 |
| 4 | 60 | 31 | 34 | 54 | 1680 |
| 5 | 70 | 52 | 56 | 63 | 2880 |
| 6 | 60 | 25 | 27 | 31 | 1000 |
| 7 | 54 | 35 | 40 | 46 | 1540 |
| 8 | 68 | 50 | 35 | 40 | 1600 |
| 9 | 72 | 26 | 30 | 31 | 1220 |
| 10 | 40 | 27 | 30 | 34 | 860 |
| Mean | 57.1 | 35.2 | 36.7 | 41.8 | 1478.0 |



| Measurements in cm of the webs in the Epigean environment | | | | | |
|---|---|---|---|---|---|
| Teia | Maximum extension | Minimum extension | Diagonal 1 | Diagonal 2 | Área (cm$^2$) |
| 1 | 40 | 35 | 26 | 28 | 760 |
| 2 | 18 | 18 | 14 | 13 | 185 |
| 3 | 38 | 35 | 35 | 20 | 780 |
| 4 | 38 | 20 | 22 | 25 | 550 |
| 5 | 43 | 26 | 24 | 33 | 790 |
| 6 | 48 | 32 | 30 | 36 | 1050 |
| 7 | 40 | 30 | 26 | 36 | 870 |
| Mean | 37.9 | 28.0 | 25.3 | 27.3 | 712.1 |

***Table 1:*** *Loxosceles web measurements in the hypogean (Laje Branca cave) and epigean (entrance of Morro Preto cave) environments (from Camargo, 2004).*

## 5. Discussion

Variations in the estimated population sizes in the studied area of Laje Branca cave (caves are defined as subterranean spaces large enough for human access) may be due, at least in part, to movements from larger spaces in the cave to inaccessible thin voids of collapsed boulders and others rock descontinuities, and vice-versa .

Female movements recorded inside the cave may be exploratory movements in search of better sites for web building and reproductive partners, or due to disturbances, for exemple.

One of these movements seemed to be for positioning the ootheca by a female in the spring of 2002: after it had been marked with ootheca, it passed out of sight during one recapture event and reappeared in a new site on the next event, with a new ootheca, 10 m far from the marking site. It is worthynote that the larger number and distance of movements during the Spring of 2001 coincided with fewer oothecae on the field, whereas the fewer movements in the spring of 2002 coincided with larger oothecae number.

When comparing 2001 and 2002, no seasonal reproductive pattern was detected. The larger oothecae number and spiderling occurrences in spring of 2002 (October to December) indicated reprodutive activity in this season. But in 2001 and February to September 2002, a lesser number of oothecae were present, except for June 2002, with four ootheca, coinciding with a major number of new adult males on the banks.

In accordance with non-quantified observations in several caves from the Alto Ribeira karst area and elsewhere, brown spiders prefer rocky substrates, avoiding open spaces, and present an aggregate distribution in the cave banks. As expected, they concentrate in sites with accumulation of organic matter, such as vegetal debris and logs carried by floods and bat guano, where prey (beetles, flies, crickes, millipeds) were more abundant.

Brown spiders are tolerant to cospecifics proximity, with adults and juveniles close to each other (10-20 cm apart), sometimes even sharing webs. The cospecific tolerance, and the sharing web behaviour may contribute to the optimization of resources, such as sparing energy on building webs and increasing the extension of bank surface cover by juxtaposing webs. If that lead to reaching best results in prey catches and surveillance, it may indicate an atual aggregate distribution on the sediment banks.

The reticulate web pattern seems to corroborate the energy optimization theory, and may spare time consuming when building them. This web pattern was also observed in Torrinha and Toca da Boa Vista caves, in the semiarid Bahia, northeastern Brazil. When compared to webs in the epigean environment, this pattern corresponds to a greater investment in web area, at the expense of threads density, representing an adaptation to feeding in habitats with lower density of prey. As well, a larger detection area favors defense against potential predators, such as the large wandering spider *Ctenus fasciatus*.

## 6. Conclusions

In caves, brown spiders prefer rocky substrates, at corners between walls and boulders and sediment floor, avoiding open spaces. They concentrate in sites with large accumulations of organic matter, where prey such as beetles (e.g., ptylodactylids), flies, crickets and millipeds were more abundant.

The population size estimated for the study area in Laje Branca Cave was around 200-300 brown spiders. Monthly variations in the estimated values are probably due, on one hand, to artifact of technique and, on the other, to spider movements between the cave and innaccessible spaces and the dynamics of the sediment banks, which is possibly the less stable site of this cave by receiving seasonal influence of epigean environment through floodings.

On the Laje Branca cave sediment banks, *L. adelaida* is moderately sedentary. While ¾ of the spiders remained in their collecting sites, ¼ moved on average 22 m in two months. The longest recorded distance between captures was 60 m. Site adjustment for new resource spots are expected as exploited spots fails to provide.

Highest longevity recorded for adult females was 14 months.

As an adaptation to lower prey abundance and to optimize defense against predators, a particular web pattern, named reticulate, is observed in caves, which differs from the webs in epigean habitats by the larger area and lower density of threads.

Brown spiders are tolerant to cospecifics proximity, with adults and juveniles close to each other (10-20 cm apart), sometimes even sharing webs. Unoccupied webs may be re-occupied by other individuals.

The moviment of the females and the flutuactions of the marked but non-recaptured individuals reveal a particular dynamic site on the sediment banks. Since these banks are the first place in cave receiving an epigean injection of organic matter and moisture trought the floodings of base stream in spring and summer season, at least a time variation for prey density and for substrate humidity are expected in there. All processes of use and colonization of received organic matter until its exhaustion by cave dwellers and other possible preys dragged to cave by waters, and the waves of extreme and receding humidity until some stability certainly influencied the presence of brown spiders on the banks, and their cycles and interactions rest to be understood. In their way, moving brown spiders can make part of the redistribution of the waterside organic matter through the cave during their cycle life.

There was no compelling evidence of seasonal reproduction of *L. adelaida* in Laje Branca Cave.

## Acknowledgments

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# In-field microbiological analyses in an ancient and remote orthoquartzite cave of Auyán-tepui (Venezuela)

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## Abstract

Orthoquartzite caves in the table mountains tepui (in Venezuela, Brazil and Colombia) are one of the most ancient, remote, and pristine subterranean environments on Earth that present unique silica stromatolite formations. When found in oligotrophic dark caves, these silica deposits represent biosignatures derived from solubilization and precipitation processes mediated by microbial activities that are still poorly understood. This work reports the composition and metabolism of microbial communities colonizing different stages of silica stromatolite formation. Oxford Nanopore analyses were conducted in situ by setting a temporary laboratory in the cave and optimizing experimental and bioinformatic procedures for limited resources and minimal laboratory settings. Taxonomy and functional analyses were conducted on the sequencing reads and on the metagenome-assembled genomes (MAGs) that were reconstructed. The results indicated that bacteria of novel lineages of Ktedonobacterales, Terriglobia, and Alphaproteobacteria are dominant in the microbial communities inhabiting the different stages of stromatolite formation. Specific genetic functions were detected in these bacteria that can be associated to the oxidation of atmospheric trace gasses (CO and $H_2$) and $CO_2$ fixation in the dark, leading to the development of complex microbial communities able to interact with the orthoquartzite rock and to colonize dark, oligotrophic and silica-based environments.

## 1. Introduction

Orthoquartzite caves in the table mountains tepui (in the Guyana Shield among Venezuela, Brazil and Colombia) are pristine, ancient and hard-to-reach subterranean environments. These caves host remarkable silica speleothems with stromatolite-like formations that exhibit diverse morphologies and impressive sizes. The formation of these deposits in geochemically stable, non-thermal and aphotic environments is associated with microbial activities that remain largely unknown (SAURO et al. 2018). These silica deposits (speleothems) result from silica solubilization and precipitation processes (from crystalline quartz to amorphous forms like opal-A and Opal-G), mediated by microbial activities interacting with the bedrock over extended periods. These unique silica deposits also serve as analogs to silica deposits on Mars, which have been interpreted as potential biosignatures of past microbial activities (RUFF & FARMER 2016).

In Imawarì Yeuta, one of the orthoquartzite caves in the Venezuelan tepuis, representing the longest, and likely oldest known cave system in the orthoquartzite subterranean landscape, previous morphological, geochemical and microbiological analyses have revealed the presence of complex and abundant microbial communities which interact with the rock substrate leading to silica stromatolite formation (GHEZZI et al. 2022). Microbiological analyses of some samples from this cave demonstrated shifts of microbial community composition across different stages of silica solubilization and stromatolite formation, suggesting the contribution and/or selection of different microbial species during the silica amorphization process (SAURO et al. 2019; GHEZZI et al. 2021). The water content was found to greatly contribute to microbial community's shaping (GHEZZI et al. 2022) and specific hydrolytic enzymes were detected in bacterial isolates which further support microbial activity contribution to some biomineralization processes occurring in the cave (GHEZZI et al. 2024). Lastly, the biotechnological potential of microorganisms isolated from Imawarì Yeuta has been demonstrated, particularly in terms of antibiotic production effective against various bacterial pathogens (GHEZZI et al. 2024).

In this work, we report the microbiological analyses we conducted in the lab by sequencing the metagenome of different samples (and therefore going beyond the only 16S rRNA gene-based analysis) to uncover the metabolic strategies involved in orthoquartzite rock modification and microbial colonization of dark, oligotrophic and silica-based environments. Furthermore, we describe microbiological analyses performed in the cave during an expedition conducted in 2023 by designing customized protocols of DNA extraction and DNA sequencing analysis. These "in cave microbiological analyses" were organized and carried out to combine the possibility to avoid sample decay and transport problems with the interest to test remote-tailored experimental approaches applicable to other scientific expeditions in other remote caves on Earth or to space mission expedition. This is one the first studies in which the entire workflow for microbial community's composition description (DNA extraction, sequencing and data analysis) is conducted in a remote subterranean environment with astrobiological, microbiological, evolutionary and biotechnological interest.

***Figure 1:*** *The huge quarzitic formations inside Imawarì Yeuta (Photo by Riccardo De Luca/La Venta.*

## 2. Materials and methods

### 2.1. Sample collection and Scanning Electron Microscopy (SEM) analysis

Microbiological samples were collected within Imawarì Yeuta from different stages of silica amorphization (a process including silica solubilization followed by amorphous silica precipitation) by using sterile tools and stored in eppendorf tubes until their use (Figure 1). Some samples were examined using Thermofisher ESEM FEG QUATTRO S under low vacuum mode to understand the grade of amorphization and the stromatolite internal layering and structures.

### 2.2. DNA sequencing with Oxford Nanopore Technology

A temporary laboratory was installed in a tent inside Imawarì Yeuta in which DNA extraction, DNA sequencing and data analysis was carried out (Figure 2). DNA extraction was carried out using PowerSoil Lyzer kit (Qiagen) as previously described (GHEZZI et al. 2022). Full-length V1-V9 sequencing (27F - AGAGTTTGATCMTGGCTCAG, 1492R -CGGTTACCTTGTTACGACTT) was performed using ONT MinION workflows (Oxford Nanopore Technologies, Oxford, UK). Full length 16S rRNA were amplified using 16S Barcoding Kit (SQK-RBK004, Oxford Nanopore Technologies) and the Phanta Max Super-Fidelity DNA Polymerase (Vazyme) following the thermocycler program as indicated by the manufacturer protocol. Amplicons were purified using AMPure® XP beads (Beckman Coulter Diagnostics, USA, CA) and ran on an electrophoresis gel for approximate quantification, before sequencing on R9 chemistry (FLO-MIN106) flow cells (Oxford Nanopore Technologies), following manufacturer's protocol. Basecalling was conducted using the "fast" mode basecalling model with Guppy v6.3.7 integrated in MinION Mk1C. Sequencing data were then transferred to Lenovo computer (32Gb RAM, 1 Tb SSD, 8 core). Fastq files were analyzed using a pipeline optimized for in-situ analyses with limited electrical power and without internet connection. The pipeline included two main modules: The first module allowed the pre-processing of the reads through the following steps: i) subsampling of the sequencing reads (s=0.25), ii) adapter removal (by Porechop), iii) length and quality filtering (by Nanofilt), iv) chimera removal (using yacrd). The second module allowed taxonomy classification running the filtered reads against the SILVA database locally. Results were lastly visualized in R using standard packages for the analysis and visualization of metabarcoding data.

### 2.3. Portable ATP detector

Adenosine triphosphate (ATP) measurements were performed to determine the presence of active microorganisms using the NBReader Portable ATP fluorescence detector (Ringbio). The portable ATP fluorescence detector utilizes the "luciferase - luciferin system", which detects ATP content. The selected areas were swabbed, then the swab was placed inside the ATP detector and the test activated. The luminometer measured the light produced by the sample processing that is directly proportional to the quantity of living organisms present in the sample. The test was repeated three times by scrubbing in a standardized way three different areas of the same type of sample.

### 2.4. Metagenomic sequencing and data analysis

A sample representative of advanced stage of silica amorphization was extracted in the laboratory using PowerLyzer DNeasy kit. The total DNA was then sequenced using ONT MinION workflows (Oxford Nanopore Technologies, Oxford, UK). The sequencing library was prepared by using the Ligation Sequencing Kit V14 (SQK-LSK114) and MinION Mk1C was used as sequencing device. Sequencing data were basecalled by using Guppy ver. 6.3.7 with "super-accurate" mode. The same total DNA was also sequenced using Illumina NextSeq. The Illumina and ONT reads were then assembled using the hybrid assembly pipeline with MEGAHIT [56]. CheckM was used for the quality check of MAGs and only MAGs with a completeness > 50% of the genome and a contamination < 10% were maintained. GTDB-Tk was used for taxonomy association.

***Figure 2:*** *Pictures showing outside (Panel A) and inside (Panel B) the temporary laboratory established inside Imawarì Yeuta to carry out the on-field DNA sequencing analyses (Photo by Vittorio Crobu/La Venta)*

## 3. Results

### 3.1. Geomorphological aspects of silica stromatolite-like structures in Imawarì Yeuta

Silica speleothems from Imawarì Yeuta were all characterized by subsequent transition from the host-rock (orthoquartzite) crystalline quartz to amorphous Opal-A and Opal-G. Several biological structures were visible through SEM in different samples representative of consecutive stages of silica stromatolite formation process including coccoid and bacilli formations. The microscopy images also indicate that the biological filaments are the locus of amorphous silica precipitation processes, forming botryoidal masses and tubular casts (Figure 3). The stromatolite selected for the in-situ microbiological analysis is laying over an orthoquartzite boulder (Figure 4). Its volume is approximately 2 m3 with a typical white porous-wet base followed upward by a transition to a darker, less-porous and drier, coralloid formation, with similar characters to the wall sequence described in SAURO et al. 2018.

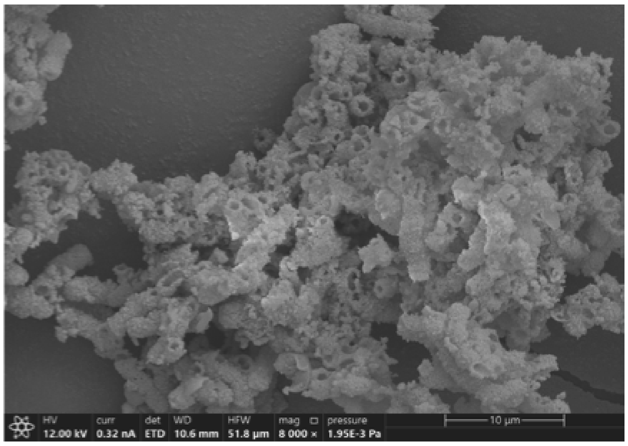

***Figure 3:*** *SEM/FESEM image of a sample representative of an advanced stage of silica amorphization in Imawarì Yeuta.*

### 3.2. On-site microbial activity detection

The ATP measurements were conducted on several samples representative of the first and advanced stages of stromatolite formation, with increasing distance from the orthoquartzite substrate. The results showed a high microbial activity value in samples in the stromatolite region that were in contact with the orthoquartzite rock suggesting that microbial cells that are in contact with the orthoquartzite rock/substrate are metabolically active and probably involved in silica solubilization processes.

***Figure 4:*** *Picture of a silica stromatolite-like structure (Photo by Martina Cappelletti/La Venta).*

### 3.3. DNA sequencing and analyses in the cave

The sequencing data analysis in the cave allowed to unravel the composition of the microbial communities present in two consecutive stages of silica stromatolite formation and in other three samples representative of first signs of microbial colonization of orthoquartzite rock (Figure 5). Oxford Nanopore sequencing data that were analyzed and visualized directly in the cave indicated the high abundance of bacteria belonging to the phylum *Acidobacteriota* in all samples although there were differences between first and advanced stages of silica amorphization at order level. Indeed, considering the *Acidobacteriota* phylum, the order Subgroup 13 characterized advanced stages of stromatolite formation (ST-w and ST-bl), while the early microbial colonization of quartz (samples P-w, and P-y) was characterized by Acidobacterales. First sign of silica amorphization (sample D-w) was also characterized by members of Ktedonobacteriales order (Chloroflexi phylum).

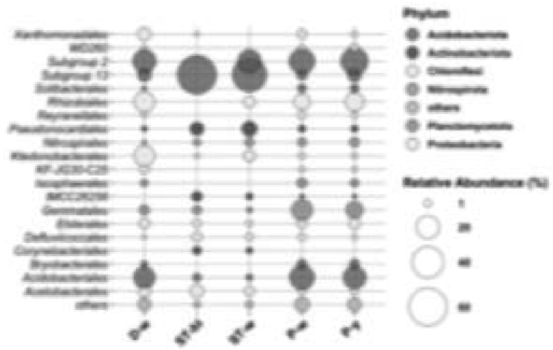

*__Figure 5:__ Relative abundances of bacterial families detected inside the five samples through in situ DNA sequencing.*

## 3.4. Metagenomic analysis of Imawarì Yeutà samples

Deep metagenomic sequencing was performed by combining Illumina HiSeq and Oxford Nanopore Technology to obtain high quality metagenome-assembled genomes (MAGs) of microorganisms present in advanced stages of silica amorphization. MAGs reconstruction allows to get insights into the genomes and genetic functions of unculturable species. A high abundance of MAGs belonging to Acidobacteriota, Alphaproteobacteria and Chloroflexi were identified (Figure 6).

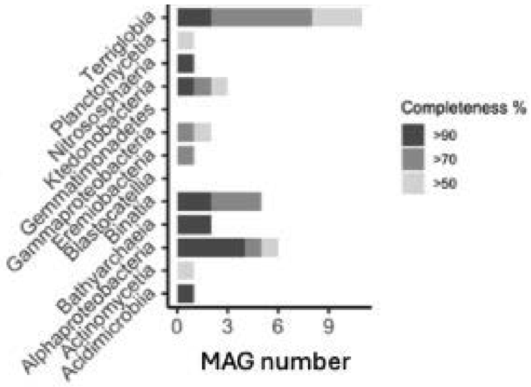

*__Figure 6:__ Number and taxonomy association (at class level) of metagenome-assembled genomes (MAGs) reconstructed from metagenomic data of a Imawarì Yeuta sample.*

The taxonomy analysis of the MAGs reconstructed from the Imawarì Yeuta sample indicated the presence of bacteria of novel taxa belonging to the phyla Acidobacteriota and Chloroflexi and to the class Alphaproteobacteria. The functional analyses showed the presence of several genes associated with the utilization of atmospheric trace gases, CO2 fixation and assimilation in the dark and gene encoding components of respiratory chains adapted to low oxygen levels.

## 4. Discussion

In this work, we carried out in situ and in lab analysis of microbiological samples from silica stromatolite-like structures collected from the ancient and remote cave Imawarì Yeuta on Venezuelan tepuis. This is one of the first study in which the entire DNA-based analysis workflow was successfully carried out in a remote subterranean environment. We therefore demonstrated the possibility to directly obtain sequencing data in cave using protocols that we designed and optimized to work with limited electrical power and no internet connection. This type of study is important to avoid difficult sample transportation logistics and material decay. Furthermore, the protocols that we developed in this study can be applicable for future expeditions in other remote and extreme places on earth and even in space missions.

Microbial activity analyses were carried out in the cave and indicated that microbial communities are active (from a metabolic point of view) at the interface between the silica stromatolite and the quartzite rock. This result supports the involvement of microbes in the silica solubilization and precipitation process that leads to the formation of the unique silica speleothems that are present in Imawarì Yeuta. In-depth sequencing analyses allowed to get insights into the taxonomy and functional features of the main microbial taxa that are present in the silica stromatolite samples. The sequencing data generally indicated that silica stromatolite-like structures host complex microbial communities of bacteria adapted to moderate acidic conditions and oligotrophy. This can be due to the low buffering capacity of quartzite rocks and to the only sporadic access of organic material inside the cave (Sauro et al. 2018, GHEZZI et al. 2022). Specifically, these microbial communities showed the presence of novel lineages of Ktedonobacterales (Chloroflexota), Acidobacteriota, and Alphaproteobacteria in samples representing first and advanced stages of microbial colonization of quartzite subterranean rock and silica solubilization. Functional analyses indicated that in the different silica stromatolite formation stages different bacteria have functions associated with the capacity to oxidize atmospheric trace gasses (CO and H2) and to fix CO2, leading to the development of complex microbial communities able to interact with the quartzite rock and to colonize dark, oligotrophic and silica-based environments.

## 5. Conclusion

In this work we provide novel insights into the composition of the microbial communities and the metabolic functions that allow the survival of microbes in this extremely oligotrophic and slightly acidic environment. The different abundance/presence of specific bacterial taxa in the different stages of silica transformation (e.g. different orders of Acidobacteriota phylum) might indicate the specific involvement of different species in the interaction with the quartz rock contributing to silica amorphization (silica solubilization and precipitation) processes. On-going studies are investigating the enrichment of specific genetic functions that can be directly or indirectly associated to biomineralization processes including genes encoding enzymes involved in metabolic activities and in bacterial stress response in relation with acidic, oligotrophic and quartz-rich environments.

## Acknowledgments

We acknowledge all members of La Venta Esplorazioni Geografiche and Theraphosa Exploring Team for the organization and support on the field. We would also like to thank the indigenous community of Kamarata and the Government of Bolivar State for granting the permit to access the cave for speleological research. The 2023 expedition was supported by Tiziano Conte, Loris Greaud Studio, Gruppe5, Ferrino, Tiberino, Amphibious, Insula. We thank Dr Maria Roberta Randi for the SEM images realized at the BIGEA Department of the University of Bologna. The movie of the expedition can be watched at the following link: https://www.zdf-studios.com/en/program-catalog/international/unscripted/science-knowledge/tepui-house-gods

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# Species Turnover of Bryophytes Driven by Rare Species and Geomorphology in Neotropical Siliciclastic Caves

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## Abstract

Caves harbor unique and specialized biodiversity, playing a critical ecological role within their ecosystems. Despite this, research on cave-dwelling plant communities, especially in tropical regions, remains limited compared to studies on cave fauna. Light, humidity, and temperature significantly influence plant growth within the different plant groups. In caves Bryophytes often dominate due to their adaptability to low-light and high-humidity conditions. These plants are essential in regulating water, influencing soil formation, and facilitating the establishment of other plant groups. This study aimed to explore the bryophyte communities associated with Brazilian caves, focusing on the effects of geomorphological features, altitude, and entrance distances using the Zeta diversity approach. Conducted at 13 cave entrances in Ibitipoca State Park (PEIB), a region known for its large quartzite caves and rich bryophyte diversity, the research identified 70 species, including 14 new records for PEIB. Bryophytes were predominantly rupicolous, terricolous, or corticicolous, with leafy, mat, tuft, and weft forms being the most common life-forms. Zeta diversity analysis revealed that species turnover is primarily driven by rare species, with entrance dimensions, slope, and colonization depth significantly predicting diversity patterns. These findings underscore the ecological importance of caves as genetic reservoirs and refuges for rare species, emphasizing the need for conservation strategies to protect these unique environments.

## 1. Introduction

The distribution and intensity of light at cave entrances directly influence the composition and distribution of plant communities, making light the primary limiting factor for flora in these environments (Dobat, 1998; Abdulin, 2011; Mazina et al., 2023). Cave entrances, often situated in diverse landscapes with varying phytogeographic contexts, create unique ecological zones that bridge external ecosystems and the oligotrophic subterranean conditions. These ecotonal areas, while well-studied for faunal communities (Prous et al., 2015; Mammola & Isaia, 2018), can also play a critical role in cave flora varying factors like its entrance dimensions, geographic orientation, and soil composition (Alves & Kolbek, 1993; Badia et al., 2021; Monro et al., 2018). Plants adapted to low-light, high-humidity conditions, such as sciophytes and ombrophiles, are more likely to thrive in these habitats (Carmo et al., 2016; Puglisi et al., 2019).

Bryophytes, with their remarkable adaptability, dominate cave ecosystems due to their resilience to low-light and high-humidity conditions (Puglisi et al., 2018, 2019; 2024; Cong et al., 2023). They play a crucial ecological role by shaping water dynamics, serving as bioindicators, supporting vegetational succession, and promoting habitats for diverse fauna. (Rodrigues et al., 2016; Ren et al., 2021). Furthermore, bryophyte diversity at cave entrances may serve as an indicator of overall plant community diversity, suggesting their importance as ecological architects (Ren et al., 2021). However, these plants are highly sensitive to climatic changes, and caves may serve as refuges for rare or threatened bryophytes, highlighting their conservation value amid global warming threats (Gabriel et al., 2004; Mammola et al., 2019a, 2019b).

This study explores the ecology of bryophytes at cave entrances in Brazil, examining how geomorphological features, altitude, colonization depth, and entrance distance shape their diversity. We hypothesize that rare species are more affected by distance due to limited dispersion, while common species respond to structural features. Insights from this research aim to inform conservation efforts to protect these unique ecosystems.

## 2. Materials and methods

**Study Area**

Ibitipoca State Park (PEIB) is a protected area located in the southeastern region of Minas Gerais, Brazil, within the municipality of Lima Duarte. Spanning 1,923.5 hectares (Fig. 1), PEIB is renowned for its large quartzite caves within a relatively small area (Corrêa-Neto, 1997; Willems et al., 2008; Wray & Sauro, 2017). Notably, it includes the largest and the third-largest quartzitic caves in Brazil: Martimiano II and Bromélias caves (Rubbiolli et al., 2019; Oliveira et al., 2024). The PEIB is known for its significant diversity of bryophytes nationwide. Over 300 bryophyte species have been recorded in Ibitipoca State Park (Amorim et al., 2011; Luizi-Ponzo et al., 2013; Yano & Luizi-Ponzo, 2014; Machado et al., 2016), of which 209 belong to liverworts (Marchantiophyta), a significant proportion of the total species reported for Brazil (Machado et al., 2016).

**Sampling Design**

Thirteen cave entrances within Ibitipoca State Park were studied between March and June 2022. At each entrance, all bryophytes plant species were surveyed and collected, starting from the speleometric closure point. Geographic data, including X and Y coordinates and altitude, were obtained using a Garmin GPSMAP 64s. Cave entrance dimensions (height, width, and slope) were measured using a handheld compass and laser rangefinder, with dimensions analyzed as height-to-width ratios.

Bryophyte samples were dried and identified by specialists at the Bryophytes Laboratory of the Federal University of Juiz de Fora (UFJF). Identification involved microscopic analysis using an Olympus SZ040 or Zeiss Stemi-200C stereoscopic microscope and Olympus BX41 or Zeiss Primo Star light microscope, following taxonomic guidelines by

Goffinet et al. (2009), Frey & Stech (2009), and Crandall-Stotler et al. (2009). Voucher specimens were deposited in the Herbarium Professor Leopoldo Krieger (CESJ) at UFJF.

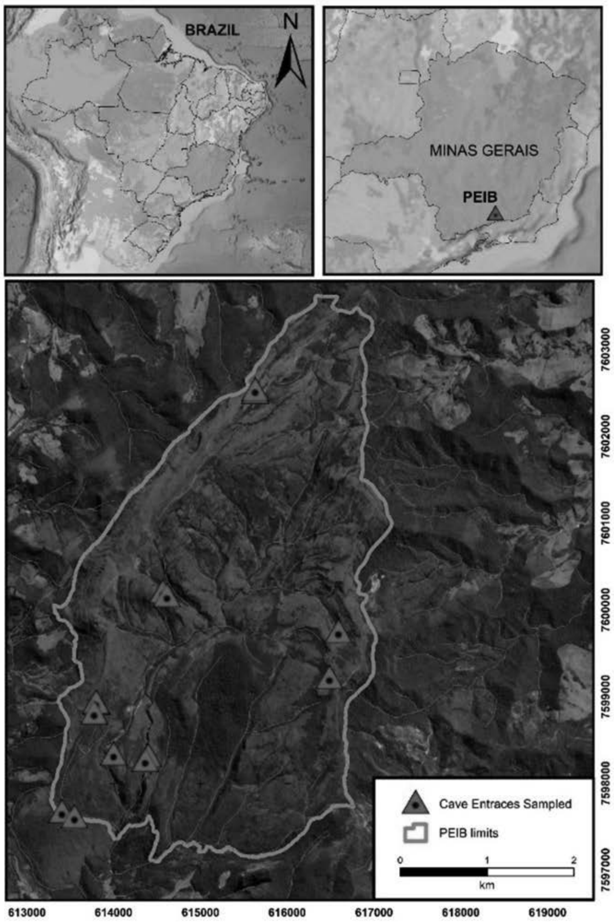

***Figure 1:*** A - Ibitipoca State Parque limits and the distribution of cave entrances sampled Ibitipoca State Parque, located in Minas Gerais state, Brazil.

**Data Analysis**

To access the life-forms and substrate preferences intrinsic to each bryophyte species, we used the information available in the Flora do Brasil database (REFLORA, 2024). To check previous records of each species in PEIB, we consulted publications on PEIB bryoflora by Yano & Luizi-Ponzo (2014) and Machado et al. (2016) and the scientific collections database SpeciesLink (SpeciesLink, 2024).

Zeta diversity (ζ) quantifies species shared across multiple assemblages, providing a framework for assessing biological diversity and species turnover patterns (Hui and McGeoch 2014; McGeoch et al. 2019). The zeta order represents the number of assemblages analyzed: ζ1 corresponds to alpha diversity, ζ2 to pairwise beta diversity, and higher orders assess species shared across more sites. We used this method to evaluate bryophyte diversity across sites, focusing on spatial variations in assemblages. Typically, zeta diversity decreases as the number of sites increases, with steep declines suggesting turnover driven by rare species, and gradual declines indicating turnover by common species. Retention rates, which measure species persistence across zeta orders, were compared across regions to understand the roles of rare and common species (Latombe et al., 2017, 2018).

To assess general diversity patterns, we calculated zeta diversity values (ζ2 to ζ6) and alpha diversity (ζ1), using normalized zeta values to address site richness differences (McGeoch et al., 2019). Simpson-equivalent zeta (ζij/Sj) and Sørensen-equivalent zeta (ζik/Søk) were employed to analyze nestedness and turnover (Baselga 2010). Zeta declines were modeled using the 'Zeta.decline' function from the 'zetadiv' R package (Latombe et al., 2018).

For environmental drivers of species turnover, we applied generalized dissimilarity models (GDM) using MS-GDM to evaluate spatial and environmental influences on zeta diversity across orders. This method accounts for non-linear environmental effects and spatial distances, exploring their impacts on turnover for both rare and common species. The analyses considered zeta orders 2 to 6, incorporating five environmental variables and inter-site distances, following Latombe et al. (2017, 2018).

## 3. Results

Our study documented 14 bryophyte species previously unrecorded in Ibitipoca State Park (PEIB), including seven liverworts and seven mosses. The liverworts, belonging to five families, included *Riccardia hymenophytoides (Aneuraceae), Cephalozia crossii, Odontoschisma brasiliense (Cephaloziaceae), Heteroscyphus marginatus, Lophocolea heterophylla (Lophocoleaceae), Symphyogyna leptothelia (Pallaviciniaceae),* and *Plagiochila aerea (Plagiochilaceae).* The mosses represented six families: *Rhynchostegium serrulatum (Brachytheciaceae), Syrrhopodon gardneri (Calymperaceae), Fissidens serratus, F. submarginatus (Fissidentaceae), Campylopus thwaitesii (Leucobryaceae), Lepidopilidium nitens (Pilotrichaceae),* and *Sphagnum magellanicum (Sphagnaceae).*

Interestingly, 11 of the 13 species previously reported by Yano & Ponzo (2014) in cave environments were absent in our study, including *Brachymenium consimile, Rhodobryum beyrichianum (Bryaceae), Holomitrium olfersianum (Dicraniaceae), Chryso-hypnum elegantulum (Hypnaceae), Lepidopilum brevipes Mitt., L. scabrisetum (Schwägr.) Steere (Bryophyta: Pilotrichaceae), Leptoscyphus amphibolius (Nees) Grolle (Marchantiophyta: Geocalycaceae), Saccogynidium caldense (Ångstr.) Grolle (Marchantiophyta: Geocalycaceae), Syzygiella trigonifolia (Steph.) Herzog, Hedwigia (Marchantiophyta: Jamesoniellaceae).*

Regarding bryophyte life forms, leafy forms were the most prevalent among cave bryophytes, followed by mats, tufts, and wefts. Substrate preferences predominantly included rupiculous, terricolous, and corticicolous types.

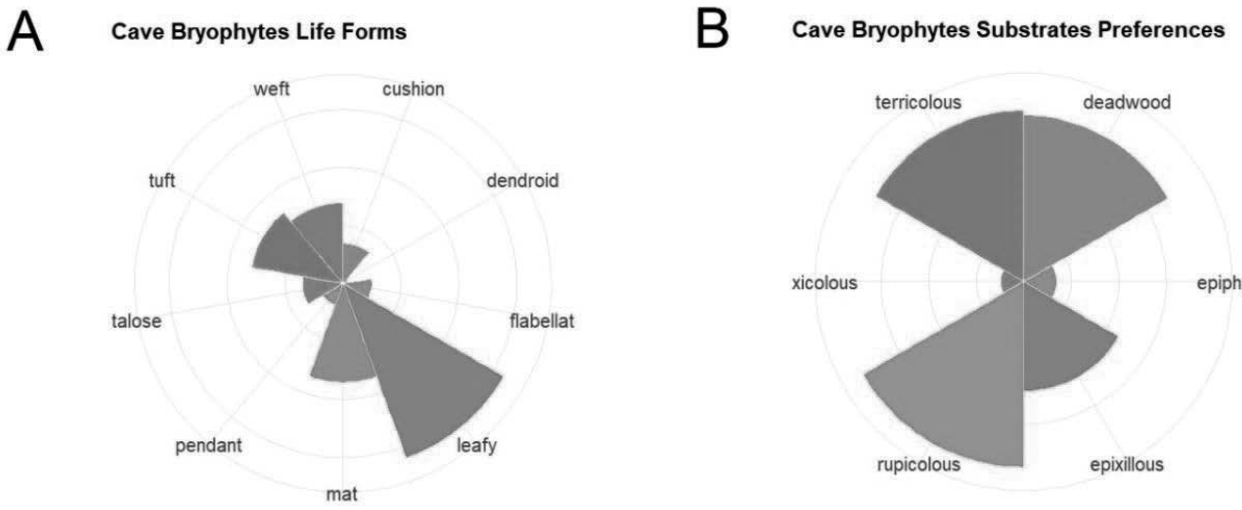

***Figure 2:*** *A) Life Forms and B) and substrate preferences of the species collected at the entrances of the caves studied.*

Zeta diversity values declined rapidly to zero, with retention rates increasing sharply, reflecting a consistent pattern across both Sørensen and Simpson analyses (Fig. 5A, C). The slightly steeper decline in Sørensen at lower zeta orders indicates minimal nestedness effects. Retention rates dropped at higher zeta orders, suggesting that even the most common species are less likely to persist as more sites are added, emphasizing the turnover driven by rare or site-specific species.

Spatial distance emerged as a key driver of turnover for rare species (ζ2,3), while common species (ζ4-6) were influenced primarily by slope and depth of colonization. For ζ2, entrance ratio, slope, and colonization depth were significant, whereas slope was the dominant factor for ζ3. The MS-GDM models explained variances of 42.9% (ζ2), 59.7% (ζ3), 62.5% (ζ4), 56.1% (ζ5), and 41.7% (ζ6), highlighting the distinct environmental and spatial variables affecting turnover across different zeta orders.

## 4. Discussion

Tropical regions, known for their rich biodiversity, face significant anthropogenic threats, including in cave ecosystems (Myers et al., 2000; Ferreira et al., 2022). While considerable conservation efforts focus on subterranean biology, the study of cave-dwelling flora in tropical areas remains underexplored. Brazil, with its vast karst systems and caves across biomes like the Cerrado and Atlantic Forest, provides an opportunity to study such ecosystems. Ibitipoca State Park (PEIB), notable for its quartzite caves and exceptional bryophyte diversity, exemplifies this potential. Our study, focusing on 13 cave entrances, identified 70 bryophyte species, including 14 not previously recorded in PEIB. This expands the known checklist of cave bryophytes, highlighting caves as vital genetic reservoirs and habitats for endemic flora (Gabriel et al., 2004; Cong et al., 2023).

Bryophyte diversity at PEIB reflects substrate availability and environmental conditions. Liverworts are typically the most diverse group in the park, but in cave habitats, mosses were slightly more diverse in our study. Substrates in cave entrances, such as tree trunks and decomposing matter, are scarce, influencing species distribution and reducing diversity differences between liverworts and mosses compared to external environments (Yano & Ponzo, 2014). Thalloid liverworts often colonize terrestrial substrates, while leafy liverworts and mosses adapt to varied substrates, with mosses showing greater habitat generalism and tolerance for low light (Shaw & Goffinet, 2000; Herrero-Borgoñón, 2024).

Subterranean conditions filter bryophyte communities, favoring specific life forms like leafy, turf, weft, and mat forms, which reflect adaptations to environmental challenges. Dense forms like cushions or turfs store water but receive less light, while mats and wefts thrive in shady, humid environments typical of caves (Vittoz et al., 2010; Bates, 1998). These functional traits underscore the ecological strategies of bryophytes in adapting to the oligotrophic, low-light, and high-humidity conditions of cave entrances.

### Turnover Patterns

Zeta diversity analyses revealed a rapid decline from the second to higher zeta orders, highlighting the dominance of rare species in the cave bryophyte communities of PEIB. This exponential decline, consistent in both Sørensen and Simpson analyses, indicates minimal nestedness effects and suggests that species retention across sites is largely stochastic (Latombe, 2017; Fonte et al., 2021). The retention rate patterns, where rates initially rise but then decrease at higher zeta orders, emphasize the influence of rare species on turnover, with even common species becoming less likely to persist across multiple sites.

These findings point to a small core of common species widely distributed and a larger pool of rare species restricted to specific locations. The unique environments of caves, shaped by geographic isolation and distinct microhabitats, foster this pattern by serving as refuges for rare, endemic, and relict species (Culver & Pipan, 2019; Oliveira & Ferreira, 2024; Gabriel et al., 2004; Monro et al., 2018; Cong et al., 2023).

### Drivers of Turnover

MS-GDM models highlighted spatial distance as a critical factor driving turnover for rare species (ζ2-3) but less influential for common species (ζ4-6). Entrance dimensions significantly affected turnover at lower zeta orders, indicating lower phylogenetic distances when species overlap increases. As zeta orders rose, factors like slope and colonization depth gained importance, particularly for common species.

Steeper slopes at cave entrances were associated with higher nutrient and moisture inputs from surface environments, creating favorable substrates for colonization. These conditions align with the requirements of bryophytes, which depend on water for metabolic processes and gamete motility during reproduction (Lemos-Michel, 2001). Thus, environmental factors at cave entrances drive the compositional differences observed between rare and common species, reflecting the critical ecological role of these unique habitats.

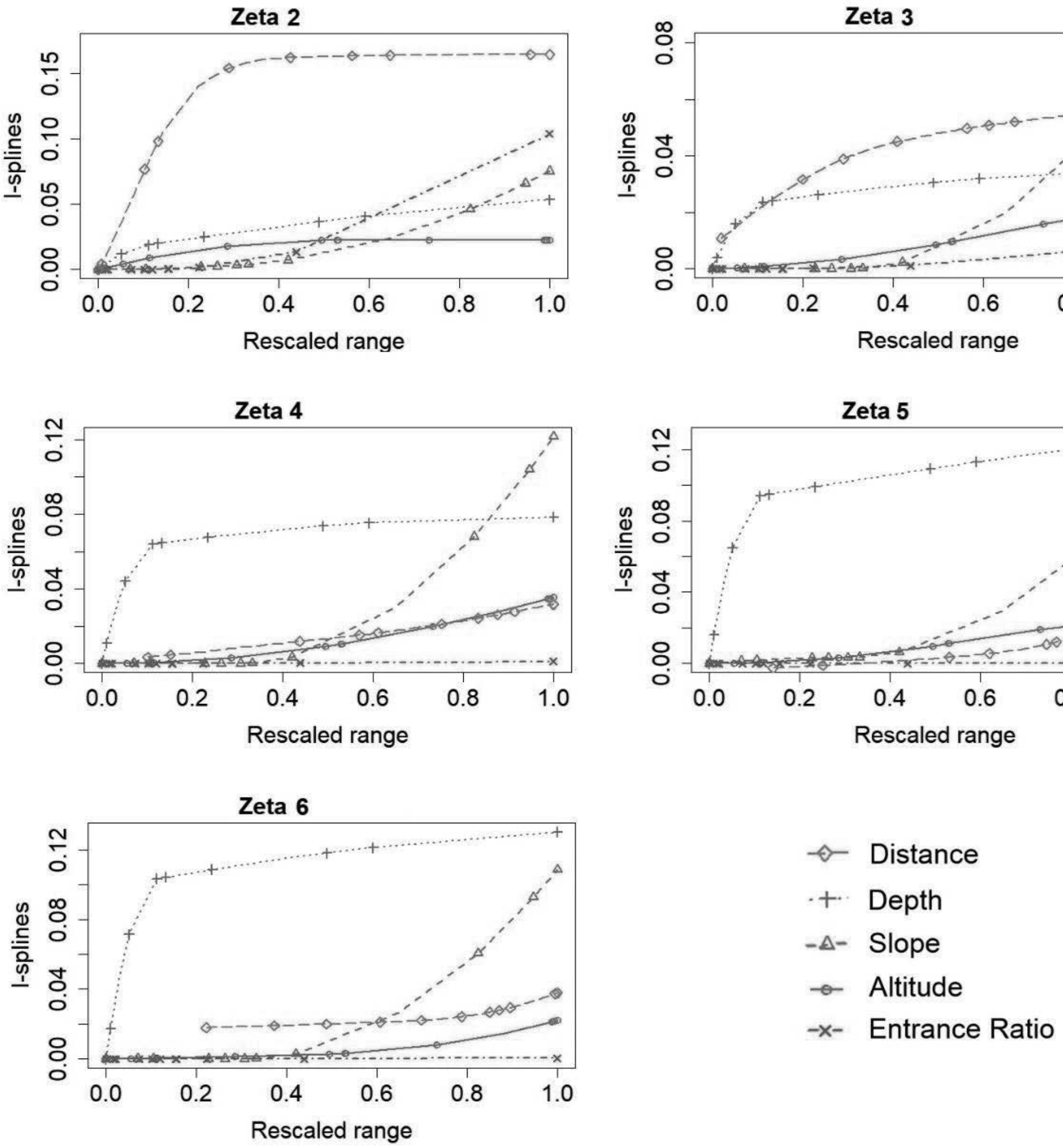

***Figure 3:*** *MS-GDM analyses for zeta orders 2-6 using environmental features and straight-line distances between sites based on their geographic coordinates of the cave entrances bryoflora communities sampled.*

## 5. Conclusion

In conclusion, our study underscores that, similar to findings in non-tropical caves, some Brazilian caves serve as important habitats for bryophyte communities, supporting highly diverse and unique assemblages. Our research in Ibitipoca State Park identified 70 bryophyte species, including 14 new records, and highlighted that cave geomorphological features (such as entrance dimensions and slope) as well as geographic distance, are significant factors in predicting bryophyte diversity and phylogenetic similarity among subterranean habitats. Furthermore, rare species appear to play a key role in driving species turnover. This research provides valuable insights into the ecology of cave flora in tropical regions and supports ongoing conservation efforts aimed at protecting cave habitats and their associated flora.

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# New species of cave-restricted *Hyalella* (Crustacea: Amphipoda) for Brazil, with the first records from the Amazon

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## Abstract

New species of the freshwater amphipod genus *Hyalella* have been discovered in groundwater habitats across Brazil. Three of these species were found in ferruginous caves (two in the state of Pará and one in Minas Gerais) while a fourth species was identified in a cave in Rio Grande do Sul. The discovery of the *Hyalella* species in Pará is particularly noteworthy, as it represents the first record of this genus in the Amazon biome, where no *Hyalella* species had been documented until now.

## Résumé

De nouvelles espèces d'amphipodes d'eau douce du genre *Hyalella* ont été découvertes dans des habitats souterrains à travers le Brésil. Trois de ces espèces ont été trouvées dans des grottes ferrugineuses, deux dans l'État du Pará et une dans celui du Minas Gerais. Une quatrième espèce a été identifiée dans une grotte de l'État du Rio Grande do Sul. La découverte des espèces du Pará est particulièrement significative, car elle constitue le premier enregistrement de ce genre dans le biome amazonien, où les espèces de *Hyalella* n'avaient jusqu'à présent jamais été documentées.

## Resumo

Novas espécies do anfípodo de água doce do gênero *Hyalella* foram descobertos para águas subterrâneas do Brasil. Três espécies foram coletadas em cavernas ferruginosas, sendo duas delas no estado do Pará, e uma no estado de Minas Gerais. A quarta espécie foi coletada em uma caverna do Rio Grande do Sul. As espécies do Pará representam um importante registro no bioma amazônico, onde até o momento não havia ocorrências de espécies deste gênero.

## 1. Introduction

The genus *Hyalella* comprises over 100 described species, with more than 80 endemic to South America (Marrón-Becerra & Hermoso-Salazar, 2023; Marrón-Becerra et al., 2023; Peralta & Verónica, 2023; Reis et al., 2023; Tomikawa et al., 2023). While most research has focused on Argentina and southern Brazil, recent discoveries in Mexico, Chile, and Paraguay suggest that this apparent distribution bias may be influenced by differences in sampling efforts and the proximity of research institutions (Reis et al., 2020, 2023; Mussini et al., 2024).

*Hyalella* is a genus of freshwater amphipods found in both epigean and hypogean environments, including caves, groundwater, and shallow subterranean habitats (Rodrigues & Bueno, 2012; Bastos-Pereira et al., 2018; Peralta & Verónica, 2023). In Brazil, six species have been recorded in subterranean habitats: *H. caeca* Pereira, 1989; *H. spelaea* Bueno & Cardoso, 2011; *H. epikarstica* Rodrigues, Bueno & Ferreira, 2014, *H. sumida* Penoni & Bueno, 2025; and *H. ceciliae* Penoni & Bueno, 2025 from São Paulo; H. formosa Cardoso & Araujo, 2014, from Paraná; *H. veredae* Cardoso & Bueno, 2014, from Minas Gerais; and *H. imbya* Rodrigues & Bueno, 2012, from a wetland in the municipality of Roque Gonzales, Rio Grande do Sul (Pereira, 1989; Cardoso et al., 2011, 2014; Rodrigues & Bueno, 2012; Penoni et al., 2025a,b). All these species are considered stygobitic.

This study significantly expands the known distribution of *Hyalella* in Brazil, providing new records for the Amazon region. Two new species were discovered in the state of Pará, collected from iron ore caves in the Serra dos Carajás region. Additionally, a third species was found in an iron ore cave in Minas Gerais, while a fourth species was identified in a carbonate cave in Rio Grande do Sul. These findings highlight the importance of subterranean environments in harboring previously undocumented *Hyalella* diversity.

## 2. Materials and methods

The newly discovered *Hyalella* species were preserved in 70% ethanol and deposited in the Collection of Subterranean Invertebrates of Lavras (ISLA) at the Federal University of Lavras (UFLA), Brazil. Specimens were dissected and mounted on slides using Hoyer's medium for detailed morphological examination. Analyses were conducted using a ZEISS Scope A1 optical microscope equipped with an Axio 105 Color camera, with species descriptions based on photographs of the paratypes. Habitus images of both males and females were captured using a ZEISS Axio ZoomV16 stereomicroscope coupled with an Axio Cam 506 Color camera. Setae nomenclature followed the classification system proposed by Zimmer et al. (2009).

### Study area

#### *Pará state (PA)*

The species *Hyalella* n. sp. 1 (Fig. 1C, 2A) (Fig. 1C) and *Hyalella* n. sp. 2 (Fig. 1D, 2B) were collected from ferruginous caves located in Serra do Tarzan and Serra Sul, respectively, in the state of Pará, Brazil. These areas belong to the Serra dos Carajás Speleological Unit (Fig. 1A,B), which contains an extensive network of over 2,000 ferruginous caves and is renowned for its vast mineral reserves, including iron, nickel, copper, and gold (Piló et al., 2015).

The region has a tropical climate, with temperatures ranging from 23°C to 25°C and an average annual precipitation of approximately 2,400 mm. The dry season extends from June to September, with monthly rainfall ranging from 10 to 90 mm, while the rainy season lasts from October to April, with precipitation levels between 160 and 340 mm per month (Sahoo et al., 2016).

Serra do Tarzan is located within two federally protected conservation units: the Campos Ferruginosos National Park and the Carajás National Forest (FLONA Carajás). Consequently, this geomorphological area remains relatively pristine compared to other parts of Carajás, which are more affected by mining activities.

In contrast, Serra Sul, despite being within FLONA Carajás, hosts the world's largest open-pit mining operation, producing up to 90 million tons of iron ore annually (Matlaba et al., 2017) (Fig. 1C, D).

#### *Minas Gerais state (MG)*

*Hyalella* n. sp. 3 (Fig. 1E,F, 2C) was identified in an iron ore cave within the Iron Quadrangle region of Minas Gerais, a major hub for iron ore and gold extraction in Brazil. This region represents a transitional zone between two global biodiversity hotspots—the Brazilian Savannah (Cerrado) and the Atlantic Forest (Myers et al., 2000). The landscape is a mosaic of native vegetation interspersed with areas impacted by human activities, including mining and agriculture (Gomes et al., 2019). The local climate is characterized by dry winters and wet summers, with monthly average temperatures ranging from 16.5°C to 23.2°C (Gomes et al., 2019).

#### *Rio Grande do Sul state*

*Hyalella* n. sp. 4 was collected in Serra Geral (Fig. 2D), within caves formed in basaltic rock formations resulting from ancient volcanic flows (Reginato et al., 2013). Although cave research in this region remains in its early stages, at least 14 species of *Hyalella* have already been described, highlighting the region's high potential for amphipod diversity (Talhaferro et al., 2021).

The regional climate is characterized by temperatures ranging from 15°C to 18°C. Peak rainfall occurs at the end of spring (September–October, 160–180 mm/month) and during summer (January, ~160 mm/month) (Buriol et al., 2007).

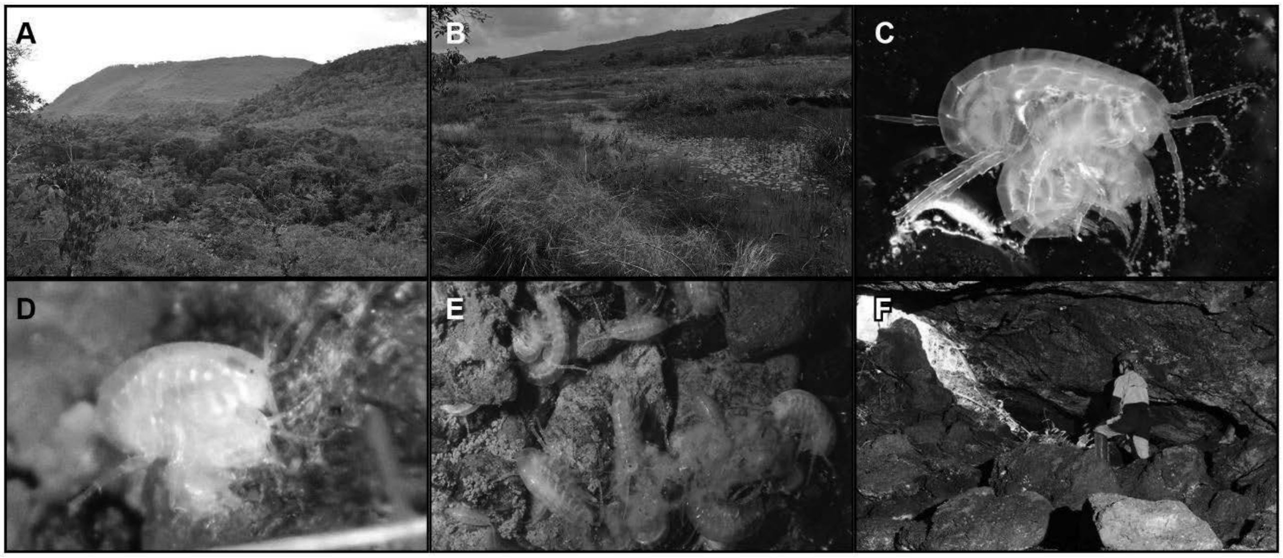

***Figure 1:*** *A, Carajás Mountain Range; B, altitude lake in Carajás Mountain Range; C, couple in amplexus of Hyalella n. sp. 1; D, couple in amplexus of Hyalella n. sp. 2; E, Hyalella n. sp. 3; F, Cave entrance where Hyalella n. sp. 3 was collected.*

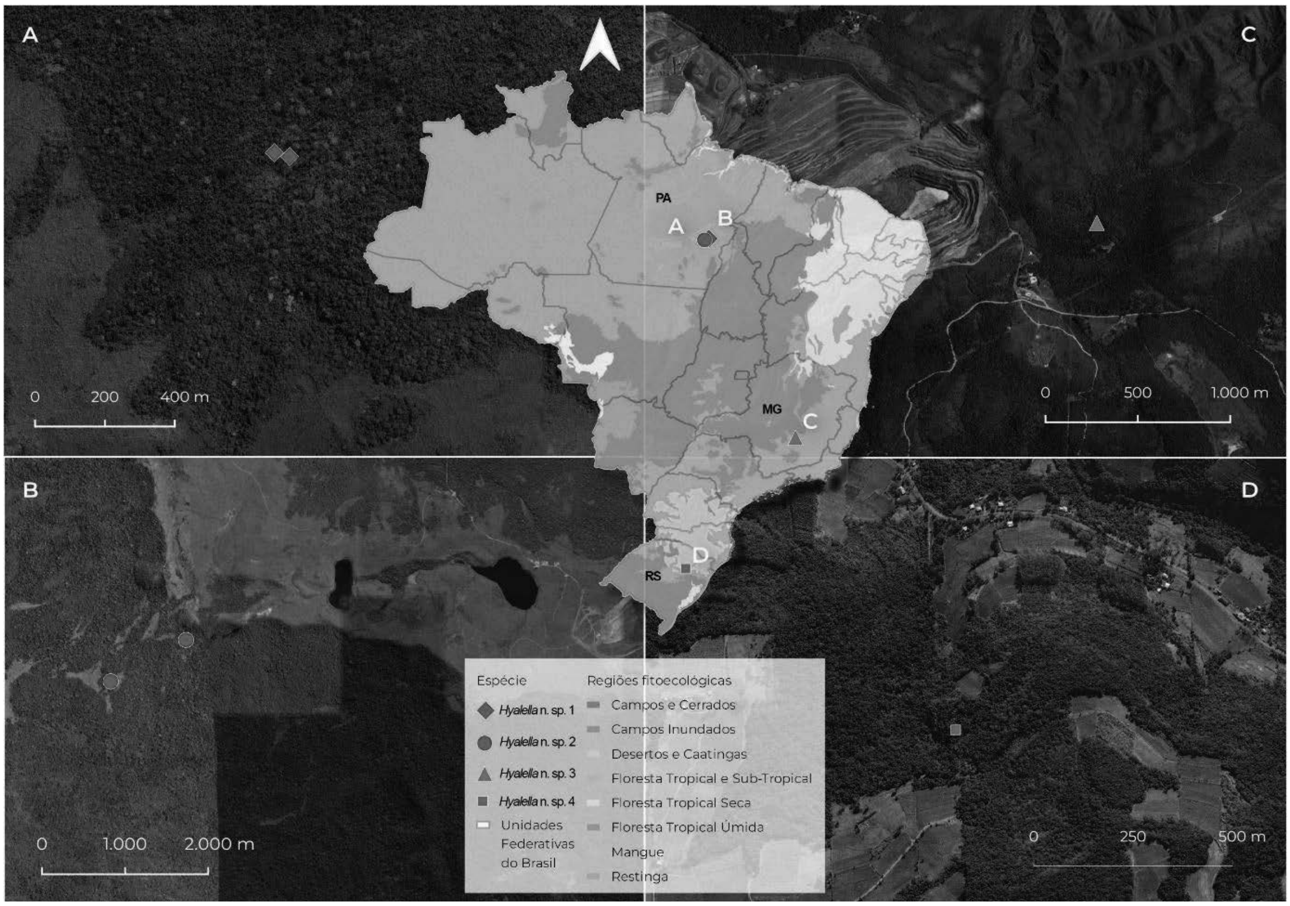

***Figure 2:*** *Distribution map of the new Hyalella species in Brazil. Abbreviations: PA, Pará state; MG, Minas Gerais state; RS, Rio Grande do Sul state.*

## 3. Results

*Hyalella* n. sp. 1 (Figs 3A, 4A) differs from the other species by the absence of eyes, by the length of the antennae 1 and 2, by the shape of male gnathopod and by the number and type of setae on telson. Comparing with the other stygobite *Hyalella* species from Brazil, *Hyalella* n. sp. 1 resembles *H. caeca* and *H. spelaea* by the absence of a curved seta on uropod 1, however differs by the number of setae on telson, with two simple setae on the middle plus one cuspidate seta on each side, while both H. caeca and H. spelaea have only two simple setae apically. By the similar length of the antennae 1 and 2, *Hyalella* n. sp. 1 resembles *H. veredae*, however, *H. veredae* presents one curved seta on uropod 1.

*Hyalella* n. sp. 2 (Figs 3B, C, 4B) differs from *Hyalella* n. sp. 1 by the reduced number of articles on the antennae (antenna 1 with 6 and antenna 2 with 8 versus antenna 1 with 14 and antenna 2 with 11 in *Hyalella* n. sp. 1), and the length of the antennae with antenna 1 shorter than antenna 2 versus antenna 1 and antenna 2 with similar length in *Hyalella* n. sp. 1. The telson differs with two cuspidate setae versus two simple and two cuspidate setae on *Hyalella* n. sp. 1.

*Hyalella* n. sp. 3 (Figs 3D, 4C) resembles *H. troglofugia*, that occurs in the nearby municipality of Nova Lima, in Minas Gerais state. Both species lack the curved setae on uropod 1, and present 2 cuspidate setae on telson. However, *Hyalella* n. sp. 3 differs from *H. troglofugia*, by the presence of reduced eyes, that are absent in *H. troglofugia*; and the number of articles on antennae, 6-7 on antenna 1 and 11 on antenna 2; while the number of articles on *H. troglofugia* are 9-11 and 9-12 respectively; the number of setae in gnathopod 1 propodus, 7 in *Hyalella* n. sp. 3 and *Hyalella* n. sp. 4 in *H. troglofugia*; and the shape of gnathopod 2 propodus, slender in *Hyalella* n. sp. 3 (1.5 times longer than wide).

*Hyalella* n. sp. 4 (Figs 3E, 4D) resembles *H. imbya* in its troglomorphic characteristics, such as the absence of eyes and body pigmentation. It also shares the relative development of antenna 1, which is longer and has more articles compared to antenna 2, as well as the presence of two simple setae on telson. However, the two species differ in the number of articles in their antennae: antenna 1 has 15–16 articles in *Hyalella* n. sp. 4 and 18–23 articles in *H. imbya*, while antenna 2 has 10 articles in *Hyalella* n. sp. 4 and 14–16 in *H. imbya*. Additionally, an important distinguishing feature is the presence of curved seta on uropod 1 in *H. imbya*, which is absent in *Hyalella* n. sp. 4.

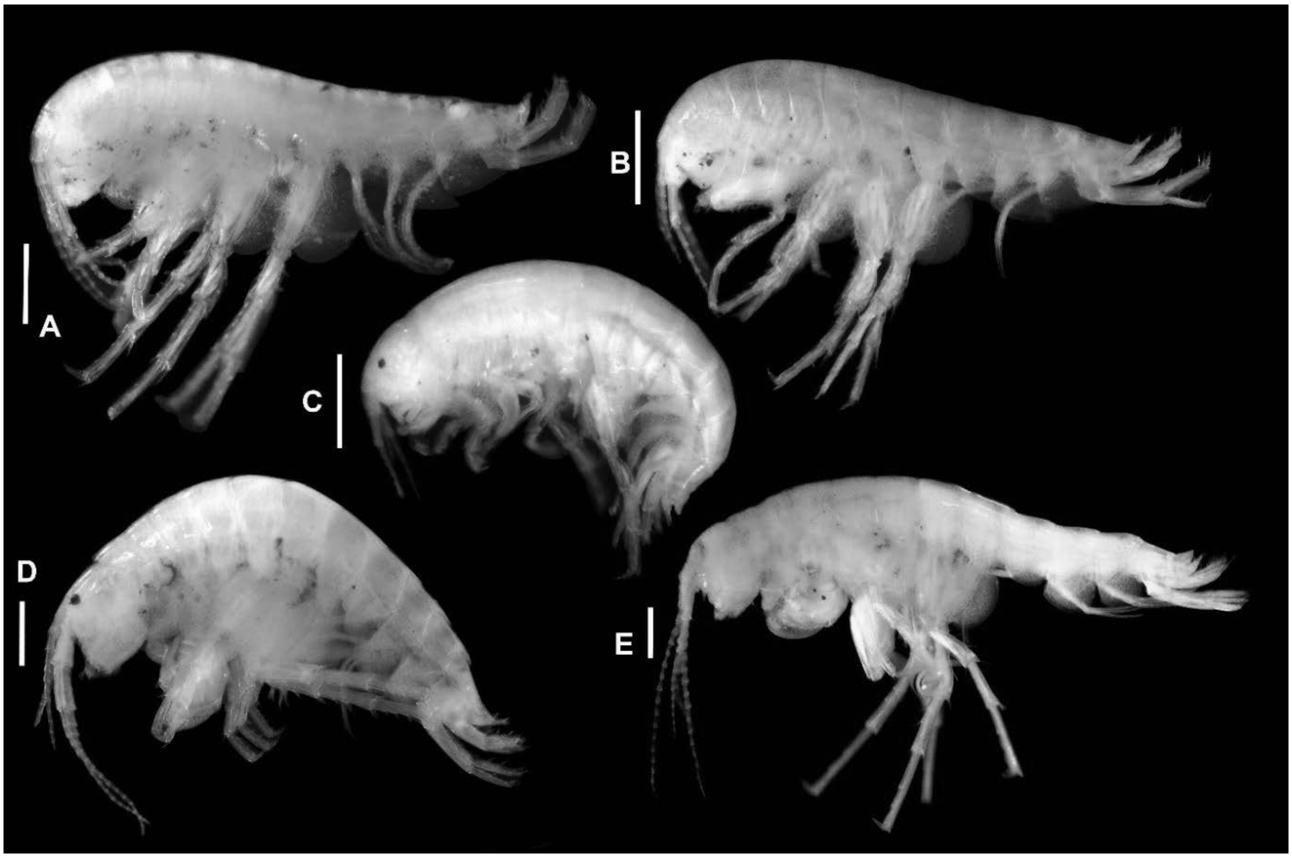

***Figure 3:*** *Subterranean species of Hyalella. A, male of Hyalella n. sp. 1; B, male of Hyalella n. sp. 2; C, female of Hyalella n. sp. 2; D, male of Hyalella n. sp. 3; E, male of Hyalella n. sp. 4. Scale bars: 0.5 mm.*

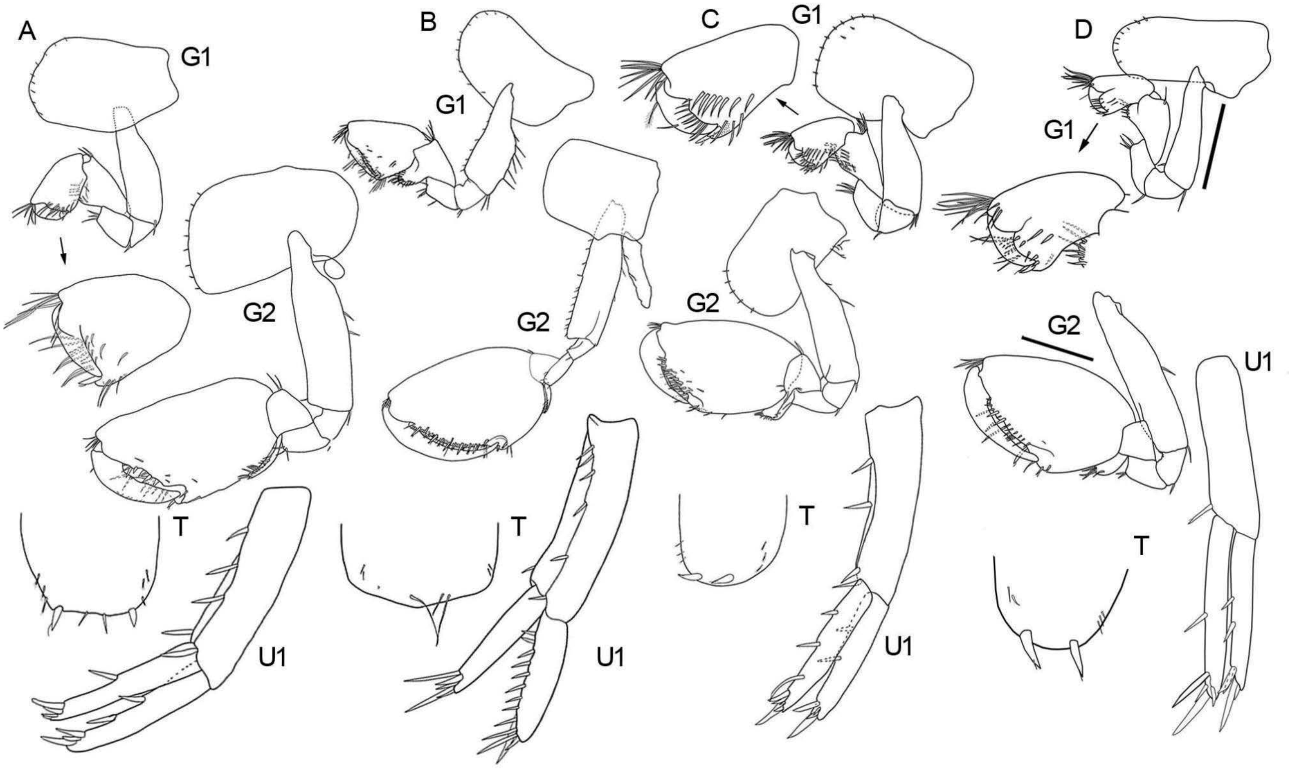

***Figure 4:*** *Subterranean species of Hyalella. A, male of Hyalella n. sp. 1; B, male of Hyalella n. sp. 2; C, male of Hyalella n. sp. 3; D, male of Hyalella n. sp. 2. Scale bars: 0.2 mm. Abbreviations: G1: gnathopod 1; G2: gnathopod 2; U1: uropod 1; T: telson.*

## 4. Discussion

Four new species are recorded in caves from Brazil. Three species are recorded in iron ore caves (Fig. 2).

*Hyalella* n. sp. 1 and *Hyalella* n. sp. 2 represent two important records for the genus in the Amazon region, and the record in the state of Pará. Providing important information on the distribution of the genus in poorly sampled locations, whether due to difficulty of access or sampling effort. *Hyalella* n. sp. 3 represents the second stygobite species recorded for Minas Gerais state, the first being *H. veredae*. However, in Minas Gerais, two epigean species present troglomorphic traits such as the variations in the eye size, H. montana and *H. troglofugia*, which indicate may suggest that such species inhabit interstitial subterranean spaces (Rodrigues et al., 2017; Bastos-Pereira et al., 2018).

*Hyalella* n. sp. 4 represents the second troglomorphic species recorded for the Rio Grande do Sul state, the first being the hypothelminorheic species, *H. imbya*.

The newly described species are considered stygobitic, displaying troglomorphic characteristics such as depigmentation and elongated appendages, particularly antenna 1 relative to antenna 2. Eye morphology varies across species: in *Hyalella* n. sp. 1 and *Hyalella* n. sp. 4, the eyes are absent; in *Hyalella* n. sp. 3, they are reduced. Interestingly, in *Hyalella* n. sp. 2, some specimens lack eyes entirely, a variation also observed in certain individuals of *H. veredae* (Zeppon et al., 2021). New records of *H. veredae* were discovered and it was observed a variation in the eyes which may reflect the degree and duration of isolation experienced by each population in subterranean environments (Zeppon et al., 2021).

## 5. Conclusion

New species are continuously being discovered, highlighting the importance of studying caves. Many of these species are restricted and endemic to specific locations, and their formal description not only advances scientific knowledge but also contributes to the preservation of the cave where they occur and the entire associated community. Therefore, conducting fauna inventories in underexplored areas is essential, as these discoveries reveal the significant potential for identifying new species.

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# Human-associated bacterial phyla and microplastic fibers are enriched in sediments of a Southern Appalachian show cave

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## Abstract

Three southern Appalachian caves were assessed in a multiproxy analysis to determine how anthropogenic impact can be recorded in cave sediment. Sediments from Daniel Boone Caverns (gated and locked with few visitors), Worley's Cave (a wild show cave), and Linville Caverns (a developed show cave) were all analyzed using Illumina sequencing for microbial community structure, as well as salt fractionation for microplastics. In addition, a combination of X-ray diffraction, whole rock geochemistry, and transmission electron microscopy (TEM) was used to determine how mineralogy affects microbial community structure, or if it records anthropogenic impact in other ways. Distinct geochemical and microbial signatures were found both within and between each cave. Samples from Linville Caverns, the most highly developed of the three caves, contained more microplastics, and more Bacteroidota and Proteobacteria (human-associated phyla) than the other two caves, while Daniel Boone caverns contained more Thermoplasmatota, Chloroflexi, and Nitrospirota (oligotrophic phyla) and fewer microplastics. The primary microplastic found across all three caves was fiber (likely from synthetic fabrics). Biological manganese oxidation was present in all three caves, with significant differences in geochemistry across sites that contained Mn oxides vs those that did not. Geochemistry and mineralogy of sediments primarily reflected the local bedrock.

## 1. Introduction

Karst aquifers are vital sources of potable drinking water on a global scale (HARTMANN et al. 2014) and they support some of the most fragile and diverse ecosystems on Earth, susceptible to human alteration (PRONK et al. 2007). Anthropogenic impact in cave ecosystems has been globally documented (SAIZ-JIMENEZ 2012). Human visitation can lead to the destruction of microhabitats (NORTHUP 2011), alteration of biogeochemical cycles, introduction of fecal bacteria (S. CARMICHAEL et al. 2013), introduction of alien or invasive species (NICOLOSI et al. 2023), and can impact sensitive cave fauna such as bats (BLEHERT et al. 2011). Natural caves are oligotrophic and maintain a relatively consistent temperature throughout the year (BARTON & JURADO 2007). Show caves disrupt this natural equilibrium by the installation of artificial lighting, stairs, and trails, and inputs from human visitors (CIGNA 2019). These human-induced alterations can contribute to changes in cave microbial biodiversity (FERNANDEZ-CORTES et al. 2011). Microplastic deposition has also been documented in cave sediments and is considered a biomarker for anthropogenic impact (HASENMUELLER et al. 2023).

Many caves contain dark brown to black manganese (Mn) oxide biofilms or ferromanganese deposits comprised of higher concentration of Mn and Fe than the surrounding bedrock (CARMICHAEL & BRÄUER 2015). Field evidence from epigenic caves in the southern Appalachian Mountains indicate that Mn oxidation is stimulated by exogenous carbon input into carbon limited environments (CARMICHAEL et al. 2015; CLOUTIER et al. 2017), although we hypothesize that cave bedrock mineralogy and geochemistry may strongly influence these processes as well. Indeed, the impact of mineral type (HUTCHENS et al. 2010) or host rock (BARTON et al. 2007) on microbial communities, and the interactions of specific microorganisms with specific minerals (KOSZNIK-KWAŚNICKA et al. 2022) have been well documented.

In this study, we sought to determine to what degree human visitation, cave minerology and/or geochemistry may influence cave microbial community assemblages, functional processes, and/or the relative abundance of biomarkers for human impact (such as microplastics).

## 2. Materials and methods

Three caves from the southern Appalachian Mountains were sampled for this study (Fig. 1). Manganese oxidation was detected using the Leucobeurbelin blue (LBB) assay. All samples were taken from horizontal surfaces such as terraces, shelves, and the cave floor, rather than from vertical cave walls. Samples were taken by scraping the collection tube along the sample site surface, and placing it on ice to be transported back to the lab. Samples were stored at -80°C, with splits taken for geochemical and mineralogical analysis. In addition to the presence or absence of Mn oxidation, traffic level and proximity to a light source were also noted (Table 1).

| Sample Site | Cave | Mn +/- | Traffic | Light |
|---|---|---|---|---|
| After Alligator | WC | (+) | High | No Light |
| Machete | WC | (+) | Low | No Light |
| Jack' O Lantern | WC | (+) | Low | No Light |
| Shimmy | WC | (-) | High | No Light |
| Dripline Ceiling | WC | (-) | Low | No Light |
| Slip' N Slide | WC | (-) | High | No Light |
| Ladder Shelf | DBC | (+) | Very Low | No Light |
| Ladder Cubby | DBC | (+) | Very Low | No Light |
| Black Coating | DBC | (+) | Very Low | No Light |
| Crawlspace Pit | DBC | (-) | Very Low | No Light |
| Sandwich Pool | DBC | (-) | Very Low | No Light |
| Mudcrack Vertebrae | DBC | (-) | Very Low | No Light |
| Rock Slide Clay | LC | (+) | Medium | No Lights |
| Laundry Chute | LC | (+) | Low | No Lights |
| Conch Shell | LC | (+) | High | Direct light |
| Side Shoot | LC | (+) | Low | No Lights |
| Passage to Bottomless Pool | LC | (-) | Low | No Lights |
| Bear Den | LC | (+) | Low | No Lights |
| Entrance Area to Bottomless Pool | LC | (-) | Medium | Lights in proximity |
| Fireplace | LC | (-) | High | Direct light |
| Small Chipped Out Puddle | LC | (-) | High | Indirect light |

***Table 1:*** *Sample site descriptions. WC- Worley's Cave; DBC- Daniel Boone Caverns; LC- Linville Caverns.*

Six samples were taken from Worley's Cave in Sullivan County, Tennessee: three from sites with little to no Mn oxidation, and three from sites that showed signs of current Mn oxidation. Worley's Cave (WC) is found within the Ordovician aged Knox Dolomite of the Valley and Ridge province and is frequently visited by recreational cavers. The cave contains a subterranean creek and is hydrologically linked to sink holes in neighboring farm fields. In contrast to WC, Daniel Boone Caverns (DBC) is rarely visited and can therefore be considered a pristine cave. It is located in Scott County, Virginia, and does not experience any agricultural or municipal runoff due to its isolation on top of a ridge. It contains several pools, but lacks a complex subsurface hydrologic system (M. J. CARMICHAEL et al. 2013). DBC is located approximately 64 km from WC and is likewise hosted within the Knox Dolomite. Six samples were taken from DBC as well: three sites with little to no Mn oxidation and three sites with Mn oxidation. Samples were collected from nine different sites in Linville Caverns (LC) with varying degrees of human traffic, manganese oxidation or light exposure (Table 1). LC is located in McDowell County, North Carolina, within the Cambrian Shady Dolomite. It is a highly trafficked show cave, receiving over 100,000 visitors a year. The cave can also be considered highly developed as a concrete path covers most of the floor in areas open to the public; stairs, handrails, and artificial lights are present in certain areas as well. Whole rock geochemistry was performed at Activation Laboratories in Ancaster, Ontario, Canada, using total ident 4E - exploration analysis. Mineralogy was assessed using a Shimadzu 6000 powder X-ray diffraction system with a Cu X-ray source, measured from 5-80°2θ in the Department of Geological and Environmental Sciences, Appalachian State University. Preliminary mineral identification was assigned via Match! Phase identification software from Crystal Impact, using the American Mineralogist database. Mineralogy was confirmed via transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM-EDS) at 120 kV and 65.5 µA, using a JEOL JEM-1400 TEM with an Oxford Xplore TEM 80 mm2 EDS detector in the Dewel Microscopy Facility at Appalachian State University. Particle size was measured on a Bettersizer S3 Plus particle size and shape analyzer with a BT-803 wet dispersion system in the Department of Geological and Environmental Sciences, Appalachian State University. DNA was extracted using MP Biomedicals FastDNA spin kit for soil. The 515/806 primers of the V4 variable region of the 16S rRNA gene were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95°C for 5 minutes, followed by 30-35 cycles of 95°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. This was followed by a final elongation step at 72°C for 10 minutes. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification as well as the relative intensity of bands. Samples were multiplexed using unique dual indices and are pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP 15 beads. Then the pooled and purified PCR product was used to prepare an Illumina DNA library. Sequencing was performed at MR DNA on a MiSeq using the 515/806 primers of the V4 variable region of the 16S rRNA gene. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, read-ends were joined and short sequences (<150bp) or those with ambiguous base reads were removed. Poor quality sequences were filtered (Emax = 1.0) and dereplicated. Chimeras were removed after dereplicating, denoising, and removal of unique sequences with sequencing or PCR errors. Taxonomic identification of zOTUs was carried out using a curated NCBI (www.ncbi.nlm.nih.gov) database and a BLASTn search. BBMap package was used to quality filter raw reads produced by Illumina MiSeq (Bushnell, 2015). Forward and reverse sequences were aligned using DADA2 and nucleotide length was trimmed to <200 and 190 for forward and reverse reads respectively (CALLAHAN et al. 2016). A minimum overlap of 12 base pairs was established, sequences were denoised and chimeras removed. The SILVA 138 SSU database was used to assign taxonomy (MCLAREN & CALLAHAN 2021). Beta-diversity (Raup-Crick Monte Carlo clustering) was used for the non-metric multidimensional scaling (NMDS) ordination plots. Alpha diversity was calculated via the Shannon and Simpson indices. Sequence data were converted to relative abundances of taxa per group as a proportion of all sequences for use in taxonomic figures (MCMURDIE & HOLMES 2013) Principal Component Analysis (PCA) was performed with the software PAST v. 1.81 (HAMMER & HARPER 2001). Eigen values, the cumulative percentage of variance explained by each, the loading coefficients for each axis and PCA scores were saved for evaluation. The relative correlation (importance) of metadata to each of the PCA axes was evaluated by examining the loading coefficients. One-way ANOVAs with the Tukey post-hoc test were used to identify any statistically significant differences in community composition at the phylum level, as well as any significant differences in geochemistry, between the three caves. Two-sample, two-tailed, unpaired t-tests were used to determine any statistically significant differences in community composition at the phyla level within Mn positive and Mn negative samples taken from a cave. Two-sample, two-tailed, unpaired t-tests were also used to determine any statistically significant differences between the geochemical factors within Mn positive and Mn negative samples taken from a cave. For all t-tests and ANOVAs, a reported p-value <0.05 was considered significant.

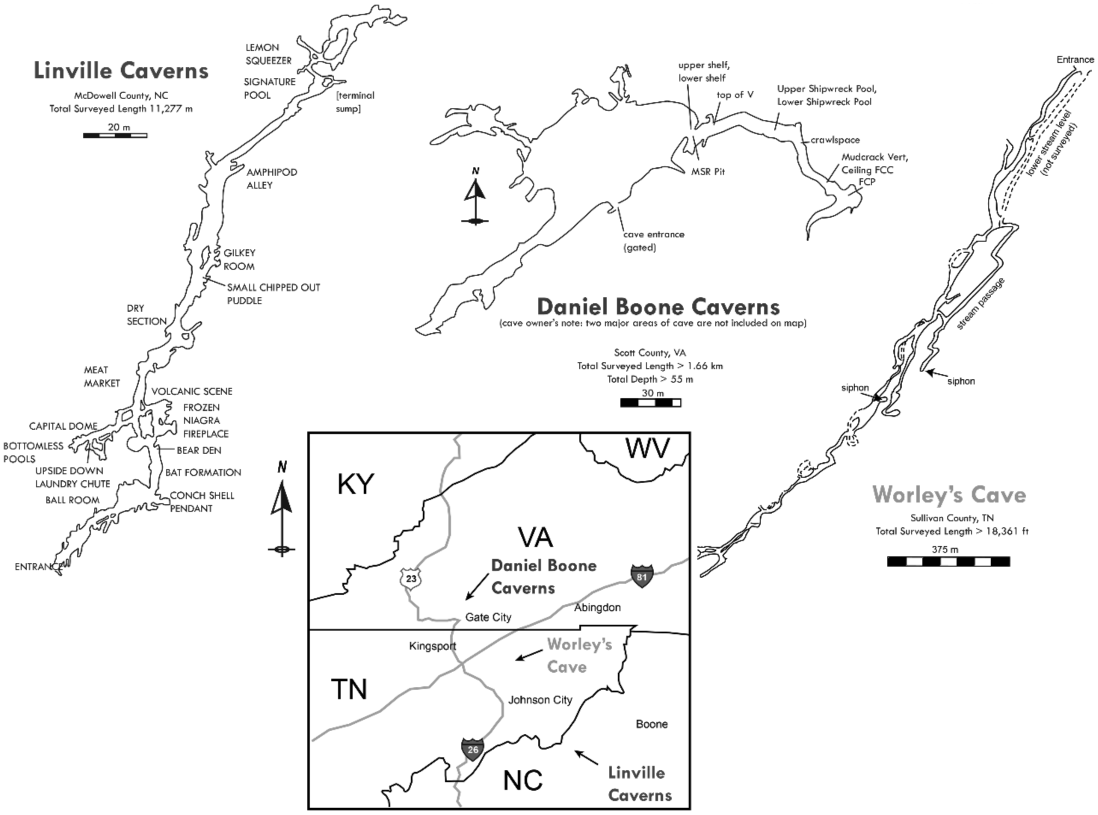

***Figure 1:*** *Maps of Linville Caverns, Daniel Boone Caverns, and Worley's Cave with the entrances and sample sites marked. Inset shows the relative Locations of the three caves within NC, TN and VA. Modified from Carmichael et al., 2013a, and Cloutier et al., 2017.*

## 3. Results

Phylum-level relative abundance demonstrated higher concentrations of Bacteroidota and Proteobacteria in Linville Caverns and lower concentrations of Crenarchaeota and Methylmirabilota; whereas Daniel Boone Caverns was enriched in Thermoplasmatota, Chloroflexi, and Nitrospirota. Site OT3 in Linville cavern also revealed a noticeable enrichment in Cyanobacteria (data not shown).

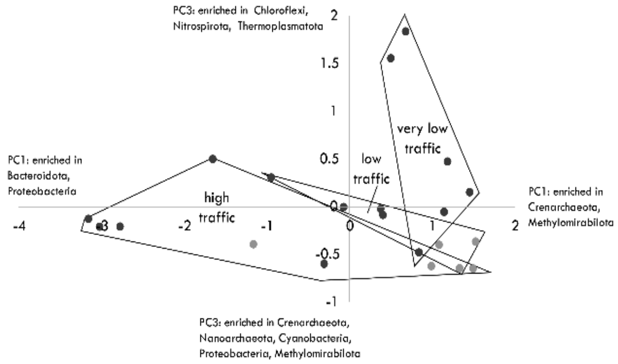

***Figure 2:*** *Arcsin transformed PCA analysis. Samples from Linville Caverns are indicated by red circles, Daniel Boone Caverns by blue circles , and Worley's Cave by green circles.*

Using arcsin tranformed PCA analysis, Phylum Data PC1 (50% of variance) vs PC3 (9% of variance) reflected degree of human traffic (Fig. 2), suggesting that human visitation may impact microbial composition. Low traffic samples were enriched in Thermoplasmatota, Chloroflexi, and Nitrospirota along PC3 and Crenarchaeota and Methylomirabilota along PC1. Interestingly, high traffic samples were enriched in two of the four human-associated phyla along PC1, namely, Bacteroidota and Proteobacteria.

Z-score transformed PCA plots of whole rock geochemistry demonstrated that geochemical variance is mostly determined by bedrock type. Mn oxidation showed a weaker signal in PCA, but was still present in PC3 and may have correlation with phosphate, perhaps related to detrital apatite or an organic source (Fig. 3).

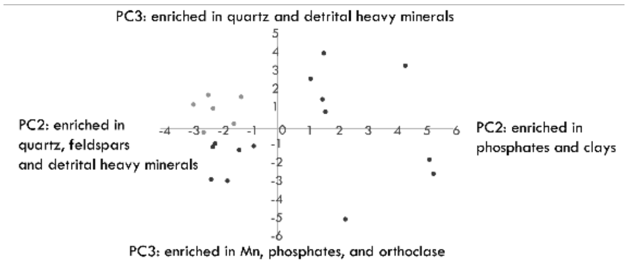

***Figure 3:*** *Z-score transformed PCA plots of whole rock geochemistry. Samples from Linville Caverns are indicated by red circles, Daniel Boone Caverns by blue circles, and Worley's Cave by green circles.*

Non-metric multidimensional scaling (NMDS) combining sediment geochemistry with phylum level community structure primarily demonstrated a clustering according to cave and possibly light content (Fig. 4). When combined with phylum data, correlation with Mn oxidation (+ or -) was not present despite the weak signal in geochemical data. When combined with geochemical data, correlation with traffic (H=high, L=low, VL=very low) was no longer present despite being a driving factor in phylum level data.

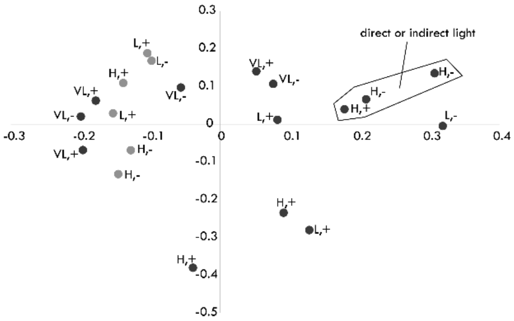

***Figure 4:*** *NMDS analysis. Samples from Linville Caverns are indicated by red circles, Daniel Boone Caverns by blue circles, and Worley's Cave by green circles. H, L and VL indicate high, low or very low traffic, respectively. Manganese oxidation is indicated by a plus sign for positive or minus sign for negative.*

An interval plot of microplastic counts revealed a positive correlation with microplastic counts and caves with higher numbers of human visitors (Fig. 5). According to the t-test, LC possessed statistically greater microplastic counts than DBC (p= 0.03), but not WC (p= 0.12). According to the ANOVA, there was no statistical difference in microplastic counts between the three cave sites. Fibrous particles were the majority of microplastic particles observed (representing 96.77% in LC, 63.64% in WC, and 100.00% in DBC), implicating synthetic fibers from the clothing of cave visitors as the primary source.

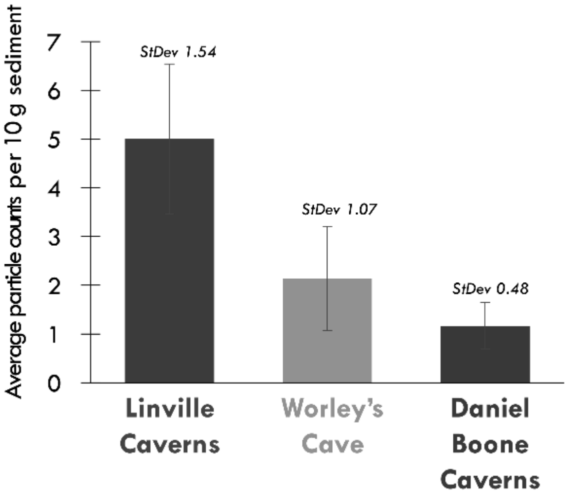

***Figure 5:*** *Interval plot of microplastic counts (per 10 g sediment).*

## 4. Discussion

Of the 1300 detectable taxa (YARZA et al. 2014) and 41 formally described bacterial phyla (PARTE et al. 2020), a mere 4 phyla predominate in and on the human body (GRICE & SEGRE 2012). Of these four human-associated phyla, two (Bacteroidetes and Proteobacteria) are enriched in the frequently-visited show cave, Linville Caverns, suggesting that human microbes represent a significant source of input into the cave microbiome. Data are also consistent with the hypothesis that Bacteroides are positively correlated and Nitrospirae are negatively correlated with anthropogenic input (BONTEMPS et al. 2022). Additionally, artificial lighting present in LC likely impacts the microbes as suggested by an enrichment of cyanobacteria in OT3 (Fig. 2), a sample site that receives direct light (Table 1), and the only sample with a visible green patina present. Enrichment of cyanobacteria in cave systems via the use of artificial lighting has been documented in previous studies (CZERWIK-MARCINKOWSKA & MASSALSKI 2018 ). Our study demonstrates that bedrock type exerts a strong influence on sediment geochemistry; as the residuum sediments differ somewhat in Linville Caverns (hosted in the Shady Dolomite) compared to the other two caves that are hosted in the Knox Dolomite. In particular, the Shady Dolomite residuum contains more clays in the silt-sized fraction compared to the Knox Dolomite, which is more quartz rich. Even the intense anthropogenic impact in Linville Caverns would not appreciably change the clastic mineralogy of the bedrock, and while there were some clear indicators of anthropogenic sediment supply in TEM analysis of particles, these particles were volumetrically insignificant. Because each of these caves differ by bedrock type and also by number of annual visitors, it's uncertain to what degree each of these factors may be driving changes in microbial community assemblages. Indeed, both the cave minerology (HUTCHENS et al. 2010; KOSZNIK-KWAŚNICKA et al. 2022) and human visitation (TOMCZYK-ŻAK & ZIELENKIEWICZ 2016) have been found to impact cave microbial communities.

Compared to this study, higher densities of microplastics were detected in sediments from show caves in Italy (BALESTRA & BELLOPEDE 2022). In Italian show caves, the microplastic abundance in the highly-trafficked tourist areas measured 1103.3, 1060.0, and 1906.7 items per kilogram in the Borgio Verezzi, Toirano, and Bossea caves respectively. In two of the caves, the microplastic count within the non-tourist sections were reduced by half (667.7 items per kilogram in Borgio Verezzi) to one third (733.3 items/kg in Bossea Cave) whereas the microplastic count in the Toirano cave remained high in non-tourist areas, averaging 1033.3 items per kilogram (BALESTRA & BELLOPEDE 2023). Our data corroborate the hypothesis that synthetic clothing is the most likely source of microplastics to cave sediments. Other studies have also found that fibrous microplastics are the predominate microplastic morphology within Italian show cave sediments, where 85% (BALESTRA & BELLOPEDE 2022) to 94% of the total microplastics detected were fibrous (BALESTRA & BELLOPEDE 2023).

## 5. Conclusion

Overall, our research shows that human visitors leave an indelible mark on cave sediments, depositing microplastic fibers and human-associated bacteria. Our data corroborate studies demonstrating that Bacteroides are positively correlated and Nitrospirae are negatively correlated with anthropogenic input, that synthetic clothing is the most likely source of microplastics to cave sediments, and that bedrock type exerts a strong influence on sediment geochemistry.

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# First records of Aracambiae crickets (Orthoptera: Phalangopsidae) in caves, with description of seven new species

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## Abstract

Phalangopsidae crickets usually inhabit humid environments and are commonly found in caves. Genera of the Aracambiae group have not yet been found in subterranean environments. The aim of this study is to present new species of *Izecksohniella*, *Marcgraviella*, and *Marliella* collected in Brazilian caves. The seven new species are primarily distinguished by the shape and position of the pseudepiphallic arms and parameres. This is the first record of phalangopsid crickets of the Aracambiae group in caves. The description of these new species provides a better understanding of the cave communities where they were found.

## Resumo

Os grilos ffalangopdeos habitam preferencialmente ambientes úmidos e são comumente achados em cavernas. Gêneros do grupo Aracambiae ainda não foram encontrados em ambientes subterrâneos. O objetivo deste estudo é apresentar novas espécies de *Izecksohniella*, *Marcgraviella* e *Marliella* coletadas em cavernas brasileiras. As sete novas espécies distinguem-se principalmente pela forma e posição dos braços e parâmeros pseudepifálicos. Este é o primeiro registro de grilos falangopsídeos do grupo Aracambiae em cavernas. A descrição dessas novas espécies permite uma melhor compreensão das comunidades cavernícolas onde foram encontradas.

## 1. Introduction

Phalangopsid crickets (Orthoptera: Ensifera: Phalangopsidae) exhibit their highest diversity in the Neotropical region, occurring predominantly in humid environments (DESUTTER-GRANDCOLAS 1995, SOUZA-DIAS 2015). These crickets occupy various microhabitats, such as crevices in tree trunks and rocks, burrows, leaf litter, spaces beneath roots, and caves (DESUTTER-GRANDCOLAS 1992, 1995). Within these environments, they contribute significantly to nutrient cycling (LAVOIE et al. 2007), playing crucial roles in ecosystem processes that are essential to the maintenance of subterranean communities. The main genera found in Brazilian caves include *Adelosgryllus* Mesa & Zefa, 2004, *Bambuina* de Mello et al., 2013, *Erebonyx* de Mello, 2021, *Endecous* Saussure, 1878, *Eidmanacris* Chopard, 1956, *Phalangopsis* Serville, 1831, and *Strinatia* Chopard, 1970. Among these, *Endecous* and *Erebonyx* are particularly notable for encompassing strictly cave-dwelling, troglobitic species (CARVALHO et al. 2023, CASTRO-SOUZA et al. 2020, DE MELLO & FERREIRA, 2021).

The Aracambiae group (Phalangopsidae: Phalangopsinae: Phalangopsini) comprises 13 species distributed across seven genera: *Aracamby* de Mello, 1992, *Cacruzia* de Mello, 1992, *Desutterella* Souza Dias et al., 2017, *Izecksohniela* de Mello, 1992, *Marcgraviella* Souza-Dias & Desutter-Grandcolas, 2014, *Marliella* Mews & Mól, 2009, and *Vanzoliniella* de Mello & Cezar dos Reis, 1994 (CIGLIANO et al. 2025). This group is characterized by male genitalia with a pair of tubular pseudepiphallic arms associated with glands, and pseudepiphallic parameres composed of both sclerotized structures and inflatable membranes (SOUZA-DIAS & DESUTTER-GRANDCOLAS 2014). Crickets of the Aracambiae group are found in the Amazon Rainforest, Atlantic Forest, and Cerrado biomes (CIGLIANO et al. 2025, DE MELLO 1992, DE MELLO & CEZAR DOS REIS 1994, MEWS & MÓL, 2009, SOUZA-DIAS & DESUTTER-GRANDCOLAS 2014, SOUZA-DIAS et al. 2017). To date, no species from the Aracambiae group have been recorded in caves.

This study provides the first records of Aracambiae crickets in caves and describes a new species of *Izecksohniela*, two new species of *Marcgraviella*, and four new species of *Marliella*, all discovered in Brazilian caves.

## 2. Materials and methods

**Collection.** Adult males of *Izecksohniella*, *Marcgraviella* and *Marliella* were collected through active searches in caves in the states of Mato Grosso, Mato Grosso do Sul, Goiás, Minas Gerais, Bahia, Sergipe, and Pernambuco. The specimens were preserved in 70% ethanol and stored in the Collection of Subterranean Invertebrates of Lavras (ISLA). All analyses and descriptions were conducted at the Center of Studies on Subterranean Biology (CEBS), at the Federal University of Lavras, Brazil.

**Examined material**. *Izecksohniella* n. sp. - Adult male, Brazil, Bahia, municipality of Iuiú, Gruta Toca Valada cave (14°29'27.8"S, 43°39'08.8"W), 13/IX/2016, Cardoso, R.C.; Adult male, same locality, 21/X/2021, Ferreira, R.L. *Marcgraviella* n. sp. 1 - Adult male, Brazil, Sergipe, municipality of Simão Dias, Toca da Raposa cave (10°43'15.7"S, 37°52'23.7"W), 11/I/2010, Ferreira, R.L.; Adult male, Brazil, Bahia, municipality of Paripiranga, Furna do Fim do Morro do Parafuso cave (10°38'25.7"S, 37°52'02.9"W), 11/I/2010, Ferreira, R.L. *Marcgraviella* n. sp. 2 - Adult male, Brazil, Minas Gerais, municipality of Morro do Pilar, SPT-0487 cave (19°12'59.8"S, 43°23'03.4"W), 26/07/2016-

13/X/2016, Carste Team. *Marliella* n. sp. 1 - Adult males, Brazil, Mato Grosso do Sul, municipality of Bodoquena, Gruta Alta Campina cave (20°36'05.0"S, 56°43'01.0"W), 02/X/2022, Ferreira, R.L. *Marliella* n. sp. 2 - Adult male, Brazil, Mato Grosso, municipality of Cuiabá, Gruta Kiogo Brado cave (15°37'04.9"S, 55°30'13.5"W), 27/X/2006, Ferreira, R.L. *Marliella* n. sp. 3 - Adult males, Brazil, Goiás, municipality of Cocalzinho de Goiás, Gruta dos Ecos cave (15°41'24.5"S, 48°24'23.9"W), 12/X/2007. *Marliella* n. sp. 4 - Adult male, Brazil, Minas Gerais, municipality of Coromandel, Gruta do Huguinho cave (18°21'06.3"S, 47°08'26.3"W), 05/X/2000, Ferreira, R.L. (Figure 1)

**Analysis and description.** Adult males were dissected, and their genitalia subjected to enzymatic treatment for at least 24 hours, following Álvarez-Padilla & Hormiga (2007). Afterward, the genitalia were prepared by carefully removing muscles and membranes, and then analyzed under a ZEISS Stemi 2000 stereomicroscope. The descriptions were based on the terminology proposed by Desutter-Grandcolas (2003) and modified by Souza-Dias et al. (2015).

Photographs of the male genitalia were obtained using a ZEISS Axiocam 506 camera attached to a ZEISS Axio Zoom V16 stereomicroscope. For the comparative diagnosis, descriptions, photographs, and illustrations available in the literature were reviewed (MEWS & MÓL 2009, SOUZA-DIAS & DESUTTER-GRANDCOLAS 2014).

Additionally, head, thorax, abdomen, and appendages were described based on observations of specimens under a ZEISS Stemi 2000 stereomicroscope.

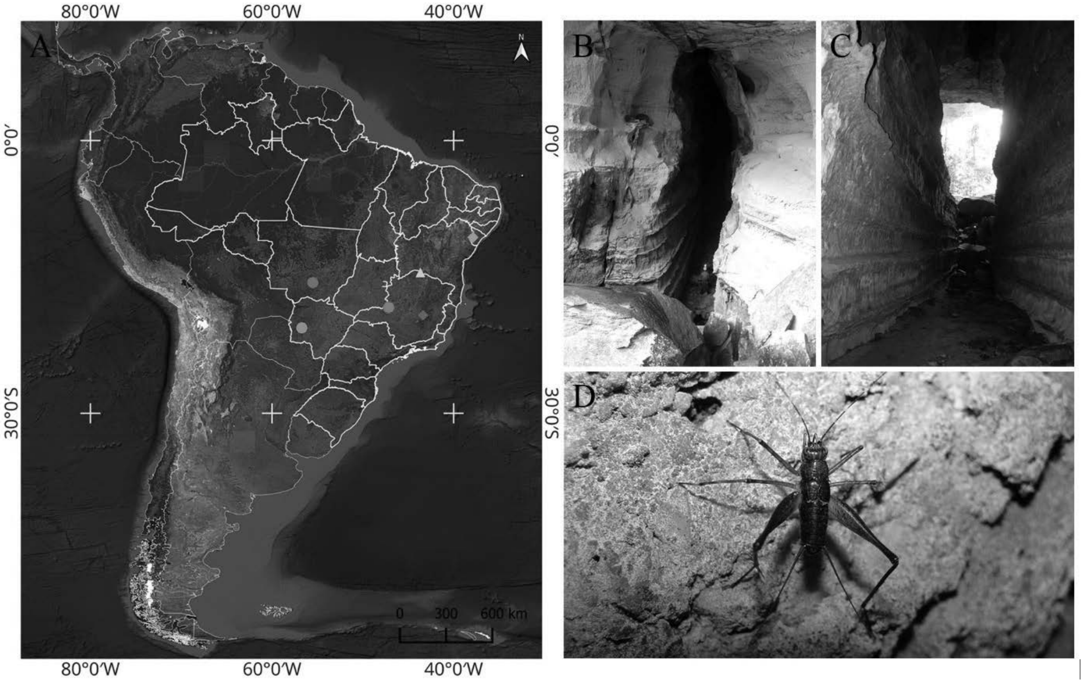

*Figure 1: A) Map of the South American continent, countries outlined with thin white lines, Brazilian states outlined with thick white lines, and colored shapes indicating the type locality of the new species of the Aracambiae group (yellow triangle - Izecksohniella n. sp., blue diamond - Marcgraviella n. sp. 1, red diamond, Marcgraviella n. sp. 2, pink circle - Marliella n. sp. 1, orange circle - Marliella n. sp. 2, purple circle - Marliella n. sp. 3, green circle - Marliella n. sp. 4); B) Entrance of Gruta Kiogo Brado cave; C) Interior of Kiogo Brado cave; D) Adult male of Marliella n. sp. 2.*

## 3. Results

*Izecksohniella* n. sp. **Type locality:** Brazil, Bahia, Iuiú, Gruta Toca Valada cave (14°29'27.8"S, 43°39'08.8"W). **Partial description of adult male:** General morphology. Head predominantly brown-orange; fastigium and frons dark brown with yellowish-brown stripes; antennae banded; segments IV and V of the maxillary palpi white, with segment V curved and truncated ventrally; pronotum brown-orange with two white spots on the anterolateral portion; metanotum with two pairs of protuberances; forewings covering approximately half of the abdomen; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs orange-brown; tibia I with a tympanum on the inner face; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, with the apex covered with a row of pointed, sclerotized teeth, the most proximal tooth being the longest, resembling an opposable thumb; pseudepiphallic ventral parameres small and sclerotized, positioned ventrally; dorsal parameres quadrangular and oblique, dorsal face predominantly membranous, featuring sclerotized distal projections in the shape of a rounded saw; ectophallic fold sclerotized and L-shaped; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 2).

*Figure 2: Izecksohniella n. sp., male genitalia (dorsal view); scale bar: 0.5 mm.*

*Marcgraviella* n. sp. 1. **Type locality:** Brazil, Bahia, Paripiranga, Furna do Fim do Morro do Parafuso cave (10°38'25.7"S 37°52'02.9"W). **Partial description of adult male:** General morphology. Head predominantly brown-orange; fastigium and frons dark brown with yellowish-brown stripes; antennae banded; maxillary palpus yellowish-brown, with segment V white distally, curved, and truncated ventrally; pronotum brown-orange with brown spots on the anterodorsal and posterolateral portions; metanotum with two pairs of protuberances; forewings covering about two-thirds of the abdomen; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs orange-brown; tibia I with a tympanum on the inner face; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, with a sclerotized apex devoid of teeth, curving ventrally; pseudepiphallic ventral parameres sclerotized, rectangular, positioned centrodorsally; dorsal parameres bilobed, each half droplet-shaped, sclerotized ventrally, with the apex sclerotized and covered with small teeth; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 3, A).

*Marcgraviella* n. sp. 2. **Type locality:** Brazil, Minas Gerais, Morro do Pilar, SPT-0487 cave (19°12'59.8"S, 43°23'03.4"W). **Partial description of adult male:** General morphology. Head predominantly brown-orange; fastigium and frons brown-orange with yellowish stripes; ocelli surrounded by dark spots; antennae banded; maxillary palpi yellowish-brown, with segment V white distally, curved, and truncated ventrally; pronotum brown-orange; metanotum with two pairs of protuberances; forewings covering approximately half of the abdomen; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs orange-brown; tibia I without a tympanum; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms short, crossing each other dorsally, with a sclerotized, pointed apex; pseudepiphallic ventral parameres sclerotized, proximal portion hook-shaped, positioned centrodorsally; dorsal parameres approximately elliptical, posterior margin sclerotized, covered with small teeth, and bearing a smooth, flattened projection with rounded margins; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 3, B).

*Marliella* n. sp. 1. **Type locality:** Brazil, Mato Grosso do Sul, Bodoquena, Gruta Alta Campina cave (20°36'05.0"S, 56°43'01.0"W). **Partial description of adult male:** General morphology. Head predominantly brown; fastigium and frons dark brown with incomplete yellowish-brown stripes; antennae banded; segments IV and V of the maxillary palpi white, with segment V curved ventrally; pronotum brown, with dark brown lateral lobes; metanotum with two pairs of protuberances; forewings short, partially covering the first tergite, and lacking distinct veins; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs yellowish-brown, covered with brown spots; tibia I without a tympanum; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, with the distal half covered with multiple rows of teeth, apex sclerotized and pointed; pseudepiphallic ventral parameres sclerotized, proximal portion hook-shaped, positioned centrodorsally; dorsal parameres bilobed, distal halves bean-shaped, proximal halves concave and bowl-shaped, with sclerotized margins covered with teeth; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 4, A).

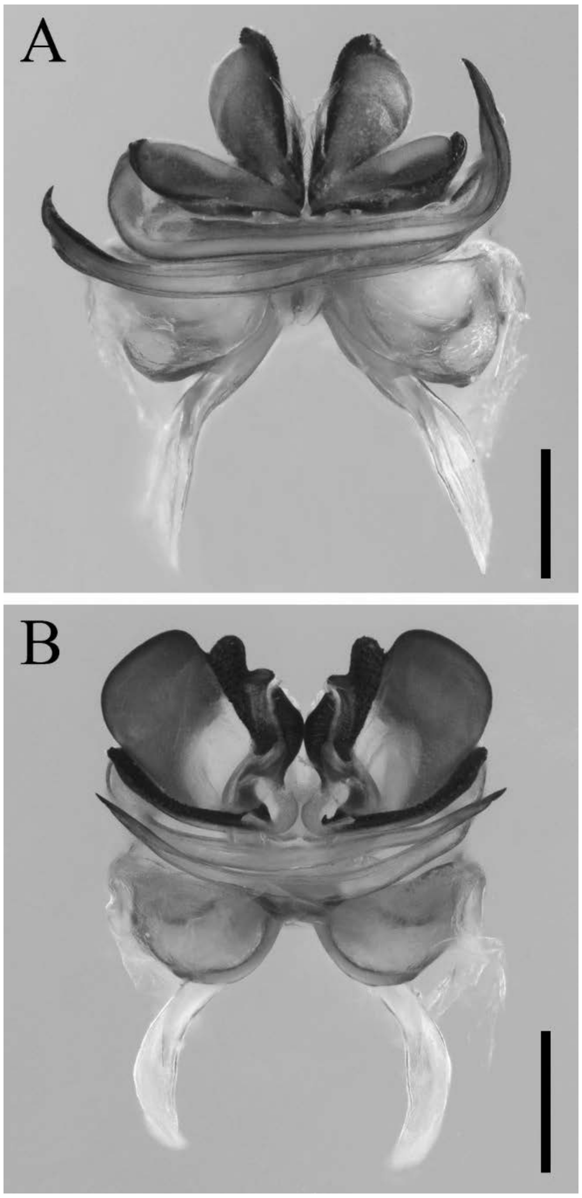

*Figure 3: A) Marcgraviella n. sp. 1, male genitalia (dorsal view); B) Marcgraviella n. sp. 2, male genitalia (dorsal view); scale bar: 0.5 mm.*

*Marliella* n. sp. 2. **Type locality:** Brazil, Mato Grosso, Cuiabá, Gruta Kiogo Brado cave (15°37'04.9"S, 55°30'13.5"W). **Partial description of adult male:** General morphology. Head predominantly brown; fastigium dark brown anteriorly; frons dark brown with yellowish-brown stripes; antennae banded; maxillary palpi brown, with segment V white distally and curved ventrally; pronotum brown; metanotum with two pairs of protuberances; forewings short, with white posterior margins, partially covering the first tergite, and lacking distinct veins; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs yellowish-brown, covered with brown spots; tibia I with a tympanum on the inner face; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, with the external distal face covered with teeth, apex sclerotized and pointed; pseudepiphallic ventral parameres sclerotized, positioned centrodorsally, proximal portion hook-shaped and covered with small teeth; dorsal parameres bean-shaped, with sclerotized distal and lateral margins covered with teeth; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 4, B).

*Marliella* n. sp. 3. **Type locality:** Brazil, Goiás, Cocalzinho de Goiás, Gruta dos Ecos cave (15°41'24.5"S, 48°24'23.9"W). **Partial description of adult male:** General morphology. Head predominantly brown; fastigium and frons dark brown with yellowish-brown stripes; antennae banded; maxillary palpi brown, with segment V white distally and curved ventrally; pronotum brown; metanotum with two pairs of protuberances; forewings short, with white posterior margins, covering the second tergite, and lacking distinct veins; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs yellowish-brown, covered with brown spots; tibia I without a tympanum; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, with the external face covered with teeth, apex sclerotized and pointed, bearing ventral teeth; pseudepiphallic ventral parameres sclerotized, positioned centrodorsally, proximal portion hook-shaped and smooth; dorsal parameres bilobed, with proximal halves concave and bowl-shaped, sclerotized margins covered with teeth; posterior margin of the distal halves sclerotized, covered with teeth, and projecting over the membranous area; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 4, C).

*Marliella* n. sp. 4. **Type locality:** Brazil, Minas Gerais, Coromandel, Gruta do Huguinho cave (18°21'06.3"S, 47°08'26.3"W). **Partial description of adult male:** General morphology. Head predominantly brown; fastigium dark brown anteriorly; frons dark brown with yellowish-brown stripes; antennae banded; maxillary palpi brown, with segment V white distally and curved ventrally; pronotum brown; metanotum with two pairs of protuberances; forewings short, with white posterior margins, partially covering the first tergite, and lacking distinct veins; supra-anal plate trapezoidal with a rounded posterior margin; subgenital plate with a median fold on the posterior margin; legs yellowish-brown, covered with brown spots; tibia I without a tympanum; tibia III with four pairs of subapical spurs on the dorsal face and two sets of apical spurs, three internal and three external; inner apical spurs longer than the outer ones. Genitalia. Pseudepiphallic arms elongated, crossing each other dorsally, bifurcated into short and long branches; external face of the short branch apex covered with teeth, long branch projecting perpendicularly, with apex curved ventrally and bearing a small tooth at the base; dorsal parameres bilobed and almost entirely sclerotized, distal halves rounded at the apex and covered with small teeth; ectophallic fold membranous; endophallic sclerite short, sclerotized, and bilobed at the apex (Figure 4, D).

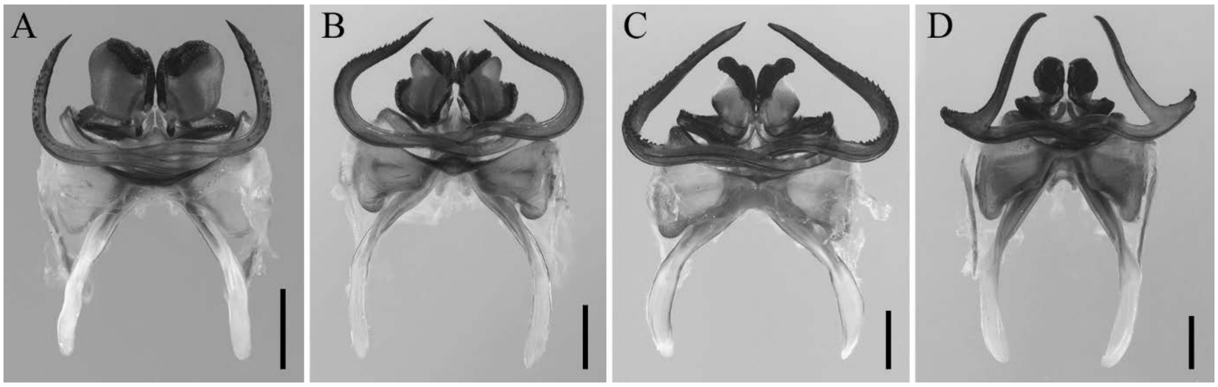

***Figure 4:*** *A) <u>Marliella</u> n. sp. 1, male genitalia (dorsal view); B) <u>Marliella</u> n. sp. 2, male genitalia (dorsal view); C) <u>Marliella</u> n. sp. 3, male genitalia (dorsal view); D) <u>Marliella</u> n. sp. 4, male genitalia (dorsal view); scale bar: 0.5 mm.*

## 4. Discussion

***Izecksohniella*** n. sp. differs from other species of the genus mainly by the quadrangular and oblique shape of the pseudepiphallic dorsal parameres, and the position and number of teeth at the apex of the pseudepiphallic arms.

***Marcgraviella*** n. sp. 1 differs from M. christianae Souza-Dias & Desutter-Grandcolas, 2014, and M. muriciensis Souza-Dias & Desutter-Grandcolas, 2014, mainly through the inclination of the pseudepiphallic dorsal parameres and the shape of their apical margins. Marcgraviella n. sp. 2 distinguishes itself from these three species mainly by the length of the pseudepiphallic arms and the shape of the pseudepiphallic dorsal parameres.

The four new species of ***Marliella*** differ from ***M. titai*** Mews & Mól, 2009, through the shape of the pseudepiphallic dorsal and ventral parameres, the curvature and ramifications of the pseudepiphallic arms, and the presence or absence of tympana. In the case of ***Marliella*** n. sp. 1, the forewings lack whitish margins.

All seven species described herein have caves as type localities, making them the first records of the Aracambiae group in subterranean environments. Phalangopsid crickets are commonly found in caves (CASTRO-SOUZA et al. 2020, DESUTTER-GRANDCOLAS 1992, 1995) and may be accidental, troglophilic, or troglobitic. None of the Aracambiae group species described herein show typical troglomorphic traits, such as tegument depigmentation, elongation of appendages, or reduction of ocular structures (CARVALHO et al. 2023, CASTRO-SOUZA et al. 2020, CHRISTIANSEN 2012, DE MELLO & FERREIRA 2021). Thus, there is no evidence that *Izecksohniella* n. sp. 1, *Marcgraviella* n. sp. 1, *Marcgraviella* n. sp. 2, *Marliella* n. sp. 1, *Marliella* n. sp. 2, *Marliella* n. sp. 3, and *Marliella* n. sp. 4 are troglobitic species. Nymphs, adult males, and females of *Izecksohniella* n. sp. 1, *Marcgraviella* n. sp. 1, *Marliella* n. sp. 1, and *Marliella* n. sp. 3 were found inside the cave, suggesting that these species are capable of completing their life cycles in subterranean environments and can, therefore, be classified as troglophilic.

## 5. Conclusion

Seven new Aracambiae species were described from crickets collected in Brazilian caves, marking the first record of the group in subterranean environments. The description of these new species provides a better understanding of cave-dwelling communities. Further studies on the habits and population dynamics of these crickets are necessary to better understand their association with the subterranean environment.

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# On the scorpion genus *Troglorhopalurus* (Arachnida: Buthidae): taxonomy and distribution

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## Abstract

This study presents evidence of a previously undescribed species of the scorpion genus *Troglorhopalurus*, expanding the known diversity of this poorly studied group. Through the morphological analysis of 16 adult specimens, significant differences were identified between *T. lacrau*, a troglophile species, and a new morphospecies, *Troglorhopalurus* sp.1. Key distinguishing features of the new species include finer carapace granulation, more elongated body and appendages, and a higher number of pectinal teeth. The new species is exclusively associated with caves in the Peruaçu district, while *T. lacrau* is distributed across both cave and epigean environments in the Irecê district and beyond, with some records possibly linked to human-mediated introductions. These findings highlight the need for a reassessment of *T. lacrau*'s conservation status, considering its expanded range and ecological flexibility. In contrast, *T. sp.1* is restricted to a small geographic area (<300 km²), underscoring its vulnerability and the importance of considering it for inclusion in the Brazilian red list of threatened species. This study not only contributes to the taxonomy and biogeography of *Troglorhopalurus* but also opens opportunities for future research into the evolutionary adaptations and phylogeography of these scorpions, particularly their relationship with cave environments.

## 1. Introduction

The Brazilian scorpion fauna is the second most species-rich in the world, with about 190 described species, in 28 genera and five families (L.S. Carvalho, in prep.). The most species-rich scorpion fauna in the world is reported from Mexico, where about 300 species are known (Santibáñez-López et al. 2015). However, these two highly diversified faunas differ in a unique characteristic: the number of cave-associated species. For Mexico, more than thirty species in five families have been identified from caves (Sissom and Reddell 2009); while only a handful species are known from Brazil (Moreno-González et al. 2021). Among the Brazilian cave scorpions, the most remarkable taxa are those belonging to the genus *Troglorhopalurus* Lourenço, Baptista & Giupponi, 2004. This genus is composed by two species, the troglobitic species *Troglorhopalurus* translucidus Lourenço, Baptista and Giupponi 2004, and the troglophile *Troglorhopalurus lacrau* (Lourenco and Pinto-da-Rocha 1997) (Moreno-González et al. 2021). *Troglorhopalurus* translucidus is known from a few caves in the limestone caves in the Chapada Diamantina region, in the state of Bahia, northeastern Brazil, without any known records outside caves. On the other hand, *T. lacrau* is reported from caves in the same region and one epigean record in the state of Ceará, 700km northwards its type-locality (Esposito *et al.* 2017).

Recently, numerous new specimens tentatively identified as *T. lacrau* have been collected in limestone caves from other localities, up to 330 km away from its type-locality. These records raise questions on the species limits of *T. lacrau*, and the possibility of the existence of additional related species. In the present study, we provide morphological evidence of an undescribed species of *Troglorhopalurus*, related to *T. lacrau* and provide an updated distribution of this species.

## 2. Materials and methods

All examined specimens were deposited in the following Brazilian museums: Centro de Estudos em Biologia Subterrânea (CEZS), Universidade Federal de Lavras, Minas Gerais; Instituto Butantan (IBSP), São Paulo; and Coleção de História Natural (CHNUFPI), Universidade Federal do Piauí, Piauí. A total of 36 measurements of body and appendage structures were taken from all available specimens, using a stereomicroscope equipped with an ocular micrometer. The specimens from two different Caatinga ecoregions were treated as different operational taxonomic units, due to a previous morphological assessment. Specimens from the Irecê district were considered to represent true *Troglorhopalurus lacrau*, as this ecoregion encompasses the type-locality of this species. Specimens from the Peruaçu district were tentatively assigned to a different morphospecies. The raw data of each measurement was also compared among morphospecies using appropriate univariate models

(T-tests, Welch's tests, or Mann-Whitney U tests). All analyses were carried out using the R programming language. Besides, the specimens of both assigned morphospecies were compared based on their general morphology, following standard taxonomic approaches.

## 3. Results

A total of 16 adult female individuals were analyzed, including 13 assigned to *Troglorhopalurus lacrau* and three assigned to *Troglorhopalurus* sp.1. Six topotype specimens from Lapa do Bode, Itaeté, state of Bahia, were measured, along with 10 specimens collected from two previously unrecorded caves. *Troglorhopalurus* sp.1 specimens were collected at four caves in the Peruaçu district, extending the geographic distribution of the genus *Troglorhopalurus* westward. Additionally, we examined specimens of *T. lacrau*, collected in epigean environments in two other municipalities of the state of Bahia

The first main observation is that the carapace of *Troglorhopalurus* sp.1 specimens presents a finer granulation, when compared to *T. lacrau* specimens. We did not observe intermediate states or polymorphism in this character within the morphospecies, which favors this character as a diagnostic feature distinguishing the two taxa.

The individual comparisons using raw measurements of each measured structure revealed some distinguishing features (Fig. 1). Specimens assigned to *Troglorhopalurus* sp.1 exhibited a significantly more elongated body, mesosoma, pedipalp (total), pedipalp femur, pedipalp patella, pedipalp chela, movable finger of the pedipalp chelae and telson. Besides, the number of pectinal teeth was greater in *Troglorhopalurus* sp.1 (Fig. 2).

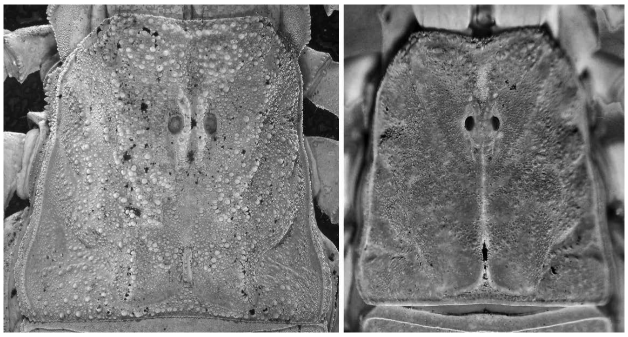

***Figure 1:** Dorsal view of the carapace of Troglorhopalurus lacrau (left) and Troglorhopalurus sp.1 (right), showing the differences in carapace granulation.*

| **Variable** | ***T. lacrau* (♀; n = 13)** | ***Troglorhopalurus.* sp.1 (♀; n = 3)** | **Statistics** |
|---|---|---|---|
| **Total length** | 39.14 - 46.50 (42.69 ± 2.74) | 44.10 - 46.00 (44.87 ± 1.00) | T = 2.275, p = 0.046 |
| **Mesosoma (length)** | 10.00 - 13.57 (11.62 ± 1.27) | 13.29 - 15.71 (14.38 ± 1.23) | T = 3.478, p = 0.039 |
| **Pedipalp (total length)** | 17.14 - 23.00 (20.06 ± 1.77) | 22.43 - 24.14 (23.38 ± 0.87) | T = 4.719, p = 0.003 |
| **Femur (length)** | 4.00 - 5.57 (4.59 ± 0.43) | 5.43 - 5.43 (5.43) | U = 36.00, p = 0.029 |
| **Patella (length)** | 4.86 - 5.86 (5.37 ± 0.35) | 6.14 - 6.43 (6.29 ± 0.14) | T = 7.13, p = 0.000 |
| **Chela (length)** | 7.36 - 10.00 (8.81 ± 0.82) | 9.71 - 10.43 (10.10 ± 0.36) | T = 4.188, p = 0.003 |
| **Chela (movable finger length)** | 4.43 - 6.43 (5.53 ± 0.53) | 6.14 - 6.57 (6.33 ± 0.22) | T = 4.148, p = 0.003 |
| **Telson (length)** | 4.00 - 4.86 (4.27 ± 0.29) | 4.36 - 5.00 (4.74 ± 0.34) | U = 34.50, p = 0.047 |
| **Pectinal tooth count** | 13 - 17 (16) | 17 - 19 (17) | U = 34.00, p = 0.049 |

***Figure 1:** Summary of significant results of univariate comparisons between measurements of both Troglorhopalurus morphospecies. Bold highlighted data represent significant comparisons. $^1$Raw measurements normalized by the own structure length. $^2$Raw measurement normalized by the carapace length.*

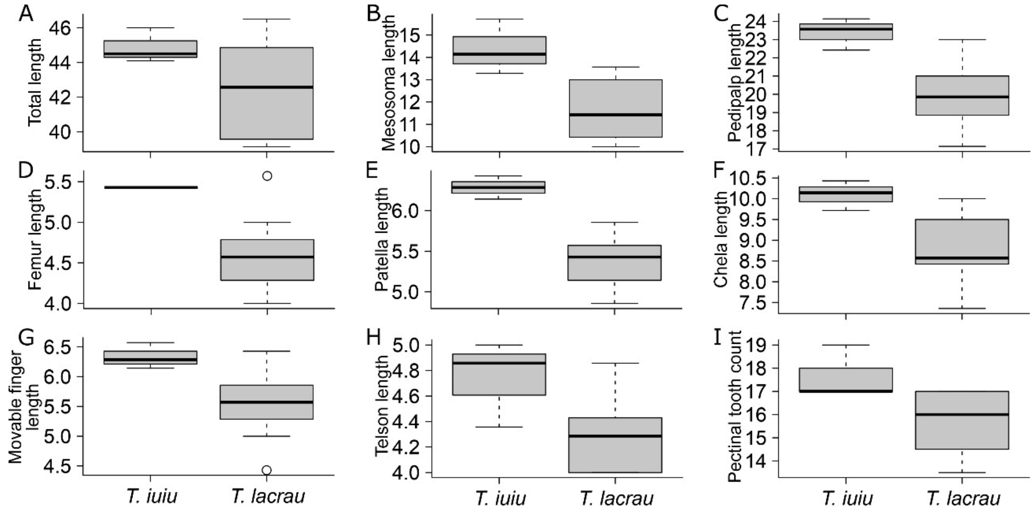

***Figure 2:** Comparison of morphometric variables between Troglorhopalurus lacrau and Troglorhopalurus sp.1. Only significant comparisons described in Table 1 are displayed. Variables: A) Total length; B) Mesosoma length; C) Pedipalp total length; D) Femur length; E) Patella length; F) Chela length; G) Chela movable finger length; H) Telson total length; I) Pectinal tooth count.*

## 4. Discussion

The data shown herein support the proposition of a new species of the scorpion genus *Troglorhopalurus*. The new species is endemic to the Peruaçu district, while *T. lacrau* is now reported from both cave and epigean environments in the Irecê district, in addition to an epigean record in the state of Ceará. Troglorhopalurus sp.1 can be distinguished from *T. lacrau* by several key features. Its carapace is mostly covered by fine granules with vestigial carinae, whereas *T. lacrau* has coarse granules and well-marked carinae. The dorsomedian carinae of the pedipalp femur and the LIM carinae on metasoma segment II are absent in T. sp.1 but vestigial in *T. lacrau*. Additionally, the DL carinae on metasoma segments II and III exhibit slightly prominent granules posteriorly in *T. sp.1*, compared to the spinoid and prominent granules on segments I-IV in *T. lacrau*. Other distinctions include the absence of retrolateral secondary and secondary accessory carinae on the pedipalp chela in *T. sp.1*, and differences in the shape of the peg sensillae on the pectinal teeth. Morphometric analyses also reveal significant differences between the two species.

Presently, *Troglorhopalurus lacrau* is considered "Data Deficient" in the threatened species Brazilian red list, owing to the existence of epigean records far from its type-locality. The new data provided herein extend its occurrence to additional caves and other epigean environments. However, it is not possible to rule out the epigean records as natural records, as they could be related to human-mediated introduction events, suggesting a species naturally endemic to three caves distributed over a 100 km² straight-line region. The known records encompass three ecoregions of the Caatinga domain: (1) the Irecê district, a very heavily deforested karstic area; (2) the Southern Depressão Sertaneja district, which encompasses deciduous caatinga vegetation and deciduous forests of the crystalline massifs; and (3) the Araripe district, an uplifted sedimentary basin located 700 km northwards of the closest record of *T. lacrau*. Further research is needed to determine whether these potentially introduced populations indeed originated from native cave populations and whether these synanthropic populations will become established or eventually disappear. However, the currently known distribution suggests that this species should be moved from data deficient to least concern in the Brazilian Red List.

The new species, however, is known exclusively from cave environments. The elongated body might be considered evidence of an initial adaptation to cave environments, but this hypothesis should be tested using phylogenetic comparative methods. Regarding its conservation status, *Troglorhopalurus* sp.1 should be considered for inclusion in the red list, owing to its limited number of locations, restricted to a narrow extent of occurrence (< 300 km²). This assessment is subject to further evaluation, following the International Union for Conservation of Nature procedures adopted by the Brazilian environmental agencies.

## 5. Conclusion

The present study provides evidence of an undescribed, potentially threatened cave-dwelling species of the poorly known scorpion genus *Troglorhopalurus*. This represents the third species in the genus. The existence of several known cave populations offers the possibility of phylogeographic studies using the genus as a model, to estimate the divergence time of species within this genus. Further studies should also focus on the distribution of epigean populations of *T. lacrau*, to provide data for reassessing the conservation status of this taxon.

## Acknowledgments

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# Strategic sites for cave prospecting and non-troglobitic faunal surveys in Brazil

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## Abstract

Subterranean environments are distributed across diverse geographical and environmental landscapes. In this study, we assume that cave occurrences areas in Brazil occupy a multidimensional large-scale space and apply a site selection model that integrates geographical and environmental factors to identify distinct locations for speleological studies. Our approach combines these findings with existing knowledge on the distribution of Brazilian caves. The results reveal 45 unique geographic-environmental sites across 19 Brazilian states, highlighting that, when accounting for geographical and environmental heterogeneity, our understanding of cave distribution in Brazil remains limited, particularly in the North and Central-West regions. This gap likely extends to the knowledge of subterranean biodiversity in these areas. Most sites identified by the model have relatively few known caves in their vicinity, emphasizing the urgent need for increased support for cave prospecting and biodiversity documentation in these regions. Additionally, the lack of a publicly accessible database containing primary records of cave biodiversity presents a significant challenge for large-scale discoveries. This limitation may contribute to the repeated documentation of biodiversity within the same geographical and environmental contexts over time.

## Resumo

Ambientes subterrâneos estão distribuídos por diversas paisagens geográficas e ambientais. Neste estudo, assumimos que as áreas de ocorrência de cavernas no Brasil ocupam um espaço multidimensional em larga escala e aplicamos um modelo de seleção de sítios que integra fatores geográficos e ambientais para identificar locais distintos para estudos espeleológicos. Nossa abordagem combina esses achados com o conhecimento existente sobre a distribuição das cavernas brasileiras. Os resultados revelam 45 locais geográfico-ambientais únicos em 19 estados brasileiros, destacando que, ao considerar a heterogeneidade geográfica e ambiental, nossa compreensão da distribuição das cavernas no Brasil continua limitada, principalmente nas regiões Norte e Centro-Oeste. Essa lacuna provavelmente se estende ao conhecimento da biodiversidade subterrânea nessas áreas. A maioria dos locais identificados pelo modelo possui relativamente poucas cavernas conhecidas em suas proximidades, o que enfatiza a necessidade urgente de maior apoio para a prospecção de cavernas e a documentação da biodiversidade nessas regiões. Além disso, a falta de um banco de dados publicamente acessível contendo registros primários de biodiversidade cavernícola representa um desafio significativo para descobertas em larga escala. Essa limitação pode contribuir para a documentação repetida da biodiversidade dentro dos mesmos contextos geográficos e ambientais ao longo do tempo.

## 1. Introduction

Subterranean environments are distributed across diverse geographical spaces and exist under a wide range of large-scale environmental conditions (MAMMOLA & LEROY, 2017). This perspective allows for the application of Hutchinson's duality theory (COLWELL & RANGEL, 2009), assuming that caves and subterranean species are organized within a multidimensional environmental space (see COELHO et al., 2023). As a result, identical environmental conditions can occur in spatially distinct caves, just as geographically proximate caves may exhibit significantly different environmental conditions. Based on this duality, it is possible to identify geographic regions with unique large-scale environmental conditions where both caves and their associated biodiversity remain unexplored and/or understudied.

Brazil hosts the largest expanse of karst areas in South America (AULER, 2004), a vast potential for cave-bearing regions (CECAV, 2024a), a wide range of climatic conditions (SPAROVEK et al., 2007), and abundant primary resources within subterranean environments. However, many caves remain undiscovered, unmapped, or lack biodiversity inventories (FICETOLA et al., 2019). The distribution of known caves has been strongly influenced by socioeconomic factors and accessibility, with records concentrated near urban centers and mining regions (Castro-Souza et al., 2023). Despite the high potential for new discoveries (ZAMPAULO & PROUS, 2022), these biases limit advancements in both scientific knowledge and conservation efforts for cave biodiversity. Therefore, it is crucial to develop strategies that redirect subterranean sampling efforts toward regions with distinct environmental conditions from those already studied, ensuring a more balanced and comprehensive sampling approach. Furthermore, no prioritization analysis for subterranean sampling has yet considered the environmental multidimensionality of cave distribution, likely because most studies treat caves as isolated systems and focus on local-scale investigations.

Since environmental diversity influences species composition (FAITH & WALKER, 1996), and regions with distinct geographic and environmental conditions tend to support unique ecological communities (HORTAL & LOBO, 2005; NUÑEZ-PENICHET et al., 2022), exploring these aspects is essential for better understanding the distribution of cave environments and subterranean biodiversity. Given this context, this study aims to: (i) identify regions in Brazil with caves occurring under distinct geographic and environmental conditions using large-scale site selection models; (ii) analyze which of these regions lack recorded caves in their surroundings, in order to recommend priority sites for new cave prospecting; and (iii) identify caves near these sites where subterranean biodiversity can be documented, contributing to a broader understanding of biodiversity across different geographic and environmental contexts. It is important to note that the approach proposed here primarily focuses on non-troglobitic species, as troglobitic species (i.e., species strictly bound to underground habitats, such as caves and subterranean waters; see Sket 2008) have distribution patterns largely shaped by historical variations, which were not considered in the models used in this study.

## 2. Materials and methods

We used polygons of Cave Occurrence Areas in Brazil (CECAV, 2024a), reprojected to the EPSG-4326 Coordinate System, to define our study area in geographic space. These data correspond to different types of potential rock formations where natural subterranean environments may exist, including shelters, underground cavities, and superficial subterranean habitats (Fig. 1a).

To represent the various climatic conditions within our study area, we extracted raster-format variables with a spatial resolution of 2.5 minutes (~4.5 x 4.5 km) for the current climate scenario, including the following: (i) *Bioclimatic variables:* We selected 15 out of the 19 bioclimatic variables available in the WorldClim 2.1 database (FICK & HIJMANS, 2017) to characterize different local climate conditions. Variables that result from the combination of temperature and precipitation data (e.g., BIO8 = Mean Temperature of the Wettest Quarter; BIO9 = Mean Temperature of the Driest Quarter; BIO18 = Precipitation of the Warmest Quarter; BIO19 = Precipitation of the Coldest Quarter) were excluded to prevent the creation of spatial mathematical artifacts, as highlighted by Escobar et al. (2014) and Nuñez-Penichet et al. (2022). (ii) *Evapotranspiration*: We used the annual potential evapotranspiration variable from the ENVIREM database (TITLE & BEMMELS, 2018) to represent local primary productivity. (iii) *Elevation*: We incorporated elevation data derived from The Shuttle Radar Topography Mission (SRTM), also available in the WorldClim 2.1 database (FICK & HIJMANS, 2017), to account for local altitude variations. Finally, all selected variables were clipped to match the boundaries of the cave occurrence polygon in Brazil (Fig. 1b).

We applied the site selection model combining environment and geography (EG) proposed by NUÑEZ-PENICHET et al. (2022) to identify cave occurrence areas with distinct geographic and environmental conditions. Initially, we conducted a Principal Component Analysis (PCA) using the selected environmental variables (Fig. 1c), aiming to use the first two principal components (PC1 and PC2) to represent, in a two-dimensional space, the environmental variation across Brazilian cave occurrence areas (Fig. 1d). Next, we divided this two-dimensional climatic space into 25 × 25 blocks, where each block represents a unique environmental interval (NUÑEZ-PENICH et al., 2022) (Fig. 1e). We then applied a random sampling process to select distinct grid cells within the environmental space. Subsequently, we analyzed the geographic distribution of all points within each selected grid cell to determine whether they were clustered into one or more groups in geographic space. This step involved measuring the geographical distances between a random sample of points within each cell, followed by a unimodality test (HARTIGAN, 1985) to assess whether the geographical distances followed a unimodal or multimodal pattern (see NUÑEZ-PENICH et al., 2022).

For unimodal patterns within a grid cell, we assumed that points were spatially clustered and selected only the point closest to the environmental centroid of that cell. For multimodal patterns, where points were distributed into distinct groups, we selected one point from each of the two largest clusters within the environmental centroid of the respective cell.

This analytical process was repeated 1,000 times, each time randomly selecting grid cells from the environmental space. The final selection was based on the replicate that exhibited the Maximum Median Geographical Distance (MMGD) between selected grid cells in the environmental space (see NUÑEZ-PENICHET et al., 2022) (Figure 1f).

Finally, we projected the best-fitting site selection model (i.e., MMGD) onto the geographic space and overlaid the recorded cave locations for Brazil, considering only those located within the boundaries of cave occurrence areas (~25,000 caves), based on data from the *‘Cadastro Nacional de Informações Espeleológicas’* (CANIE, 2024b) (Fig. 1g). Additionally, we compared the distances between the selected sites and the two nearest known caves, using the previously filtered CANIE data (Table 1). We defined caves located within 8 km of a selected site as «nearby,» considering our macroecological working scale (i.e., ~4.5 × 4.5 km grid cells), as proximity may facilitate accessibility and/or the feasibility of cave detection and sampling. Finally, we graphically represented the distances (in kilometers) for the selected sites across Brazilian states.

All analyses were conducted in the R environment (R CORE TEAM, 2024). The site selection models combining environment and geography for Brazilian subterranean regions were implemented using the ‘biosurvey: Tools for Biological Survey Planning’ package (NUÑEZ-PENICHET et al., 2022). Distance comparisons between selected sites and known caves were performed using the ‘geosphere: Spherical Trigonometry’ package (HIJMANS, 2022).

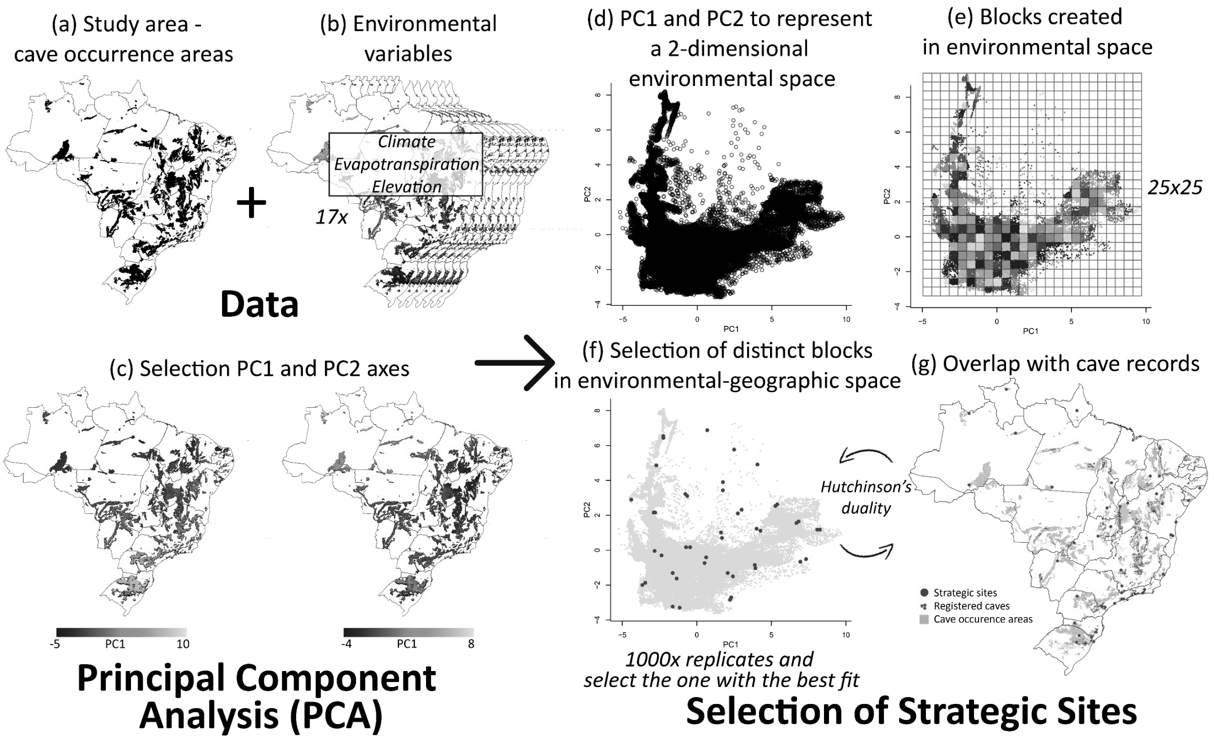

***Figure 1:** Workflow for Selection of Strategic Site for Cave Prospecting and Subterranean Biodiversity Surveys. (a) We defined our study area based on the boundaries of the occurrence areas of Brazilian caves; (b) We represented the environmental conditions of this area using different environmental variables (i.e., climate, evapotranspiration, and elevation); (c) We performed a principal component analysis (PCA) with the selected environmental variables; (d) We used the first two PCA axes to represent the two-dimensional environmental space of subterranean regions; (e) We divided the two--dimensional environmental space into 25 × 25 blocks; (f) We selected distinct blocks in both environmental and geographical space through 1,000 replicates to identify the best model; (g) We projected our model onto the geographical space and overlaid it with the records of cataloged caves in Brazil.*

## 3. Results

The first two axes of the PCA (i.e., PC1 and PC2) explained 73.6% of the environmental variance across the analyzed areas, considering climate variables, evapotranspiration, and elevation. The division of the environmental space represented by PC1 and PC2 into blocks allowed us to identify 353 distinct environmental blocks within our study area. The strategic site selection model, which integrated environmental and geographic conditions across cave occurrence areas, identified 45 unique space-environment sites distributed across 19 Brazilian states (Fig. 2).

Overall, the Central-West region of Brazil lacks any known caves near the selected sites (i.e., more than 8 km away). In the North region, only 11% of the identified sites have nearby caves, corresponding to a single site in Amazonas out of a total of nine. In the Southeast, 27.3% of the sites have nearby caves, concentrated in Rio de Janeiro and a single site in São Paulo, out of a total of eleven. In the Northeast and South regions, 33% of the sites have caves in close proximity. In the Northeast, three sites are in Bahia and one in Piauí, out of a total of twelve. In the South, one site is in Rio Grande do Sul, one in Santa Catarina, and one in Paraná, out of a total of nine (Fig. 2).

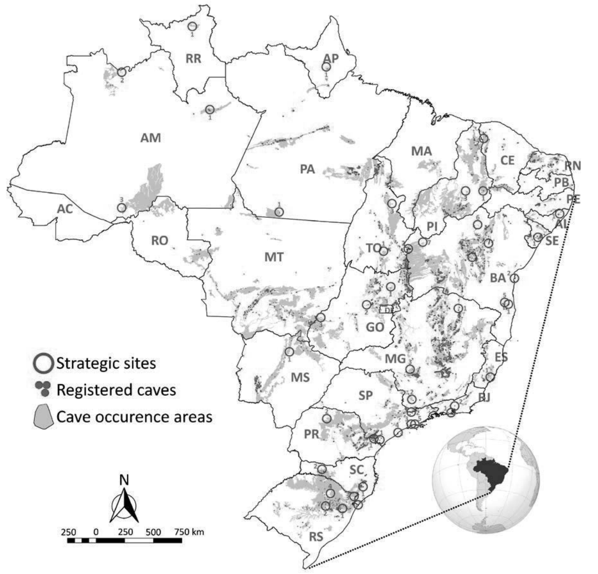

***Figure 2:** Map of distribution of strategic sites for cave prospecting and/or subterranean biodiversity surveys selected within Brazil's environmental and geographic space of cave occurrence areas. The numbers associated with the sites (in blue) indicate their relative proximity to other caves within the same state. However, this ranking does not necessarily imply the presence of nearby caves (see Table 1 and Figure 3).*

The states with the highest number of sites identified by the model were Bahia (7), São Paulo (5), and Rio Grande do Sul (4), followed by Amazonas (3), Goiás (3), Minas Gerais (3), Santa Catarina (3), and Tocantins (3). Piauí (2), Paraná (2), and Rio de Janeiro (2) had an intermediate number of selected sites. In contrast, Alagoas (1), Amapá (1), Ceará (1), Espírito Santo (1), Mato Grosso do Sul (1), Pará (1), Roraima (1), and Sergipe (1) had the lowest number of selected sites. The states of Acre, Mato Grosso, Paraíba, Pernambuco, Rio Grande do Norte, and Rondônia did not have any selected sites (Fig. 2).

Considering the cataloged caves near the selected sites, only the state of Rio de Janeiro has caves within close proximity (i.e., less than 8 km) of all the sites identified by the model. In Bahia, three out of the seven selected sites have nearby caves. In São Paulo, Rio Grande do Sul, Santa Catarina, Piauí, and Paraná, only one of the indicated sites has caves in its vicinity. The same applies to one of the selected sites in Amazonas. In Pará and Sergipe, the single site identified in each state is also located near caves. Conversely, in Goiás, Minas Gerais, Tocantins, Alagoas, Amapá, Ceará, Espírito Santo, Mato Grosso do Sul, and Roraima, the selected sites are more than 8 km away from any known caves (Fig. 3).

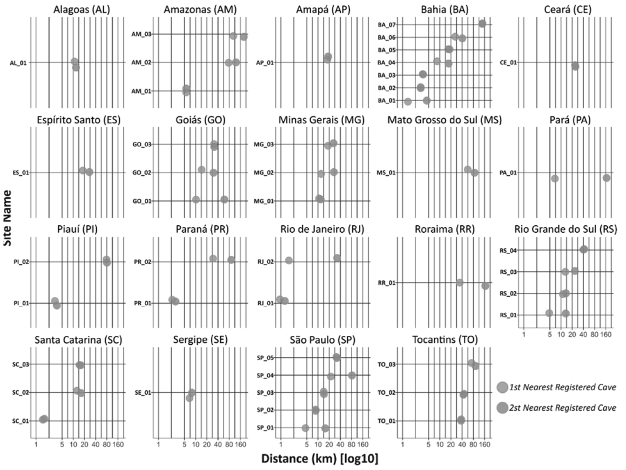

***Figure 3:*** *Distribution of distances (km) [log 10] to the two nearest caves of each selected strategic site across different states of Brazil.*

## 4. Discussion

Our strategic site selection model, which integrates large-scale geographical and environmental conditions, identified several regions with limited knowledge regarding cave distribution, particularly in the Central-West and Northern regions of Brazil. Even in areas where caves are known to exist, such as the South, Southeast, and Northeast, only about one-third of the sites identified by the model have documented caves. These findings highlight, for the first time, that when considering large-scale geographic and environmental heterogeneity, knowledge of cave distribution in Brazil remains limited, likely impacting the currently cataloged subterranean biodiversity.

Brazilian environmental legislation mandates cave prospecting, cataloging, and fauna documentation for activities that may impact cave environments (CRUZ & PILO, 2019; CECAV, 2022b; SION, 2022). While this requirement has contributed to mapping cave distribution across the country (CECAV, 2024b), it has also led to a strong bias in cave records, concentrating them near mining areas and urban centers (CASTRO-SOUZA et al., 2023). Consequently, this bias is reflected in the environmental space occupied by cave occurrences, revealing significant gaps when applying an unbiased model to select geographically and environmentally distinct sites.

To investigate the regions surrounding the unexplored sites identified in this study (primarily in the North and Central-West) greater investment in cave prospecting projects and biodiversity research in these areas is essential. In recent years, research on Brazilian cave heritage has advanced significantly, supported by funding programs for speleological groups and research institutions across the country. Notable initiatives include the Terms of Commitment for Speleological Compensation, the Speleological Project Management System, and the National Action Plan for the Conservation of Brazilian Cave Heritage (https://www.gov.br/icmbio/pt-br/assuntos/centros-de-pesquisa/cavernas).

For sites with already known caves (i.e., AM_02, BA_01, BA_02, BA_03, PA_01, PI_01, PR_01, RJ_01, RJ_02, RS_01, SC_01, SE_01, and SP_01), we recommend conducting new biological surveys in these caves (see Table 01). Whenever possible, making existing biological data publicly available online could be a viable alternative, as many caves have likely been studied for environmental impact assessments. Unfortunately, such information remains largely inaccessible to the scientific community.

The lack of an integrated and publicly accessible database on Brazilian subterranean biodiversity remains one of the greatest obstacles to research and conservation efforts. In this study, this limitation prevented the integration of biological data with our model, unlike the approach used by Nuñez-Penichet et al. (2022). While licensing and environmental agencies require that biodiversity data from impact assessments and cave studies be deposited in scientific collections and research institutions, there is no standardized digital format requirement (e.g., Darwin Core) nor an obligation to make these data publicly available through the primary government agency responsible for cave conservation in Brazil, the National Center for Research and Conservation of Caves (CECAV). Moreover, many specimens documented within caves may not have been taxonomically identified by specialists in the group due to the shortage of taxonomists and the lack of oversight regarding the taxonomic accuracy of identifications conducted in Brazilian environmental impact assessments. This lack of taxonomic refinement, transparency and accessibility restricts access to primary biodiversity records and hinders large-scale investigations and comprehensive conservation strategies.

## 5. Conclusion

Overall, our findings offer critical insights into identifying speleological sites in geographically and environmentally unexplored regions through a probabilistic approach. This methodology could also be applied to specific lithologies or areas with limited cave coverage, ensuring randomness and independence in site selection for future studies. Furthermore, we emphasize the urgent need for a public database documenting primary biodiversity records from Brazilian caves, similar to the existing national cave registry (CECAV, 2024b). Without transparent access and data integration, different research groups and environmental licensing teams may repeatedly document the same geographic and environmental spaces over time, limiting broader scientific and conservation efforts.

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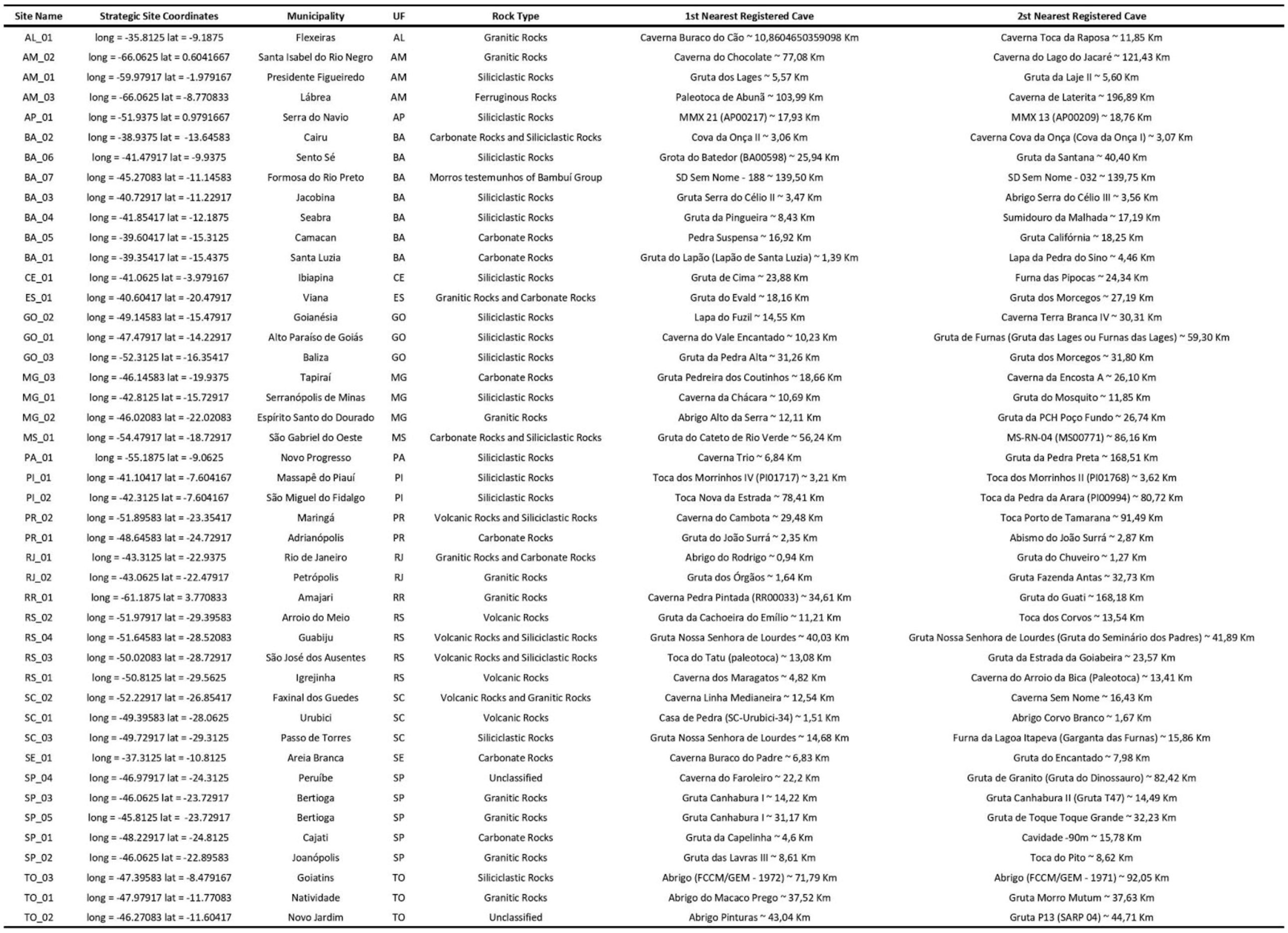
***Table 1:*** *Geographic information of the selected strategic sites and the distances to the two nearest caves, according to data from the 'Cadastro Nacional de Informações Espeleológicas' (CANIE).*



| Site Name | Strategic Site Coordinates | Municipality | UF | Rock Type | 1st Nearest Registered Cave | 2st Nearest Registered Cave |
|---|---|---|---|---|---|---|
| AL_01 | long = -35.8125 lat = -9.1875 | Flexeiras | AL | Granitic Rocks | Caverna Buraco do Cão ~ 10,8604650359098 Km | Caverna Toca da Raposa ~ 11,85 Km |
| AM_02 | long = -66.0625 lat = 0.6041667 | Santa Isabel do Rio Negro | AM | Granitic Rocks | Caverna do Chocolate ~ 77,08 Km | Caverna do Lago do Jacaré ~ 121,43 Km |
| AM_01 | long = -59.97917 lat = -1.979167 | Presidente Figueiredo | AM | Siliciclastic Rocks | Gruta dos Lages ~ 5,57 Km | Gruta da Laje II ~ 5,60 Km |
| AM_03 | long = -66.0625 lat = -8.770833 | Lábrea | AM | Ferruginous Rocks | Paleotoca de Abunã ~ 103,99 Km | Caverna de Laterita ~ 196,89 Km |
| AP_01 | long = -51.9375 lat = 0.9791667 | Serra do Navio | AP | Siliciclastic Rocks | MMX 21 (AP00217) ~ 17,93 Km | MMX 13 (AP00209) ~ 18,76 Km |
| BA_02 | long = -38.9375 lat = -13.64583 | Cairu | BA | Carbonate Rocks and Siliciclastic Rocks | Cova da Onça II ~ 3,06 Km | Caverna Cova da Onça (Cova da Onça I) ~ 3,07 Km |
| BA_06 | long = -41.47917 lat = -9.9375 | Sento Sé | BA | Siliciclastic Rocks | Grota do Batedor (BA00598) ~ 25,94 Km | Gruta da Santana ~ 40,40 Km |
| BA_07 | long = -45.27083 lat = -11.14583 | Formosa do Rio Preto | BA | Morros testemunhos of Bambuí Group | SD Sem Nome - 188 ~ 139,50 Km | SD Sem Nome - 032 ~ 139,75 Km |
| BA_03 | long = -40.72917 lat = -11.22917 | Jacobina | BA | Siliciclastic Rocks | Gruta Serra do Célio II ~ 3,47 Km | Abrigo Serra do Célio III ~ 3,56 Km |
| BA_04 | long = -41.85417 lat = -12.1875 | Seabra | BA | Siliciclastic Rocks | Gruta da Pingueira ~ 8,43 Km | Sumidouro da Malhada ~ 17,19 Km |
| BA_05 | long = -39.60417 lat = -15.3125 | Camacan | BA | Carbonate Rocks | Pedra Suspensa ~ 16,92 Km | Gruta Califórnia ~ 18,25 Km |
| BA_01 | long = -39.35417 lat = -15.4375 | Santa Luzia | BA | Carbonate Rocks | Gruta do Lapão (Lapão de Santa Luzia) ~ 1,39 Km | Lapa da Pedra do Sino ~ 4,46 Km |
| CE_01 | long = -41.0625 lat = -3.979167 | Ibiapina | CE | Siliciclastic Rocks | Gruta de Cima ~ 23,88 Km | Furna das Pipocas ~ 24,34 Km |
| ES_01 | long = -40.60417 lat = -20.47917 | Viana | ES | Granitic Rocks and Carbonate Rocks | Gruta do Evald ~ 18,16 Km | Gruta dos Morcegos ~ 27,19 Km |
| GO_02 | long = -49.14583 lat = -15.47917 | Goianésia | GO | Siliciclastic Rocks | Lapa do Fuzil ~ 14,55 Km | Caverna Terra Branca IV ~ 30,31 Km |
| GO_01 | long = -47.47917 lat = -14.22917 | Alto Paraíso de Goiás | GO | Siliciclastic Rocks | Caverna do Vale Encantado ~ 10,23 Km | Gruta de Furnas (Gruta das Lages ou Furnas das Lages) ~ 59,30 Km |
| GO_03 | long = -52.3125 lat = -16.35417 | Baliza | GO | Siliciclastic Rocks | Gruta da Pedra Alta ~ 31,26 Km | Gruta dos Morcegos ~ 31,80 Km |
| MG_03 | long = -46.14583 lat = -19.9375 | Tapiraí | MG | Carbonate Rocks | Gruta Pedreira dos Coutinhos ~ 18,66 Km | Caverna da Encosta A ~ 26,10 Km |
| MG_01 | long = -42.8125 lat = -15.72917 | Serranópolis de Minas | MG | Siliciclastic Rocks | Caverna da Chácara ~ 10,69 Km | Gruta do Mosquito ~ 11,85 Km |
| MG_02 | long = -46.02083 lat = -22.02083 | Espírito Santo do Dourado | MG | Granitic Rocks | Abrigo Alto da Serra ~ 12,11 Km | Gruta da PCH Poço Fundo ~ 26,74 Km |
| MS_01 | long = -54.47917 lat = -18.72917 | São Gabriel do Oeste | MS | Carbonate Rocks and Siliciclastic Rocks | Gruta do Cateto de Rio Verde ~ 56,24 Km | MS-RN-04 (MS00771) ~ 86,16 Km |
| PA_01 | long = -55.1875 lat = -9.0625 | Novo Progresso | PA | Siliciclastic Rocks | Caverna Trio ~ 6,84 Km | Gruta da Pedra Preta ~ 168,51 Km |
| PI_01 | long = -41.10417 lat = -7.604167 | Massapê do Piauí | PI | Siliciclastic Rocks | Toca dos Morrinhos IV (PI01717) ~ 3,21 Km | Toca dos Morrinhos II (PI01768) ~ 3,62 Km |
| PI_02 | long = -42.3125 lat = -7.604167 | São Miguel do Fidalgo | PI | Siliciclastic Rocks | Toca Nova da Estrada ~ 78,41 Km | Toca da Pedra da Arara (PI00994) ~ 80,72 Km |
| PR_02 | long = -51.89583 lat = -23.35417 | Maringá | PR | Volcanic Rocks and Siliciclastic Rocks | Caverna do Cambota ~ 29,48 Km | Toca Porto de Tamarana ~ 91,49 Km |
| PR_01 | long = -48.64583 lat = -24.72917 | Adrianópolis | PR | Carbonate Rocks | Gruta do João Surrá ~ 2,35 Km | Abismo do João Surrá ~ 2,87 Km |
| RJ_01 | long = -43.3125 lat = -22.9375 | Rio de Janeiro | RJ | Granitic Rocks and Carbonate Rocks | Abrigo do Rodrigo ~ 0,94 Km | Gruta do Chuveiro ~ 1,27 Km |
| RJ_02 | long = -43.0625 lat = -22.47917 | Petrópolis | RJ | Granitic Rocks | Gruta dos Órgãos ~ 1,64 Km | Gruta Fazenda Antas ~ 32,73 Km |
| RR_01 | long = -61.1875 lat = 3.770833 | Amajari | RR | Granitic Rocks | Caverna Pedra Pintada (RR00033) ~ 34,61 Km | Gruta do Guati ~ 168,18 Km |
| RS_02 | long = -51.97917 lat = -29.39583 | Arroio do Meio | RS | Volcanic Rocks | Gruta da Cachoeira do Emílio ~ 11,21 Km | Toca dos Corvos ~ 13,54 Km |
| RS_04 | long = -51.64583 lat = -28.52083 | Guabiju | RS | Volcanic Rocks and Siliciclastic Rocks | Gruta Nossa Senhora de Lourdes ~ 40,03 Km | Gruta Nossa Senhora de Lourdes (Gruta do Seminário dos Padres) ~ 41,89 Km |
| RS_03 | long = -50.02083 lat = -28.72917 | São José dos Ausentes | RS | Volcanic Rocks and Siliciclastic Rocks | Toca do Tatu (paleotoca) ~ 13,08 Km | Gruta da Estrada da Goiabeira ~ 23,57 Km |
| RS_01 | long = -50.8125 lat = -29.5625 | Igrejinha | RS | Volcanic Rocks | Caverna dos Maragatos ~ 4,82 Km | Caverna do Arroio da Bica (Paleotoca) ~ 13,41 Km |
| SC_02 | long = -52.22917 lat = -26.85417 | Faxinal dos Guedes | SC | Volcanic Rocks and Granitic Rocks | Caverna Linha Medianeira ~ 12,54 Km | Caverna Sem Nome ~ 16,43 Km |
| SC_01 | long = -49.39583 lat = -28.0625 | Urubici | SC | Volcanic Rocks | Casa de Pedra (SC-Urubici-34) ~ 1,51 Km | Abrigo Corvo Branco ~ 1,67 Km |
| SC_03 | long = -49.72917 lat = -29.3125 | Passo de Torres | SC | Siliciclastic Rocks | Gruta Nossa Senhora de Lourdes ~ 14,68 Km | Furna da Lagoa Itapeva (Garganta das Furnas) ~ 15,86 Km |
| SE_01 | long = -37.3125 lat = -10.8125 | Areia Branca | SE | Carbonate Rocks | Caverna Buraco do Padre ~ 6,83 Km | Gruta do Encantado ~ 7,98 Km |
| SP_04 | long = -46.97917 lat = -24.3125 | Peruíbe | SP | Unclassified | Caverna do Faroleiro ~ 22,2 Km | Gruta de Granito (Gruta do Dinossauro) ~ 82,42 Km |
| SP_03 | long = -46.0625 lat = -23.72917 | Bertioga | SP | Granitic Rocks | Gruta Canhabura I ~ 14,22 Km | Gruta Canhabura II (Gruta T47) ~ 14,49 Km |
| SP_05 | long = -45.8125 lat = -23.72917 | Bertioga | SP | Granitic Rocks | Gruta Canhabura I ~ 31,17 Km | Gruta de Toque Toque Grande ~ 32,23 Km |
| SP_01 | long = -48.22917 lat = -24.8125 | Cajati | SP | Carbonate Rocks | Gruta da Capelinha ~ 4,6 Km | Cavidade -90m ~ 15,78 Km |
| SP_02 | long = -46.0625 lat = -22.89583 | Joanópolis | SP | Granitic Rocks | Gruta das Lavras III ~ 8,61 Km | Toca do Pito ~ 8,62 Km |
| TO_03 | long = -47.39583 lat = -8.479167 | Goiatins | TO | Siliciclastic Rocks | Abrigo (FCCM/GEM - 1972) ~ 71,79 Km | Abrigo (FCCM/GEM - 1971) ~ 92,05 Km |
| TO_01 | long = -47.97917 lat = -11.77083 | Natividade | TO | Granitic Rocks | Abrigo do Macaco Prego ~ 37,52 Km | Gruta Morro Mutum ~ 37,63 Km |
| TO_02 | long = -46.27083 lat = -11.60417 | Novo Jardim | TO | Unclassified | Abrigo Pinturas ~ 43,04 Km | Gruta P13 (SARP 04) ~ 44,71 Km |

# *Aspergillus* and *Penicillium* from quartzite and ferruginous caves in minas gerais

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## Resumo

A ordem Eurotiales compreende gêneros economicamente importantes, como *Aspergillus* e *Penicillium*, amplamente distribuídos em habitats terrestres, incluindo cavernas. O estado de Minas Gerais abriga a maioria das cavernas conhecidas no Brasil; no entanto, sua micobiota cavernícola é pouco estudada. Este estudo investiga a presença de espécies de *Aspergillus* e *Penicillium* em cavernas ferruginosas e quartzíticas da porção sul da Serra do Espinhaço, em Minas Gerais, Brasil. Amostras foram coletadas de vários substratos cavernícolas e analisadas por métodos de filogenia molecular. Foram obtidos 330 isolados, com *Penicillium* sendo o gênero dominante. As análises filogenéticas das regiões gênicas da beta-tubulina e calmodulina identificaram 4 espécies de Aspergillus e 14 de *Penicillium*, com 16 espécies potencialmente novas detectadas. Oito espécies foram relatadas em cavernas no mundo pela primeira vez, enquanto três constituem novos registros para cavernas brasileiras. O estudo destaca a importância do estudo da micobiota de cavernas na elucidação da diversidade fúngica e no aprimoramento das estimativas globais de fungos. Além disso, enfatizamos a necessidade de esforços de conservação, já que esses ecossistemas cavernícolas estão ameaçados pela expansão das atividades de mineração na região.

## Abstract

The Eurotiales order comprises economically important genera, such as *Aspergillus* and *Penicillium*, that are ubiquitously distributed in terrestrial habitats, including caves. Minas Gerais state harbours most of the caves known in Brazil; however, its cave mycobiota is poorly studied. This study investigates the presence of *Aspergillus* and *Penicillium* species in ferruginous and quartzite caves of the Southern Espinhaço Mountain Range in Minas Gerais, Brazil. Samples were collected from various cave substrates and analysed using molecular phylogenetic methods. A total of 330 isolates were obtained, with *Penicillium* being the dominant genus. Phylogenetic analyses of beta-tubulin and calmodulin gene regions identified 4 Aspergillus and 14 *Penicillium* species, with 16 potentially new species detected. Eight species were reported in caves worldwide for the first time, while three were new records for Brazilian caves. The study highlights the importance of cave mycobiota research in uncovering fungal diversity and improving global fungal estimates. It also emphasizes the need for conservation efforts, as these cave ecosystems are threatened by expanding mining activities in the region.

## 1. Introduction

The order *Eurotiales* comprises diverse and economically important fungal genera such as *Aspergillus* and *Penicillium* (*Aspergillaceae*). *Penicillium* is known for producing penicillin, revolutionising bacterial infection treatment (HOUBRAKEN et al., 2011). *Aspergillus oryzae* is used in food fermentation, while *P. camemberti* and *P. roqueforti* are used in cheese production (COTON et al., 2020; LESSARD et al., 2014; ABE & GOMI, 2007). However, both genera can cause food spoilage, produce harmful mycotoxins, and include pathogenic species (YU et al., 2020; FRISVAD et al., 2019).

Members of the *Eurotiales* order are widely distributed in terrestrial habitats. They are found in indoor environments (VISAGIE et al., 2014b), natural ecosystems (BARBOSA et al., 2020, 2022; VISAGIE et al., 2014a), and caves (VANDERWOLF et al., 2013). *Aspergillus* and *Penicillium* are among the most abundant fungi in caves worldwide (ZHANG et al., 2021). Members of *Eurotiales* in Brazilian caves have been reported in different states, including Goiás (OLIVEIRA et al., 2024a), Minas Gerais (CONDÉ et al., 2022), Pará (NÓBREGA et al., 2024), Pernambuco (CUNHA et al., 2020), Rio Grande do Norte (ALVES et al., 2022b), and Sergipe (LIMA et al., 2024).

The state of Minas Gerais harbours 11,029 caves, representing almost half of the caves registered in Brazil (CRUZ & COSTA NETO, 2023). Many of these caves are located in the Southern Espinhaço Mountain Range (SEMR) (AULER et al., 2015), including the quartzite and ferruginous caves found in the municipality of Conceição do Mato Dentro and its vicinities. The Monumento Natural da Serra da Ferrugem (MNSF) is an integral conservation unit which preserves an area of approximately 867 ha. However, iron mining activities that surround the MNSF threaten the caves and biodiversity of this pristine environment (DIAS & MADEIRA FILHO, 2020), including its cave mycobiota. This study aimed to identify fungal isolates belonging to the order *Eurotiales* from ferruginous and quartzite caves in the SEMR. Phylogenetic analyses using the DNA sequences of the beta-tubulin and calmodulin gene regions were performed to identify fungal isolates.

# 2. Methodology

## 2.1. Study area and sample collection

Samples were collected at three different locations. In Alvorada de Minas (Itapanhoacanga), sampling was performed in one ferruginous cavity. In Conceição do Mato Dentro, sampling was performed in the Gruta Curral de Pedras (quartzite/ferruginous), which is approximately 400m long; and in the MNSF, where sampling was performed in five ferruginous cavities.

Samples of airborne fungal particles, rocks, sediment, leaf litter, roots, and animal dung were collected from the caves. Airborne fungal particles were collected using the Koch sedimentation method (KUZMINA et al., 2012). Petri plates (90 mm) containing Potato Dextrose Agar (PDA) amended with chloramphenicol (2 mg/L), Dichloran-Rose Bengal-Chloramphenicol Agar (DRBC), and Dichloran Glycerol Agar (DG18) culture media were exposed to the cave environment for 15 minutes. Rock samples were collected with the aid of a sterile swab. Sediment samples were collected at a depth of 1–5 cm after removing the surface layer. Leaf litter and root samples were collected and stored in paper bags, while animal dung samples were collected and stored in 50 mL Falcon tubes.

## 2.2. Fungal isolation

The serial dilution method was used to isolate fungi from rocks, sediments, and animal dung (ZHANG et al., 2017). The root samples were processed as described by OLIVEIRA et al. (2024b). PDA, DRBC, and DG18 culture media were used for fungal isolation. All plates were incubated at 25 °C for 30 days, during which fungal growth was observed daily, and new fungal colonies were periodically transferred to fresh PDA plates. Litter samples were washed in running tap water and then incubated in a humid chamber (CASTAÑEDA-RUIZ et al., 2005), where they were periodically observed under a stereoscopic microscope and reproductive fungal structures were transferred to fresh PDA plates.

Pure cultures were obtained using the hyphal tip method (TUITE, 1969). Fungal cultures were stored in 2 mL microtubes containing 10% glycerol solution at −20 °C. All isolates were stored in the cave fungal collection of Micolab/UFV.

## 2.3. DNA isolation, amplification and sequencing

Total genomic DNA was isolated from fungal mycelia grown in PDA for seven days using a commercial Wizard Genomic DNA Purification kit (Promega®). DNA amplification of the internal transcribed spacers 1 and 2 and the intervening 5.8S subunit (ITS) using primer pairs ITS5/LR6 (VILGALYS & HESTER, 1990; WHITE et al., 1990) was performed for all isolates. Subsequently, a smaller set of representative isolates of *Eurotiales* was chosen, and the partial beta-tubulin (*BenA*) and calmodulin (*CaM*) gene regions were amplified using the primers Bt2a/Bt2b (GLASS & DONALDSON, 1995) and CMD5 and CMD6 (HONG et al., 2006), respectively. DNA amplicon sequencing was performed by Macrogen Inc. (South Korea). The sequencing results were visualised and trimmed using FinchTV software (Geospiza Inc.).

## 2.4. Phylogenetic analyses

A DNA sequence dataset was constructed using *BenA* and *CaM* sequences generated in this study and those stored

in the National Center for Biotechnology Information (NCBI). DNA alignments were performed using MAFFT v.7 (KATOH & STANDLEY, 2013). Phylogenetic analyses were performed using maximum likelihood (ML) in IQ-TREE software (MINH et al., 2020), with 10,000 ultrafast bootstrap (BS) replicates. Only BS values of ≥ 90 were plotted at the nodes. Phylogenetic trees were visualized using FigTree v. 1.4.3 and exported to graphics programs for editing.

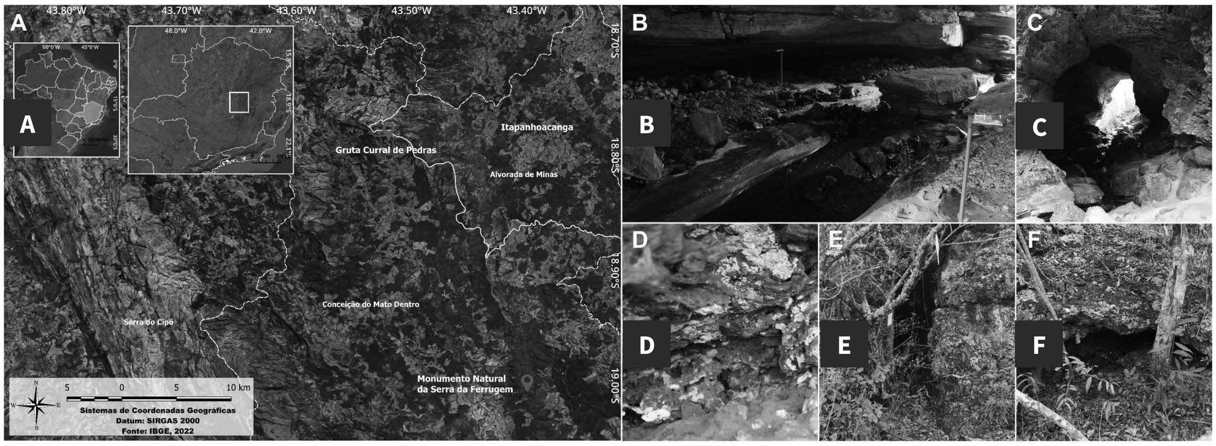

***Figure 1:*** *A: Location of the caves analysed in this study. B–C: Gruta Curral de Pedras airborne fungi sampling and cave entrance. D: Fungal colonisation of cave walls in Itapanhoacanga. E–F: Ferruginous cavities in Monumento Natural da Serra da Ferrugem.*

# 3. 3. Results

A total of 330 isolates belonging to *Aspergillus* and *Penicillium* were found in the caves sampled, of which 15 were identified as *Aspergillus* and 315 as *Penicillium*, according to ITS sequencing. From the samples analysed, 143 isolates were recovered from sediments, 80 from airborne particles, 52 from animal dung, 39 from rocks, 11 from leaf litter, and five from plant roots (Fig. 2A)

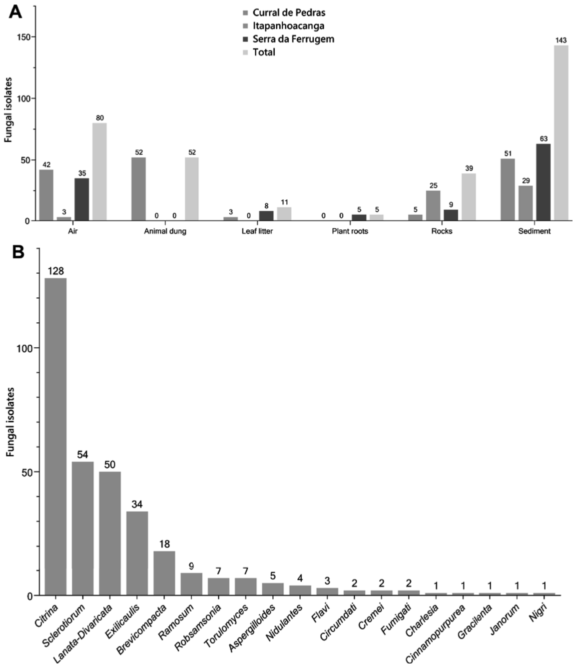

***Figure 2:*** *Statistics of Aspergillus and Penicillium isolates obtained in this study.* ***A:*** *Number of isolates obtained from different substrates in the three localities.* ***B:*** *Abundance of fungal isolates obtained by section in Aspergillus and Penicillium.*

Of these, 153 isolates were recovered from the Gruta Currral de Pedras, 120 from the Serra da Ferrugem caves, and 57 from Itapanhoacanga cave. In the caves analysed, section *Citrina* was the most abundant with 128 isolates, followed by *Sclerotiorum*, *Lanata-Divaricata*, *Exilicaulis*, and *Ramosum* with 54, 50, 34, and 18 isolates, respectively (Fig. 2B). The other sections are depicted in Figure 2B.

Phylogenetic trees were constructed using a small subset of isolates which were subjected to DNA sequencing of more phylogenetically informative regions, *i.e*. *BenA* and *CaM* (Figs 2 and 3). Four species of *Aspergillus* were identified, namely *A. bertholletiae* (sect. *Flavi*), *A. stramenius* (sect. *Fumigati*), *A. tubingensis* (sect. *Nigri*), and *A. versicolor* (sect. *Nidulantes*), whereas strains CF 3851 and CF 4138, belonging to sections *Circumdati* and *Flavi*, respectively, could not be identified to the species level (Fig. 3).

In the genus *Penicillium*, 14 strains and 14 species were identified, whereas the other 17 strains, representing 14 species, could not be assigned to any known species (Fig. 4). Isolates identified were *P. fusisporum*, *P. brevicompactum*, *P. nothofagi*, *P. paxilli*, *P. steckii*, *P. shearii*, *P. melinii*, *P.* cf. *sichuanense*, *P.* cf. *carajasense*, *P.* cf. *stangiae*, *P. viridissimum*, *P. virgatum*, *P.* cf. *xyleborini*, and *P.* cf. *austricola* (Fig. 4). *Penicillium* isolates were distributed in sections *Aspergilloides*, *Brevicompacta*, *Charlesia*, *Citrina*, *Exilicaulis*, *Gracilenta*, *Lanata-Divaricata*, *Ramosum*, *Robsamsonia*, *Sclerotiorum*, and *Torulomyces* (Fig. 4).

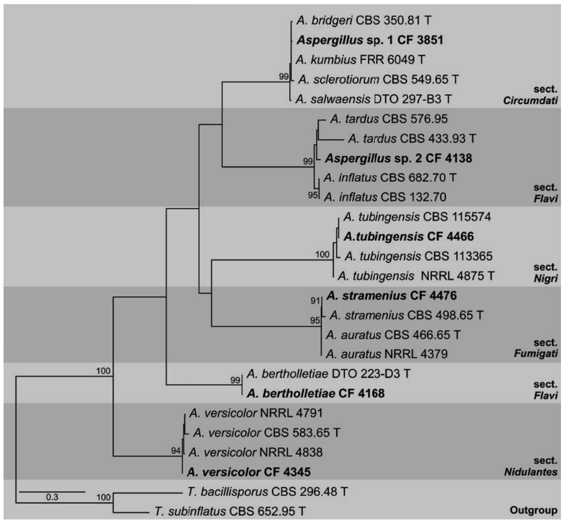

***Figure 3:*** *Maximum-likelihood phylogenetic tree of Aspergillus based on CaM sequences. Isolates found in this study are shown in bold. Ex-type isolates are marked with ‘‘T’’. Only bootstrap (bs) values ≥ 90 % are shown at branches. The tree is rooted with T. bacillisporus CBS 296.48 and T. subinflatus CBS 652.95.*

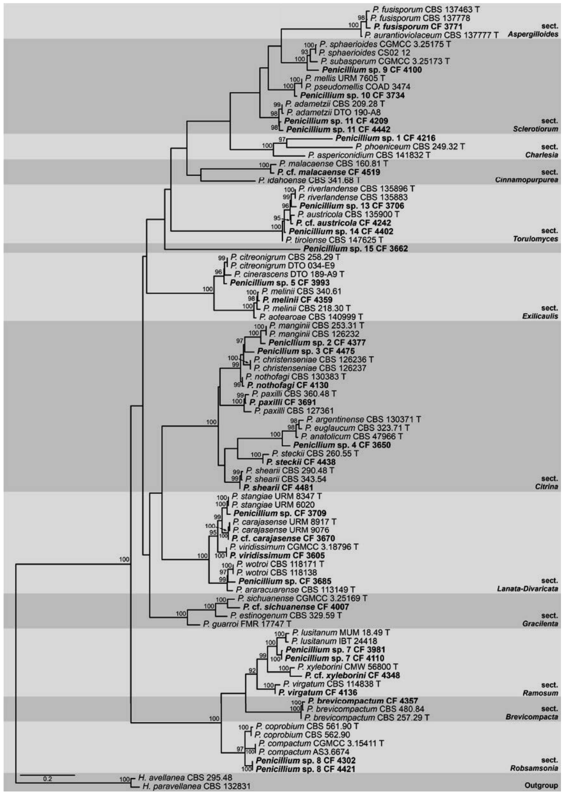

***Figure 4:*** *Maximum-likelihood phylogenetic tree of Penicillium based on BenA sequences. Isolates found in this study are shown in bold. Ex-type isolates are marked with ‘‘T’’. Only bootstrap (bs) values ≥ 90 % are shown at branches. The tree is rooted with H. avellanea CBS 295.48 and H. paravellanea CBS 132831.*

The isolates that could not be identified may represent new species of *Aspergillus* and *Penicillium*. They will be further analysed using a polyphasic approach, combining multigene molecular phylogeny and morphological analyses of vegetative and reproductive structures, to determine whether these isolates represent new species. Table 1 depicts the species found in *Aspergillus* and *Penicillium* according to phylogenetic analyses of the *CaM* and *BenA* sequences.

***Table 1:*** *List of Aspergillus and Penicillium species found in the caves studied using BenA and CaM gene regions*

| Species | | Section | Isolate | Cave substrate | Locality |
|---|---|---|---|---|---|
| *Aspergillus* sp. 1 | | *Circumdati* | CF 3851 | Air | Serra da Ferrugem |
| *Aspergillus* sp. 2 | | *Flavi* | CF 4138 | Sediment | Curral de Pedras |
| *A. bertholletiae* | | *Flavi* | CF 4168 | Sediment | Curral de Pedras |
| *A. stramenius* | ■ | *Fumigati* | CF 4476 | Sediment | Itapanhoacanga |
| *A. tubingensis* | | *Nigri* | CF 4466 | Cave wall | Itapanhoacanga |
| *A. versicolor* | | *Nidulantes* | CF 4345 | Animal dung | Curral de Pedras |
| *P. fusisporum* | ■ | *Aspergilloides* | CF 3771 | Plant roots | Serra da Ferrugem |
| *P. brevicompactum* | | *Brevicompacta* | CF 4357 | Air | Curral de Pedras |
| *Penicillium* sp. 1 | | *Charlesia* | CF 4216 | Leaf litter | Curral de Pedras |
| *P. nothofagi* | ■ | *Citrina* | CF 4130 | Sediment | Curral de Pedras |
| *P. paxilli* | ● | *Citrina* | CF 3691 | Cave wall | Serra da Ferrugem |
| *P. steckii* | ● | *Citrina* | CF 4438 | Sediment | Itapanhoacanga |
| *P. shearii* | | *Citrina* | CF 4481 | Sediment | Itapanhoacanga |
| *Penicillium sp.* 2 | | *Citrina* | CF 4377 | Animal dung | Curral de Pedras |
| *Penicillium sp.* 3 | | *Citrina* | CF 4475 | Sediment | Itapanhoacanga |
| *Penicillium sp.* 4 | | *Citrina* | CF 3650 | Sediment | Serra da Ferrugem |
| *Penicillium sp.* 5 | | *Exilicaulis* | CF 3993 | Air | Curral de Pedras |
| *P. melinii* | ● | *Exilicaulis* | CF 4359 | Sediment | Curral de Pedras |
| *P.* cf. *sichuanense* | ■ | *Gracilenta* | CF 4007 | Air | Curral de Pedras |
| *P.* cf. *carajasense* | | *Lanata-Divaricata* | CF 3670 | Sediment | Serra da Ferrugem |
| *P.* cf. *stangiae* | ■ | *Lanata-Divaricata* | CF 3709 | Sediment | Serra da Ferrugem |
| *P. viridissimum* | ■ | *Lanata-Divaricata* | CF 3605 | Air | Serra da Ferrugem |
| *Penicillium* sp. 6 | | *Lanata-Divaricata* | CF 3685 | Cave wall | Serra da Ferrugem |
| *P. virgatum* | | *Ramosum* | CF 4136 | Sediment | Curral de Pedras |
| *P.* cf. *xyleborini* | ■ | *Ramosum* | CF 4348 | Animal dung | Curral de Pedras |
| *Penicillium* sp. 7 | | *Ramosum* | CF 3981 | Air | Curral de Pedras |
| *Penicillium* sp. 7 | | *Ramosum* | CF 4110 | Air | Curral de Pedras |
| *Penicillium* sp. 8 | | *Robsamsonia* | CF 4302 | Animal dung | Curral de Pedras |
| *Penicillium* sp. 8 | | *Robsamsonia* | CF 4421 | Animal dung | Curral de Pedras |
| *Penicillium* sp. 9 | | *Sclerotiorum* | CF 4100 | Sediment | Curral de Pedras |
| *Penicillium* sp. 10 | | *Sclerotiorum* | CF 3734 | Sediment | Serra da Ferrugem |
| *Penicillium* sp. 11 | | *Sclerotiorum* | CF 4209 | Cave wall | Curral de Pedras |
| *Penicillium* sp. 11 | | *Sclerotiorum* | CF 4442 | Sediment | Itapanhoacanga |
| *P.* cf. *austricola* | ■ | *Torulomyces* | CF 4242 | Animal dung | Curral de Pedras |
| *Penicillium* sp. 12 | | *Torulomyces* | CF 3706 | Sediment | Serra da Ferrugem |
| *Penicillium* sp. 13 | | *Torulomyces* | CF 4402 | Sediment | Curral de Pedras |
| *Penicillium* sp. 14 | | undefined | CF 3662 | Sediment | Serra da Ferrugem |

■ = first report in caves worldwide; ● = first report in Brazilian caves.

## 4. Discussion

Caves are environments characterised by partial or total absence of light, high humidity, stable temperature, and limited amount of organic matter (except for bat caves) (BARTON; JURADO, 2007; POULSON; WHITE, 1969). Despite these challenging conditions, fungi can thrive in these ecosystems, and many studies have reported the presence of these organisms, including the discovery of new species and genera (ALVES et al., 2022b; CONDÉ et al., 2023; ZHANG et al., 2017). According to estimates of fungal diversity in caves worldwide, nearly 2,000 species of fungi have been reported in these environments (VANDERWOLF et al., 2013; ZHANG et al., 2021), of which *Aspergillus* and *Penicillium* are often the most abundant genera.

Based on phylogenetic analyses, we identified four species in the genus *Aspergillus* and 14 species in *Penicillium* from ferruginous and quartzite caves in Minas Gerais. Additionaly, we identified 16 fungal lineages that may represent new species in these genera. Cunha et al., (2020) found that *Aspergillus* and *Penicillium* were predominately isolated from different substrates in the Meu Rei bat cave in Pernambuco. Caves in Minas Gerais also harbour *Aspergillus* and *Penicillium* (CONDÉ et al., 2022; TAYLOR et al., 2014; TAYLOR et al., 2013). In the Lapa Nova cave, these genera were the most abundant in air and bat guano samples (TAYLOR et al., 2013), including species found in our study, such as *A. versicolor* and *P. brevicompactum*. In the RM3 ferruginous cave located in the Iron Quadrangle, these genera were also abundant, and *A. fumigatus*, the most common causal agent of pulmonary aspergillosis, was isolated (TAYLOR et al., 2014). Although we did not find *A. fumigatus* in our survey, we identified one strain, *A. stramenius* (CF 4476), which belonged to the same section as *A. fumigatus* (section *Fumigati*).

In this study, eight species are reported in caves worldwide for the first time, namely *A. stramenius*, *P. fusisporum*, *P. nothofagi*, *P.* cf. *sichuanense*, *P.* cf. *stangiae*, *P. viridissimum*, *P.* cf. *xyleborini*, and *P.* cf. *austricola*), Additionally, three species are reported in Brazilian for the first time, namely *P. paxilli*, *P. steckii*, and *P. melinii*. Interestingly, *P. stangiae* is a species discovered in a soil sample from the Atlantic Forest in Pernambuco (ALVES et al., 2022a), whereas our strain CF 3709 was also found in the soil of Serra da Ferrugem, which is located in the Cerrado biome, thus expanding the known distribution of this fungus in Brazil.

We identified phylogenetic lineages of *Aspergillus* and *Penicillium* that may represent new species. For example, isolate CF 3662 could not be identified in any section of *Penicillium*, and further analyses will determine whether this isolate represents a new section in the genus. Studies have revelead new taxa of fungi in caves from Minas Gerais, including the discovery of a new fungal genus and seven new species (CONDÉ et al., 2023; DUTRA et al., 2023; LEÃO et al., 2024), which emphasise the potential of caves to harbour undiscovered fungi.

Ferruginous caves in the MNSR are threatened because of the expansion of mining activities in the region (DIAS; MADEIRA FILHO, 2020). Therefore, studies on cave mycobiota are important to uncover the fungi that inhabit these pristine and threatened environments, which helps to improve global fungal estimates. This finding reinforces the need to include fungi in conservation efforts, which can lead to the protection of fungal species and subterranean environments.

## 5. Conclusion

In this study, we provide information on the distribution of fungi belonging to *Aspergillus* and *Penicillium* in the caves of Minas Gerais. Additionally, we provide the perspective for finding new species of *Eurotiales* fungi in Brazilian ferruginous and quartzite caves. These reports on *Aspergillus* and *Penicillium* in Brazilian caves are important to improve our knowledge of cave biodiversity, as fungi have historically been neglected in this field.

These discoveries are of utmost importance for filling the knowledge gap in current estimates of fungal species on Earth. Currently, these estimates indicate that less than 10% of the 2–3 million fungal species inhabit our planet. Therefore, studies on cave mycobiota can help to enhance these numbers.

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# Systematic Conservation Planning of subterranean habitats: approaches and key challenges

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## Abstract

Despite their ecological significance, subterranean ecosystems are often underrepresented in conservation efforts. We aim to review the current applications of systematic conservation planning and other prioritization tools in the conservation of subterranean ecosystems, focusing on approaches employed, their effectiveness, and the challenges faced in their implementation. By synthesizing the current literature from 1964 to 2021, we assess where and how prioritization has been applied and critically analyse the extent to which prioritization frameworks effectively address biodiversity protection.

Our findings reveal that prioritization in subterranean conservation remains largely species-focused, with an emphasis on bats and arthropods. Other taxonomic groups remain critically underrepresented. Additionally, we identify a significant bias toward caves in prioritization efforts, with limited focus on groundwater, anchialine, and fissural systems. Furthermore, while threat assessments are increasingly incorporated into conservation planning, our review shows that visitor impacts and habitat change receive greater attention than climate change, alien species and pollution, which are rarely considered.

By advancing prioritization methodologies and ensuring their alignment with conservation needs, we can enhance the effectiveness of subterranean habitat protection and contribute to broader biodiversity conservation targets.

## 1. Introduction

Subterranean habitats are critical environment for biodiversity MAMMOLA (2019), hosting species with unique adaptations to life in darkness, low-energy environments, and stable climatic conditions GIBERT & DEHARVENG (2002). These ecosystems provide vital ecological services, such as groundwater filtration and nutrient cycling GRIEBLER & AVRAMOV (2015), yet they are often overlooked in conservation planning FERNÁNDEZ et al. (2021); MAMMOLA et al. (2022). Systematic Conservation Planning (SCP) refers to the process of identifying and ranking sites, species, or ecosystems based on their biodiversity value, level of threat, and feasibility of protection KUKKALA et al. (2013). It is an essential tool for optimizing resource allocation and ensuring that conservation efforts have the greatest possible effect.

The importance of this optimization approach is further underscored by the ambitious conservation targets set by the European Union (EU) for the coming decades, such as those outlined in the EU Biodiversity Strategy for 2030 GIAKOUMI et al. (2025); MAMMOLA et al. (2020); MAMMOLA et al. (2019). This strategy emphasizes the need to protect at least 30% of Europe's land and sea, focusing on areas of high biodiversity value, including subterranean systems. However, achieving these targets is especially challenging given the limited resources available for conservation efforts CULVER & PIPAN, (2019). Without optimization algorithms and systematic prioritization strategies, conservation initiatives may fall short of the EU's ambitious goals LINKE et al. (2019).

This review assesses the implementation of SCP approaches in subterranean ecosystems. We aim to evaluate where and how these approaches are applied, identifying the methods most used along with their advantages and limitations. By doing so, we seek to highlight the main existing biases in perspectives on the use of prioritization solutions, ensuring that conservation efforts are scientifically tailored and effectively contribute to biodiversity protection.

## 2. Materials and methods

To achieve our objectives, we conducted a systematic literature review based on the framework established by MAMMOLA et al. (2022) and MEIERHOFER et al. (2024). Their studies employed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) methodology to identify conservation-related studies focusing on subterranean ecosystems. The dataset created in that review consisted of 708 publications spanning from 1964 to 2021, documenting 1954 conservation interventions (see MAMMOLA et al. (2022) for details). Given our focus on prioritization, we filtered this dataset to isolate studies explicitly addressing optimization approaches in subterranean habitat conservation.

This allowed us to identify studies that applied systematic conservation planning, spatial prioritization frameworks, and cost-effective resource allocation in subterranean habitats. Additionally, we aim to expand the dataset by incorporating newly published literature from 2022 to 2025 following the same PRISMA-based search methodology to ensure consistency and comparability with the original dataset.

Our analysis assessed the geographical distribution of the application of prioritization approaches. We then examined the taxonomic coverage of prioritization efforts, followed by an evaluation of how these frameworks were applied across different subterranean systems, including caves, groundwater, and anchialine environments. Finally, we quantified the extent to which threat assessments were integrated into prioritization methodologies.

By systematically categorizing prioritization strategies, we identified key methodological trends, assessed their advantages and limitations, and highlighted critical gaps requiring further research and refinement. This analysis provides an evidence-based foundation to enhance prioritization methodologies in subterranean conservation and support more effective biodiversity protection strategies.

## 3. Results

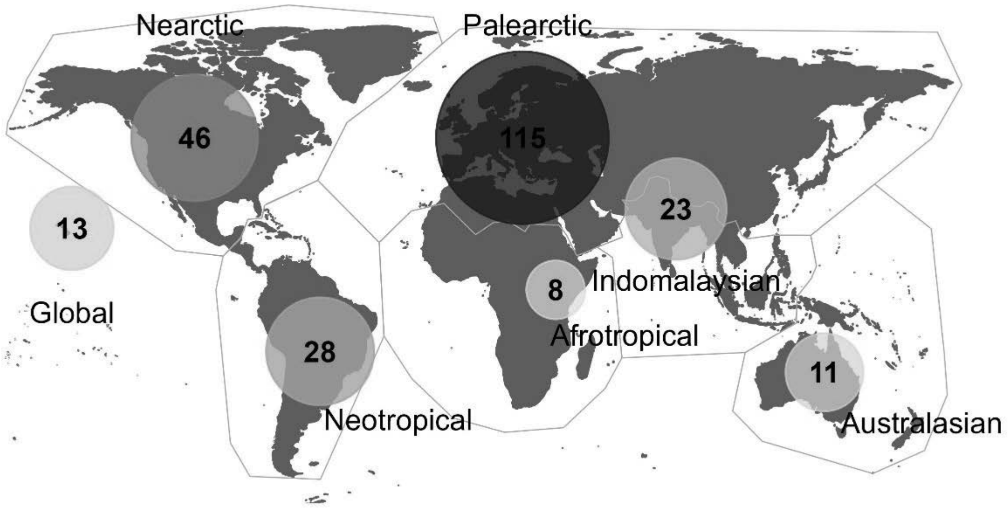

***Figure 1:*** *Distribution of the studies involving SCP across biogeographic regions and globally. The size of each circle represents the number of applications per region, with numerical labels indicating the exact count.*

**Overview of the reviewed studies**

The review resulted in 152 unique papers that explicitly addressed SCP, subset from the original database of 708 publications. These studies documented a total of 246 prioritization interventions within subterranean habitats.

**Geographic distribution**

Prioritization approaches in subterranean habitats are unevenly distributed geographically (Fig. 1). Most prioritization interventions were concentrated in karst regions of Europe (47.1%) and North America (18.8%). Other regions were comparatively underrepresented, highlighting a major bias in subterranean conservation efforts worldwide.

**Target taxa**

Taxonomic focus within prioritization studies also varied widely (Fig. 2A). The most frequently studied taxa were bats (85 applications) and arthropods (47 applications), while other invertebrates and vertebrates each accounted for 21 applications. A considerable number of applications (48) did not focus on any specific taxonomic group. This taxonomic bias suggests that subterranean prioritization efforts have largely centered on certain appealing or ecologically significant taxa, leaving other important subterranean organisms underrepresented in conservation planning.

**Targeted systems**

An analysis of the types of subterranean systems covered in the reviewed studies revealed significant variation in focus (Fig. 2B). Most of the prioritization applications (127) concentrated on caves, followed by groundwater systems (50 applications), anchialine/marine systems (27 applications). Other systems, including fissural systems (4 applications), artificial habitats (7 applications), and show caves (3 applications), were comparatively underrepresented. Only 14 of the applications addressed all types of subterranean systems collectively. This disparity highlights the preference toward cave ecosystems, while other types of subterranean environments remain underexplored in conservation prioritization strategies.

**Targeted threats**

Threat-based prioritization was found to be frequently evaluated (69.1%), with only a subset of applications incorporating explicit spatial modeling of threats, such as pollution, groundwater extraction, and land-use changes. Furthermore, prioritization efforts rarely accounted for the ecological processes that sustain subterranean ecosystems, such as hydrological connectivity and species dispersal mechanisms. In fact, as shown in Fig. 2C, our analysis showed that only 76 out of the reviewed approaches explicitly integrated multiple forms of threat data into their prioritization approaches, while an equal number of studies did not incorporate any form of threat assessment. Among those that considered singular threats, the most addressed factors were visitor impact (26 approaches), surface habitat change (19 approaches), subterranean habitat change (18 approaches), pollution (10 approaches), alien species and pathogens (10 approaches), and climate change (9 approaches). Overexploitation was rarely considered, with only two applications including it in prioritization frameworks. This distribution suggests that while threats are recognized as important, their integration into prioritization methodologies remains inconsistent and often incomplete.

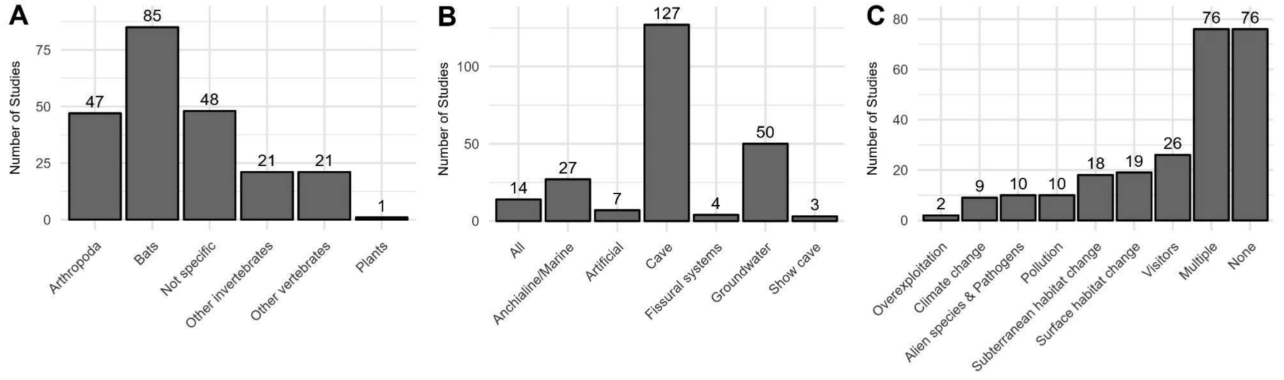

***Figure 2:** (A) Taxonomic focus of studies, categorized into major biological groups. (B) Habitat types investigated in the studies. (C) Threats assessed in subterranean ecosystems. The numbers above the bars indicate the number of SCP applications in each category.*

## 4. Discussion

The intense research focus on Europe and North America has resulted in geographic biases that limit the global applicability of prioritization frameworks. While these regions are rich in subterranean biodiversity, many other subterranean-rich areas, particularly in tropical arid regions, remain understudied TRAJANO et al. (2016). Expanding conservation research into less-studied regions would enhance global subterranean biodiversity protection and provide a more equitable distribution of conservation efforts.

Species-based prioritization has dominated conservation strategies, yet this approach often neglects the broader ecological and evolutionary dynamics of subterranean biodiversity CARDOSO et al. (2021). The focus on bats and arthropods, while important, has led to significant gaps in the protection of other taxa, such as plants and less studied invertebrates MAMMOLA et al. (2019). Future prioritization models should account for these overlooked taxonomic groups and explicitly incorporate a diversity-driven perspective. To enhance conservation effectiveness, prioritization frameworks should integrate functional and phylogenetic diversity, ensuring that entire ecological networks and evolutionary processes are preserved rather than just individual species MAMMOLA et al. (2022).

The biases in habitat focus also indicate a need for more comprehensive prioritization frameworks. The overwhelming focus on caves compared to groundwater systems and anchialine systems reveals a clear research gap. Many conservation efforts target the most visible subterranean environments, but prioritization frameworks must expand to include fissural systems, artificial habitats, and lesser-known subterranean networks. Such an approach would provide a more accurate representation of conservation needs across different subterranean ecosystems.

The limited use of threat-based prioritization is another significant shortcoming. Although many studies recognize the importance of pollution, climate change, and surface habitat change, fewer incorporate predictive threat modeling into prioritization frameworks. The tendency to focus on immediate and observable threats such as visitor impacts and habitat degradation rather than long-term and inevitable pressures like climate change suggests a need for more forward-looking conservation strategies. This imbalance suggests that certain threats are underestimated in conservation planning. By incorporating spatial modeling and predictive tools, conservation strategies can become more forward-looking and adaptive, enabling them to address emerging risks. This is particularly important for climate change impacts, as even small shifts in subterranean temperature and precipitation could drastically affect these highly specialized ecosystems MAMMOLA et al. (2019).

Another important insight from our review is that the practical application of SCP frameworks remains limited. Existing methods often rely on species richness, endemism, or rarity to prioritize underground systems NITZU et al. (2018); RABELO et al. (2018); PIPAN et al. (2020), but these approaches are rarely implemented NITZU et al. (2019); MOLDOVAN et al. (2019). Conservation effectiveness is typically assessed indirectly CARDOSO et al. (2021); BORGES et al. (2012), with little direct empirical validation.

Additionally, successful conservation also requires integrating ecosystem services GIRARDELLO et al. (2019) and protecting natural resources (Martin Jung et al., 2021), to align conservation goals with broader ecological functions. While expanding underground protected areas is vital FERNANDEZ et al. (2021) , real-world implementation faces challenges POLLOCK et al. (2020), particularly in assessing effectiveness.

To address these challenges, standardized assessment frameworks and adaptable conservation strategies are needed. Advances in ecological modelling, remote sensing applications, and automated monitoring can improve efficiency testing, bridging the gap between theory and practice. Future research should focus on long-term monitoring and predictive tools to ensure prioritization translates into tangible biodiversity protection. By incorporating these elements, conservation efforts can become more scientifically robust, adaptable, and capable of mitigating the impacts of climate change and other anthropogenic pressures on subterranean biodiversity.

## 5. Conclusion

Prioritization is essential for the effective conservation of subterranean habitats, which face conservation challenges. Our review highlights key gaps in current prioritization approaches, including the need to integrate functional and phylogenetic diversity, the importance of threat-based assessments, and the lack of efficiency testing. Addressing these gaps will require innovative methodologies, increased funding, and collaborative efforts among researchers, policymakers, and stakeholders. By adopting a more comprehensive and adaptive approach in prioritization, we can ensure the protection of subterranean biodiversity these habitats provide, contributing to the achievement of global and regional conservation targets.

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# Cenotes, windows to the underground aquifer, link between geomorphology and environmental characteristics: different types of ecosystems

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## Abstract

Cenotes are windows to the underground aquifer, the main source of fresh water in the Yucatan Peninsula. They have a wide variety of shapes and a great diversity of reservoirs known generically as cenotes. Some have lake-like shapes, others have vertical walls, and others develop extensive systems of grottos and caves, to name a few. Understanding the morphological and environmental differences of these unique ecosystems can facilitate the prediction of biodiversity and carrying capacity, and contribute favorably to resource management and conservation. This work carries out an exhaustive sampling and analysis of the morphology and environmental characteristics that allows a deeper categorization of the different ecosystems present in these systems, proposing a differentiation and typification of the different ecosystems observed in cenotes, which is an essential tool for their study, management, and conservation..

## Resumen

Los cenotes son ventanas al acuífero subterráneo, principal fuente de agua dulce en la península de Yucatán, presentan una amplia variedad de formas donde existe una gran diversidad de embalses conocidos en forma genérica como cenotes, algunos tienen formas semejantes a un lago, otros tienen paredes verticales y otros desarrollan extensos sistemas de grutas y cuevas, por mencionar algunos. El comprender las diferencias morfológicas y ambientales de estos ecosistemas únicos puede facilitar la predicción de la biodiversidad, la capacidad de carga, y contribuir favorablemente a la gestión de los recursos y conservación. El presente trabajo, realiza un muestreo exhaustivo y un análisis de la morfología y características ambientales que permite una categorización más profunda de los diferentes ecosistemas presentes en estos sistemas, proponiendo una diferenciación y tipificación de lo distintos ecosistemas que se observan en los cenotes, lo cual es herramienta esencial para su estudio, manejo y conservación.

## 1. Introducción

En la Península de Yucatán (PY) los cenotes son “ventanas al acuífero subterráneo” que es la principal reserva de agua dulce, siendo los ecosistemas acuáticos superficiales predominantes en la parte central y norte de la PY que esta desprovista de ríos y arroyos superficiales, esto hace que los cenotes sean realmente importantes en los ámbitos ecológico, económico y cultural; algunos cenotes forman parte de los sistemas de cuevas inundadas más extensos del mundo. (Pohlman et al., 1997; Torrescano-Valle & Islebe, 2015). Los cenotes son sistemas acuáticos bastante heterogéneos, la conjunción de agua dulce y marina definen diversos patrones de circulación y estratificación en los sistemas, que, en conjunto con la diversidad de geomorfologías, profundidad, volumen, incidencia lumínica y otros factores más, generan una gran diversidad de ecosistemas acuáticos conocidos como cenotes, los cuales proporcionan hábitats para gran diversidad de fauna y flora (Aguilar, 2021; Rissolo, n.d.; Schmitter-Soto, Suárez-Morales, et al., 2002c; Zambrano et al., 2006). (Cabadas-Báez et al., 2010). Los cenotes son los únicos sitios influenciados por la luz solar, las regiones superficiales influenciadas por el sol dan acceso a fuentes de carbono y otros aportes orgánicos derivados de la fotosíntesis como oxígeno disuelto que posiblemente no lleguen a los pasajes más profundos de los sistemas (sección cueva). También las entradas de los cenotes constituyen sumideros para los aportes alóctonos de materia orgánica, representando un aporte de nutrientes importante a estos ecosistemas acuáticos subterráneos (Chávez-Solís et al., 2020; Zambrano et al., 2006, Schmitter-Soto, Comín, et al., 2002a). El identificar los diferentes tipos de cenotes y/o tener una clasificación con base en sus diferencias geomorfológicas y ambientales permite comprender de manera integral los ecosistemas y sus dinámicas. Factores como la posición geográfica, las características geomorfológicas de las dolinas y entradas, así como las diferencias en la forma en que la luz solar directa entra y llega a la superficie del agua, influyen directamente en los factores físicos, químicos y bióticos del cuerpo de agua (temperatura, pH, turbidez, oxígeno disuelto, fotosíntesis, etc.) (Camargo-Guerra et al., 2013). Estas diferencias ambientales a su vez pueden explicar los patrones de diversidad y distribución de diferentes organismos y la estructura de las comunidades (Alcocer et al., 1999; Camargo-Guerra et al., 2013; Chávez-Solís et al., 2020

La diferenciación de lo distintos ecosistemas que se observan en los cenotes y la clasificación de los diferentes tipos, es una herramienta esencial para su estudio, manejo y conservación. El comprender las diferencias morfológicas y ambientales de estos ecosistemas únicos puede facilitar la predicción de la biodiversidad, la capacidad de carga, y contribuir favorablemente a la gestión de los recursos y conservación, así mismo, facilitará la identificación de aquellos que requieren implementación de medidas de manejo específicas (capacidad de carga de cada cenote, sobreexplotación, la contaminación, protección).

Al categorizar los diferentes tipos de cenotes, se pueden establecer programas de investigación y monitoreo más eficientes, así como facilitar la creación de materiales educativos y de divulgación más precisos y completos. Esto permite recopilar datos específicos sobre cada tipo de cenote, lo que a su vez ayuda a identificar tendencias y riesgos ambientales.

## 2. Materiales y métodos

*Evaluación geomorfológica y ambiental de los cenotes:* Debido a que los cenotes presentan una amplia variedad de morfologías heterogéneas, se utilizaron como base las clasificaciones de Hall (1936) y Schmitter-Soto et al. (2002) a los que se le sumaron algunos parámetros y características. Para seleccionar las localidades se revisaron diferentes bases de datos, los registros de macrofauna estigobionte obtenidos de bases de datos publicas y programas de ciencia ciudadana, así como de la literatura científica existente, registros de investigadores y colecciones, también mediante la comunicación personal con instructores de buceo o guías de la región. Las campañas de trabajo de campo se llevaron acabo entre mayo del 2022 y octubre del 2024, logrando obtener datos morfometricos de 170 localidades llamadas “cenotes y aguadas”. Los diferentes tipos de cenotes visitados se evaluaron y describieron morfométricamente y ecológicamente para determinar que características de los cenotes podrían influir en los patrones de diversidad y distribución de las especies acuáticas. Se consideraron tanto variables métricas como cualitativas. Con un distanciometro (Disto X, Leica) se tomaron las medidas del sistema y se calculo el área y perímetro tanto de la entrada como del espejo de agua. Las características del hábitat en las localidades también fueron documentadas mediante esquemas, topografías, fotografías y videos.

Del universo de variables obtenidas en las 170 localidades, se seleccionaron 6 medidas métricas para realizar la evaluación de la variación morfométrica en los diferentes tipos de cenotes encontrados, por medio de un Análisis de Componentes Principales utilizando el software PRIMER, los datos fueron transformados y estandarizados.

Los cenotes tipo manantial, aguadas o con zona de inundación no fueron considerados en el análisis debido a que las mediciones tomadas en estos sistemas difieren por sus propias características, por lo que el PCA se realizó con los datos de 130 localidades.

*Evaluación del cuerpo de agua:* Se seleccionaron 60 cenotes de diferentes morfologías que fueron buceados para registrar las características ambientales del cuerpo de agua y la dentificación de cambios en el ecosistema, se realizo la evaluación de la entrada y morfología del cenote, estimación de la profundidad, medición de temperatura superficial, evaluación de transparencia del agua y turbidez, incidencia luminica del sol, entre otras. Las características del hábitat en las localidades también fueron documentadas mediante esquemas, fotografías y videos. Cabe mencionar que la evaluación científica de estos cuerpos de agua requiere una metodología sistemática y multidisciplinaria que combine técnicas de buceo especializado en caverna con protocolos de investigación ambiental, y con protocolos de seguridad.

## 3. Resultados

Los diferentes cuerpos de agua visitados presentaron una amplia variedad de morfologías y distintas condiciones ambientales, se observó que las condiciones bióticas y abióticas cambian entre ellos y entre regiones de la península.

Se obtuvieron 13 variables métricas y cualitativas de las 170 localidades, que contribuyen a las diferencias ecológicas. Con base en la clasificación de Hall (1936) y la evaluación morfométrica realizada a los diferentes tipos de cenotes (170 localidades), se consideraron algunos factores geomorfológicos que podrían incidir en diferentes parámetros ambientales importantes para los organismos. Por ejemplo, uno de los factores importantes considerados es la incidencia lumínica solar, las diferencias en la morfología de la ventana kárstica (ancho, largo, área, perímetro, profundidad, desarrollo, etc.) que determinan la manera en que la luz y materia orgánica externa ingresa al sistema y llega a la superficie del cuerpo de agua, que a su vez podría determinar la supervivencia de los productores primarios fotosintéticos capaces de modificar la estructura de la red alimentaria y los procesos en la estructura de otras comunidades (J. E. Cohen, 1989).

Un factor importante fue el tamaño de la entrada en relación del espejo de agua (aVK/aEA) y fue utilizado como un categorizador para algunos de los tipos de cenotes. También se consideró el desarrollo vertical y horizontal del sistema, la ubicación del cenote, altitud del terreno, distancia a la costa y presencia de flujo de agua. Una vez asignadas las 170 localidades a un tipo de cenote (ecosistema), se mapearon para observar si existía algún patrón en su distribución, encontrando que la morfología de los diferentes tipos de cenotes esta relacionado a la altitud del terreno adyacente, donde se pudo observar que la mayoría de los cenotes de tipo Cueva, Acceso restringido, Semiabiertos y Cilíndricos, con desarrollo principalmente vertical, se encuentran en zonas por arriba de los 10 m.s.n.m. aproximadamente, mientras que los cenotes tipo lago, aguada y con zona de inundación se ubican en zonas bajas y cerca de las costas. Por otro lado, los cenotes tipo Manantial (Surgencia) se encontraron en las zonas costeras o de ribera de la península.

Descripción del cuerpo del agua: Se encontró que el cuerpo de agua de los cenotes presenta diferentes formas independientemente de la morfología de la ventana kárstica, es decir, cenotes con un mismo tipo de entrada pueden presentar cuerpos de agua muy distintos. De igual manera las Hidroregiones varían dependiendo de la morfología. El sustrato que se encuentra en el fondo varia a lo largo del sistema (sustrato heterogéneo) dependiendo del tipo de sistema, generalmente los sistemas abiertos se encuentra lodos y MO, donde el fondo cambia gradualmente.

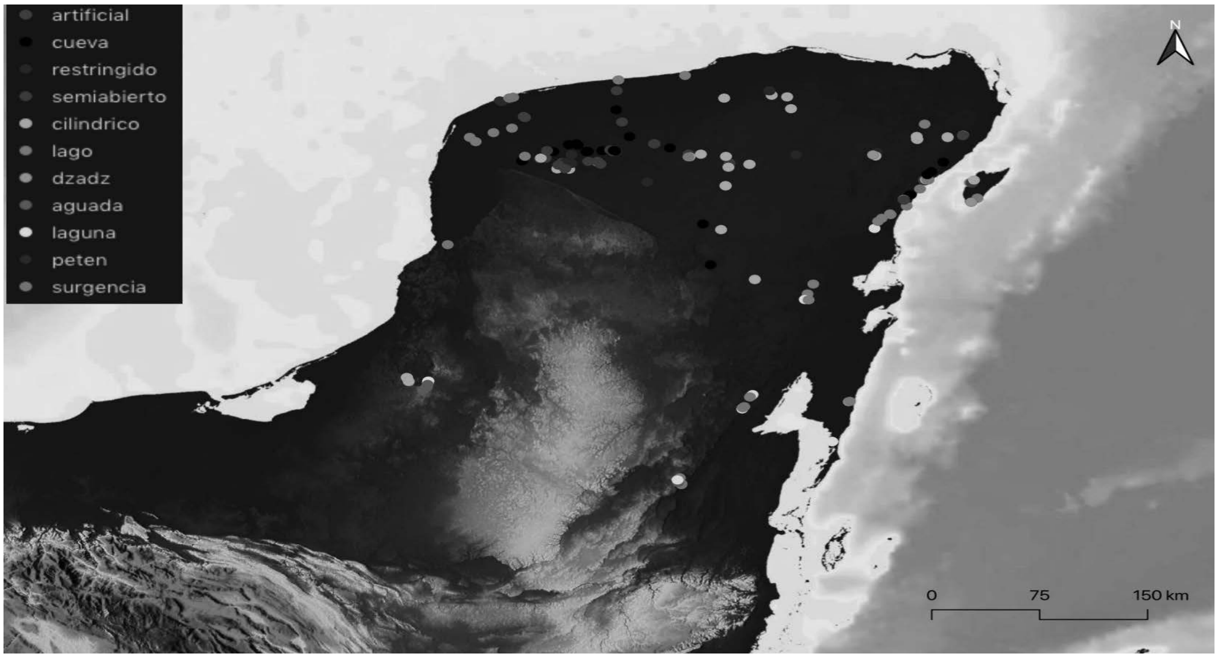

***Figura 1:*** *Mapa de la de elevación de la PY con las 170 localidades visitadas, el color de los puntos indica el tipo de sistema.*

## 4. Discusión

Se encontró que la geomorfología de la entrada influye de manera importante en la incidencia lumínica, estos cambios podrían explicar algunas variaciones en diferentes factores ambientales como temperatura, producción fotosintética, estratificación del cuerpo de agua, O2 disuelto, etc., los cuales a su vez pueden determinar algunos patrones en las especies de la fauna, por ejemplo, ocupación diferencial del habitad, movimiento diarios (migraciones diurnas y actividad nocturna), o podrían tener un papel relevante en el reloj circadiano.

Por otro lado, las variables geomorfológicas como pendiente de la orilla, forma de las paredes, tipo de desarrollo, etc., que normalmente no se tienen en cuenta en los estudios biológicos, pueden afectar la estructura de las comunidades en los cenotes, por ejemplo, variables como la concentración de nutrientes, la temperatura y la cobertura de macrófitos pueden cambiar, dependiendo de la presencia y la longitud de la zona de la orilla. Se ha visto que aquellos cenotes con un área costera pueden tener mayores fuentes de alimentación autóctonas debido a la cantidad y variedad de productores primarios albergados, a diferencia de los cenotes verticales en donde generalmente los nutrientes se depositan directamente en el fondo, fuera del alcance de estos (Camargo-Guerra et al., 2013). Por lo que se esperaría encontrar comunidades más complejas en las regiones ligadas a la superficie y orillas (concretamente en las piscinas de los cenotes y en las cavernas), en comparación con la región de la cueva.

En la actualidad de los sistemas acuáticos subterráneos de la PY albergan gran variedad de especies, la singularidad de los sistemas provee diferentes condiciones ambientales. La fauna terrestre asociada a los sistemas también puede que tenga un papel importante, por ejemplo, dos de las localidades visitadas tipo gruta donde la entrada era pequeña y lejana al cuerpo de agua, y aparentemente la MO alóctona difícilmente llega, presentaron aguas eutrofizadas y turbias (color café), posiblemente esto se debía a la gran cantidad de murciélagos presentes (en una de ellas hematófagos), sugiriendo eutrofización por el guano.

Si el tipo de cenote es un factor importante que influye en los patrones espaciales de los conjuntos de especies, se esperaría que la diversidad de organismos variará entre los diferentes tipos de cenotes, donde los patrones resultantes variarían de un sistema a otro dependiendo de las características geomorfológicas y ambientales tanto de la entrada del cenote, como de la microcuenca. Encontrando diferencias consistentes y significativas de número de especies y abundancia en los diferentes tipos de cenotes, independientemente de la época del año (estación) o la variación intrínseca natural (Nieves-Ortiz et al., 2021).

## 5. Conclusiones

Los cenotes presentan una amplia variedad de formas donde existe una gran diversidad de embalses conocidos en forma genérica como cenotes, algunos tienen formas semejantes a un lago con una zona de “playa”, otros tienen paredes verticales sin zonas de orilla, y otros desarrollan extensos sistemas de grutas y cuevas, por mencionar algunos (Schmitter-Soto, Comín, et al., 2002a). La diferenciación y tipificación de lo distintos ecosistemas que se observan en los cenotes, es una herramienta esencial para su estudio, manejo y conservación. El comprender las diferencias morfológicas y ambientales de estos ecosistemas únicos puede facilitar la predicción de la biodiversidad, la capacidad de carga, y contribuir favorablemente a la gestión de los recursos y conservación, así mismo, contribuiría a la identificación de aquellos que requieren implementación de medidas de manejo específicas, establecer programas de investigación y recopilar datos específicos sobre cada tipo de cenote.

## Agradecimientos

*Estamos completamente agradecidos con todos* los dueños, administradores de cenotes y autoridades gubernamentales que nos permitieron y facilitaron realizar la toma de datos en las localidades. También agradecemos a todos los investigadores,, grupos de investigación, prestadores de servicios turísticos y buzos que contribuyeron con valiosa información que ayudó a caracterizar los diferentes tipos de ecosistemas que se encontraron. Por último y no menos importante, a la UNAM que financió todos los muestreos.

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# This cave is mine: potential competitive exclusion between two species of *Rowlandius* (Schizomida: Hubbardiidae) in the Caatinga

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## Abstract

The order Schizomida comprises small arachnids, with the genus *Rowlandius* being the most diverse, including 66 described species. In Brazil, two species, *Rowlandius potiguar* and *Rowlandius* **sp. n. 1**, are both endemic to the Caatinga biome and inhabit the caves of the Jandaíra Formation, exhibiting similar morphologies. Preliminary observations suggested that each cave hosts only one of these species. Based on an extensive sample size, molecular data, and morphological identification, we hypothesized a pattern of competitive exclusion between these species. This study employed phylogeographic, phylogenetic, and ecological niche modeling approaches to investigate whether there is a high degree of niche overlap between the two species or if they exhibit significant niche specialization. Specimens were collected from 27 caves, including 14 newly recorded locations. Molecular analyses, utilizing mitochondrial and nuclear markers, revealed no instances of cave sympatry among the different lineages. Ecological niche modeling indicated that *R. potiguar* demonstrates greater specialization in restricted habitats, while *Rowlandius* **sp. n. 1** shows higher ecological flexibility and adaptability to varying environmental conditions. Furthermore, the soil water index emerged as the most significant environmental variable influencing the distribution of both species, consistent with their hygrophilic nature. The findings strongly support the hypothesis of competitive exclusion, with no sympatry observed between the species based on morphological, molecular, and ecological data. This pattern likely reflects an eco-evolutionary process of niche partitioning, where the two species may have evolved divergent strategies to reduce competition in resource-limited environments like caves. While some caves are located within the Furna Feia National Park, the majority lie outside protected areas. As a result, most populations of both species face ongoing threats, highlighting the urgent need for conservation efforts.

## 1. Introduction

The order Schizomida comprises small arachnids, with the genus *Rowlandius* being the most speciose, encompassing 66 described species (World Arachnida Catalog, 2025). These species exhibit a predominantly Neotropical distribution and are found in diverse habitats such as leaf litter, soil, and caves, particularly in the Caatinga, a semi-arid biome.

In Brazil, two *Rowlandius* species are endemic to the Jandaíra Formation (spanning Rio Grande do Norte and Ceará), including caves located within the Furna Feia National Park (FFNP). The park is a significant spot for troglobitic species in the Caatinga. The two species, *Rowlandius potiguar* (Fig. 1A) and *Rowlandius* **sp. n. 1** (Fig. 1B, currently under description), share similar distributions and exhibit comparable external morphologies. Notably, these species have never been recorded coexisting in the same cave, suggesting a possible case of competitive exclusion. Based on this observation, we hypothesize that there is either a high degree of niche overlap between these two species or that both exhibit pronounced niche specialization.

To investigate this, we employed a phylogeographic and phylogenetic approach, sampling schizomids across their known distribution. Additionally, we conducted ecological niche modeling (ENM), a method that integrates known occurrence data with environmental variables to characterize species' ecological niches. ENM is widely applied to both terrestrial and aquatic organisms and has been increasingly explored for cave-dwelling fauna. However, studies of this kind remain scarce in Brazil, particularly for schizomids (Peterson *et al.,* 2011).

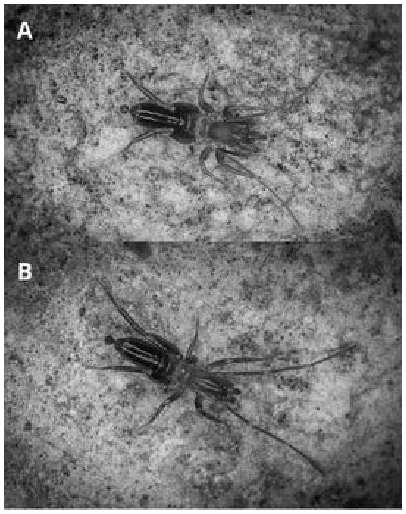

***Figure 1:*** *Specimens observed live in Felipe Guerra, Rio Grande do Norte, Brazil. (A) Male of Rowlandius potiguar, Carrapateira Cave; (B) Male of Rowlandius* ***sp. n. 1****, Pinga Cave. Source: Diego Bento, 2024.*

## 2. Materials and methods

We collected schizomids from 27 caves, including 14 new records. The specimens were deposited in the Natural History Collections of the Federal University of Piauí (CHNUFPI) and the Subterranean Invertebrates Collection of Lavras (ISLA) at the Federal University of Lavras.

For molecular analyses, total DNA was extracted from 97 specimens using the DNeasy Blood & Tissue Kit (Qiagen). Two molecular markers, cytochrome c oxidase I (cox1) and rDNA 28S, were sequenced following amplification via polymerase chain reaction (PCR). *Rowlandius* ubajara was used as the outgroup for phylogenetic analyses.

Consensus sequences were assembled using Geneious Prime software and aligned with ClustalW (Thompson et al., 1994) implemented in MEGA 11.0 (Tamura et al., 2021). Phylogenetic relationships were inferred through multilocus Bayesian inference (BI) analysis performed in BEAST (Drummond et al., 2012).

Ecological niche modeling (ENM) was performed using Maxent models in R to evaluate the habitat suitability of both species. Occurrence data were obtained from the literature, biological collections, and the results of morphological and molecular analyses. A total of 30 occurrence points for *R. potiguar* and 16 for *Rowlandius* **sp. n. 1** were included in the modeling. Bioclimatic, soil, and vegetation variables were used, with a Pearson correlation filter (P > 0.7) applied to select four final variables. Calibration parameters were optimized using AIC, AUC, and omission rate values. The best-performing models were then used to generate species distribution maps in QGIS.

Additionally, a Principal Component Analysis (PCA) was conducted to examine similarities and differences in the relationships between species and the selected environmental variables, providing further insights into their habitat preferences.

## 3. Results

The multilocus phylogenetic tree constructed using Bayesian inference (BI) revealed three major clades with strong branch support values (Fig. 2). The clade originating from the type locality of *Rowlandius potiguar* (Carrapateira Cave) also included specimens from 13 additional localities. This clade was identified as the sister group to a second clade comprising individuals of *Rowlandius* **sp. n. 2**, collected from Casa de Pedra Cave in Martins. Together, these two clades formed the sister group to the third clade, representing *Rowlandius* **sp. n. 1**, which included individuals from nine different caves. Notably, no sympatry was observed among the distinct lineages.

The ecological niche modeling results demonstrated strong predictive accuracy (AUC > 0.8), highlighting similarities in habitat suitability between *R. potiguar* and *Rowlandius* **sp. n. 1** (Fig. 3). However, *Rowlandius* **sp. n. 1** exhibited a broader niche amplitude and greater adaptability to varying environmental conditions, whereas *R. potiguar* showed higher habitat specialization, resulting in a more restricted distribution.

The Soil Water Index emerged as the most influential variable in model construction, contributing over 50% to the results. Temperature and precipitation variables played key roles in defining niche partitioning between the species, while cation exchange capacity (CEC) was less significant and primarily highlighted differences in microhabitat preferences (Fig. 4).

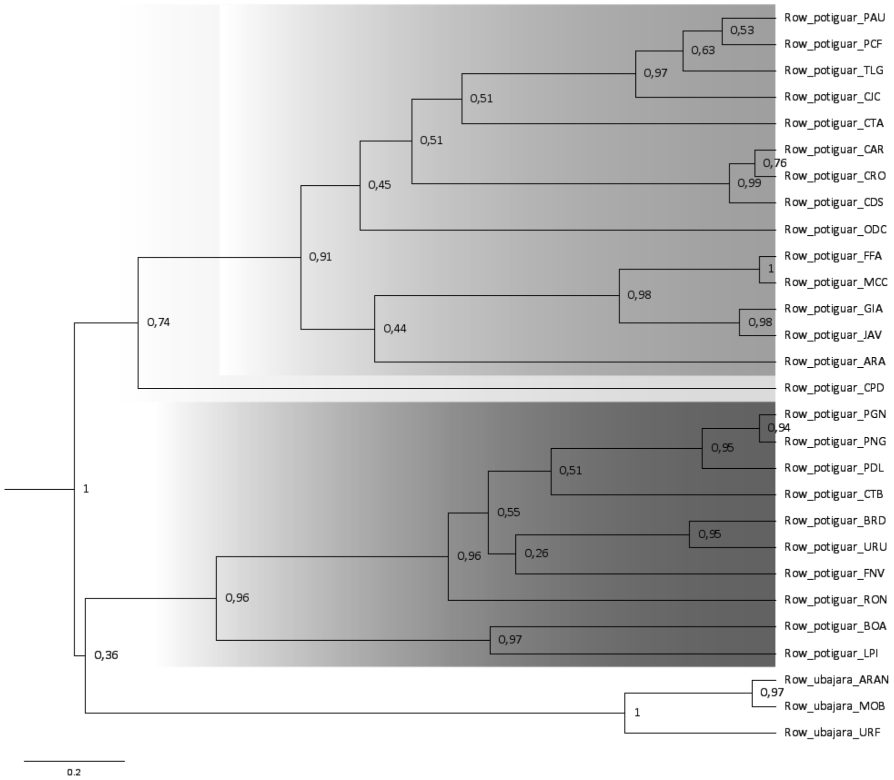

***Figura 2:*** *Multilocus consensus phylogenetic tree resulting from Bayesian inference. The colors represent each clade: blue for R. potiguar, yellow for Rowlandius sp. n. 2, and red for Rowlandius sp. n. 1. Each branch of the tree displays the posterior probability.*

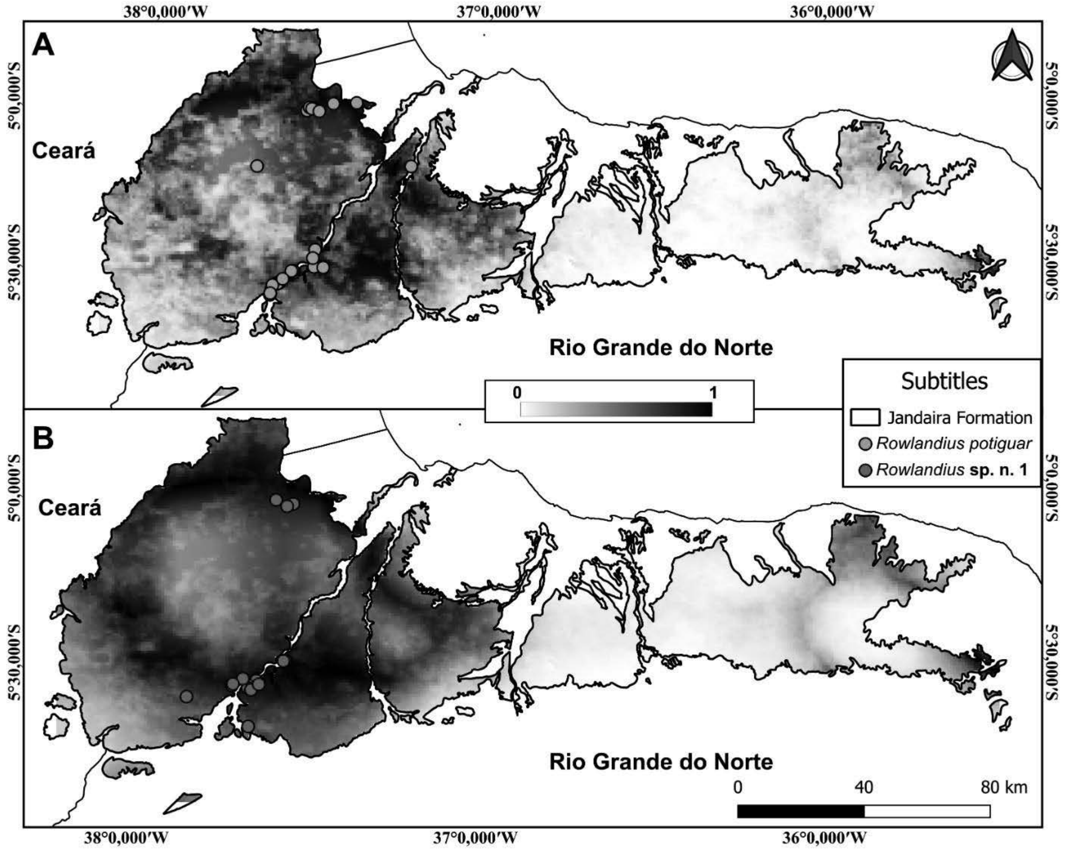

*Figure 3:* *Habitat suitability, with the gradient representing the degree of suitability. (A) R. potiguar; (B) Rowlandius* ***sp. n. 1****.*

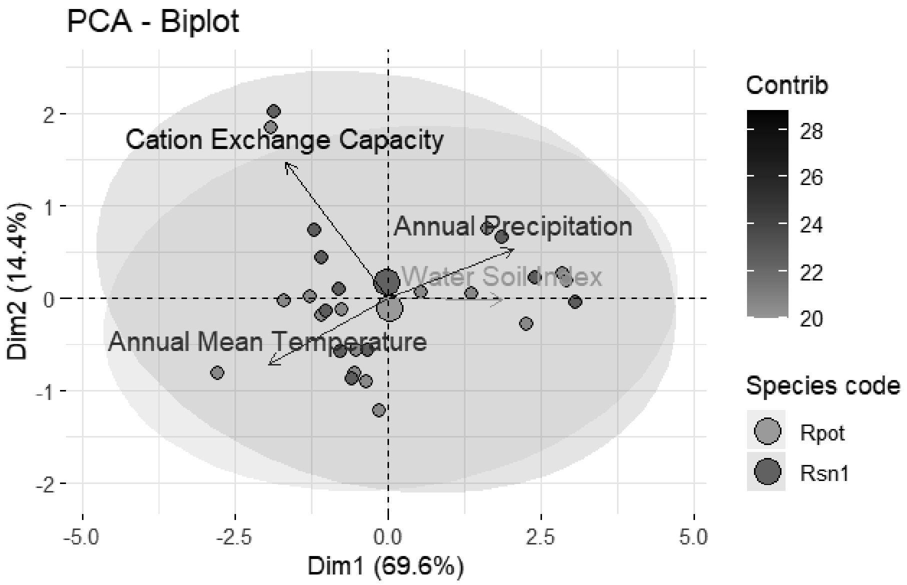

*Figure 4:* *Principal Component Analysis (PCA), with larger circles representing the centroids, which are very close to each other. Contribution: Gradient of environmental variable contributions, where darker shades indicate higher contributions.*

## 4. Discussion

The expanded collections of *Rowlandius* from Caatinga caves, including new records, have provided a more comprehensive understanding of the genus's geographic distribution, significantly enhancing the knowledge base beyond what was previously reported in the literature (Santos *et al.,* 2013; Bento *et al*., 2021).

Molecular and morphological analyses consistently revealed the existence of a species complex, further emphasizing the diversity of this genus in the semiarid region. The lack of a clear geographic pattern between the two species found in the Jandaíra Formation (*R. potiguar* and *Rowlandius* **sp. n. 1**) suggests population isolation driven by geological fragmentation of the rocky outcrops in which they occur. This pattern aligns with observations of the troglobitic planthopper *Kinnapotiguara troglobia* in the western Jandaíra Formation (Bento *et al*., 2024). Caves within the same rocky outcrop may function as interconnected systems, offering refuge and higher humidity compared to the external environment, contributing to the isolation of cave-dwelling populations.

The absence of sympatry between the studied species and the niche modeling results suggest highly similar environmental suitability, reinforcing the hypothesis of competitive exclusion in resource-limited environments such as caves.

Niche modeling further indicated that *Rowlandius* **sp. n. 1** exhibits greater ecological flexibility and adaptability to varying environmental conditions, while *R. potiguar* displays more specialized habitat requirements, favoring restricted environments. This pattern suggests a potential dominance of *Rowlandius* sp. n. 1 in overlapping areas, which may highlight the ecological vulnerability of *R. potiguar* to environmental changes and competitive pressures.

Although some populations of *R. potiguar* and *Rowlandius* **sp. n. 1** are located within caves protected by the Furna Feia National Park (FFNP), the majority of localities and populations lie outside protected areas. This makes them vulnerable to numerous threats, including unregulated visitation, mining, deforestation, and agricultural expansion (Bento *et al*., 2024).

## 5. Conclusion

This study revealed a broader geographic distribution of the genus *Rowlandius* in Caatinga caves. Molecular analyses uncovered a species complex, including two new species, highlighting the genus's remarkable diversity in the region. The absence of sympatry between *R. potiguar* and *Rowlandius* **sp. n. 1**, combined with niche modeling results, supports the hypothesis of competitive exclusion between the two species.

*Rowlandius* **sp. n. 1** demonstrated a more generalist ecological niche, while *R. potiguar* exhibited a more specialized ecological niche. Niche partitioning appears to be influenced by gradients of temperature and precipitation, with cation exchange capacity (CEC) playing a key role in microhabitat differentiation.

Although some caves within the Furna Feia National Park (FFNP) offer protection to both species, the majority of localities are situated outside protected areas. This leaves the species vulnerable to various threats, including mining, deforestation, and agricultural activities, emphasizing the need for expanded conservation efforts.

## Acknowledgments

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# Contribuição do banco de ovos para a diversidade de zooplâncton em ambientes aquáticos subterrâneos

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## Resumo

O zooplâncton tem a capacidade de formar de ovos ou formas de resistência quando as condições ambientais não estão favoráveis, sendo esta uma estratégia fundamental para a manutenção da diversidade de espécies em ambientes aquáticos subterrâneos. Aqui nós realizamos um experimento para avaliar a eclosão de zooplâncton a partir do sedimento de ambientes aquáticos subterrâneos do bioma Caatinga. Foram registradas 164 eclosões de ciliados, 14 de rotíferos, 8 de ostrácodes e 3 de cladóceros, além de 129 eclosões de zooplâncton no tratamento com fotoperíodo de 12 h de luz/ 12 h de escuro e 60, no tratamento de 24 h de escuro. Portanto, mostramos o potencial que o banco de ovos e formas dormente do sedimento de ambientes aquáticos subterrâneos tem para contribuir com a colonização do zooplâncton.

## Abstract

Zooplankton have the ability to form eggs or dormant forms when environmental conditions are unfavorable, which is a fundamental strategy for maintaining species diversity in subterranean aquatic environments. In this study, we conducted an experiment to assess zooplankton hatching from sediment samples collected from subterranean aquatic environments in the Caatinga biome. A total of 164 hatchings of ciliates, 14 of rotifers, 8 of ostracods, and 3 of cladocerans were recorded, in addition to 129 zooplankton hatchings under a 12-hour light/12-hour dark photoperiod treatment and 60 hatchings under a 24-hour dark treatment. Therefore, we demonstrate the potential of the egg bank and dormant forms in the sediment of subterranean aquatic environments to contribute to zooplankton colonization.

## 1. Introdução

O estado do Rio Grande do Norte (RN) ocupa o 4º lugar em número de cavernas no Brasil (1374 cavidades catalogadas), estando presentes principalmente em rochas calcárias, de clima semiárido e bioma Caatinga (CECAV/ICMBio 2022, Bento et al., 2017). Estudos realizados nesta região indicam áreas com alta relevância biospeleológica, porém estima-se que apenas 5% das cavernas conhecidas têm a sua fauna registrada, sendo a maioria espécies terrestres (Bento et al. 2021).

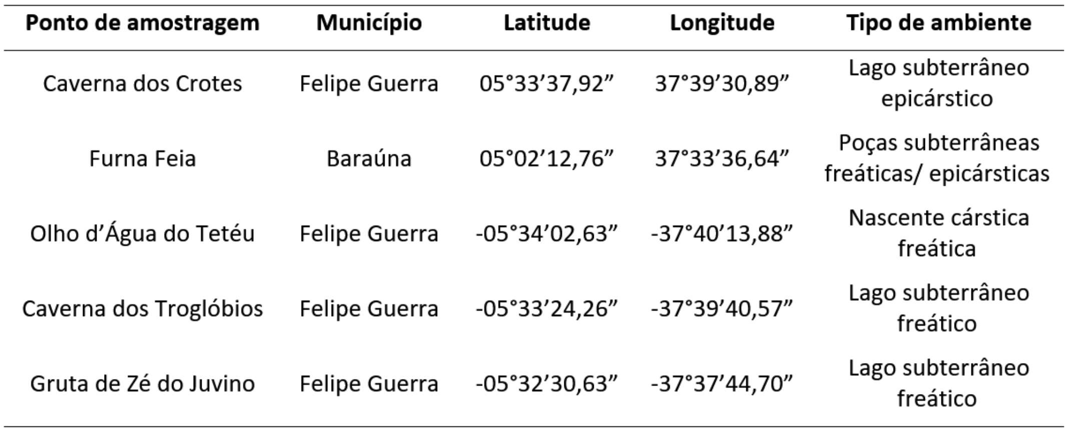
Nos ecossistemas aquáticos, o zooplâncton apresenta alta diversidade de espécies e funcional, sendo elo da teia trófica e atuando na ciclagem de matéria e energia (Litchman et al., 2013). Mais especificamente para os ambientes aquáticos subterrâneos da Caatinga foram registradas 100 espécies de zooplâncton, incluindo protozoários testáceos, rotíferos, cladóceros e copépodes (Puppin-Gonçalves et al., 2024). Este número de espécies é elevado quando comparado a outros estudos no mundo (36 espécies em Simões et al. 2013; 41 espécies em Baković et al., 2022, 24 espécies em Bozkurt, 2019) realizados em ambientes aquáticos subterrâneos.

Uma caracteristica importante do zooplâncton é a capacidade de apresentar formas dormentes em condições ambientais não favoráveis (i.e. ovos de resistência ou indivíduos em baixa atividade metabólica) (Vargas et al., 2019, Fortaneto, 2019). Esta estratégia é ainda mais importante em ambientes aquáticos subterrâneos que tendem a ser afóticos, dependentes da matéria orgânica alóctone e com recursos alimentares limitados (Culver & Pipan 2009). O acesso ao banco de formas dormentes ocorre através do sedimento e reflete a comunidade passiva que habita o ecossistema. Tais bancos são cruciais para a compreensão da dinâmica das comunidades, por serem fontes de colonização e essenciais à resiliência dos ambientes que passam por impactos antrópicos ou naturais (Brendonck & Meester 2003, Brendonck 2017). É, portanto, uma forma de acessar a composição futura da comunidade (Brendonck & Meester 2003, Piscia et al. 2020). Como alguns ambientes subterrâneos são difíceis de amostrar, o acesso ao banco de formas dormentes contribui para melhor conhecermos a biodiversidade e dinâmica dos ecossistemas subterrâneos, sendo possível o registro de novos táxons. Este é o primeiro estudo avaliando a contribuição do banco de ovos e formas dormentes para a comunidade zooplânctonica em ambientes cavernícolas, portanto, avançará no conhecimento da biodiversidade e dinâmica dos ecossistemas subterrâneos, sendo possível o registro de novas espécies. O objetivo deste estudo foi avaliar experimentalmente a contribuição do banco de ovos e formas dormentes do sedimento para a diversidade do zooplâncton em ambientes aquáticos subterrâneos do bioma Caatinga.

## 2. Materiais e métodos

Foram realizadas amostragens de sedimento em cinco ambientes aquáticos subterrâneos do bioma Caatinga no Estado do RN (Figura 1). Em cada local de coleta, o sedimento foi coletado de forma aleatória com auxílio de uma pá e com profundidade de 5 cm. A coleta foi realizada em dezembro de 2022 que corresponde ao período de seca no bioma.

Em laboratório, o sedimento foi homogeneizado, seco em estufa a 40°C e, posteriormente, acondicionado em potes plásticos no escuro à temperatura ambiente. Os microscosmos artificiais foram criados com a adição de 100g de sedimento em recipientes plásticos e hidratados com 300ml de água destilada (proporção 1:3, simulando inundação persistente). Os microcosmos foram mantidos em duas simulações distintas de fotoperíodo: 12h luz/ 12h escuro e de 24h escuro para simular as cavernas afóticas (Figura 2). O período de incubação do experimento foi de 35 dias (13 de novembro a 15 de dezembro de 2023), com coleta dos organismos que aclodiram a cada 2 dias. Para a coleta foi filtrada toda a água dos microscosmos em rede de plâncton de malha de 50-µm. Após a coleta, foi reposta a água destilada em cada um dos microscosmos. Os organismos coletados foram fixados com formol 4% e identificados até o menor nível taxonômico possível.



| Ponto de amostragem | Município | Latitude | Longitude | Tipo de ambiente |
|---|---|---|---|---|
| Caverna dos Crotes | Felipe Guerra | 05°33’37,92” | 37°39’30,89” | Lago subterrâneo epicárstico |
| Furna Feia | Baraúna | 05°02’12,76” | 37°33’36,64” | Poças subterrâneas freáticas/ epicársticas |
| Olho d’Água do Tetéu | Felipe Guerra | -05°34’02,63” | -37°40’13,88” | Nascente cárstica freática |
| Caverna dos Troglóbios | Felipe Guerra | -05°33’24,26” | -37°39’40,57” | Lago subterrâneo freático |
| Gruta de Zé do Juvino | Felipe Guerra | -05°32’30,63” | -37°37’44,70” | Lago subterrâneo freático |

***Figura 1:*** *Locais de amostragem dos sedimentos utilizados no experimento de eclosão de ovos e formas dormentes de zooplâncton.*

***Figura 2:*** *Experimento mostrando os microcosmos artificiais no fotoperíodo permanentemente escuro (24 h) e 12 h luz/12 h escuro.*

## 3. Resultados

Durante o nosso experimento, eclodiram ciliados, rotíferos, cladóceros e ostrácodas a partir do banco de ovos e formas dormentes do sedimento. Ciliado foi o grupo com maior número de eclosões (164), seguido por rotíferos (14), ostrácodas (8) e cladóceros (3). Foi possivel identificar a espécie de cladócero Leydigia ipojucae com três eclosões na caverna da Furna Feia no fotoperíodo de 12 h de luz/ 12 h de escuro. Ainda neste local, foi identificado o gênero de rotífero Lepadella sp. que eclodiu nos dos fotoperíodos (dois indivíduos). Os demais grupos não puderam ser identificados em nível de espécie.

As eclosões começaram a ser observadas a partir da segunda semana de experimento (Figura 3). O maior número de eclosões foi observado na caverna da Furna Feia (134), seguido por Gruta de Zé do Juvino (28), Caverna dos Crotes (15) e Olho d'agua do Tetéu (12). Nenhuma espécie de zooplâncton eclodiu do sedimento da Caverna dos troglóbios.

Foram registradas 129 eclosões de zooplâncton no tratamento com fotoperíodo de 12 h de luz/ 12 h de escuro e 60, no tratamento de 24 h de escuro. Não foram observadas eclosões de cladóceros no fotoperíodo de 24 h de escuro, mas foi observado um aumento na proporção de rotíferos e ostrácodes que aclodiram neste tratamento (Figura 4).

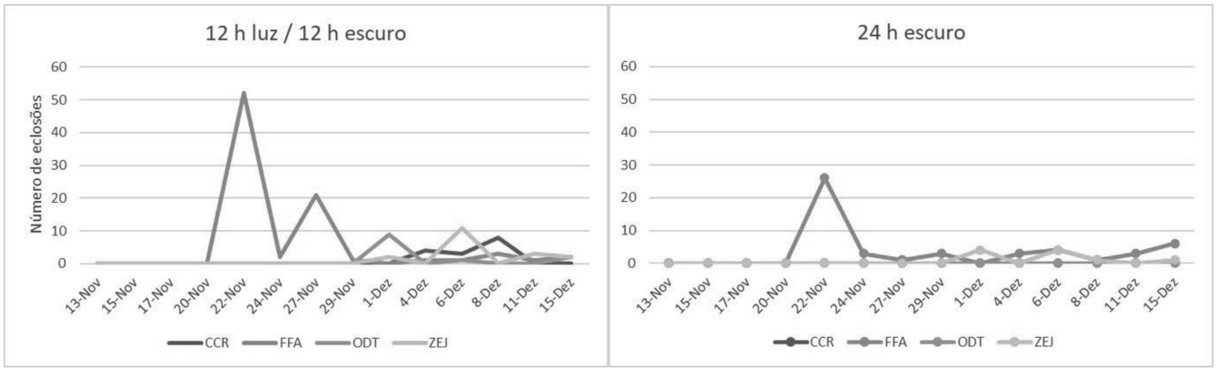


***Figura 3:*** *Número de eclosões de zooplâncton ao longo dos dias de coleta nos fotoperíodos de 12 h luz/12 h escuro (esquerda) e 24 h escuro (direita). As cores representam os locais de coleta (CCR = Caverna Crotes ; FFA = caverna da Furna Feia ; ODT = Olho d'agua do Tetéu ; ZEJ = Gruta de Zé do Juvino).*

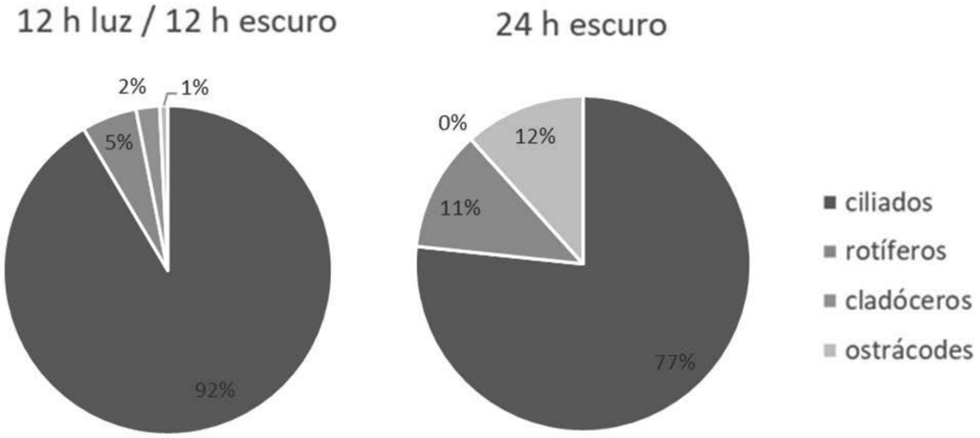


***Figura 4:*** *Porcentagem de eclosões (%) de zooplâncton por grupos nos fotoperíodos de 12 h luz/ 12h escuro (esquerda) e 24 h de escuro (direita).*

## 4. Discussão

Nosso experimento mostrou que as eclosões de zooplâncton a partir do banco de ovos e formas dormentes do sedimento tem um grande potencial para contribuir com a diversidade desses organismos em ambientes aquáticos subterrâneos. Registramos uma maior proporção de eclosões de ciliados que constituem o microzooplâncton, mas também registramos a ocorrência de espécies maiores como cladóceros. No estudo de Berger et al. (2007), os ciliados foram os primeiros organismos a eclodirem no experimento, seguidos por fitoplâncton e organismos zooplanctônicos surgiram por volta do 20º dia (i.e. *Daphnia*). Os protozoários ciliados são os organismos mais importantes do loop microbiano, atuando como link entre a produção microbiana e os níveis tróficos superiores. Os Gastrotricha, ciliados encontrados no experimento, se alimentam de bactérias, protistas, biofilme e matéria orgânica (Balsamo et al. 2020), recursos abundantes na caverna da Furna Feia, a qual possui entrada significativa de matéria orgânica proveniente do guano das colônias de morcegos.

O maior número de eclosões ocorreram no fotoperíodo de 12 h luz/ 12 h escuro. Para a inclusão deste tratamento, nos baseamos em metodologias de eclosão de ovos e formas dormentes de ambientes superficiais do semiárido (Vendramin et al. 2023, Pires et al. 2023). Entretanto, este foi o primeiro estudo a mostrar que as eclosões de espécies de zooplâncton ocorrem também com 24 h de escuro, como observada nos ambientes cavernícolas. A única espécie de cladócero que eclodiu foi restrita ao fotoperíodo de 12 h luz/ 12 h escuro. Isso pode ser devido ao fato de ser uma espécie mais sensível as condições cavernícolas e de ser uma espécie da superfície que acidentalmente foi levada para o ambiente subterrâneo. Portanto, mostramos com nosso experimento o potencial que o banco de ovos tem para contribuir com a colonização do zooplâncton em ambientes aquáticos subterrâneos.

## 5. Conclusão

Com este experimento, mostramos que o sedimento do ambientes aquáticos subterrâneos abriga ovos e formas dormentes de zooplâncton que podem contribuir com a diversidade nesses ambientes e auxiliar na identificação de espécies, incluindo estigóbias. Estudos futuros incluindo mais locais de coleta e com um tempo maior de incubação, podem ser importantes para a eclosão de outros organismos do zooplâncton, como os copépodes.

## Agradecimentos

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# Global evolution of cave animal of the year campaigns

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## Abstract

Several member countries of the International Union of Speleology UIS are joining the Cave Animal of the Year campaigns since the International Year of Caves and Karst 2021. International Cave Animals will be launched every 4 years according the International Congresses of Speleology. For 2025 the International Cave Animal of the Year is Lepidoptera butterflies and moths.Beside the international species UIS members can provide national campaigns to highlight underground biodiversity in their countries and beyond. Every campaign has its own approach and highlights. They range from card games, special designer objects, articles in newspapers, events for children and to conferences. Some countries share the same species every year. The actions thus not only show the wide range of cave animals, but also the great ideas for bringing the protection of cave animals to the attention of the public. The overview of all cave animals honoured in the last four years is intended to inspire further action and promote cooperation.

## Résumé

Plusieurs pays membres de l'Union internationale de spéléologie (UIS) se joignent aux campagnes de l'animal des cavernes de l'année depuis l'Année internationale des grottes et du karst 2021. International Cave Animals sera lancé tous les 4 ans conformément aux Congrès internationaux de spéléologie. D'ici 2025, les papillons et les lépidoptères seront les animaux des cavernes de l'année. En plus des espèces internationales, les membres de l'ISU peuvent organiser des campagnes nationales pour mettre en évidence la biodiversité des eaux souterraines dans leurs pays et au-delà. Chaque campagne a sa propre approche et ses propres points forts. Ils vont des jeux de cartes, des objets de design spéciaux, des articles de journaux, des événements pour enfants et des conférences. Certains pays partagent la même espèce chaque année. Par conséquent, les actions montrent non seulement le large éventail d'animaux des cavernes, mais aussi les grandes idées pour attirer l'attention du public sur la protection des animaux des cavernes. L'aperçu de tous les animaux des cavernes honorés au cours des quatre dernières années vise à inspirer de nouvelles actions et à promouvoir la coopération.

## Resumen

Varios países miembros de la Unión Internacional de Espeleología (UIS) se suman a las campañas del Animal de Cueva del Año desde el Año Internacional de las Cuevas y el Karst 2021. International Cave Animals se lanzará cada 4 años de acuerdo con los Congresos Internacionales de Espeleología. Para 2025, el Animal de Cueva Internacional del Año son las mariposas y polillas lepidópteras. Además de las especies internacionales, los miembros del IEU pueden organizar campañas nacionales para poner de relieve la biodiversidad subterránea en sus países y fuera de ellos. Cada campaña tiene su propio enfoque y aspectos destacados. Van desde juegos de cartas, objetos especiales de diseño, artículos en periódicos, eventos para niños y conferencias. Algunos países comparten la misma especie todos los años. Por lo tanto, las acciones no solo muestran la amplia gama de animales de las cavernas, sino también las grandes ideas para llamar la atención del público sobre la protección de los animales de las cuevas. La visión general de todos los animales de las cavernas homenajeados en los últimos cuatro años tiene como objetivo inspirar nuevas acciones y promover la cooperación.

## 1. Introduction

Caves represent critical yet often overlooked ecosystems that house specialized and fragile species. The "Cave Animal of the Year" campaign was initiated to bring these environments into public focus. Beginning in **Germany**, the campaign's influence has grown, with **Italy, Australia, and the USA** in a first stage participating now under the UIS's umbrella. Each year, a selected species serves as a focal point for educational and conservation activities. For 2025, Lepidoptera take center stage, emphasizing their ecological importance and vulnerability, but this 2025 campaign will promote also other species to be elected in order to encourage other countries in participate globally to the campaign.

## 2. Materials and Methods

To foster the "International Cave Animal of the Year 2025" campaign, UIS encourages participating countries to select a regionally significant butterfly or moth species or any other special cave animal and present it to the public and policymakers. Campaigns include educational materials, exhibitions, and outreach activities aimed at fostering cave conservation awareness. These initiatives often involve collaborations with speleological societies, conservation organizations, and citizen scientists.

Field studies on cavernicolous Lepidoptera are integral to understanding their ecological roles and adaptations. Researchers document species that utilize caves seasonally or year-round. For example, some species feed on guano deposits, contributing to nutrient recycling in cave ecosystems. Efforts are also focused on assessing the impacts of climate change and human activities on these fragile habitats.

***Figure 1:*** *A group of the Tissue Moth (Triphosa dubitata) on a cave wall. Photo: Klaus Bogon.*

The campaign employs a combination of public outreach and scientific research:

**Web site and Press release:** as first step of the campaign the national be sites are updated at the end of the year and a specific press release created with key information of the selected species and importance for cave biodiversity

**Educational Materials:** Posters, brochures, and interactive tools like games and puzzles are distributed to schools, tourist caves, and online platforms. These materials are oriented to a variety of age groups, aiming to make learning about subterranean biodiversity engaging and accessible.

**Public Engagement:** Events such as workshops, webinars, and field trips are organized to engage diverse audiences. School programs and collaborations with local communities ensure that the message reaches even remote areas.

**Scientific Research:** Monitoring cave-dwelling species like Triphosa dubitata involves non-invasive methods, environmental data collection, and collaboration with speleologists and conservationists. Studies focus on understanding the ecological roles of these species and the threats they face.

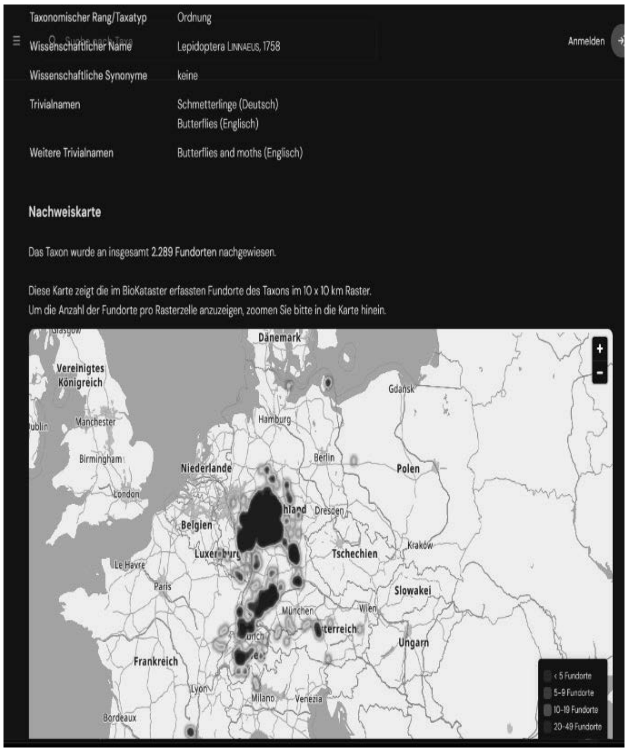
**Collaborative Efforts:** Partnerships with local and international organizations enhance resource sharing and amplify impact. Collaborations with policymakers also aim to integrate cave conservation into national and regional agendas.

## 3. Results

Global campaigns have already demonstrated significant impacts. Awareness programs, such as those for cave beetles and bats, have led to increased public engagement and policy advancements. Similarly, campaigns in Australia and the USA have highlighted the importance of endemic and threatened species, such as the Ghost Bat (*Macroderma gigas*) and the Little Brown Bat (*Myotis lucifugus*). In Italy during the Covid 19 pandemic the Campaign has attracted interest on cave biodiversity and provide clear information on importance of bats and cave biodiversity.

In Germany campaign yearly reach over 50 newspapers and 40 showcaves receive 150 flyers and one poster, some ask for more. Over all about 2000 flyer and 100 posters are distributed. At cavers level the brochure and posters are distributed also at VdHK's annual meeting.

For Lepidoptera, preliminary results indicate diverse cave usage patterns. Species like *Triphosa dubitata* and guano-feeding moths exemplify the ecological significance of Lepidoptera in caves. Their presence underscores the need to conserve both the species and their habitats.



| | |
|---|---|
| Taxonomischer Rang/Taxatyp | Ordnung |
| Wissenschaftlicher Name | Lepidoptera Linnaeus, 1758 |
| Wissenschaftliche Synonyme | keine |
| Trivialnamen | Schmetterlinge (Deutsch)<br>Butterflies (Englisch) |
| Weitere Trivialnamen | Butterflies and moths (Englisch) |

**Nachweiskarte**

Das Taxon wurde an insgesamt 2.289 Fundorten nachgewiesen.

Diese Karte zeigt die im BioKataster erfassten Fundorte des Taxons im 10 x 10 km Raster.
Um die Anzahl der Fundorte pro Rasterzelle anzuzeigen, zoomen Sie bitte in die Karte hinein.

***Figure 2:*** *Distribution of cavernicolous Lepidoptera from German Biokataster, Lepidoptera was found at 2289 places so this map shows more or less the activity of cavers in regard to biospeleology, some areas and not karst areas only, because the kadastre is also counting cave related species in other underground habitats.*

The “Cave Animal of the Year” campaign has achieved several significant results:

- **Public Awareness:** The campaign’s educational initiatives have reached thousands globally. For example, interactive games and social media campaigns have successfully engaged younger audiences.
- **Policy Advancements:** Germany’s efforts led to caves being recognized as protected biotopes under national law. Italy’s campaigns have similarly influenced local conservation policies.
- **Scientific Discoveries:** Research initiatives have uncovered new insights into the behavior and ecological significance of species like Italodytes stammeri and Speleomantes salamanders. The role of Lepidoptera in nutrient recycling within cave ecosystems is now better understood.
- **Global Collaboration:** The UIS’s coordination has fostered partnerships across countries, creating a unified approach to cave biodiversity conservation in 10 countries with a coordinate effort.

1. Australia
2. Austria
3. Brazil
4. France
5. Germany
6. Greece
7. India
8. Italy
9. Spain
10. Switzerland
11. USA

## 4. Discussion

Caves provide essential shelters for species, offering refuge from extreme weather and predators. However, these habitats face threats from human activities such as mining, tourism, and pollution. Climate change exacerbates these challenges by altering cave microclimates and ecosystems.

Collaborative conservation efforts are crucial to mitigating these threats. Projects like the “Caves as Shelters of Life” initiative, where cavers and cave visitors can inform about the presence of lepidoptera in caves by the platform I naturalist (a pilot activity started in Italy in the Lazio Regio https://www.inaturalist.org/projects/triphosa-dubitata-nel-lazio.), and get in touch with local experts, emphasize education, research, and sustainable caving practices. Citizen science initiatives, such as butterfly and moth surveys, play a pivotal role in documenting species diversity and distribution.

Through global partnerships, the UIS Biology Commission aims to strengthen conservation policies and foster a deeper understanding of subterranean biodiversity. Highlighting Lepidoptera as the “Interantional Cave Animal of the Year” in 2025 provides a unique opportunity to engage diverse audiences and advocate for the protection of these vital habitats and present the outcome at the International Conference of Speleology.

***Figure 3:*** *Example of educational and outreach materials distributed during the various campaign. https://hoehlentier.de/en/press-releases/*

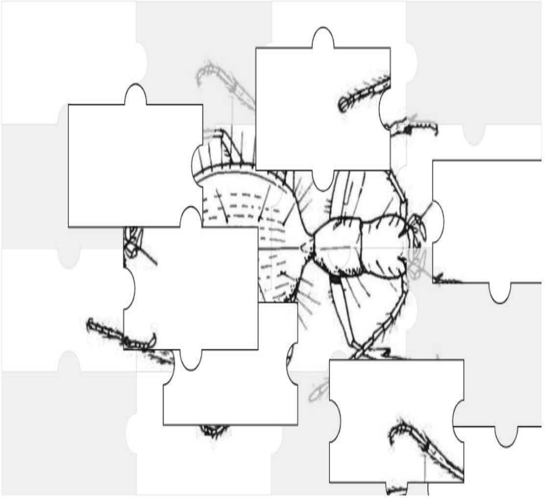


***Figure 4:*** *Italodytes Stammeri Puzzle from https://animalidigrotta.speleo.it/italodytes-stammeri/*

***Figure 5:*** *Complete set of outreach materials distributed in Australia https://www.caveanimaloftheyear.org.au/2023/glow-worms*

The International Cave Animal of the Year campaign can grow significantly through the inclusion of national campaigns that highlight regionally significant cave-dwelling species while maintaining the shared aim of promoting cave protection. By aligning national efforts with the overarching international goal, the campaign can create a cohesive global movement. Here's how this framework can be derived and expanded: Global-Local Synergy

Through global partnerships, the UIS Biology Commission aims to integrate insights from national campaigns into the international framework. By showcasing national outcomes, such as regional educational programs and conservation strategies, at global events like the International Congress of Speleology, these campaigns can foster a deeper understanding of subterranean biodiversity and strengthen international conservation policies.

The campaign's success lies in its ability to combine public education with actionable conservation strategies. However, several challenges remain:

- **Funding and Resources:** The campaign's reliance on volunteer efforts underscores the need for sustainable funding and institutional support.
- **Long-Term Engagement:** While annual campaigns effectively raise awareness, maintaining public interest between events requires continuous initiatives and innovative strategies.

Future directions for the campaign include expanding its reach through digital platforms, integrating new technologies like virtual reality to simulate cave environments, and fostering stronger collaborations with academic and governmental organizations.

## 5. Conclusion

The "International Cave Animal of the Year" campaign has emerged as an effective tool for raising awareness and promoting conservation. By focusing on Lepidoptera in 2025, UIS aims to shed light on the ecological importance of these insects and the challenges they face. Through education, research, and policy advocacy, the campaign seeks to ensure the long-term preservation of cave biodiversity.

The campaign not only raises awareness but also drives policy changes and scientific advancements, ensuring the protection of these fragile ecosystems for future generations.

## Acknowledgments

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Additional materials and updates available at:

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Austria: https://hoehle.org/hoehlentier

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Greece: https://www.inaturalist.org/projects/cave-animal-of-the-year--2021-greece

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Swiss: https://cavernicola.ch/

# Cave Animal of the Year in Italy: a communication-action campaign for the knowledge and protection of biodiversity in caves.

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## Abstract

The "Cave Animal of the Year" campaign in Italy, introduced in 2018 by the Italian Speleological Society SSI ETS, aims to raise awareness about subterranean fauna. The initiative promoted globally by the German Speleological Society, highlights a specific cave species each year, showcasing its ecological significance and the need to protect its unique habitat. In 2019, the campaign in Italy began by featuring the common cave fly, *Limonia nubeculosa*, followed by the millipede *Plectogona sanfilippoi* in 2020 and by the cave beetle Italodytes stammeri in 2021. The following year, the bat *Miniopterus schreibersii* was highlighted to stress the importance of preserving cave ecosystems. In 2023, the genus *Niphargus*, small crustaceans, underscored the importance of subterranean aquatic biodiversity.

For 2024, the campaign focuses on Speleomantes cave salamanders. These amphibians face significant threats, such as chytridiomycosis, a harmful fungal disease, necessitating careful conservation effort.

In 2025 the UIS Biology commission propose *Lepidoptera* – butterflies and moths, and choosing the cave moth *Triphosa dubitata*, the SSI wants to underline that there is still a huge need in the research of underground ecosystems. The initiative's development includes brochures, workshops, school lessons, and collaborations with tourist caves to educate the public and promote biodiversity monitoring.

## Résumé

**Animal des Cavernes de l'Année en Italie: une campagne de communication-action pour la connaissance et la protection de la biodiversité souterraine.** Lancée en 2018 par la Société Spéléologique Italienne SSI ETS, cette campagne vise à sensibiliser le public à la biologie souterraine. L'initiative, promue au niveau mondial par la Société Allemande de Spéléologie, met chaque année en lumière un animal des grottes, pour soligner l'importance et la nécessité de protéger son habitat. En 2019, la campagne en Italie a commencé par mettre en avant la mouche des cavernes commune, Limonia nubeculosa, suivie du mille-pattes Plectogona sanfilippoi en 2020 et du scarabée des cavernes Italodytes stammeri en 2021. L'année suivante, la chauve-souris Miniopterus schreibersii a été mise en avant pour souligner l›importance de la préservation des écosystèmes. En 2023 le crustacés du genre Niphargus, a souligné l'importance de la biodiversité aquatique souterraine. Pour 2024, la campagne se concentre sur les salamandre des grottes Spéléomantes. En 2025, la commission de biologie de l'UIS propose les lépidoptères et la SSi sélectionne Triphosa dubitata. Le développement de l'initiative comprend des brochures, des ateliers, des cours scolaires et des collaborations avec des grottes touristiques pour éduquer le public et promouvoir la surveillance de la biodiversité.

## 1. Introduction

Cave Animal of the Year is an initiative born in Germany in 2008 with the aim of increasing and spreading knowledge and awareness of the faunal peculiarities of the underground environment, generally little known both by the authorities responsible for environmental protection and by the general public.

For this reason, the German Speleological Society (Verband der Deutschen Höhlen VdHK), during its annual convention, decided that starting from 2009 every year a "Cave Animal of the Year" would be designated and a series of initiatives and activities aimed at the knowledge and protection of the designated animal would be introduced.

In 2014, the Cave Animal of the Year initiative was awarded the France HABE award by the Department of Karst and Cave Protection of the International Union of Speleology (UIS).

The Italian Speleological Society, with its adhesion to this campaign, in collaboration with VdHK and under the aegis of the European Federation of Speleology (FSE), intends to raise awareness of the importance of underground ecosystems and the animals that live there, emphasizing the need to carry out research and conservation activities in this field of extreme scientific and environmental interest. Among the actions, a website has been created and maintained, put online since November 2018, which is part of the dissemination campaign which also includes dissemination by means of a poster and flyers, distributed to Italian tourist caves (on the site you can find downloadable versions and a press review as part of the communication kit).

The authors of this article are part of the promotion team of the Cave Animal of the Year campaign. The working group on cave animals has grown progressively and the following participate: Enrico Lana, Valentina Balestra, Fabio Stoch, Paolo Agnelli, Alessandra Tommasini, Salvatore

Inguscio, Danilo Russo, Enrico Lunghi, Fabio Mosconi and Gaia Salvi who have contributed with texts and photographs for the creation of various contents. Also the Italian association of touristic caves AGTI and other specific partners have enriched each year the campaign.

## 2. Materials and methods

**Materials**

Biological Specimens: The campaign involves studying various cave species, such as *Limonia nubeculosa* (2019), *Plectogona sanfilippoi* (2020), *Italodytes stammeri* (2021), and others. These organisms were selected based on their ecological importance and representativeness of cave ecosystems.

Promotional Materials: Posters (Fig. 1), brochures, flyers, and interactive educational tools, including puzzles and games, were developed to raise public awareness.

Digital Resources: Websites and social media platforms served as hubs for campaign dissemination, offering downloadable materials and information.

Monitoring Equipment: Tools for observing and documenting cave fauna, including cameras for photography and environmental sensors for measuring temperature and humidity.

**Methods**

Species Selection: Each year, a cave-dwelling species was selected based on its ecological role, vulnerability, and potential for raising public interest.

Public Engagement:

Distribution of educational materials to tourist caves and schools.

Organization of webinars, workshops, and public events.

Development of interactive games to engage diverse audiences.

Field Research: Regular inspections of caves were conducted to observe and monitor selected species. Techniques included:

Non-invasive observation of fauna.

Use of bait traps to attract specific species for study.

Recording environmental conditions in caves.

Collaborative Efforts: Involvement of speleologists, biologists, local authorities, and environmental organizations to coordinate research and public education.

Awareness Campaigns:

Integration with events like International Biodiversity Day.

Creation of multimedia content, including videos and livestreams, to extend outreach.

***Figure 1:*** *First Year Italy CAOY Poster.*

## 3. Results

The "Cave Animal of the Year" (CAOY) campaign in Italy has achieved remarkable results in its mission to raise awareness about subterranean biodiversity, promote conservation, and foster public engagement through education, research, and collaboration. Below is a detailed summary of the outcomes across various dimensions of the initiative with more than 59 events related to the campaign:

### 3.1. Public Engagement and Awareness

**Educational Reach**

School Programs: The campaign engaged thousands of students through remote and in-person lessons, workshops, and educational games tailored to different age groups. These activities not only increased awareness but also inspired curiosity and commitment to environmental conservation among younger audiences.

Tourist Cave Guides: Training programs for tourist cave guides enhanced their ability to communicate the importance of cave ecosystems, further extending the campaign's reach to visitors.

**Campaign Visibility**

Promotional Materials: Posters, flyers, and brochures distributed to tourist caves, schools, and local organizations effectively spread key messages. Materials were made available both physically and online (Fig. 2) for greater accessibility.

Interactive Tools: Engaging resources, including puzzles, crosswords, and online games, allowed audiences to learn about cave biodiversity in an enjoyable and memorable way.

Social Media and Digital Outreach: Extensive use of social media platforms amplified the campaign's message, reaching national and international audiences and creating a sustained digital presence.

**Event Participation**

The campaigns were integrated with significant environmental events, such as International Biodiversity Day, which served as focal points for public engagement. Live webinars, virtual workshops, and interactive livestreams during these events drew broad participation from diverse audiences.

### 3.2. Conservation and Research Outcomes

**Monitoring of Cave Ecosystems**

For the 2021 campaign regular field studies were conducted to observe and document the biodiversity of Castellana Grotte Caves. These included:

Monthly inspections of tourist and non-tourist sections.

Non-invasive observation and photographic documentation of cave fauna, including key species like *Italodytes stammeri*.

Recording environmental parameters, such as temperature and humidity, to assess habitat conditions.

**Biodiversity Insights**

- Monitoring efforts provided new insights into the resilience of cave ecosystems, even in tourist-heavy areas, and contributed to a deeper understanding of the ecological roles of selected species.
- The research highlighted specific threats to cave fauna, such as chytridiomycosis in *Speleomantes salamanders*, emphasizing the need for preventive measures like equipment disinfection and careful handling of species.

## 3.3. Collaborative and Organizational Achievements

**Partnership Building**

- The campaign established strong collaborations with local administrations, tourist cave associations (AGTI), research institutions, and international organizations like the UIS and FSE. These partnerships increased resources, expertise, and reach, allowing for comprehensive campaign execution.
- Speleologists, biologists, and conservationists worked together on multi-year initiatives, such as long-term monitoring of *Italodytes stammeri* populations, fostering a model of sustainable and cooperative research.

**Integration with Global Initiatives**

- The campaign aligned with international efforts, such as the International Year of Caves and Karst (2021-2022), amplifying the visibility of Italian contributions to subterranean conservation and in 2025 follow the decision of the UIS Biology Commission to launch an International Cave Animal all 3 years within the frame of the International Congress of Speleology (ICS).

**Recognition**

- By tying annual campaigns to global events like World Biodiversity Day, the CAOY initiative gained recognition from environmental organizations and the public, reinforcing its credibility and impact.

## 3.4. Behavioral and Societal Impacts

**Increased Awareness**

- The campaign successfully shifted public perception, emphasizing the importance of caves as critical ecosystems rather than mere geological formations.
- Visitors to tourist caves became more aware of their ecological footprint and the need to adopt responsible behaviors during cave explorations.

**Improved Practices**

- Enhanced awareness among cavers led to better adherence to hygiene protocols, such as equipment disinfection, reducing the risk of spreading diseases like chytridiomycosis.

**Sustainable Tourism**

- - Tourist cave operators adopted improved practices to balance ecological preservation with visitor access, ensuring long-term sustainability of these unique environments.

## 3.5. Broader Environmental Impacts

**Policy Influence**

- Data generated through the campaign support calls for stronger protections for cave ecosystems and their inhabitants.
- The initiative fostered greater collaboration between conservationists and policymakers, promoting sustainable management of karst regions.

**Public Engagement Beyond Caves**

- By connecting cave conservation to broader environmental themes, such as water resource protection and biodiversity loss, the campaign successfully linked its objectives to global ecological challenges, inspiring wider environmental action.

La Società Speleologica Italiana partecipa anche quest'anno con questa campagna alla **Giornata Mondiale per la Biodiversità 2023** con una serie di eventi ed azioni sul web, in città, grotte e parchi naturali italiani. IDB 2023 in Italy | Convention on Biological Diversity (cbd.int).

***Figure 2:*** *Some communication action between 2020 and 2023.*

# 4. Discussion

The "Cave Animal of the Year" (CAOY) campaign in Italy has proven to be an exemplary model for integrating public education, scientific research, and collaborative action to promote subterranean biodiversity conservation. This discussion highlights the strengths, challenges, and broader implications of the campaign, referencing specific outcomes and activities to provide a nuanced analysis.

## 4.1. Raising Awareness and Public Engagement

The CAOY campaign's primary goal has been to foster public understanding of the ecological importance of caves and their inhabitants. By engaging diverse audiences, from schoolchildren to speleologists, the initiative has successfully bridged the gap between scientific knowledge and public awareness.

**Educational Impact**

Educational efforts, such as school programs and workshops, have been central to the campaign's success. The creation of interactive games and educational materials (Fig. 3) (e.g., puzzles, crosswords, and quizzes) proved effective in engaging younger audiences, as evidenced by the widespread participation in these activities. These tools not only raised awareness but also cultivated a long-term interest in biospeleology and conservation.

**Social Media and Digital Outreach**

Social media platforms played a pivotal role in extending the campaign's reach. Livestreams, webinars, and video releases were particularly impactful during COVID-19 restrictions, ensuring continuity in public engagement. For example, the webinars on *Niphargus spp*. in 2023 and Speleomantes salamanders in 2024 highlighted specific conservation challenges while reaching a broader audience than traditional events.

## 4.2. Advancing Scientific Research and Conservation

**Species-Specific Contributions**

Each annual campaign focused on a selected species, yielding valuable insights into its ecology, behavior, and conservation needs. For instance:

*Limonia nubeculosa* (2019) underscored the ecological links between surface and subterranean ecosystems.

*Plectogona sanfilippoi*, (2020), is a well-depigmented, almost transparent animal, with eyes that are no longer functional reduced to small triangular areas on the sides of the head. The general appearance and elongation of the antennae and legs are characteristic of its specialization in underground life.

*Italodytes stammeri* (2021) provided an opportunity to develop a long-term monitoring program, revealing that diverse troglobian fauna persist even in tourist-accessible caves.

*Miniopterus schreibersii* (2022) Due to the COVID-19 pandemic, the International Year of Caves and Karst, initially scheduled for 2021, has been extended until 2022. This initiative aimed to increase awareness and understanding of karst landscapes and to promote their protection from an ecosystem point of view. One of the communication actions is focused on the promotion of a "Cave Animal of the Year" to stimulate a shared responsibility in the conservation of karst ecosystems. The campaign highlighted bats considered symbolic mammals of speleology.

*Niphargus spp.* (2023) Water is life and for the Italian Speleological Society and Italian speleologists, knowing and protecting the water in our caves means understanding and defending the life it contains, focusing on the genus of crustaceans Niphargus as "Cave Animal of the Year". This genus is widely spread with over 425 species in Europe, from the Iberian Peninsula to Great Britain in the north and reaches the mountain massifs of Iran to the east. In Italy there are over a hundred known species or in the process of being described and our peninsula is, together with the Balkan area, one of the centers of greatest biodiversity for this genus.

***Speleomantes salamanders*** (2024) highlighted the threat of chytridiomycosis, emphasizing the importance of preventive measures like equipment disinfection.

***Triphosa dubitata***, (2025) there is still a huge need for action, especially in the research of underground ecosystems and the species that inhabit them. The cave is not only a shelter, but a vital element that helps us better understand the biodiversity and ecological dynamics that allow the survival of many forms of life.

**Monitoring Efforts**

The campaign's commitment to regular field studies, including non-invasive observation and environmental data collection, has established a robust foundation for ongoing conservation. For example, monthly inspections in the Castellana Caves revealed rich populations of troglobian species despite decades of tourism, demonstrating the resilience of some cave ecosystems

## 4.3. Collaborative Achievements

**Multistakeholder Approach**

The campaign's success can be attributed to its collaborative framework, involving the Italian Speleological Society (SSI ETS), tourist cave associations, local administrations, and international bodies like the UIS. This network facilitated knowledge exchange, resource sharing, and coordinated action.

The partnership with AGTI ensured effective distribution of educational materials and direct engagement with cave visitors.

The collaboration with academic experts, and specific stakeholders, added scientific rigor to the campaign's outreach and research efforts.

**Integration with Global Initiatives**

Aligning the CAOY campaign with global events, such as the International Year of Caves and Karst (IYCK) and World Biodiversity Day, amplified its impact. These synergies not only boosted visibility but also positioned Italian efforts within a broader international context, enhancing the campaign's credibility and reach.

## 4.4. Challenges and Areas for Improvement

Despite its successes, the campaign faced several challenges:

Balancing Tourism and Conservation: While tourist caves were vital for outreach, managing the ecological impact of increased human activity remains a concern. The need for stricter visitor management protocols is evident from studies showing how human presence can disrupt cave ecosystems.

Limited Resources: The reliance on volunteer efforts and partnerships underscores the campaign's vulnerability to resource constraints. Expanding funding and institutional support would enhance its sustainability.

Public Engagement Beyond Events: While annual campaigns effectively raise awareness, maintaining consistent public interest between events remains a challenge. More year-round initiatives could help sustain momentum.

## 4.5. Broader Implications and Future Directions

The CAOY campaign highlights the potential of combining research, education, and public engagement to address conservation challenges. Its emphasis on caves as ecosystems, rather than mere geological formations, has reshaped public perception and policy priorities in Italy.

**Policy Influence**

The campaign's data-driven approach supports calls for stronger legal protections for cave ecosystems. For example, highlighting the endemic and vulnerable nature of species like Niphargus spp. and Speleomantes salamanders has underscored the need for sustainable karst management.

**Scaling the Model**

The Italian experience offers a replicable model for other regions with significant subterranean biodiversity. Expanding international collaborations, particularly within the UIS network, could further enhance the campaign's impact.

***Figure 3:*** *Biospeleo Cards Photo @Luisa Dainelli .*

## 5. Conclusion

The CAOY campaign in Italy represents a groundbreaking effort in cave conservation, education, and public engagement. Its achievements demonstrate the power of combining scientific research with innovative communication strategies to foster lasting environmental awareness and action. The initiative serves as a model for similar campaigns globally, proving that a multi-stakeholder approach can effectively protect delicate ecosystems while inspiring a deeper appreciation for the natural world.

Strategic elements of the Cave Animal of the Year Campaign in Italy:

Emphasis on **protecting cave biodiversity through public education and scientific research**.

Collaboration with **various organizations** to promote sustainable management of cave ecosystems.

Workshops, webinars, and interactive activities to engage the public and educate about cave biodiversity.

Special events on International Biodiversity Day to raise awareness of the winning twin **cave and biodiversity**.

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Interactive games :
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https://puzzel.org/it/matching-pairs/play?p=-M_oXh3Z2nuJsKqMpGEQ
https://puzzel.org/it/quiz/play?p=-M_X-effH6DH_H3yZQjn

Find out where he lives in Puglia: Puzzle
https://puzzel.org/it/slidingpuzzle/play?p=-MWY145N9CY9KQVVbdwg

See what he does in the cave: Insect Description and Puzzle
https://puzzel.org/it/jigsaw/play?p=-MXrzhzc43Q6sZvK7jKY

Get to know Biospeleogia: Presentation and Crossword
https://puzzel.org/it/crossword/play?p=-MXs1qAp9XTHl14u4suX

# Mitochondrial genomes and phylogenetic relationships of six cave cricket species of *Endecous* Saussure, 1878 (Orthoptera: Phalangopsidae)

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## Abstract

Crickets of the genus *Endecous*, comprising 27 species distributed across five subgenera, are frequently found in caves, with some species being exclusive to subterranean environments and exhibiting troglomorphic adaptations. However, molecular data for this genus remain scarce. In this study, we sequenced and characterized six new mitochondrial genomes of *Endecous*, representing three subgenera. The mitogenomes showed similar genome sizes were recorded for *E. (Pedroecous) didymus* (16,458 bp), *E. (Notendecous) potiguar* (16,737 bp), *E. (P.) troglobius* (16,261 bp), and *E. (Endecous) ubajarensis* (16,401 bp). In contrast, *E. (E.) painensis* (15,851 bp) and *E. (E.) peruassuensis* (17,627 bp) exhibited the smallest and largest mitogenomes reported to date for the family, respectively. Phylogenetic analysis, based on the available mitogenomes of Phalangopsidae, supported the monophyly of the genus *Endecous*, but the monophyly of the subgenera *E. (Endecous)* and *E. (Notendecous)* was not recovered. *Endecous* is one of the most representative cricket groups in Brazilian caves, and investigating their mitogenomes can provide valuable insights into their biology while contributing to future conservation efforts, particularly for troglobitic species.

## Resumo

Grilos do gênero *Endecous*, com 27 espécies organizadas em cinco subgêneros, são frequentemente encontrados em cavidades, e algumas espécies são exclusivas dos ambientes subterrâneos, apresentando adaptações troglomórficas. Entretanto, dados moleculares para o gênero são escassos. No presente trabalho, foram sequenciados e caracterizados seis novos genomas mitocondriais de *Endecous* com representantes de três subgêneros. Os mitogenomas apresentaram tamanhos semelhantes das sequências entre as espécies *E. (Pedroecous) didymus* (16.458 pb), *E. (Notendecous) potiguar* (16.737 pb), *E. (P.) troglobius* (16.261 pb) e *E. (Endecous) ubajarensis* (16.401 pb). Por outro lado, *E. (E.) painensis* (15.851 pb) e *E. (E.) peruassuensis* (17.627 pb) apresentaram, o menor e o maior mitogenoma descrito até o momento para a família, respectivamente. Na análise filogenética no contexto dos mitogenomas disponíveis para Phalangopsidae, o monofiletismo do gênero foi suportado; no entanto, os subgêneros *E. (Endecous)* e *E. (Notendecous)* não foram recuperados como monofiléticos. *Endecous* é um dos grupos de grilos mais representativos em cavidades brasileiras e estudar os mitogenomas pode fornecer novas informações sobre a biologia das espécies e contribuir para estudos futuros de conservação, especialmente de espécies troglóbias.

## 1. Introduction

Crickets of the family Phalangopsidae BLANCHARD (1845) (Orthoptera, Grylloidea) exhibit a spider-like appearance, characterized by elongated appendages and a brown-toned coloration pattern. They are distributed across tropical and subtropical regions worldwide (DESUTTER-GRANDCOLAS, 1995; CIGLIANO et al., 2025). These species occupy various forest strata, with some occurring sporadically or permanently in caves (DESUTTER-GRANDCOLAS, 1995). In Brazil, approximately 40 cave-dwelling cricket species have been identified to date, all belonging to the Phalangopsidae family (BOLFARINI et al., 2022), with *Zebragryllus aphonus* TAVARES et al. (2024), being the only exception, as it belongs to the Gryllidae family (TAVARES et al., 2024).

The genus *Endecous* SAUSSURE (1878) is endemic to South America, with most described species occurring in Brazil across various regions and biomes, except in the northern region. It is also found in Argentina, Bolivia, and Uruguay (BOLFARINI et al., 2022; CIGLIANO et al., 2025). Among Neotropical phalangopsids, *Endecous* is the most representative genus in subterranean environments and the surroundings of caves. Additionally, it includes the highest number of troglomorphic species restricted to caves in Brazil (e.g., *E. vitreus, E. infernalis*, and *E. troglobius*). Currently, the genus comprises 27 species grouped into five subgenera: *E. (Endecous), E. (Pedroecous), E. (Notendecous)* (GOROCHOV, 2014), and the recently described *E. (Bodoquenaecous)* and *E. (Ramalhoecous)* (CARVALHO et al., 2023a, b). Variations in male genital morphology are used to differentiate subgenera and describe species (GOROCHOV, 2014;

CASTRO-SOUZA et al., 2017; CARVALHO et al., 2023a, b).

These characteristics reinforces the potential for biological studies on *Endecous* species. Although the genus is among the best-known cave crickets in the Neotropics, particularly in terms of taxonomic studies (GOROCHOV, 2014; CASTRO-SOUZA et al., 2017, 2020), troglomorphic adaptations (CAMPOS et al., 2023; CARVALHO et al., 2023), bioacoustics, and cytogenetics (CASTRO-SOUZA et al., 2020; TIMM et al., 2021), however, molecular studies remain scarce, with few exception (COSTA 2022; SILVA et al., 2023). There is an increasing need to integrate taxonomic information with phylogenetic studies to assess species relationships and troglomorphic traits, aiding in the classification of troglobitic species, as suggested by DESUTTER-GRANDCOLAS (1997). Mitochondrial markers have been widely used in molecular studies of animals (GALTIER et al., 2009). However, for Endecous, complete mitogenomes have been published for only two species to date: *E. (E.) chape* and *E. (N.) onthophaphus* (SILVA et al., 2023).

Given the scarcity of molecular data and mitochondrial genome sequences, this study aims to sequence and annotate the mitogenomes of six Endecous species from three subgenera: *E. (P.) didymus, E. (P.) troglobius, E. (E.) painensis, E. (E.) peruassuensis, E. (E.) ubajarensis*, and *E. (N.) potiguar*. Additionally, it seeks to characterize their mitogenomes and investigate phylogenetic relationships within the context of the available data for Phalangopsidae.

## 2. Materials and Methods

**Sampling and DNA extraction**

Endecous individuals were collected from caves across different regions of Brazil (Fig. 1). Six species representing three subgenera were sampled: *E. (P.) didymus* (ITV09621), *E. (P.) troglobius* (ITV09606), *E. (E.) painensis* (ITV02066), *E. (E.) peruassuensis* (ITV02060), *E. (E.) ubajarensis* (ITV39487), and *E. (N.) potiguar* (ITV35228). *Endecous (P.) didymus* and *E. (P.) troglobius* were collected from the same cave (Fig. 1). All specimens were deposited in the Subterranean Invertebrate Collection of Lavras (ISLA/UFLA). Genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol for insects. The extracted DNA was stored at -20 °C for further analyses.

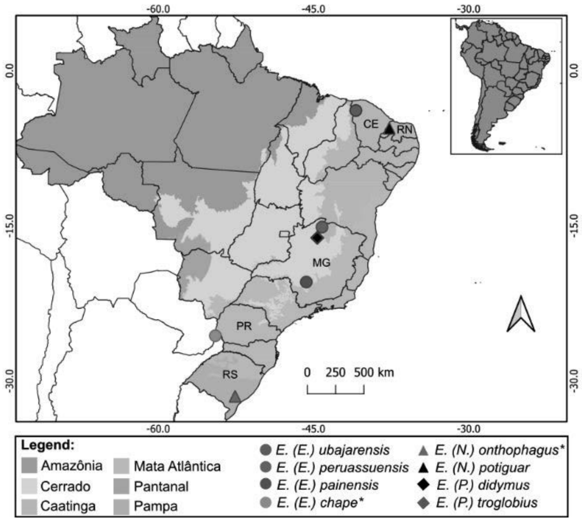


***Figure 1:*** *Map showing the locations of the caves where eight Endecous species were collected. Two of these species are based on data available in databases (indicated with *).*

**Sequencing, Assembly, and Annotation of Mitogenomes**

Low-coverage sequencing was performed using the NextSeq 2000 P1 kit (Illumina) with 300 cycles (2 × 150 bp) on the Illumina NextSeq 2000 platform. Genome assembly was conducted with NovoPlasty 4.3 (DIERCKXSENS et al., 2017), utilizing paired-end Illumina reads and the cox1 gene as the seed. Annotation was performed using MITOS 2 (BERNT et al., 2013). Nucleotide composition was calculated for each mitogenome using MEGA11 (TAMURA et al., 2021), while strand asymmetry was determined using the formulas AT-skew = (A% − T%)/(A% + T%) and GC-skew = (G% − C%)/(G% + C%) (PERNA & KOCHER, 1995).

**Phylogenetic Analysis**

To reconstruct phylogenetic relationships, available mitogenomes for Phalangopsidae were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/): Subfamily Cacoplistinae: *Cacoplistes rogenhoferi* (GenBank: NC039664) and *Meloimorpha japonica* (NC039665); Subfamily Phaloriinae: *Vescelia pieli* (NC087743); Subfamily Phalangopsinae: *E. (E.) chape* (OQ935836) and *E. (N.) onthophagus* (OQ935837). Outgroup taxa included representatives from the families Gryllidae and Oecanthidae: *Gryllus bimaculatus* (MT993975) and *G. lineaticeps* (NC057052) from Gryllidae, and *Oecanthus sinensis* (NC034799) from Oecanthidae.

The phylogenetic tree was inferred using nucleotide sequences of the 13 protein-coding genes (PCGs), and each gene was aligned separately with MAFFT v7.4 (KATOH & STANDLEY, 2013) using the *Auto* alignment algorithm. The alignments were then concatenated in Geneious Prime 2022.0.2 (KEARSE et al., 2012). Maximum likelihood (ML) analysis was performed in RAxML v8.2 (STAMATAKIS, 2014) using the GTR+GAMMA model with 1,000 rapid bootstrap replicates.

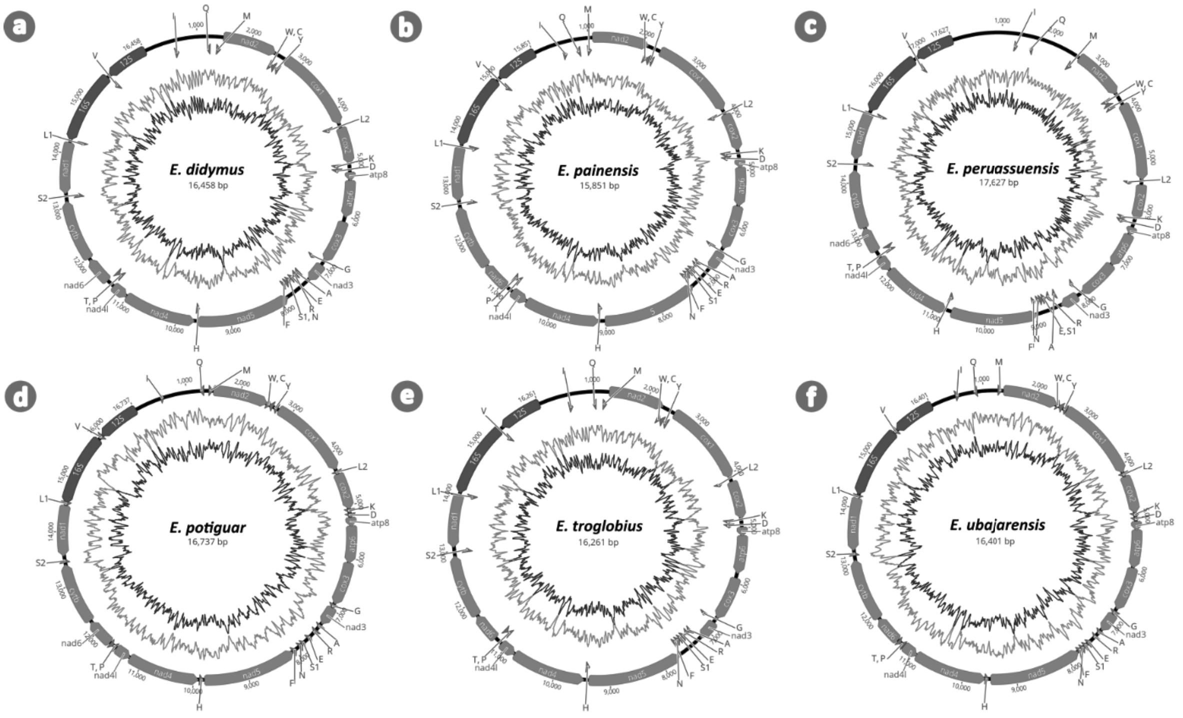


***Figure 2:*** *Circular maps of the complete mitochondrial genomes of six Endecous species. Annotated genes are arranged in a counterclockwise direction, starting with the two rRNA (rrnS [12S] and rrnL [16S]) shown in blue, the 13 PCGs in green (nad1, cytb, nad6, nad4l, nad4, nad5, nad3, cox3, atp6, atp8, cox2, cox1, and nad2). Additionally, the 22 tRNA in pink, labeled with their corresponding IUPAC amino acid codes (trnV, trnL1, trnS2, trnT, trnP, trnH, trnF, trnN, trnE, trnS1, trnR, trnA, trnG, trnD, trnK, trnL2, trnY, trnW, trnC, trnM, trnQ, and trnI).*

## 3. Results

**Characterization of Mitogenomes**

Among the complete sequences generated in this study, *E. (E.) painensis* and *E. (E.) peruassuensis* exhibited the smallest and largest mitogenomes, measuring 15,851 bp and 17,627 bp, respectively. In comparison, the other species had genome sizes exceeding 16,000 bp (Figs. 2–3). This variation is primarily attributed to intergenic regions and, in particular, the size of the control region, as observed in *E. (E.) peruassuensis* (Fig. 2c).

All mitogenomes generated in this study have a circular structure and a similar genetic composition, containing 37 genes. These include 13 protein-coding genes (PCGs), consisting of two ATPase subunits (*atp6* and *atp8*), one cytochrome b gene (*cytb*), three cytochrome c oxidase subunits (*cox1–3*), and seven NADH dehydrogenase subunits (*nad1–6* and *nad4l*). Additionally, there are two ribosomal RNA (rRNA) genes, *rrnS* (12S) and *rrnL* (16S), along with 22 transfer RNA (tRNA) genes *(trnA, trnR, trnN, trnD, trnC, trnQ, trnE, trnG, trnH, trnI, trnL1, trnL2, trnK, trnM, trnF, trnP, trnS1, trnS2, trnT, trnW, trnY,* and *trnV*) (Fig. 2).

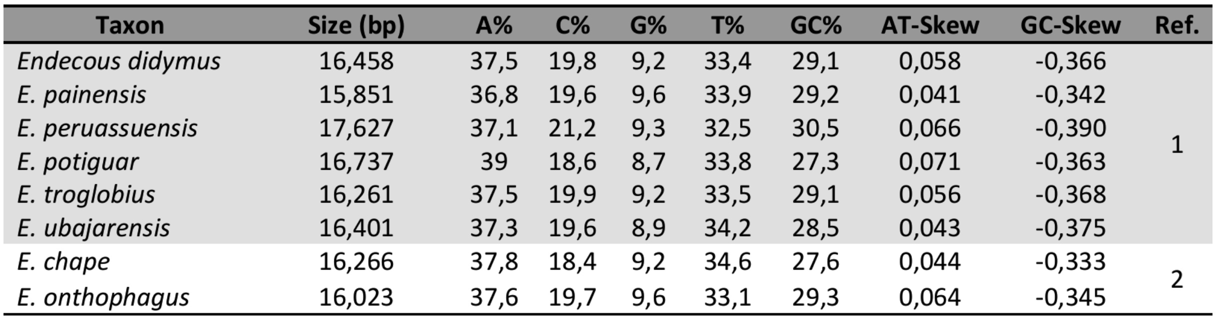
Gene organization was consistent across all six analyzed species, with 20 genes (nine PCGs and 11 tRNAs) encoded on the heavy strand (H-strand) and 17 genes (four PCGs, two rRNAs, and 11 tRNAs) on the light strand (L-strand) (Fig. 2). All 13 PCGs used ATN (N = A/C/T/G) as the start codon, except for *E. (E.) ubajarensis* and *E. (N.) potiguar*, which had an ACG start codon in cox1, and *E. (E.) peruassuensis*, which had CGA. Additionally, nad1 in *E. (N.) potiguar* started with TTG. Conversely, the stop codon was predominantly TAA, with variations in nad4 and nad5 in *E. (E.) painensis* and *E. (E.) peruassuensis*, and in cox3 in *E. (E.) ubajarensis*, where the stop codon was TAG. Incomplete stop codons (T) were found in nad5 for *E. (P.) didymus* and *E. (P.) troglobius*, and in cox2 for all species.

The GC content (%) was similar among Endecous species, ranging from 27,3% (E. (N.) potiguar) to 30,5% (*E. (E.) peruassuensis*) (Fig. 3). Regarding strand asymmetry, AT-skew values were positive, indicating an excess of adenine (A) relative to thymine (T) in the sequences, while negative GC-skew values suggested a higher abundance of cytosine (C) over guanine (G) (Fig. 3).

**Phylogenetic Analysis**

The topology of the maximum likelihood (ML) phylogenetic tree showed strong support for most clades (bootstrap support [BS] = 100), with Phalangopsidae forming a well-supported clade comprising representatives of the subfamilies Phalangopsinae, Phaloriinae, and Cacoplistinae (Fig. 4). The eight *Endecous* species clustered into a monophyletic group with maximum support, positioned as the sister group to *V. pieli* (Phaloriinae) (BS = 100), followed by representatives of Cacoplistinae (Fig. 4).

Two internal nodes within *Endecous* exhibited low support values (BS < 50), particularly in groupings that included representatives of *E. (Endecous)* and *E. (Notendecous)*, indicating that these subgenera were not recovered as monophyletic, except for the clade formed by the species *E. (P.) didymus* and *E. (P.) troglobius* (BS = 100; Fig. 4).



| Taxon | Size (bp) | A% | C% | G% | T% | GC% | AT-Skew | GC-Skew | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| *Endecous didymus* | 16,458 | 37,5 | 19,8 | 9,2 | 33,4 | 29,1 | 0,058 | -0,366 | 1 |
| *E. painensis* | 15,851 | 36,8 | 19,6 | 9,6 | 33,9 | 29,2 | 0,041 | -0,342 | |
| *E. peruassuensis* | 17,627 | 37,1 | 21,2 | 9,3 | 32,5 | 30,5 | 0,066 | -0,390 | |
| *E. potiguar* | 16,737 | 39 | 18,6 | 8,7 | 33,8 | 27,3 | 0,071 | -0,363 | |
| *E. troglobius* | 16,261 | 37,5 | 19,9 | 9,2 | 33,5 | 29,1 | 0,056 | -0,368 | |
| *E. ubajarensis* | 16,401 | 37,3 | 19,6 | 8,9 | 34,2 | 28,5 | 0,043 | -0,375 | |
| *E. chape* | 16,266 | 37,8 | 18,4 | 9,2 | 34,6 | 27,6 | 0,044 | -0,333 | 2 |
| *E. onthophagus* | 16,023 | 37,6 | 19,7 | 9,6 | 33,1 | 29,3 | 0,064 | -0,345 | |

***Figure 3:*** *Mitochondrial genome sizes and nucleotide composition of Endecous species, with highlighted species from the present study. Ref. 1. Present study, and 2. Silva et al. (2024).*

## 4. Discussion

**Mitogenomes and Characterization**

The mitogenomes generated in this study exhibit a structure consistent with those of other Orthoptera species (MA & LI, 2018; GAUGEL et al., 2023). Compared to previously available mitogenomes for Phalangopsidae, *M. japonica* (15,880 bp) and *E. (E.) chape* (16,266 bp) (MA & LI, 2018; SILVA et al., 2023) represented the smallest and largest genomes, respectively. However, with the newly sequenced data, the mitogenome size range for the family now extends from 15,851 bp in *E. (E.) painensis* to 17,627 bp in *E. (E.) peruassuensis*. This variation is primarily attributed to differences in intergenic regions, particularly the non-coding control region, which is rich in AT bases.

In Orthoptera, all 13 protein-coding genes (PCGs) typically initiate with ATN start codons, although exceptions have been observed in *cox1* and *nad1*, as seen in *C. rogenhoferi*, *M. japonica*, *Ornebius fuscicercis*, and *Polionemobius taprobanensis*, as well as in other Grylloidea species (MA & LI, 2018; MA et al., 2019; SILVA et al., 2023). Similarly, alternative stop codons, such as TAG, have been reported in different genes of *E. (N.) onthophagus* and certain representatives of the Trigonidiidae family (MA et al., 2019; SILVA et al., 2023). In *Endecous*, incomplete stop codons have been described in *nad4-5* and *cox2* (SILVA et al., 2023). These incomplete stop codons are typically followed by tRNA genes and are presumably completed (TAA) through post-transcriptional polyadenylation after tRNA cleavage (OJALA et al., 1981; MA et al., 2019).

The GC content of the six newly sequenced mitogenomes falls within the expected range for Phalangopsidae. Negative GC-skew and positive AT-skew values, which quantify sequence asymmetries, indicate similar patterns across different subfamilies, including *Endecous* (*Phalangopsinae*), *C. rogenhoferi* and *M. japonica* (*Cacoplistinae*), and *V. pieli* (*Phaloriinae*) (MA & LI, 2018; SILVA et al., 2023). However, nucleotide composition can vary across groups, such as Trigonidiidae, exhibiting distinct asymmetry patterns among subfamilies (MA et al., 2019).

**Phylogenetic Reconstruction**

The phylogenetic analysis recovered previously observed relationships within Phalangopsidae, with the *Endecous* mitogenomes (Phalangopsinae) forming a well-supported monophyletic group closely related to *V. pieli* (Phaloriinae). Meanwhile, species from Cacoplistinae (*C. rogenhoferi* and *M. japonica*) represented the earliest diverging lineage within the family, a relationship consistent with expected subfamily classifications (CIGLIANO et al., 2025).

*Endecous* is one of only two cricket genera in Brazil with known troglobitic species (MERLO et al., 2022; BOLFARINI et al., 2022; CAMPOS et al., 2023; CARVALHO et al., 2023a). Geographically isolated cave refuges may contribute to speciation through troglomorphic adaptations (CASTRO-SOUZA et al., 2020; CAMPOS et al., 2023). In the maximum likelihood (ML) tree topology, *E. (P.) didymus* and *E. (P.) troglobius* were recovered as sister species. Both are sympatric and occur in Lapa Sem Fim cave, the largest cave in the state of Minas Gerais, Brazil, with *E. (P.) troglobius* being a strictly troglobitic species confined to the aphotic zone of the cave (CASTRO-SOUZA et al., 2020). However, genetic distance analysis revealed minimal separation between the two species (Fig. 4), with mitochondrial gene variations of less than 2%, a threshold generally associated with intraspecific variation (AVISE, 2000).

In some groups, such as *Oecanthus* (*Oecanthidae*) and *Gryllus* (*Gryllidae*) (SILVA et al., 2022; 2023), certain species exhibit very close genetic relationships. For instance, *Gryllus pennsylvanicus* and *G. firmus* show clear morphological differences and occur in sympatric populations. In hybrid zones, individuals with *G. firmus* nuclear ancestry frequently possess *G. pennsylvanicus* mtDNA, suggesting unidirectional introgression (BYERLY et al., 2023). Given the morphological and ecological characteristics of *E. (P.) didymus* and *E. (P.) troglobius*, additional genetic data are essential to refine genetic distance analyses and determine whether their low genetic divergence results from a shared organellar lineage or potential unidirectional or bidirectional hybridization. Moreover, in situ and ex situ behavioral studies, particularly those examining courtship rituals and mate attraction strategies, could provide valuable insights into the mechanisms driving the separation of these two sympatric species.

Furthermore, the phylogenetic relationships among representatives of *E. (Endecous)* and *E. (Notendecous)*, the two most diverse subgenera of the genus, with 18 and four species, respectively (CIGLIANO et al., 2025), suggest a paraphyletic infrageneric classification. Incorporating additional nuclear markers, along with sampling and sequencing the mitochondrial genomes of other *Endecous species,* will be crucial for reconstructing the phylogenetic relationships and testing the monophyly of *Endecous* subgenera with greater robustness. Additionally, integrating evolutionary analyses and mapping troglomorphic traits could provide valuable insights into the characteristics associated with troglobitic adaptations, particularly in cladistic analyses (DESUTTER-GRANDCOLAS, 1997; BOLFARINI et al., 2022).

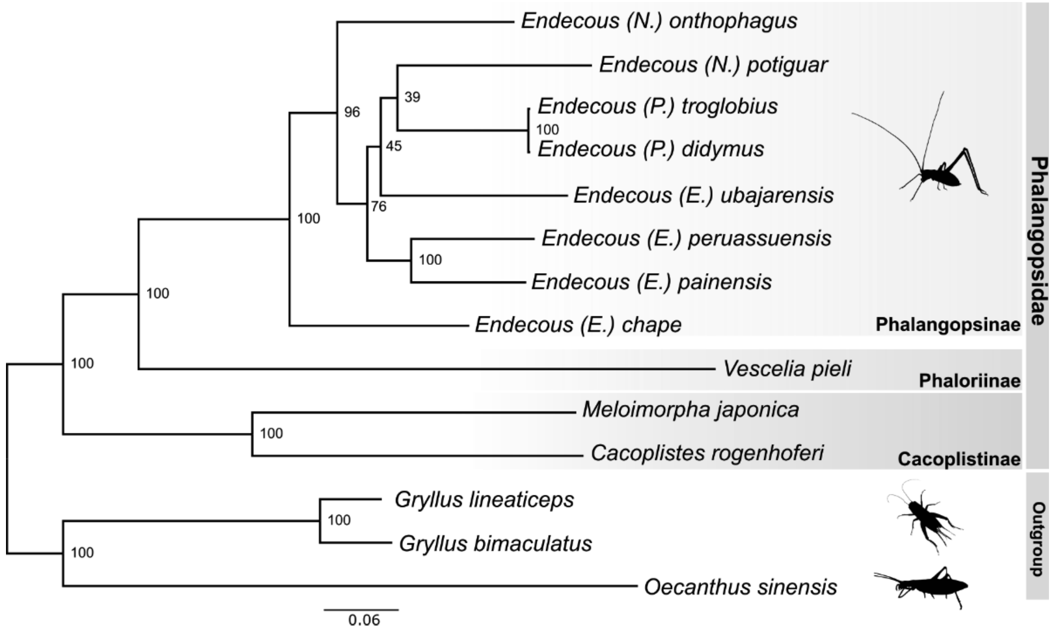


***Figure 4:*** *ML tree showing the relationships of the generated mitogenomes and those available for the family Phalangopsidae, based on nucleotide sequences of the 13 PCGs. The outgroup consists of species from the families Gryllidae and Oecanthidae. Bootstrap values are displayed at the nodes.*

## 5. Conclusion

The *Endecous* mitogenomes generated in this study exhibited structural and compositional characteristics consistent with those previously reported for Phalangopsidae. In the phylogenetic analysis, *Endecous* mitogenomes formed a monophyletic group; however, the classification of subgenera requires further investigation. Future studies should include the currently unsampled subgenera *E. (Bodoquenaecous)* and *E. (Ramalhoecous)*, as well as additional species from *E. (Endecous)*, *E. (Pedroecous)*, and *E. (Notendecous)*.

The data generated in this study provide valuable genetic insights into cave-dwelling crickets of the genus *Endecous* and will contribute to future phylogenetic and demographic research. For understudied species facing extinction threats, particularly troglobitic species, the characterization of genetic resources is crucial for conservation efforts and population diversity assessments.

## Acknowledgments

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# Influence of habitat heterogeneity and sampling scale on invertebrates cave fauna

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## Abstract

The Serra do Ramalho karst in Brazil remains poorly understood, but previous research has shown that the area is location of an endemic fauna that is threatened with extinction. This study was conducted to contribute to understanding the ecology and conservation of subterranean fauna in the region. Specifically, we aimed to evaluate the influence of habitat heterogeneity, including physical, trophic, and microclimatic traits, on species richness and composition at different sampling scales. Additionally, the study sought to determine whether the response to these variables differed between troglobitic and non-troglobitic fauna. Sampling was conducted in 26 caves across three municipalities in Serra do Ramalho, with two sampling events conducted in 2021 and 2022; however, each cave was only sampled once. Invertebrate fauna in the caves was sampled using sectors measuring 10x3m and quadrats measuring 1x1m, which were placed along the cave floor. A total of 9178 individuals were recorded, distributed in 416 morphospecies, of which 72 were considered troglobitic. Inside sectors, non-Troglobitic (n-TB) species composition was determined by temperature, moisture, trophic diversity, and availability. Whilst for the troglobitic (TB), distance from the entrance, moisture, and trophic diversity were the best predictors. The non-troglobitic species richness was better explained by temperature, moisture, and trophic availability, while for the troglobitic species richness, the moisture was the variable that explained it's variation. Regarding the quadrats, non-troglobitic and troglobitic respond to shelter diversity, trophic availability, and distance. The non-troglobitic species richness was better explained by trophic availability and for the troglobitic, none of the variable's measures explained its richness variations. The study concluded that physical, trophic, and microclimatic features are important for structuring the cave fauna in isolation or at different scales. These features also have distinct effects on non-troglobitic and troglobitic species.

## 1. Introduction

Understanding the elements that influence the invertebrate communities through space and time has been a greater paradigm of community ecology (HARMS et al., 2000; CRIST et al., 2003; STIREMAN, 2008). In this context, cave characteristics such as permanent absence of light, stability in temperature and humidity conditions, and scarcity of food resources act as filters in determining the structure of invertebrate communities, promoting a gradient of resources and conditions that vary from the entrance to the deeper regions (POULSON & WHITE, 1969; PECK, 1974; PROUS et al., 2004; NICOLOSI et al., 2021).

The external environments highly influence cave entrances, consequently sheltering species (troglophiles) with more tolerance to climatic fluctuations. In deeper areas, conditions of temperature and humidity are stable, and the availability of organic resources is low. In consequence, deeper regions shelter specialized-restricted species (troglobites) that are more sensitive to climate variations and possess adaptations to live in an oligotrophic, dark area.

To comprehend patterns of richness, composition, and species distribution patterns, distinct factors should be taken into consideration: resource availability, temperature, moisture, and different light zones (TOBIN et al., 2013; MAMMOLA et al., 2017; LUNGHI et al., 2017). These features combined with different sampling scales are essential to identify and understand the characteristics and patterns for future management and conservation of cave environments since models get more accurate at larger scales once it is possible to incorporate variables that are not present in smaller scales and combine different scales can provide a more precise understanding of invertebrate fauna and its structure (PELLEGRINI et al., 2016a).

Therefore, this study aims to investigate the responses of cave invertebrate fauna (composition and richness) to physical, trophic, and microclimatic variations, substrate heterogeneity/diversity, and substrate features within caves at multiple scales, including microscale (1 $m^2$ quadrats), mesoscale (30 $m^2$ sectors). For this purpose, (i) we hypothesized that higher substrate diversity will support higher invertebrate species richness in the sectors and quadrats; (ii) troglobitic species tend to respond differently to the sampled variables compared to non-troglobitic species due to their preference for more stable environments combined to higher levels of specialization to subterranean habitats.

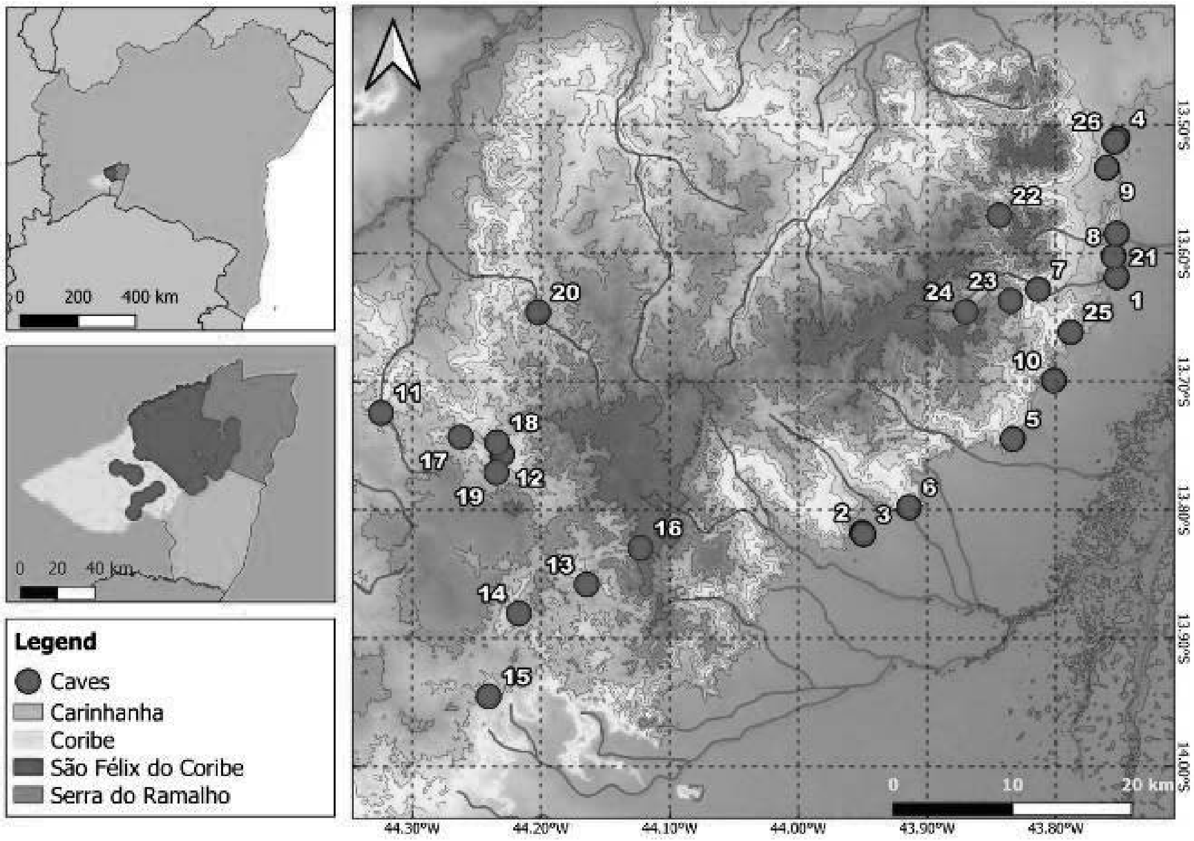

*Figure 1: Caves location in Serra do Ramalho Karstic area, Bahia state, Brazil. Municipalities of Carinhanha, Coribe, São Felix do Coribe and Serra do Ramalho.*

## 2. Method and Materials

The study was conducted in 26 caves along Serra do Ramalho karst, Bahia state, Brazil (Figure 1), and it holds a variety of limestone outcrops formed by the Bambuí Group. The region comprehends important cave systems.

To evaluate the richness and composition of invertebrate communities, 145 sectors were established in the 26 caves, with each sector (3 x 10m) comprising 3 quadrats (1 x 1m), here considered "mesoscale" and "microscale", respectively (Figure 2). All transects were placed on the cave floor at least 100m apart (when possible).

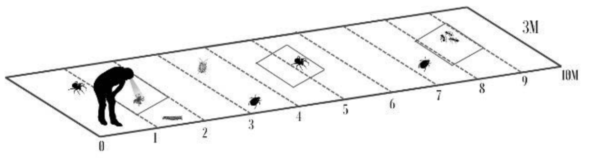

***Figure 2:*** *Illustrative representation of the sampling scales. Sectors with 3 x 10 meters (Black square), quadrats with 1 x 1m (blue square).*

The invertebrates of each scale were registered through detailed visual research and hand sampling (SOUZA-SILVA et al., 2021; OLIVEIRA FURTADO et al., 2022). To minimize the impact of the sampling on the invertebrate communities, some specimens were collected, and their abundance accounted.

All individuals were identified at the lowest taxonomic level possible with the assistance of taxonomic keys and other literature. The troglobitic species were separated by traits that indicate their evolution and adaptability in subterranean environments. Some troglomorphisms observed were lack or reduction in ocular structure and pigmentation and elongation of appendices and sensorial structures (CULVER & PIPAN, 2009).

A thermohygrometer was used to measure temperature and humidity in each sector (SOUZA-SILVA et al., 2021).

To measure the abiotic attributes in mesoscale, the sectors were divided into 10 sections of 1 m each, and the percentual of substrate was estimated visually (PELLEGRINI et al., 2016a). Later, the sum of these proportions and the average of every substrate and resource proportion in the sector were calculated, as well as the Shannon-diversity Index (H') for each sector. Regarding the quadrats, the measurement of abiotic attributes was made using photographs. Digital photographs were taken in the field in a vertical position (camera positioned at 90º relative to the cave floor) using the Canon Powershot SX50, HS. The photos were analyzed in the laboratory through ImageJ software (RASBAND, 1997), and the arithmetic average of every abiotic attribute was calculated (SOUZA-SILVA et al., 2021).

Counting individuals and morphotypes of each sample unit (sectors and quadrats) acquired the abundance and richness of non-troglobitic (n-TB) and troglobitic (TB).

The substrates were classified, and based on this, we obtained the physical features, which included distance from the entrance, substrate diversity, shelter diversity, and trophic resource diversity. All diversities were calculated using the Shannon-Weaver Index (BUTTIGIEG & RAMETTE, 2014). The availability of each abiotic attribute was also included, encompassing physical, trophic, and microclimatic characteristics. The microclimatic variables considered were temperature and humidity, however, were not included in the quadrat's models. Linear models

(GLMM) were performed to predict the influence of local abiotic variables on non-troglobitic and troglobitic species richness (response variables), using transects and quadrats as sample units, performed in RStudio (R CORE TEAM, 2023). To compare the model results with the null models, the ANOVA function from 'Vegan' package was made. To obtain r² values of the GLMMs, we used the function r.squaredGLMM from the 'MuMIn' package, while to obtain r² values of the GLM's, we used the function r.squaredLR from the 'piecewiseSEM' package. Before running the GLM's and GLMM's, we tested the correlation of all variables in their respective model through CHART. CORRELATION from the 'PerformanceAnalytics' package. Variables with high correlation values (>0.65) were excluded from the model (ZUUR et al., 2010). To explain the possible relationship, strength, and direction (- or +) between overall fauna, non-troglobitic and troglobitic species composition with the physical, trophic, and microclimatic variables, it was performed a Distance-based redundancy analysis (dbRDA) based on the Bray-Curtis similarity matrix (CLARKE et al., 2014). To perform this analysis, we used the two models as explained before.

## 3. Results

A total of 9178 individuals were recorded, distributed in 43 orders, 123 families, and 416 morphospecies, of which 72 were considered troglobitic (17.3%) (Figure 3).

Regarding the sectors, the dbRDA of non-Troglobitic (n-TB) showed that temperature, moisture, trophic diversity and availability were the best predictors, explaining 46% of the variation in species composition (p-value ≤ 0.05) (Figure 4A). Whilst for the troglobitic (TB), the dbRDA showed that distance from the entrance, moisture, and trophic diversity were the best predictors, explaining 52% of the variation in species composition (p-value ≤ 0.05) (Figure 4B). The non-troglobitic species richness was better explained by temperature, moisture, and trophic availability ($R^2$= 0.31). While for the troglobitic species richness, the moisture was the variable that explained its variation ($R^2$= 0.77, p-value ≤ 0.05).

Regarding the quadrats, the dbRDA of non-Troglobitic and troglobitic showed that shelter diversity, trophic availability, and distance were the best predictors, explaining 2.28% and 2.84%, respectively, of the variation in species composition (p-value ≤ 0.05) (Figures 4C, 4D). The non-troglobitic species richness was better explained by trophic availability ($R^2$= 0.09, p-value ≤ 0.05). For the troglobitic, none of the variable's measures explained its richness variations.

Temperature, humidity, shelter and resources diversity, and resource availability were differed in averages among all 26 caves. Regarding the sectors, the humidity indicated a positive relationship with the distance from the entrance ($R^2$ = 0.08, p-value ≤ 0.05), while the substrate diversity ($R^2$ = 0.07, p-value ≤ 0.05), shelter diversity ($R^2$ = 0.05, p = 0.006), resource availability ($R^2$ = 0.06, p-value ≤ 0.05) and shelter availability ($R^2$ = 0.04, p-value ≤ 0.05), showed a negative relationship with the distance from the entrance ($R^2$ = 0.07, p-value ≤ 0.05).

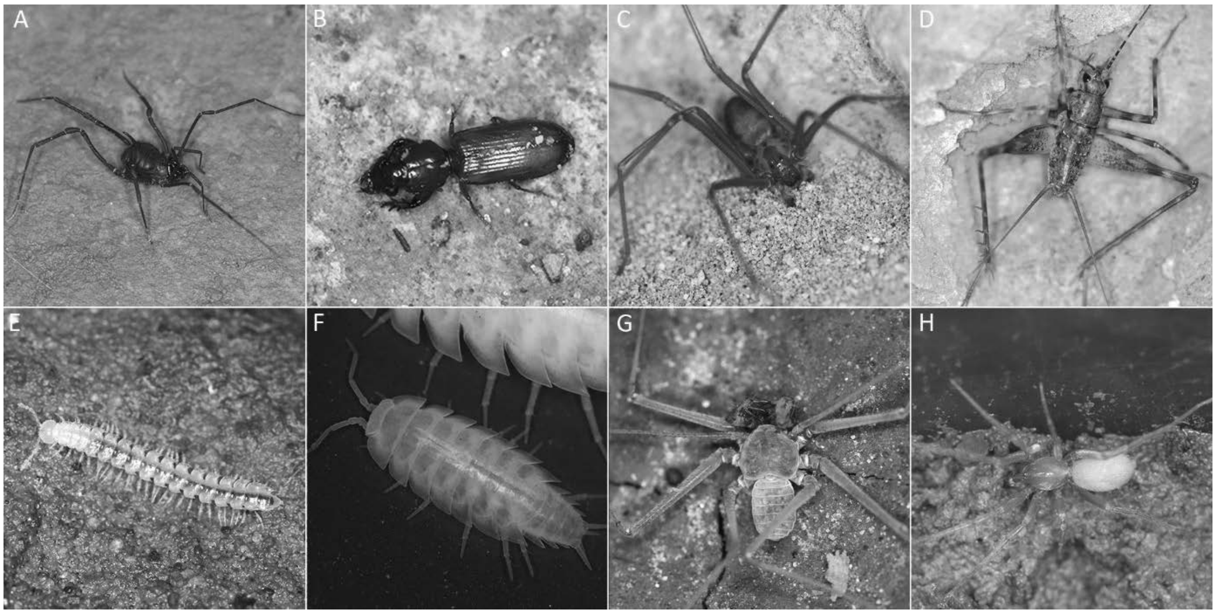

***Figure 3:** Some of the species found in Serra do Ramalho karst: Opiliones: Gonyleptidae (Eusarcus sp.) (A); Coleoptera: Carabidae (Clivinina sp). (B); Araneae: Sicariidae (Loxoceles sp.) (C); Ensifera: Phalangopsidae (Eidmanacris sp.) (D); Diplopoda: Polydesmida (Phaneromerium sp.) (E); Isopod: Styloniscidae (F); Amblypygi: Charontidae (Charinus troglobius) (G); and Araneae: Ochyroceratidae (H).*

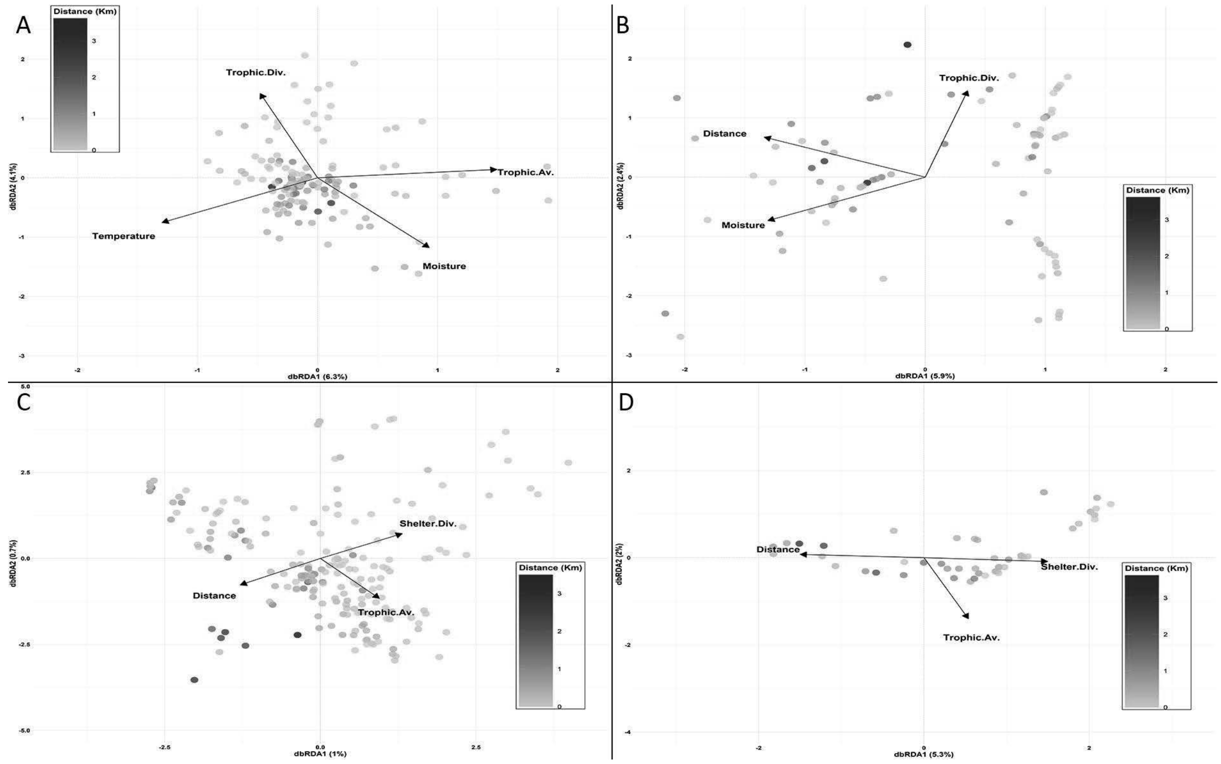

***Figure 4:*** *Spatial variation in species composition based on dbRDA. (A) Non-troglobitic species in sectors, with temperature, moisture, trophic diversity, and availability as main predictors. (B) Troglobitic species in sectors are influenced by distance from the entrance, moisture, and trophic diversity. (C) Non-troglobitic species in quadrats are explained by shelter diversity, trophic availability, and distance. (D) Troglobitic species in quadrats, explained by the same environmental variables as the non-troglobitic.*

## 4. Discussion

Our study highlights the importance of different environmental factors, including sampling scales, in explaining variations in substrate structure, species richness, and the composition of the invertebrate fauna on the cave floor. The results revealed that distance from the entrance, temperature, moisture, and substrate heterogeneity play a key role in shaping the composition of cave invertebrate species. However, its impact varied between troglobitic and non-troglobitic species and across different scales.

The different responses of fauna in relation to the sampling scales may be related to the influence of the variables analyzed here. This may occur because, as the scale increases, the number of variables capable of explaining the variations that occur in composition and richness also increases. At larger scales, we can observe greater environmental complexity, allowing the identification of different factors that affect faunal diversity. Thus, we highlight the importance of considering multiple sampling scales to more fully understand the structuring factors of cave communities (FERREIRA & MARTINS, 2009; RABELO et al., 2020).

As one enters the cave, the decrease in the diversity and availability of shelter and resources causes a gradient of conditions and resources from the entrance to deeper locations. This gradient suggests a high heterogeneity of habitats throughout the cave. Thus, changes in the composition of cave species can occur both in space and time, in response to variations in habitat characteristics. (PELLEGRINI & FERREIRA, 2016). This is corroborated by our results, which showed that non-troglobites and troglobites responded differently to environmental variations. Non-troglobites prefer high trophic availability, low humidity, and low temperature. In contrast, troglobites prefer environments further from the inlet with high humidity, characteristic of stable environments.

In this context, the characteristics of the permanent absence of light, stability in temperature and humidity conditions, and scarcity of food resources (POULSON & WHITE, 1969; PECK, 1974), the structure of invertebrate communities. Thus, restrictions imposed by the environment and the availability of resources occur, and this is a primary factor in structuring the community and determining the composition and richness of cave species (POULSON, 2005). This occurs due to the tendency of richness to be proportional to the availability of these resources (DOUBE, 1986).

This pattern was also observed by SOUZA-SILVA et al. (2021) and DEHARVENG & BEDOS (2000), who found that troglobitic species are more abundant in the deeper zones of caves. On the other hand, POULSON & WHITE (1969) and PELLEGRINI et al. (2016b) found that a greater richness of invertebrates is found in regions close to entrances when compared to deeper areas. The combination of the absence of light and greater microclimatic stability in the deeper zones of the cave creates ecological niches suitable for the survival of troglobitic species, as they have adaptations to live in these environments, such as reduced pigmentation, elongation of appendages, reduction or absence of eyes, and development of sensory structures (RACOVITZA, 1907; SKET, 2008).

These results demonstrate the great richness and diversity in the caves, showing that these environments are not as homogeneous as previously believed. On the contrary, the cavities present a high environmental complexity, which reflects the variations generated by the microclimate, resources, and structure of the substrate, which will directly or indirectly influence the composition and richness of cave invertebrates. Furthermore, the use of different scales helps to understand this complexity, considering the subterranean ecosystem as a whole, at different spatial and temporal scales, revealing the importance of local and regional factors in determining biodiversity.

## 5. Conclusion

The study highlights the importance of habitat heterogeneity and different scales of sampling in understanding subterranean fauna. While non-troglobitic fauna may have a wider range of resources and tolerance to microclimatic features, troglobitic fauna requires more specific conditions. Also, the different sampling scales can lead to distinct responses from the fauna, and may drive unlike decisions on environmental protection. Deforestation around cave entrances can negatively impact epigean and cave fauna by reducing fauna movement and affecting temperature and humidity conditions, which highly treat restricted subterranean fauna. Biological research is crucial for understanding cave biodiversity and helps in decision-making.

## Acknowledgments

The authors would like to thank the Programa de Pós-Graduação in Applied Ecology (UFLA), Center of Study in Subterranean Biology (CEBS/UFLA) for providing structure for the development of the study. We would also like to thank CNPq for the research support, the Instituto Chico Mendes de Conservação e Biodiversidade (ICMBio-Vale S.A.) regarding the implementation of compensation for the irreversible negative impacts of natural underground cavities with a high degree of relevance, through the project: “Dispersion versus confinement: compositional analysis and habitat structure as a subsidy to the understanding of mechanisms responsible for subterranean fauna identity”.

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# Survey of fungi from the air and sediment of the Lapa da Cachoeira do Funil Cave in Mambaí, Goiás, Brazil: Preliminary data

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## Resumo

Este estudo teve como objetivo investigar a diversidade de fungos presentes no ar e no sedimento/solo da caverna Lapa da Cachoeira do Funil, localizada na Área de Proteção Ambiental (APA) das Nascentes do Rio Vermelho, no Cerrado. Foram obtidos 150 isolados pertencentes a 43 gêneros, com variações na abundância relacionadas a fatores ambientais. Gêneros de importância médica, agrícola e biotecnológica, como *Aspergillus, Fusarium, Trichoderma* e *Penicillium*, foram identificados. Os resultados ressaltam a relevância dos inventários fúngicos para a conservação da biodiversidade cavernícola e para a compreensão do papel ecológico desses microrganismos em ambientes subterrâneos.

## Abstract

This study aimed to investigate the diversity of fungi present in the air and sediment/soil of the Lapa da Cachoeira do Funil cave, located in the Environmental Protection Area (APA) of the Nascentes do Rio Vermelho, in the Cerrado. A total of 150 isolates belonging to 43 genera were obtained, with variations in abundance related to environmental factors. The medical, agricultural, and biotechnologically important genera, including *Aspergillus, Fusarium, Trichoderma*, and *Penicillium*, were identified. The results highlight the relevance of fungal inventories for cave biodiversity conservation and understanding these microorganisms' ecological role in protected subterranean environments.

## 1. Introdução

As cavernas são ambientes únicos e isolados que fornecem condições favoráveis ao desenvolvimento de comunidades de fungos que prosperam nesses ecossistemas oligotróficos, caracterizados por uma alta umidade, temperaturas estáveis e especialmente pela ausência de luz ZHANG et al. (2023); JIANG et al. (2017). A diversidade fúngica é fundamental para os ecossistemas das cavernas, pois os fungos atuam no parasitismo, na ciclagem de nutrientes e sendo fontes de recursos orgânicos para outros organismos VANDERWOLF et al. (2013); RAWAT et al. (2017); CUNHA et al. (2020); CARVALHO et al. (2022). Tais condições características do ambiente cavernícola estão vulneráveis aos impactos das mudanças climáticas e ameaçam a ecologia das cavernas, devido à baixa tolerância térmica de algumas espécies subterrâneas e à alteração das condições microclimáticas MAMMOLA et al. (2019); PALLARÉS et al. (2020).

Portanto, o impacto das mudanças climáticas pode ocasionar alterações metabólicas em muitas espécies, principalmente em táxons fúngicos, que podem se tornar novos patógenos, patógenos incomuns ou adaptados, e levantam preocupações quanto às consequências na saúde e diversidade CASADEVALL et al. (2019); NNADI & CARTER (2021). Além disso, atividades humanas como o turismo desregulado, a exploração mineral, as práticas agrícolas e as construções indevidas ameaçam a conservação de ecossistemas subterrâneos, bem como as espécies que os habitam FERREIRA & MARTINS (2001); MAMMOLA et al. (2019).

O levantamento microbiológico de espécies, com ênfase em fungos, é fundamental para subsidiar a elaboração de plano de manejo espeleológico devido à alta diversidade de fungos nesses ambientes, incluindo espécies patogênicas e oportunistas, que podem impactar a saúde humana e a conservação ambiental, além de revelar potencial biotecnológico TAYLOR et al. (2013); ALVES et al. (2022), PRAZERES et al. (2025). O Cerrado, conhecido como Savana brasileira, é considerado um *hotspot* de biodiversidade. Além disso, apesar dos estudos de fungos cavernícolas no Brasil terem aumentado nos últimos anos com cerca de 30 cavernas estudadas, isso representa apenas 0,1% das cavernas existentes no País PRAZERES et al. (2025).

Na região do Cerrado, a Área de Proteção Ambiental (APA) das Nascentes do Rio Vermelho abriga mais de 150 cavernas documentadas, incentivando práticas de uso sustentável, ecoturismo e agroecologia. No entanto, essa área enfrenta significativa pressão antrópica ESBÉRARD et al. (2005). Assim, com o intuito de regular a ocupação humana, a APA foi instituída pelo decreto de 13 de setembro de 2001. No entanto, essa medida não assegura a proteção total da região MIRANDA (2016). Como resultado, os impactos provocados por atividades humanas comprometem a estabilidade das comunidades biológicas, incluindo os fungos, e limitam o conhecimento e a descoberta de novas espécies.

A caverna Lapa do Funil abriga um dos mais impressionantes conjuntos naturais da região da APA Nascentes do Rio Vermelho (NRV). Nela, um grande volume de água que deságua em um vale cristalino que se forma ao seu final, criam um roteiro turístico singular atraindo cerca de 2 mil turistas anualmente para ecoturismo VIEIRA & VIEIRA (2009); Prefeitura MUNICIPAL DE MAMBAÍ (2023). (Fig.1). Considerando esses fatores, o levantamento da micodiversidade da caverna é essencial tanto para garantir a segurança dos turistas quanto para a conservação do ambiente natural.

Nesse sentido, o objetivo deste estudo foi realizar um estudo preliminar para inventariar a diversidade de fungos isolados do ar e do sedimento/solo da Caverna turística Lapa da Cachoeira do Funil, por meio de análises morfológicas e moleculares, fornecendo dados iniciais para pesquisas e ações de manejo espeleológico.

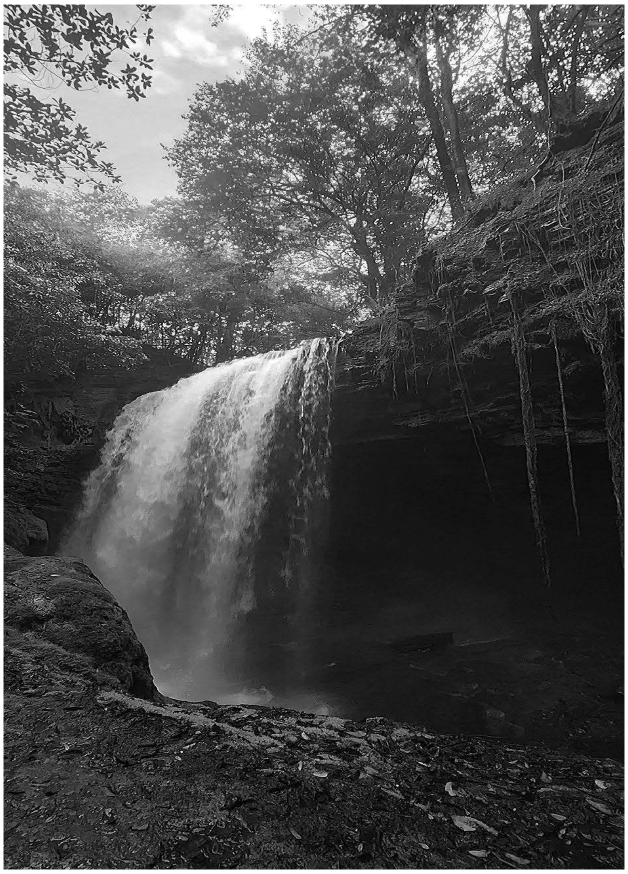

***Figura 1:*** *Cachoeira do Funil em Mambaí, Goiás, Brasil (vista próxima ao 3º ponto de coleta).*

## 2. Materiais e Métodos

A Lapa da Cachoeira do Funil, localizada na Área de Proteção Ambiental (APA) das Nascentes do Rio Vermelho, em Goiás (UTM: 375.925 S / 8.399.403 E), encontra-se no Cerrado e é uma caverna com desenvolvimento de 350 metros, banhada pelo Rio Ventura LINHARES (2002). É uma das cavernas formadas pela unidade geomorfológica do Grupo Bambuí (que forma o maior grupo de eventos calcários favoráveis à existência de cavernas no território brasileiro), o qual corresponde cerca de 105.200 km2, de acordo com AULER & FARRANT (1996); AULER et al. (2009).

Os fungos do ar foram isolados pelo método de sedimentação em placas de Petri de 90 mm de diâmetro, contendo 20 mL dos meios DRBC, BHI ou ASC, expostas a 1 m do solo por 20 minutos CUNHA et al. (2020). As placas foram incubadas no escuro a 28°C ± 2°C por até sete dias para contagem das unidades formadoras de colônias (UFC). As colônias foram purificadas e transferidas para tubos de preservação e microtubos para extração de DNA. Para o isolamento de fungos do sedimento/solo, amostras (10 g) foram coletadas, na camada superficial (0-5 cm), em triplicata e acondicionadas em recipientes estéreis. No laboratório, 1 g do sedimento/solo foi suspenso em 9 mL de água destilada estéril, agitado a 100 rpm por 20 minutos e submetido a diluições seriadas até $10^{-4}$. Dessa diluição, 1 mL foi inoculado nos meios DRBC e ASC em placas de Petri, incubadas no escuro a 25 °C por até 14 dias. As colônias foram purificadas e transferidas para tubos contendo ASC ou outros meios específicos, como BDA. O número de UFC/g de sedimento/solo foi calculado pela média das réplicas CUNHA et al. (2020).

A identificação morfológica foi baseada na análise de estruturas macro e micromorfológicas, seguindo metodologias especializadas CROUS et al. (2009); SAMSON et al. (2010); SEIFERT et al. (2011). Para a identificação molecular, o DNA genômico foi extraído conforme o protocolo do Wizard® Genomic DNA Purification Kit (Promega, USA). As regiões ITS e LSU foram amplificadas com os *primers* ITS1/ITS4 WHITE et al. (1990) e LR0R/LR5 VILGALYS & SUN (1994); VILGALYS & HESTER (1990). Outros lócus, como *tef1*, *RPB1*, *RPB2*, calmodulina, β-tubulina e actina, foram analisados conforme o gênero ou grupo taxonômico. As reações de amplificação, a purificação dos amplicons e as reações de sequenciamento foram realizadas seguindo o protocolo de BEZERRA et al. (2017). As sequências obtidas foram comparadas com as depositadas no *GenBank* do *NCBI* por meio da ferramenta BLASTn para a anotação da identificação (%) genérica.

## 3. Resultados

Os resultados do levantamento de fungos do ar e do sedimento/solo revelaram uma riqueza significativa de gêneros entre os diferentes pontos de coleta e substratos. No total, foram obtidos 149 isolados, sendo 44 do ar e 105 do sedimento/solo. A identificação dos isolados revelou a presença de 42 gêneros, dos quais alguns foram exclusivos para cada substrato, enquanto outros ocorreram em ambos.

A coleta de fungos anemófilos nos diferentes pontos da caverna revelou uma variação no número de isolados. Foram obtidos 20 isolados no Ponto 1, localizado na entrada da caverna, em uma zona de transição entre a zona de iluminação direta e o início da zona de penumbra. No Ponto 2, situado no interior da caverna, local que não havia presença de luz, forneceu um número de isolados consideravelmente menor (3). O Ponto 3, um ambiente externo próximo à cachoeira, onde havia a presença direta de iluminação, foram obtidos 21 isolados. O sedimento/

solo apresentou maior variedade do número de isolados nos diferentes pontos. No Ponto 1, foram obtidos 64 isolados, número maior em comparação com o ar para o mesmo ponto. No Ponto 2 foram obtidos 12 isolados, (Fig. 2) e no Ponto 3, o número de isolados foi de 29.

A identificação dos fungos resultou em 18 gêneros para o ar, sendo eles: *Absidia, Allophoma, Apiospora, Aspergillus, Cladosporium, Colletotrichum, Curvularia, Fusarium, Fusicolla, Meira, Myxospora, Neopestalotiopsis, Penicillium, Pseudopithomyces, Purpureocillium, Trichoderma, Waltergamsia* e *Zygosporium*. Na identificação dos fungos do sedimento/solo, foram obtidos 24 gêneros: *Aspergillus, Cladosporium, Debaryomyces, Epicoccum, Eutypella, Fusarium, Hypocrea, Leptosphaeria, Marquandomyces, Microsphaeropsis, Mucor, Musidium, Penicillium, Phialemonium, Pleurostoma, Pochonia, Purpureocillium, Pyrenochaetopsis, Rhodotorula, Saturnispora, Talaromyces, Thyridium, Trichoderma* e *Waltergamsia*. Dentre os gêneros identificados, pode-se observar que sete foram comuns a ambos os substratos: *Aspergillus, Cladosporium, Fusarium, Penicillium, Purpureocillium, Trichoderma* e *Waltergamsia*. A ocorrência desses gêneros tanto no ar quanto no sedimento/solo sugere sua ampla adaptabilidade às diferentes condições ambientais da caverna. Por outro lado, 11 gêneros foram exclusivos ao ar, incluindo *Absidia, Allophoma, Apiospora, Colletotrichum, Curvularia, Fusicolla, Meira, Myxospora, Neopestalotiopsis, Pseudopithomyces* e *Zygosporium*, enquanto 17 foram exclusivos do sedimento/solo: *Debaryomyces, Epicoccum, Eutypella, Hypocrea, Leptosphaeria, Marquandomyces, Microsphaeropsis, Mucor, Musidium, Phialemonium, Pleurostoma, Pochonia, Pyrenochaetopsis, Rhodotorula, Saturnispora, Talaromyces* e *Thyridium*. (Fig. 3).Esses resultados destacam a complexidade da distribuição fúngica em ambientes cavernícolas, demonstrando a possível influência de fatores ambientais como luz, umidade e substrato na composição da micodiversidade local, bem como a movimentação de animais (pássaros) e a influência da atividade turística como um vetor ativo de propágulos fúngicos. Estudos adicionais podem aprofundar a compreensão sobre o papel ecológico desses fungos e suas interações com o ambiente cavernícola.

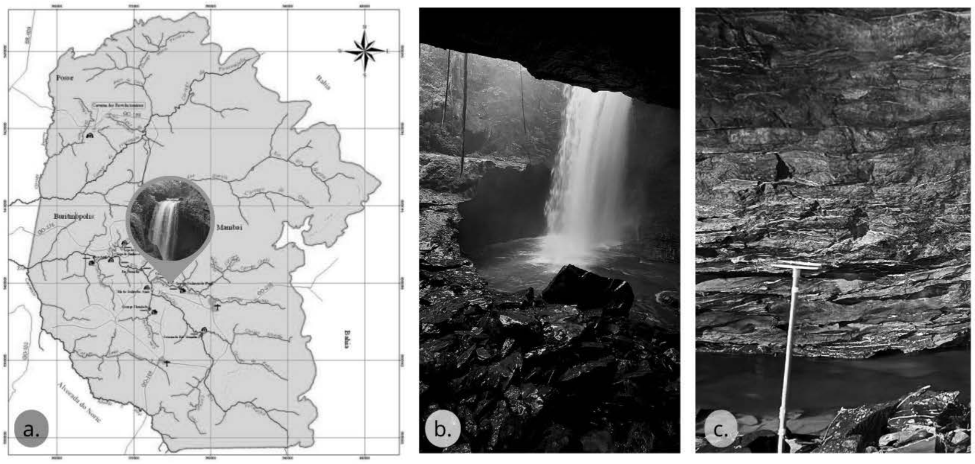

***Figura 2:*** *A. Mapa turístico da APA das Nascentes do Rio Vermelho, Fonte: Adaptado de SOCIEDADE BRASILEIRA DE ESPELEOLOGIA (2021); B. Vista interna da cachoeira; C. Ponto de coleta interno (2) com suporte para técnica de sedimentação em meio de cultura (fungos do ar).*

## 4. Discussão

O levantamento de espécies fúngicas nesta caverna do Cerrado revelou uma comunidade diversificada, possivelmente influenciada pela umidade, luz e matéria orgânica. Gêneros como *Aspergillus, Cladosporium, Fusarium, Penicillium* e *Trichoderma* foram encontrados para ambos os substratos estudados, indicando adaptabilidade às condições cavernícolas. Essa ampla distribuição se assemelha com estudos em outras cavernas, como PEREIRA et al. (2022), que destacaram a presença de *Cladosporium* como um gênero comum em cavernas brasileiras, incluindo a descrição de novas espécies. A exclusividade de certos gêneros em substratos específicos, como ar ou sedimento/solo, indica que diferentes nichos ecológicos favorecem grupos fúngicos distintos, um padrão também observado por LIMA et al. (2024). Vários gêneros identificados possuem relevância para saúde humana, agricultura e biotecnologia. Por exemplo, *Aspergillus* e *Fusarium* incluem espécies patogênicas para humanos imunocomprometidos e plantas cultivadas PATERSON & LIMA (2017), tais gêneros foram observados em cavernas da Caatinga CUNHA et al. (2020) e também em cavernas do Cerrado PRAZERES et al. (2025). Além dos aspectos patogênicos, muitos gêneros apresentam potencial biotecnológico e agrícola, tais como *Trichoderma*, amplamente utilizado como agente de biocontrole contra patógenos TYŚKIEWICZ (2022); este gênero foi identificado em nosso estudo, reforçando seu papel ecológico em ambientes cavernícolas. Gêneros como *Talaromyces* e *Mucor*, que produzem enzimas de interesse industrial e farmacêutico ALVES et al. (2002); LI et al. (2017); LIMA et al. (2024), foram igualmente identificados no nosso estudo. Essa descoberta reforça a importância ecológica e o possível uso biotecnológico desses microrganismos em ambientes subterrâneos. Estudos recentes destacam a diversidade fúngica em cavernas brasileiras como essencial para a manutenção desses ecossistemas e descobertas taxonômicas CUNHA et al. (2020); ALVES et al. (2022); LIMA et al. (2024); PRAZERES et al. (2025). Os dados preliminares deste estudo demonstram a importância de fungos cavernícolas e reforçam o impacto deste grupo na manutenção do ambiente cavernícola. Além disso, a identificação de gêneros com potencial biotecnológico e ecológico demonstram que cavernas do Cerrado são hotspot da diversidade de fungos.

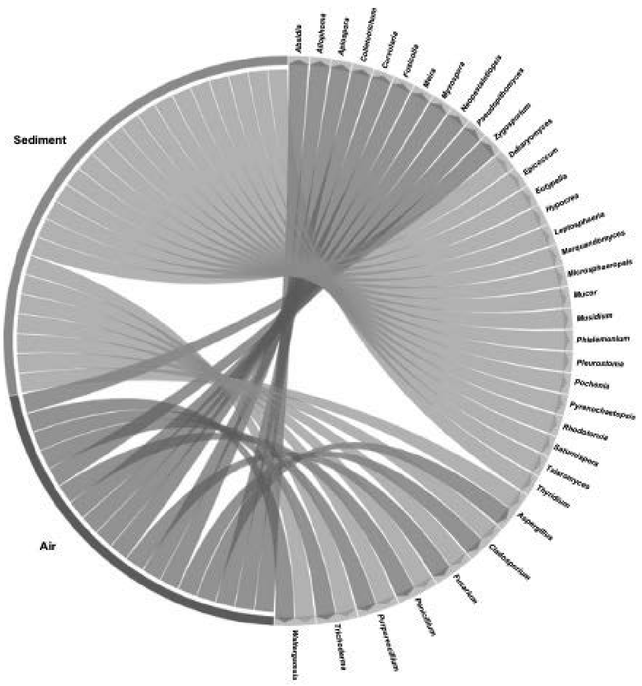

***Figura 3:*** *Gêneros fúngicos identificados do ar (em azul) e sedimento/solo (em laranja) na Caverna Lapa da Cachoeira do Funil, Mambaí, Goiás, Brasil.*

## 5. Conclusão

O presente estudo revelou uma riqueza de 42 gêneros de fungos na caverna Lapa da Cachoeira do Funil, demonstrando a possível influência de fatores ambientais na distribuição de fungos em diferentes pontos da caverna. A presença de gêneros de relevância médica, agrícola e biotecnológica evidencia a importância dos inventários fúngicos para o conhecimento da diversidade subterrânea e para o potencial aproveitamento desses organismos. Além disso, a identificação de espécies do ambiente cavernícola reforça a necessidade de estratégias de conservação, especialmente diante da crescente exploração turística e das mudanças ambientais. Estudos futuros podem aprofundar a caracterização funcional dessas comunidades fúngicas e avaliar seu papel ecológico na manutenção dos ecossistemas subterrâneos.

## Agradecimentos

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# Phage-host dynamics in the Wind Cave Lakes, South Dakota

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## Abstract

Due to their isolation and limited nutrient input, the Wind Cave Lakes (WCL) contain a bacteria-dominated community with one of the lowest microbial cell densities known. Despite this nutrient limitation, the microbial community of the lakes is remarkably diverse. Given the low biomass, it was assumed that very few bacteriophages would be present, but they were found in numbers comparable to surface environments. In other environments, bacteriophages have been shown to provide their hosts with beneficial genes, which can enhance survival under challenging conditions. To investigate whether phage-host mutualism similarly impacts host survival within these nutrient-limited lakes, we examined the viral genomes present within the WCL community. The results indicate the lakes contain a high level of previously undescribed viral diversity with a dominantly lytic lifestyle. Nonetheless, there were some genes carried by the phages that suggest that some of these infections have the potential to benefit the host. Together these data suggest that the majority of phages may not play a specifically beneficial role though the transfer of genes, but may carry out other roles, such as carbon turnover. A more detailed analysis of the virome of the lakes is needed to answer this question.

## 1. Introduction

The Wind Cave Lakes are an incredibly low-nutrient environment that supports one of the lowest cell numbers of an aquatic environment measured on Earth, at 2.3 x $10^3$ cells/mL. Only water in deep South African gold mines and Antarctic accretion ice contains lower cell densities. Despite this low biomass, the WCL support a taxonomically and metabolically diverse community dominated by bacteria (HERSHEY, 2021). Viruses that infect bacteria, known as bacteriophages (or phages), have many effects on microbial communities: some phages kill their host cells, but phages also recycle nutrients, drive an increase in host genetic diversity, and transfer genes to their hosts. Past work has suggested that in low-nutrient environments, phages are more likely to form mutually beneficial relationships with their hosts, where in return for sheltering the phage from challenging environmental conditions, they may enhance host survival by transfering beneficial genes.

The Wind Cave Lakes are an ideal study site for phage-host dynamics, since the lakes represent an isolated ecosystem. Wind Cave is one of the longest (218 km) and oldest caves in the world. The WCL are isolated, located 124 m below the surface and 3.5 hours of travel time from the entrance. Unlike in many other cave lakes, the water enters the cave through artesian flow. Given their isolation and nutrient limition, no multicellular life has been observed, and archaea constitute <2% of the population. As a result, phages are the top predator in the lakes (HERSHEY et al., 2018).

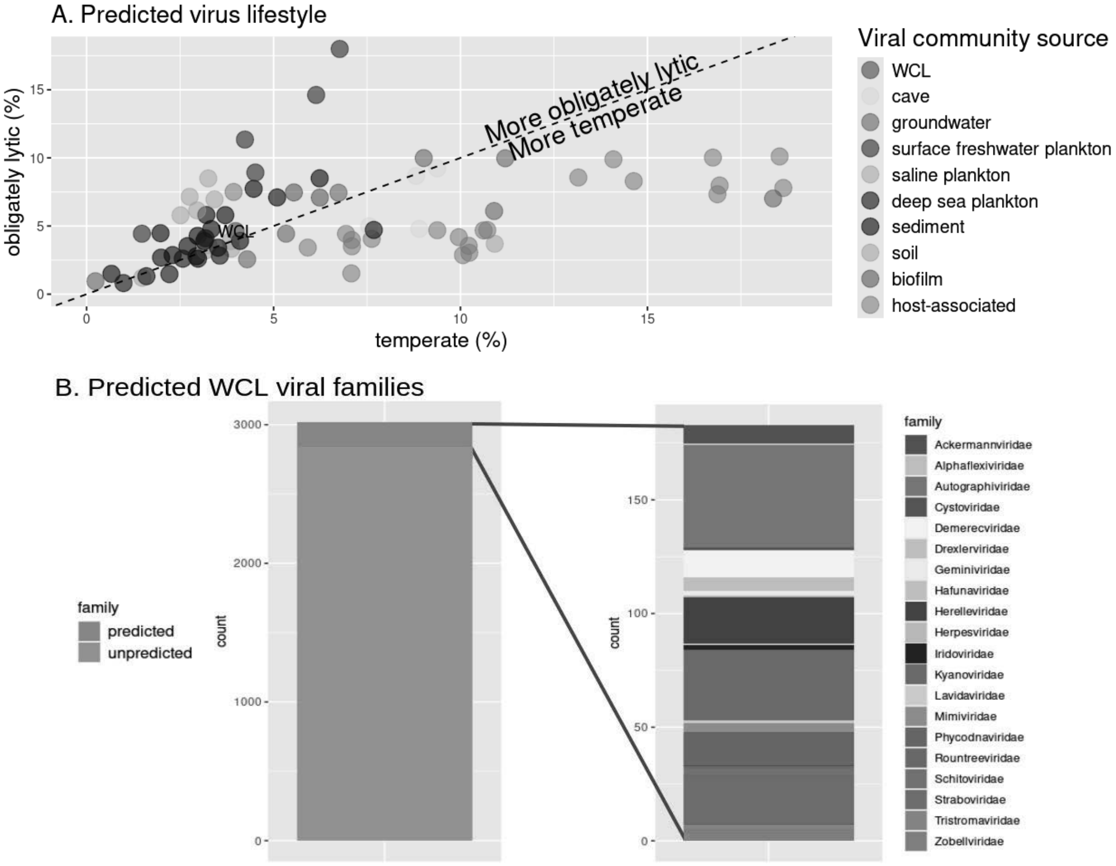
While there has been siginificant research on bacteria in caves, only a handful have investigated cave viruses (bacteriophages), and to date none have investigated a role they may play in adaptation. Investigating the phages of the WCL may help explain whether they play a the role in the high microbial diversity observed, and provide important clues as to whether phages help microbes adapt to the ultraoligotrophic conditions. Here, we use partial phage genomes obtained from the metagenome of WCL to investigate phage-host dynamics compared to other environments to determine their role in host diversity.

## 2. Materials and methods

Partial viral genomes (contigs) were identified from within a mixture of cellular (bacterial) and bacteriophage DNA sequenced from within the WCL. Viral contigs were also identified within 130 publicly-available comparison datasets from environments including other caves, high- and low-nutrient surface environments, and high- and low-nutrient ocean samples. For each of the 131 datasets, the partial viral genomes were identified via using three tools, VIBRANT, VirSorter2, and geNomad (CAMARGO et al., 2024; GUO et al., 2021; KIEFT et al., 2020).

Locations of genes within the viral contigs were predicted via Prodigal (HYATT et al., 2010), and the identities of the genes were determined via matching the unknown genes against six gene databases: KEGG, COG, Pfam, Vfam, TIGR, and PADLOC (BATEMAN et al., 2004; KANEHISA et al., 2025; LI et al., 2021; PAYNE et al., 2022 ; TATUSOV et al., 2000). Viral families were assigned via geNomad. Lifestyle was predicted via a combination of prophage prediction via CheckV and lifestyle prediction via PhaTYP (NAYFACH et al., 2021; SHANG et al., 2022). For individual genes of interest, structure was predicted using AlphaFold3 (ABRAMSON et al., 2024).

## 3. Results


A. Predicted virus lifestyle

B. Predicted WCL viral families

***Figure 1:*** *(A). The proportion of temperate vs obligately lytic phages within the WCL and comparison datasets. Viral contigs were identified from mixed microbial metagenomes via three tools, VIBRANT, Virsorter2, and Genomad, and lifestyles were predicted via PhaTYP. Obligately lytic phages outnumber temperate phages in the WCL, and the WCL has a lower proportion of temperate phages than 65.5% of all comparison communities. (B). Counts of predicted viral families for WCL viral contigs. Viral taxonomy was predicted via geNomad. Viral family was able to be predicted for 183 (4.9%) of WCL viral contigs. Of the contigs with predicted families, 154 (84.1%) are within the class Caudoviricetes.*

**Characteristics of WCL bacteriophages**

A total of 3,739 viral contigs were identified within the Wind Cave Lakes, of which 282 had a predicted lifestyle; 173 (61.3%) represented obligately lytic viruses (not capable of phage-host mutualism), while 109 (38.7%) were predicted to represent temperate viruses (potentially capable of phage-host mutualism). Among the comparison datasets, the proportions of obligately lytic and temperate viruses varied from 88.9% obligately lytic in Quebec groundwater to 77.0% temperate in a frog microbiome sample. The WCL viruses had a higher proportion of obligately lytic phages than average, but a lower proportion compared to planktonic communities, with more obligately lytic phages than 65.5% of all comparison datasets and 45.1% of planktonic datasets (fig. 1a).

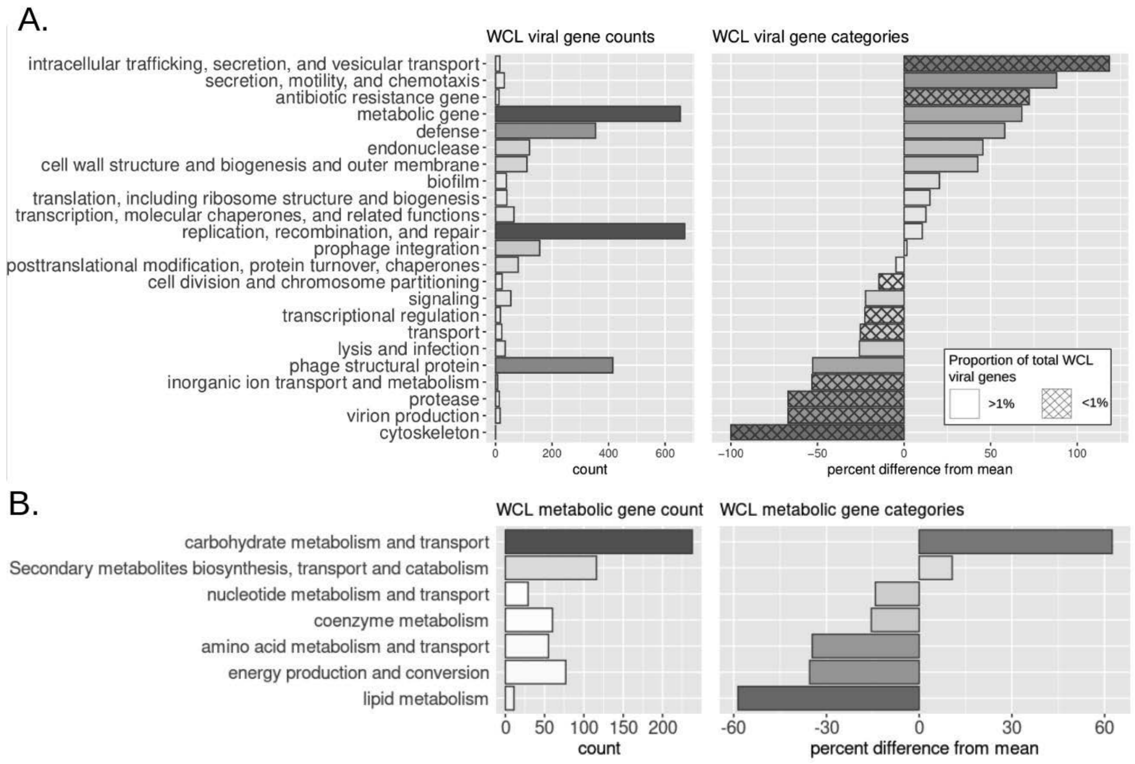


***Figure 2:*** *(A). Enrichment of viral gene categories in the WCL compared to comparison datasets. Location of genes within the partial viral genomes were predicted via Prodigal, and the identities of the genes were determined via matching the unknown genes against six gene databases: KEGG, COG, Pfam, Vfam, TIGR, and PADLOC. Gene categories enriched in the WCL phages include defense and metabolic genes, while genes encoding structural proteins, as well as lysis and infection genes, were underrepresented. (B). Enrichment of metabolic gene categories in the WCL vs comparison datasets. Among the categories of metabolic genes, genes involved in carbohydrate metabolism and transport were enriched compared to the other communities. Genes involved in the biosynthesis, transport, and catabolism of secondary metabolites were slightly enriched, while all other categories of metabolic genes were underrepresented.*

It was only possible to predict the viral family for 183 (4.9%) of WCL viral contigs. For comparison datasets, the proportion of predicted families ranged from 29.1% within a Norway surface freshwater planktonic metagenome to 1.3% in a human microbiome sample. WCL viruses represent slightly less uncharacterized viral diversity than average, with a lower proportion of predicted viral families than 34.1% of comparison datasets (fig. 1b).

The majority of WCL viral genes (9,299, or 68.4%) did not have a match in the databases used. Of the genes that did, the majority (3,649, or 84.8%) had a known function. None of the comparison datasets had a majority of genes with a database match. The proportion of genes with a match ranged from 54.2% in a human microbiome sample to 24.7% in Lake Baikal sediment. Nonetheless, the WCL viruses had more uncharacterized genetic diversity than average; the WCL partial viral genomes had fewer genes with a database match than 84.4% of comparison datasets.

**Enrichment of viral genes**

Metabolic genes, which have the potential to benefit the host cell, were enriched in the WCL viruses, with 72% more genes than the average. Also enriched (with 65% more genes than average), were defense genes, which may indicate parasitic rather than mutualistic phage infections. Other enriched categories included biofilm formation, prophage integration, and genes involved in secretion, motility, and chemotaxis (fig. 2a).

Metabolic genes as a whole were enriched; however, not all metabolic subcategories were enriched equally; genes involved in carbohydrate metabolism and transport represented a greater proportion (65%) of all metabolic genes compared to other datasets; metabolic genes involved in biosynthesis, transport, and catabolism of secondary metabolites were slightly enriched (8% greater than the mean). All other metabolic gene categories, including energy production and conversion, as well as nucleotide, amino acid, and coenzyme metabolism, were underrepresented (fig. 2b).

Individual genes of interest detected include *kilR*, a cell division inhibitor that, when carried by a phage infecting *Escherichia coli*, increases cell viability during treatment with two antibiotics (WANG et al., 2010). Structural prediction indicates that the protein product of the WCL *kilR* gene is near-identical to the homolog in the database. Potentially beneficial genes with partial protein structure matches to database proteins included phoH, a gene that is involved in responding to phosphate starvation (HSIEH & WANNER, 2010). Other genes include copA, a copper shock gene; a magnesium transporter; and norQ, a gene involved in nitrite reduction.

## 4. Discussion

**Phages are present, active, and genetically diverse in WCL**

The presence of viral partial genomes in the WCL metagenome indicates that viruses are present and actively infecting cellular hosts. Bacteriophages in general are poorly characterized and genetically diverse, but the WCL viruses have a greater uncharacterized genetic diversity and lower proportion of known genes than many comparison datasets. Interestingly, they have a lower potential diversity than average, although this may be related to sample size (we were only able to sequence infected cells using this approach).

**Potential indicators of phage-host mutualism vs parasitism**

Under low-nutrient conditions, mutualistic (temperate) interactions would be expected to dominate, as the host cell provides a refuge from poor environmental conditions. Of WCL viral contigs with a predicted lifestyle, only 38.7% were predicted to be temperate. This ratio of temperate to obligately lytic phages is lower than average; however, given the lack of complete viral contigs, lifestyle prediction tools may underestimate the proportion of temperate phages. Since the majority of WCL and comparison viral contigs are low-completeness, the actual proportions of temperate phages may be higher than indicated here.

Some phages carry genes to defend against the host cell's «immune system.» If mutualism is common, cells may employ fewer defense genes, and thus phages may carry fewer anti-host defense genes. If mutualism were the dominant virus-host dynamic, it would be expected that defense genes would be underrepresented, while potentially beneficial categories would be enriched. Our data indicate that defense genes are enriched in general, in both temperate and obligately lytic viral genomes. Metabolic genes, which have the potential to benefit the host, are also enriched, along with other potentially beneficial categories, including: biofilm formation, secretion, motility, and chemotaxis. The only potentially beneficial gene category underrepresented are signaling genes.  While the limited proportion of temperate phages suggests that mutalism may not be the dominant phage-host dynamic in the lakes, the presence of individual potentially beneficial genes suggests that it may co-occur with parasitism.

## 5. Conclusion

Phages are present and active within the WCL microbial community. The viruses observed are mostly uncharacterized, with significant uncharacterized genetic diversity. The enrichment of anti-host defense genes within the viral genome fragments indicates the prevalence of parasitic bacteriophage infections, while the enrichment of potentially beneficial gene categories, including environmental adaptation, suggest a mutually beneficial relationship from certain phage-host interactions. Such data may suggest that the primary role of phages within the lakes is not one of information exchange, but may be related to carbon turnover to enhance the release of nutrients that are otherwise bound up in biomass. Given that the metagenome only provided a snapshot of active infections in the cells, obtaining additional information through the isolation and sequencing of free phages in the lakes may improve our understanding of their role in the WCL microbial community.

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# A fauna subterrânea nas Avaliações de Impactos Ambientais: Uma abordagem qualitativa dos estudos apresentados em processos de licenciamento ambiental no Brasil

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## Resumo

A Avaliação de Impacto Ambiental (AIA) é uma ferramenta legal utilizada para regulamentar os impactos de projetos de desenvolvimento humano em diversos países, incluindo o Brasil. No entanto, a integração de considerações sobre biodiversidade nos processos de AIA tem sido alvo de críticas devido à baixa qualidade dos estudos e aos desafios para fundamentar decisões em evidências científicas robustas. Essa questão é especialmente preocupante na espeleologia, onde os estudos biospeleológicos são frequentemente escassos ou ausentes dos Estudos de Impacto Ambiental (EIA). Este estudo analisou 19 AIAs associadas a EIAs realizados nos estados de Minas Gerais e Pará, com foco em projetos minerários que apresentam potenciais impactos sobre a fauna subterrânea. Com base nos critérios propostos por Dias (2019), avaliamos a qualidade metodológica, a relevância ecológica dos dados coletados e as abordagens analíticas empregadas. Embora alguns estudos apresentassem descrições metodológicas satisfatórias, a maioria revelou deficiências nas análises ecológicas e na conexão entre os impactos e a fauna subterrânea. Além disso, enfrentamos limitações no acesso a documentos relevantes.

A insuficiência de informações sobre biodiversidade comprometeu as avaliações de impacto, resultando em descrições generalizadas sobre os efeitos dos empreendimentos na fauna subterrânea. Este estudo destaca as limitações identificadas nos AIAs analisados no que se refere à biospeleologia e propõe recomendações para apoiar a tomada de decisão baseada em evidências, aprimorar a qualidade dos dados sobre biodiversidade e aumentar a transparência no processo de AIA.

## Abstract

Environmental Impact Assessment (EIA) is a legal tool used to regulate the impacts of human development projects in various countries, including Brazil. However, the integration of biodiversity considerations in EIA processes has faced criticism due to the low quality of studies and the challenges in grounding decisions on robust scientific evidence. This issue is particularly concerning in speleology, where biospeleological studies are often scarce or absent from Environmental Impact Statement (EIS). This study analyzed 19 EIA associated with EISs conducted in the states of Minas Gerais and Pará, focusing on mining projects with potential impacts on subterranean fauna. Based on the criteria proposed by Dias (2019), we assessed the methodological quality, ecological relevance of the collected data, and the analytical approaches employed. While some studies demonstrated satisfactory methodological descriptions, most revealed deficiencies in ecological analyses and in connecting impacts to subterranean fauna. Additionally, we encountered limitations in accessing relevant documents. The insufficient information on biodiversity compromised impact assessments, leading to generalized descriptions of how developments affect subterranean fauna. This study highlights the limitations identified in the analyzed EIAs concerning biospeleology and proposes recommendations to support evidence-based decision-making, enhance the quality of biodiversity data and improve transparency in the EIA process.

## 1. Introdução

A preservação da biodiversidade é crucial para manter serviços ecossistêmicos vitais, como a regulação climática e a polinização, mas está ameaçada pelas atividades humanas, como agricultura, pecuária e mineração, que causam perda de habitats e declínio da biodiversidade (WWF, 2020). No Brasil, apesar de sua alta diversidade biológica, muitas espécies enfrentam risco de extinção devido a ações antrópicas (ICMBio, 2018). Nesse contexto, marcos legais, como a Política Nacional do Meio Ambiente, instituída na década de 1980 pela Lei Federal 6.938/81, e a Avaliação de Impacto Ambiental (AIA), foram criados para prevenir, mitigar e gerenciar impactos ambientais. A AIA foi introduzida nos Estados Unidos em 1969 é adotada globalmente, inclusive no Brasil. Atualmente, as AIA's são reconhecidas como uma ferramenta essencial para orientar decisões sobre a aprovação ou não de atividades de desenvolvimento propostas que podem causar danos aos recursos naturais (Sánchez, 2013). O principal objetivo das AIAs é identificar os potenciais efeitos negativos que um projeto a ser realizado pode causar ao meio ambiente, de modo a permitir que esses impactos sejam evitados ou mitigados durante o planejamento, a construção e a execução da atividade (Geneletti, 2002). O processo de AIA também se aplica ao patrimônio espeleológico e, dentro deste contexto, nos casos em que cavidades naturais subterrâneas podem ser alvo de impactos, a realização da AIA é exigida como parte do processo de licenciamento ambiental. No entanto, estudos destacam falhas recorrentes no processo, como levantamentos insuficientes e medidas mitigadoras limitadas, comprometendo a qualidade das decisões sobre licenças ambientais. Além disso, a identificação e mensuração dos impactos ambientais são subjetivos, o que gera desafios significativos para a aplicação eficaz desses instrumentos metodológicos no processo de tomada de decisão (SÁNCHES, 2013). Essas deficiências podem levar a decisões mal fundamentadas sobre a concessão de licenças ambientais comprometendo a integridade dos ecossistemas. Diante desse cenário, este estudo teve como objetivo analisar um conjunto de AIA realizadas no escopo de estudos espeleológicos, com foco específico na área de bioespeleologia, visando identificar limitações e oportunidades para aprimorar as práticas de avaliação.

## 2. Materiais e Métodos

**Área de estudo**

Considerando a alta correlação entre áreas destinadas à mineração e a de cavernas, a escolha das regiões para este estudo foi direcionada a locais com significativa atividade mineradora, já que mais de 40% das cavernas brasileiras possui alguma relação com este tipo de atividade (AMDA, 2024). A mineração no Brasil é uma atividade de interesse público, pelo seu papel na economia do país. Em 2024, a Produção Mineral Brasileira alcançou US$ 42 bilhões, como um dos principais motores da economia nacional (IBRAM, 2024). O estado de Minas Gerais, líder na atividade mineradora no Brasil, concentra 40 das 100 maiores minas do país. Localizado no centro-sul do estado, o Quadrilátero Ferrífero é uma das regiões de maior relevância para tal atividade, com extração de minerais como ouro, manganês, ferro, e que responde por aproximadamente 60% da produção nacional de minério de ferro. As reservas minerais do norte do Brasil também possuem grande diversidade de minerais, com destaque para a Serra de Carajás, no estado do Pará, que abriga a maior jazida de minério de ferro do mundo, sendo um ponto crucial para a produção mineral brasileira, especialmente de ferro. Portanto, as áreas selecionadas para este estudo são representativas de regiões com intensa exploração mineral e significativa ocorrência de cavernas, garantindo uma análise mais abrangente dos impactos dessa atividade sobre os ecossistemas subterrâneos.

No Brasil, atividades econômicas com potencial de causar impactos ambientais significativos devem passar por Avaliação de Impacto Ambiental (AIA), integrada ao Estudo de Impacto Ambiental (EIA), como requisito para a obtenção de licenças ambientais, cujo nível (federal, estadual ou municipal) depende da extensão dos impactos potenciais (SÁNCHEZ, 2013; CONAMA, 1997). O licenciamento para projetos de grande impacto ocorre em três etapas: Licença Prévia, Licença de Instalação e Licença de Operação (SÁNCHEZ, 2013). Os documentos de AIA são públicos por lei (CONAMA, 1997). Para este estudo, foram analisados processos de licenciamento ambiental disponíveis em plataformas públicas, como o site do IBAMA-MMA (https://licenciamento.ibama.gov.br/) para nível federal e o Sistema de Licenciamento Ambiental da SEMAD (https://ecosistemas.meioambiente.mg.gov.br/sla/#/acesso-visitante) para nível estadual. Os documentos aqui foram referenciados com a sigla «P» (Projeto) e numerados, de forma a preservar a confidencialidade das informações dos empreendimentos analisados. No nível federal, foi adotada uma abordagem sistemática para identificar projetos com potencial de impactar cavidades naturais subterrâneas A pesquisa utilizou palavras-chave relacionadas a espeleologia, como: cavidades, cavernas, espeleologia e bioespeleologia — tanto nos Estudos de Impacto Ambiental (EIA), como em outros tipos de documentos e analisou os relatórios encontrados integralmente. As informações relevantes foram organizadas em uma planilha-base para posterior análise. Foram considerados inconsistentes e, portanto, não incluídos na análise final, os relatórios que: (1) Abordavam a fauna cavernícola, mas indicavam que os estudos de bioespeleologia ainda estavam em processo de análise; (2) Mencionaram cavidades ou cavernas sem estabelecer uma relação direta com o contexto da avaliação de impacto ambiental referente a bioespeleologia e (3) Utilizavam termos relevantes, como ‹cavidades›, em contextos não relacionados ao tema central, como, por exemplo, ‹cavidades em árvores›. Relatórios com informações parcialmente relevantes foram sinalizados adequadamente para análise complementar, evitando a exclusão prematura de dados potencialmente úteis. A busca na plataforma do Sistema de Licenciamento Ambiental foi direcionada aos principais municípios que possuem atividades minerárias instaladas. Em parte dos processos encontrados os documentos possuíam o título “Avaliação dos impactos da atividade ou do empreendimento sobre o patrimônio espeleológico”, o que facilitou o acesso, quando o mesmo não ocorria, era necessário consultar a quase totalidade dos documentos existentes no processo. Diferentemente do que foi feito para o nível federal, aqui foram selecionados apenas os documentos específicos sobre Avaliação de Impacto Ambiental em cavidades, portanto, não foi necessário estabelecer critérios de exclusão. As buscas realizadas nas esferas federal e estadual apresentaram diferenças devido à variação entre os sistemas e termos de referência dos diferentes órgãos ambientais, o que exigiu métodos de busca distintos. Os dados obtidos também foram tabulados na planilha-base digital.

**Avaliação das informações sobre a bioespeleologia nos estudos analisados**

Avaliação da qualidade dos estudos: A análise dos dados seguiu a metodologia proposta por Dias (2019), com adaptações para o conjunto de dados utilizados. Avaliamos a qualidade dos estudos por meio de um conjunto de 19 critérios, em que os critérios de 1 a 13 estão relacionados ao desenho amostral, e os critérios de 14 a 21 são relacionados a relevância ecológica dos dados coletados e análises utilizadas:

1. O desenho amostral está vinculado às perguntas;
2. Pesquisas de campo foram conduzidas;
3. Cronograma de campo é relatado;
4. Unidades amostrais foram georreferenciadas;
5. O método de busca ativa foi empregado;
6. A aplicação dos métodos é descrita;
7. Apresenta justificativas da conservação das espécies e dos métodos empregados embasados na literatura;
8. O número de unidades de amostragem é relatado;
9. A distância entre as unidades de amostragem é relatada;
10. Tem mapa com a localização das cavidades;
11. Sazonalidade foi considerada;
12. Área de influência avaliada (250m ou AI proposta);
13. Dados secundários foram considerados;
14. Tem listas de espécies;
15. O status de conservação das espécies foi relatado;
16. A importância ecológica das espécies foi relatada e possui conexão com os impactos;

17. A relação ambiente-espécie foi identificada;

18. Conclusões e medidas de mitigação foram citadas;

19. Impacto genérico.

De acordo com Dias et al. (2019), esses critérios foram compilados com base em uma revisão das recomendações da literatura, juntamente com critérios extraídos de Termos de Referência (TRs). Para isso, adotamos a metodologia de geração de valores, que atribui uma pontuação para cada critério, sendo: Em relação à fauna: Pontuação 0 (Ausente): Nenhuma menção aos impactos sobre a fauna subterrânea; Pontuação 1 (Genérico): Houve menção a impactos sobre a fauna cavernícola, mas sem especificar quais espécies ou grupos seriam afetados; Pontuação 2 (Satisfatório): Houve uma explicação detalhada sobre a fauna potencialmente afetada pelo empreendimento. Em relação aos impactos: Avaliamos o nível de detalhamento apresentado nos relatórios sobre os tipos de impactos que os projetos poderiam causar à fauna cavernícola utilizando a mesma escala de pontos (Tabela 1). Pontuação 0 (Ausente): Uma lista de impactos foi apresentada, mas sem qualquer descrição referente à fauna cavernícola; Pontuação 1 (Genérico): As descrições dos impactos foram apresentadas, mas não estabelecem conexão entre as descrições dos impactos e as atividades do empreendimento; Pontuação 2 (Satisfatório): As descrições indicaram como a fauna cavernícola seria impactada, conectando as descrições dos impactos às atividades do empreendimento. Essa abordagem permitiu avaliar não apenas se os impactos eram mencionados, mas também se eram explicados de forma clara e com a profundidade adequada, evidenciando as conexões entre as atividades do projeto e os potenciais efeitos sobre a fauna cavernícola. Essas pontuações foram então utilizadas para gerar um valor para cada estudo analisado. Dessa forma, relatórios com pontuações mais altas indicaram um nível satisfatório de detalhamento, tanto na identificação das espécies/grupos afetados, quanto na descrição dos mecanismos pelos quais os impactos ocorreriam. Por outro lado, pontuações mais baixas refletem deficiências na clareza das atividades do empreendimento, com ausência ou baixa conexão entre os impactos descritos e as medidas mitigadoras propostas. Assim, os relatórios que atingiram valores acima de 70% foram considerados satisfatórios, permitindo uma visão mais rica e detalhada dos dados CRESWELL (2017).

## 3. Resultados

No total, foram analisados 19 estudos relacionados a processos de licenciamento ambiental, dos quais 20 são de âmbito federal e 11 de âmbito estadual, sendo 12 referentes a Minas Gerais e sete ao Pará. No entanto, dentre os projetos federais, referentes aos estados de Minas Gerais e Pará, 12 foram excluídos por: (1) Abordar a fauna cavernícola, porém, os estudos de bioespeleologia ainda estavam em processo de análise (n=3); (2) Mencionaram cavidades ou cavernas sem estabelecer uma relação direta com o contexto da avaliação de impacto ambiental referente a bioespeleologia (n=5) e utilizavam termos relevantes, como ‘cavidades’, em contextos não relacionados ao tema central (n=4). No âmbito estadual, foram analisados 11 estudos espeleológicos, totalizando 19 estudos considerados na análise. As principais atividades identificadas incluíram extração e beneficiamento de minério de ferro, pilhas de rejeitos e minerodutos. Os principais impactos na fauna foram perda de habitat, afugentamento e efeito de borda. Dos 19 estudos, apenas 3 (16%) tiveram pontuação acima de 70% (26 pontos) (Figura 1).

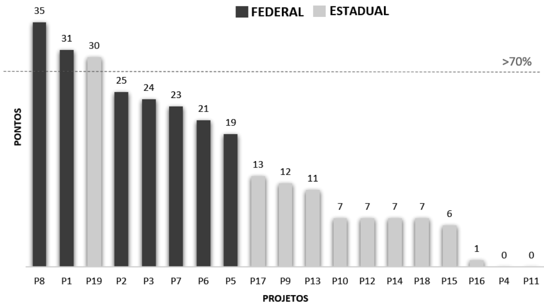

***Figura 1:*** *Gráfico com a Pontuação total de cada projeto analisado.*

Os critérios que obtiveram maior pontuação estão relacionados ao desenho amostral, enquanto aqueles relacionados às informações sobre a fauna subterrânea apresentaram pontuação inferior. Estas informações estão detalhadas na Tabela 1.

## 4. Discussão

### Sub Avaliação da qualidade dos estudos

A maioria das AIA’s apresentou um detalhamento genérico, abaixo de 70% quanto à abrangência dos impactos sobre a fauna cavernícola, e apenas três relatórios atingiram pontuação acima de 70% (Gráfico 1). Estes resultados sugerem que as avaliações de impacto ambiental, quanto a fauna associada às cavidades, tendem a ter limitações generalizadas, bem como corroboram as preocupações de diversos autores que destacam que avaliações deficientes podem comprometer significativamente a acurácia das previsões dos impactos (TREWEEK et al. 1993; MANDELIK et al. 2005; DIAS, 2019). Uma previsão adequada dos impactos sobre a biodiversidade exige informações robustas e bem fundamentadas. Caso contrário, interpretações equivocadas podem resultar em falhas no suporte a decisões de preservação e conservação, aumentando os riscos para a fauna ao orientar ações inadequadas (DIAS, 2019). Entre as duas categorias de critérios analisados, os relacionados ao desenho amostral obtiveram as maiores pontuações (Tabela 2). Como é possível constatar em nossos resultados, os critérios relacionados à fauna cavernícola tiveram pontuações baixas (Tabela 2), bem como é possível observar que critérios como a relevância ecológica das espécies e sua associação com os impactos, o reconhecimento da relação entre o ambiente e as espécies, a identificação dos status de ameaça das espécies e a inclusão de listas completas de espécies. Muitos estudos não apresentam dados sobre a fauna cavernícola e, quando o fazem, apresentam somente os vertebrados, especialmente morcegos, anuros e serpentes — sendo os dois últimos frequentemente classificados como fauna acidental (TRAJANO & MARCELO, 2017; CULVER & PIPAN, 2019). Os morcegos possuem importância ecológica amplamente reconhecida, desempenhando funções essenciais como polinização, dispersão de sementes e controle natural de populações de insetos. Além disso, sua presença nas cavernas contribui significativamente para a dinâmica desses ecossistemas, fornecendo recursos tróficos, como guano e restos orgânicos, fundamentais para a base alimentar dos organismos subterrâneos (SAKOUI et al. 2020). Entretanto, os invertebrados também desempenham papéis cruciais na manutenção dos ecossistemas cavernícolas, sendo os principais componentes das comunidades faunísticas nesses ambientes. A escassez de dados sobre esses organismos é particularmente preocupante, considerando também sua importância ecológica, como na decomposição da matéria orgânica, no controle populacional e na sustentação das cadeias alimentares subterrâneas. Além disso, os invertebrados cavernícolas são potenciais bioindicadores ambientais, refletindo a saúde dos ecossistemas subterrâneos e de suas áreas adjacentes (MAMMOLA et al. 2019). A coleta de dados sobre esses organismos é essencial não apenas para preencher lacunas no conhecimento científico, mas também para subsidiar importantes medidas de conservação, como a definição de áreas de influência ambiental, conforme estabelecido na Instrução de Serviço SISEMA 08/2017. Esse normativo regula os procedimentos para a instrução de processos de licenciamento ambiental de empreendimentos com potencial de impacto sobre cavidades naturais subterrâneas e suas áreas de influência, destacando a necessidade de avaliações mais abrangentes e criteriosas da fauna cavernícola. Em relação aos critérios associados ao desenho amostral, alguns obtiveram pontuações elevadas, em alinhamento com as diretrizes do TR de espeleologia da Secretaria de Estado de Meio Ambiente e Desenvolvimento Sustentável – SEMAD – do estado de Minas Gerais (Instrução de Serviço Sisema 08/2017), que ressaltam a importância de metodologias bem definidas para assegurar a precisão, confiabilidade e comparabilidade dos dados. A adoção de métodos rigorosos, como coletas em diferentes sazonalidades, é crucial, pois as variações temporais podem influenciar os ecossistemas cavernícolas, alterando a composição da fauna em diferentes épocas do ano. Um cronograma de pesquisa de campo bem estruturado garante que as atividades sejam realizadas de maneira organizada e dentro dos prazos adequados, permitindo o acompanhamento contínuo e o planejamento eficaz de cada etapa. A utilização de métodos como a busca ativa permite mapear a fauna cavernícola, compreender suas dinâmicas e interações (FERREIRA, 2005; HUNT & MILLAR 2001). Por fim, a descrição clara dos critérios metodológicos utilizados é essencial para garantir a transparência dos processos, promovendo a confiabilidade dos resultados e facilitando a replicação dos estudos. Além disso, permite a avaliação da necessidade de ajustes no desenho amostral, conforme os objetivos do estudo (MYERS et al. 2000; CULVER & SKET 2000). Apesar dos critérios metodológicos básicos, como as unidades amostrais foram georreferenciadas com mapas, pesquisa de campos, consideração da sazonalidade e a adequação do cronograma, terem obtido pontuações superiores (Tabela 2), outras dimensões metodológicas, de maior complexidade e igualmente essenciais, apresentaram desempenho inferior. Alguns exemplos marcantes estão relacionados ao desenho amostral, onde parte dos critérios obteve uma baixa pontuação, como a relação entre o desenho amostral vinculado às perguntas e conclusões e se medidas de mitigação foram citadas.

***Tabela 1:** Pontuações obtidas para cada critério.*

| CATEGORIAS | N | CRITÉRIOS | TOTAL DE PONTOS |
|---|---|---|---|
| **Desenho amostral** | 1 | Unidades amostrais foram georreferenciadas | 28 |
| | 2 | Tem mapa com a localização das cavidades | 26 |
| | 3 | Área da influência avaliada (250m ou AI proposta) | 25 |
| | 4 | Impacto genérico | 24 |
| | 5 | O número de unidades de amostragem é relatado | 21 |
| | 6 | Pesquisas de campo foram conduzidas | 17 |
| | 7 | Cronograma de pesquisa de campo é relatado | 17 |
| | 9 | O método de busca ativa foi empregado | 17 |
| | 10 | Sazonalidade foi considerada | 14 |
| | 11 | Dados secundários foram considerados | 14 |
| | 12 | A aplicação dos métodos é descrita | 13 |
| | 13 | Conclusões e medidas de mitigação foram citadas | 13 |
| **Relevância ecológica dos dados coletados e análises utilizadas** | 14 | A distância entre as unidades de amostragem é relatada | 9 |
| | 15 | Tem lista de espécies | 8 |
| | 16 | Apresenta justificativas da conservação das espécies e dos métodos empregados embasados na literatura | 8 |
| | 17 | A importância ecológica das espécies foi relatada e possui conexão com os impactos | 7 |
| | 18 | O desenho amostral está vinculado às perguntas | 6 |
| | 19 | A relação ambiente-espécies foi identificada | 5 |
| | 20 | Os status de conservação das espécies foram relatados | 4 |

O emprego de um desenho amostral adequadamente estruturado e robusto, permite a coleta de dados de forma sistemática e minimiza erros e interpretações ambíguas, assim como viabiliza a identificação e mensuração objetiva dos impactos ambientais sobre os ecossistemas cavernícolas. A tomada de decisões embasadas, assegura a implementação de medidas de mitigação efetivas e o cumprimento das normas legais voltadas à proteção e conservação do patrimônio natural e científico associado às cavernas (BARKER & WOOD, 1999; MPU, 2004; GONTIER et al., 2006; SODERMAN, 2006). É provável que os critérios que não atingiram pontuações superiores a 70% estejam relacionados a restrições financeiras, à limitação de tempo, à falta de mão de obra especializada e falta de informações sobre as espécies (COSCARON et al, 2009; DIAS 2004; BRAGAGNOLO & PINTO DA ROCHA, 2003). Apesar dessas limitações, alguns estudos recorreram ao uso de registros secundários de espécies, uma abordagem válida que complementa os dados primários, ampliando o banco de informações disponíveis e permitindo uma representação mais fiel da realidade local, além de possibilitar comparações espaciais e temporais. Adicionalmente, vale destacar que os estudos mais antigos foram os que obtiveram as pontuações mais baixas, o que evidencia que, ao longo do tempo, a qualidade das pesquisas tem melhorado, refletindo avanços nas metodologias, a atuação de profissionais especializados e o maior aperfeiçoamento na abordagem dos impactos ambientais em cavernas. Foram empenhados esforços para identificar o maior número possível de estudos usando os repositórios online, contudo, apesar de os documentos relacionados ao licenciamento ambiental serem públicos (CONAMA, 1997), a organização e a acessibilidade destes apresentaram desafios consideráveis (FERNÁNDEZ et al., 2018; DIAS et al., 2019). Dessa maneira, esses problemas provavelmente demonstram que houve projetos relevantes que não puderam ser incluídos em nosso estudo. Cabe destacar que não esgotamos as possibilidades de pesquisas em tais repositórios, portanto, é possível ampliar o n amostral em estudos vindouros. Ademais, parte dos processos encontrados no Sistema de Licenciamento Ambiental (SEMAD-MG) continham documentos que estavam indisponíveis em decorrência de problemas técnicos do próprio site. Os dados sobre as AIA's em cavidades obtidos junto ao IBAMA, foram majoritariamente aqueles contidos nos Estudos de Impacto Ambiental/ Relatório de Impacto Ambiental (EIA/RIMA), com apenas um Plano de Conservação Ambiental (PCA), ao passo que as informações adquiridas junto ao SLA-SEMAD vieram de estudos específicos sobre AIA em cavidades. Em ambos os casos, os estudos sobre espeleologia são apresentados, preponderantemente, separados do EIA/RIMA e, principalmente, os dados destes estudos não são correlacionados. Destaca-se que as cavidades não estão apartadas do restante da paisagem onde se inserem, ao contrário, o entorno é fundamental para a manutenção dos processos ambientais e do ecossistema cavernícola. Contudo, ainda que a especificidades dos ecossistemas cavernícolas tragam a necessidade de tratá-los com particularidade, se faz sobremaneira importante a integração destes dados, contribuindo inclusive para a melhor organização e acesso aos dados, uma vez que foram encontradas dificuldades para localizar documentos nas plataformas de busca, mesmo quando citados no texto principal. Outra limitação identificada foi a abordagem restrita de alguns relatórios, que apresentaram dados limitados ao patrimônio espeleológico geológico e arqueológico. Informações sobre a fauna subterrânea encontravam-se em estágio inicial de análise ou eram incompletas, dificultando uma avaliação abrangente desse componente. Foi observado que muitos estudos sugerem o monitoramento como uma medida mitigadora, todavia, ele por si só não constitui uma ação mitigatória, mas sim uma ferramenta para avaliar a eficácia das medidas mitigadoras implementadas, verificar o cumprimento das previsões de impacto e acompanhar a evolução das condições ambientais ao longo do tempo. Assim, destaca-se a necessidade de uma abordagem mais integrada e abrangente nos estudos de impacto ambiental que envolvem cavernas, o que não se restringe a incorporação das informações espeleológicas no corpo principal dos EIA, mas também o aprofundamento nos dados sobre a fauna, especialmente os invertebrados. Tais medidas contribuem para uma compreensão mais completa e eficaz dos impactos ambientais e à formulação de planos de conservação mais robustos, promovendo a proteção desses ecossistemas subterrâneos tão singulares e relevantes. É importante reconhecer que avaliamos os documentos assumindo que a qualidade dos relatórios representa a qualidade dos estudos e avaliações em si. Além disso, a análise qualitativa que usamos para avaliar a qualidade dos estudos e a abrangência das previsões de impacto foi influenciada pelas categorias que escolhemos avaliar (Tabela 1). Nossa abordagem considerou um conjunto de elementos para fornecer uma visão importante sobre os componentes incluídos nos relatórios de impacto ambiental e destaca os elementos essenciais que devem ser levados em conta na elaboração de um relatório de avaliação robusto e transparente.

## 5. Conclusão

A integração dos estudos de espeleologia e do Estudo de Impacto Ambiental, tanto analiticamente como estruturalmente (no mesmo documento) é o principal apontamento, pois como foi arguido, permite uma avaliação mais consistente dos processos ambientais. Os dados de geoespeleologia e de biologia subterrânea deveriam integrar os diagnósticos do meio físico e biótico, respectivamente, que são tópicos obrigatórios na estruturação dos Estudos de Impacto Ambiental. A implementação de uma base de dados nos órgãos responsáveis pelos licenciamentos, com a disponibilização integral dos documentos de forma acessível, seria um repositório fundamental de dados secundários para todos os futuros processos de licenciamento. A construção de parcerias com instituições de pesquisas e as empresas de consultoria e/ou empreendedores, para o fomento de estudos direcionados às incertezas e subjetividades que orbitam os estudos ambientais nos mais diferentes setores.

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# Two new troglobitic species of the genus *Spelaeogammarus* da Silva Brum, 1975 (Amphipoda, Artesiidae) from Brazil

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## Abstract

Recently, two new subterranean species of the genus Spelaeogammarus da Silva Brum, 1975 were found from two distinct regions in Brazil: Serra do Ramalho, Bahia, and Montes Claros, Minas Gerais, increasing the total number of known species in the genus to 10. Both species present all diagnostic characters of the genus, however Spelaeogammarus sp. nov. 2 has some unique distinctive characters such as the accessory flagellum 3-articulate and the antenna 2 flagellum 5-articulate, as well as Spelaeogammarus. sp. nov. 1. that distinguishes by the antenna 2 flagellum 5-articulate and by the absence of plumose setae on apical margin of inner plate of maxilliped.

## 1. Introduction

The order Amphipoda stands out among Brazilian subterranean crustaceans, with species distributed across five distinct families. The genus *Spelaeogammarus* da Silva Brum, 1975, part of the family Artesiidae, is composed entirely of aquatic amphipods species exclusively adapted to subterranean environments (Bastos-Pereira & Ferreira, 2017; Bueno et al., 2022).

To date, eight species have been described within the genus. Seven are endemic to caves in Bahia: *Spelaeogammarus bahiensis* da Silva Brum, 1975 (type species), *S. spinilacertus* Koenemann & Holsinger, 2000, *S. trajanoae* Koenemann & Holsinger, 2000, *S. santanensis* Koenemann & Holsinger, 2000, *S. titan* Senna et al., 2014, *S. sanctus* Bastos-Pereira & Ferreira, 2015, and *S. ginae* Bueno et al., 2022. Until 2017, the genus was believed to be exclusive to Bahia, but the discovery of *S. uai* Bastos-Pereira & Ferreira, 2017 in Minas Gerais expanded its known range (Figures 1 and 4).

Recent expeditions to western Bahia (Serra do Ramalho) and northern Minas Gerais (Montes Claros) have revealed two additional species of *Spelaeogammarus*. This study describes these new species, provides an updated identification key and morphological comparison table for the genus, and revises its diagnosis. Additionally, considerations on the habitat and conservation threats to the newly discovered species are discussed.

## 2. Materials and methods

**Study Area**

The species described were discovered in two distinct karst regions formed by limestone from the São Francisco Carbonate Supergroup, specifically the Bambuí Group. The Serra do Ramalho karst system (Bahia) (Figure 2) spans approximately 12,000 km², located between the Carinhanha and Corrente Rivers, tributaries of the São Francisco. It features karst landscapes with dolines, closed basins, limestone towers, and underground rivers, with recharge occurring through polygonal depressions and diffuse infiltration. The eastern margin is notable for striking cliffs and well-developed epikarst areas. The Montes Claros region (Minas Gerais) (Figure 3), also within the São Francisco River basin, has a tropical semi-arid climate with Cerrado vegetation, including deciduous and semideciduous forests. It ranges in altitude from 600 to 1,000 meters and is characterized by rugged terrain, prominent limestone outcrops, and diverse karst formations.

**Field sampling and laboratory**

Specimens were collected using a hand net and preserved in vials containing 70% ethanol. In the laboratory, they were measured and photographed using a ZEISS Axio Zoom V16 stereomicroscope with an Axio Cam 506 color camera. The analyzed specimens were stained with Bengal rose dye, dissected, and mounted on slides with Hoyer's medium. Drawings were created based on the holotype, with final illustrations produced using GIMP software (v. 2.10) and a Wacom Cintiq drawing pad (Montesanto 2015, 2016). One paratype of *Spelaeogammarus* **sp. nov. 2** was dissected and photographed under an SEM. Additional material was deposited in the Collection of Subterranean Invertebrates at the Federal University of Lavras (ISLA/UFLA).

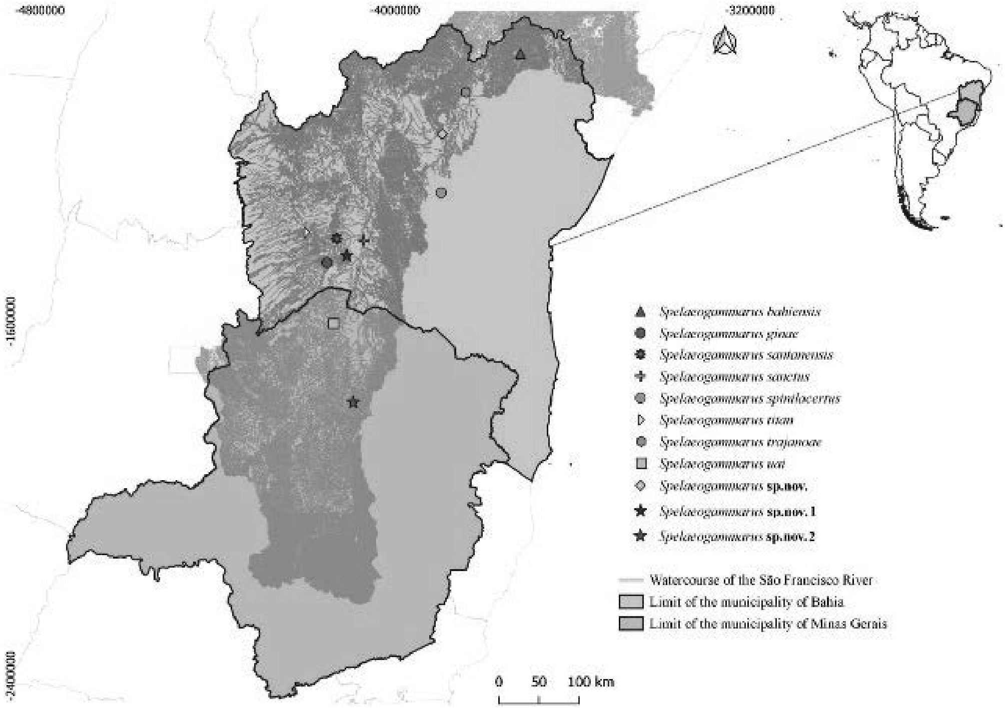

***Figure 1:*** *Map showing the distribution of Spelaeogammarus species in Brazil.*

## 3. Results

*Spelaeogammarus* **sp. nov. 1** (Figure 2) is characterized by the flagellum of the antenna 1 with 10 articles; accessory flagellum 4 articulate; maxilla 1 outer lobe apical margin with 7 simple robust setae; maxilliped inner plate apical margin without plumose setae; gnathopod 1 basis anterior margin with 2 small setae, posterior margin bearing 2 long setae; gnathopod 2 posterior margin with 3 long setae; uropod 3 outer ramus bearing 7 dorsal bifid setae; telson with 1 apical and 3 subapical stout setae.

*Spelaeogammarus* **sp. nov. 2** (Figure 3) is distinguished by the accessory flagellum 3-articulate and by the absence of bifid setae on outer ramus of uropod 3. It is also characterized by the antenna 1 flagellum with 11 articles; the antenna 2 flagellum 5-articulate; maxilla 1 outer lobe apical margin with 6 simple robust setae and 1 plumose setae; maxilliped inner plate apical margin with 3 plumose setae; gnathopod 1 basis anterior margin with 4 small setae, posterior margin with 4 simple long setae; gnathopod 2 posterior margin with 6 long setae; telson with 1 apical and 2 subapical stout setae.

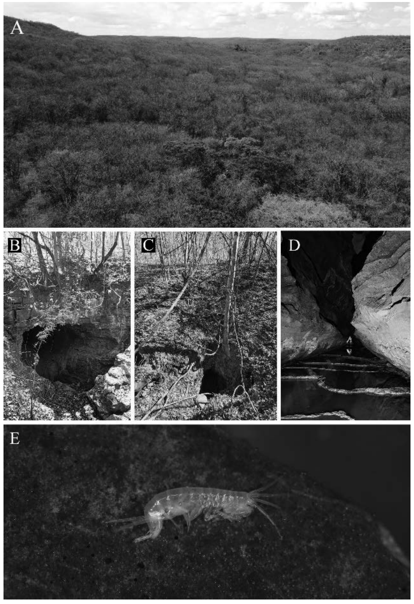

***Figure 2 :*** *A) Overview of the external karst area of Serra do Ramalho, Bahia; B, C) cave entrance of Spelaeogammarus* ***sp. nov. 1****; D) Inner section of the cave; E) Spelaeogammarus* ***sp. nov. 1*** *living specimens.*

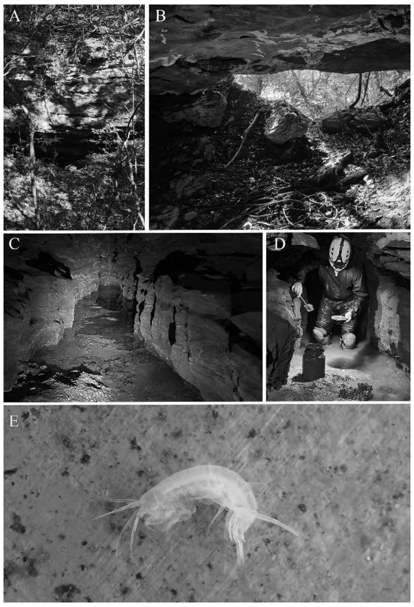

***Figure 3:*** *A,B) Cave's entrance of Spelaeogammarus* ***sp. nov. 2****; C) Inner section of the cave, showing the ponds where specimens were found; D) collection of specimens using a hand net; E) Spelaeogammarus sp. nov. 2 living specimens.*

## 4. Discussion

*Spelaeogammarus* **sp. nov. 1** share morphological similarity with *S. spinilacertus, S. bahiensis* and *Spelaeogammarus*. **sp. nov. 2.** These species share common features such as the number of articles in the accessory flagellum of antenna 1, number of setae on the apical margin of the outer plate of maxilla 1, absence of plumose setae on the apical margin on the inner plate of maxilliped and number of setae on coxa 6.

*Spelaeogammarus*. **sp. nov. 2** is similar to *S. spinilacertus, S. bahiensis* and *Spelaeogammarus*. **sp. nov.** by the 4-articulate accessory flagellum. It resembles *S. santanensis, S bahiensis, S uai* and *S. sanctus* by 7 multi-cuspidate stout setae on the outer plate of maxilla 1. Resembles S. bahiensis and Spelaeogammarus. sp. nov. by the absence of plumose setae on the inner plate of maxilliped. It resembles *S. spinilacertus, S. trajanoae* and *Spelaeogammarus*. **sp. nov.** by 1 stout setae on coxa 6. Finally, by 1 apical + 3 subapical stout setae per lobe on telson it bears a resemblance to *S. uai* and *S. titan*.

*Spelaeogammarus* **sp. nov. 2** resembles *S. sanctus* by 1 apical + 2 subapical stout setae per lobe on telson. By the number of plumose setae on the apical margin of the inner plate of maxilliped it resembles *S. uai* and *S. ginae*. With 4 small setae on anterior margin of gnathopod 1 basis it resembles *S. ginae* and *S. santanensis*. Finally, it resembles *Spelaeogammarus* **sp. nov.**, *S. spinilacertus* and *S. trajanoae* by 1 stout seta on coxa 6.

Although *Spelaeogammarus* **sp. nov. 2** shares similarities with *Spelaeogammarus* **sp. nov. 1**, such as 5-articulate antenna 2 flagellum and 1 stout seta on coxa 6, it is characterized by being the first species of the genus that presents a 3-articulate accessory flagellum and by the absence of bifid setae on the dorsal margin of the uropod 3 outer ramus.

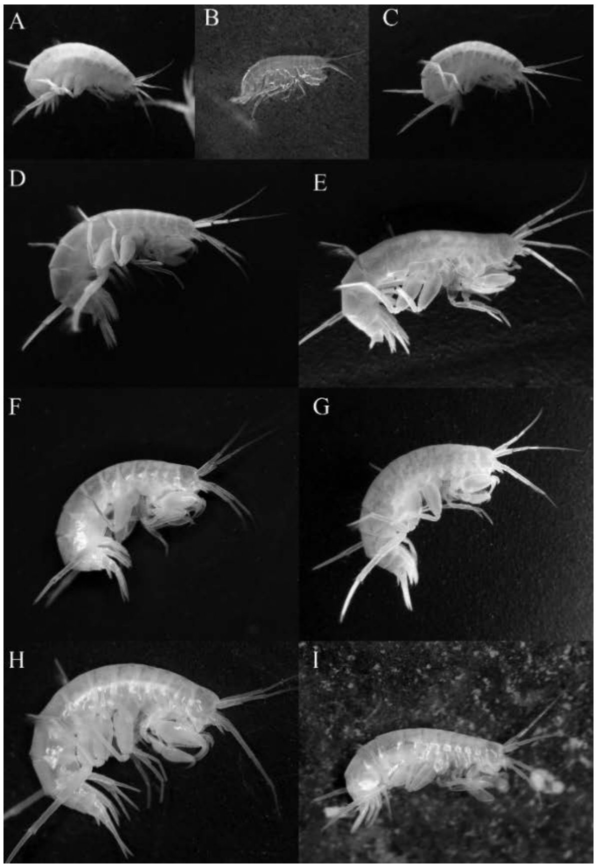

***Figure 3:*** *A,B) Cave's entrance of Spelaeogammarus sp. nov. 2; C) Inner section of the cave, showing the ponds where specimens were found; D) collection of specimens using a hand net; E) Spelaeogammarus sp. nov. 2 living specimens.*

## 5. Conclusion

The increasing number of new described species within the genus *Spelaeogammarus* highlights the vast diversity that remains unexplored. With the recent discovery of a second species from Minas Gerais, the potential for expanding the known distribution of this genus has become increasingly evident.

The state of Bahia, in particular, stands out for its exceptional subterranean biodiversity, as reflected in the number of species described from the region. Within the Serra do Ramalho karst system, two major hotspots of subterranean biodiversity have been identified: ACCS and the Pedro Cassiano Cave (Ferreira et al., 2023; Vaz et al., 2024), which are located in close proximity

The discovery of these two new species significantly enhances our understanding of the genus' distribution and underscores the importance of further exploratory efforts. Notably, the discovery of new species associated with the São Francisco River Basin suggests that their distribution may be closely linked to the basin's boundaries. Ongoing research in these karstic regions is likely to reveal additional species and provide deeper insights into the ecological dynamics of subterranean environments, emphasizing the urgent need for conservation initiatives to protect these unique ecosystems.

## Acknowledgments

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# Caves as a crucial refuge: large colony of the endangered species *Natalus macrourus* (Gervais, 1856) in an anthropized landscape

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## Abstract

*Natalus macrourus* (GERVAIS, 1856), a bat species of the family Natalidae, is classified as “Vulnerable” in Brazil and is considered a cave-restricted species. Its endangered status is primarily attributed to habitat destruction and cave degradation, which are critical threats to its survival. Records of this species in southeastern Brazil are sparse and sporadic. We report the discovery of a large colony in Taboa Cave, Sete Lagoas, Minas Gerais, in an anthropized area. Using non-invasive methods, we documented and estimated the population at approximately 100 to 120 individuals, which probably represents the largest recorded population of *N. macrourus* in southeastern Brazil to date. The identification of such a significant, previously unrecorded colony in close proximity to urbanized areas highlights the importance of caves as a crucial refuge and the urgent need for conservation policies aimed at protecting this endangered species.

## 1. Introduction

Habitat loss is a well-recognized factor strongly associated with the risk of species extinction in their natural environments (FAHRIG 2003). In caves and other subterranean habitats, this decline is often linked to intense anthropogenic pressures leading to strongly negative effects on bat communities (GUIMARÃES & FERREIRA 2014, DELGADO-JARAMILLO et al. 2017). Caves are important roosts for many bat species, offering protection and suitable conditions for roosting and reproduction (KUNZ 1982). Among these, the insectivorous *Natalus macrourus* (Gervais, 1856) stands out for its strong dependence on cave environments. This species is distributed south of the Amazon River, spanning Bolivia, Brazil, and Paraguay (TEJEDOR 2011, GARBINO & TEJEDOR 2012). Globally, it is classified as “Near Threatened” by the International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species (TEJEDOR & DAVALOS 2016), while in Brazil, it holds the status of “Vulnerable” due to population decline linked to habitat degradation (BRASIL/ICMBio 2024, DELGADO-JARAMILLO et al. 2017).

The reliance of *N. macrourus* on cave ecosystems underscores its dependence on these environments for shelter, reproduction, and protection, making it particularly vulnerable to habitat destruction and ecosystem degradation (GUIMARÃES & FERREIRA 2014, DELGADO-JARAMILLO et al. 2017, BARROS & BERNARD, 2023). Habitat loss and cave degradation pose significant threats to the species, severely affecting its distribution and abundance (BARROS & BERNARD 2023a). Despite its conservation status, knowledge of the species distribution within Brazil remains limited, with notable spatial and temporal gaps in occurrence records. These data deficiencies complicate conservation planning and hinder a deeper understanding of the population ecology of *N. macrourus* (DELGADO-JARAMILLO et al. 2017).

In southeastern Brazil, *N. macrourus* has been reported in the states of Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo. However, these records are infrequent and scattered, and no large colonies (>100 individuals) have been documented in the literature for this region. This lack of information impedes the development of effective conservation strategies for the species.

In this study, we report the discovery of a large colony of *N. macrourus* in southeastern Brazil, specifically in a cave in Minas Gerais. We provide a detailed description of the species’ habitat and the surrounding landscape, offering valuable insights to guide future conservation efforts.

## 2. Materials and methods

**Study Area**

Taboa cave is located in the municipality of Sete Lagoas, Minas Gerais, Brazil (19°28’30.0”S, 44°19’41.7”W) (Figure 1). The cave extends approximately 1.000 meters and is geologically situated within the southern São Francisco Craton, part of the Neoproterozoic sedimentary sequence of the Bambuí Group (ALKMIM & MARTINS-NETO 2001). The natural vegetation in the area consists of deciduous tropical forests growing on carbonate outcrops and well-developed, fertile soils. The surrounding landscape is a mosaic of pastures, agricultural fields, forest fragments, lakes, and carbonate rock outcrops.

The cave environment is characterized by moisture gradients. Its small entrance (1.5 meters high by 2 meters wide) restricts light penetration and helps maintain a stable microclimate. A subterranean stream flows through the distal sections of the cave, contributing to consistently high humidity levels (FERREIRA et al. 2015).

Biologically, the cave is notable for its endemic troglobitic fauna, which includes invertebrate species such as arachnids (*Tisentnops onix* BRESCOVIT & SANCHEZ-RUIZ 2016; *Charinus taboa* VASCONCELOS, GIUPPONI & FERREIRA 2016), isopods (*Spelunconiscus septemlacuum* BASTOS-PEREIRA et al. 2022), and diplopods (*Pseudonannolene taboa* INIESTA & FERREIRA 2014).

Despite its ecological significance, Taboa Cave is located outside any protected area. However, it is near a state conservation unit the Monumento Natural Estadual Gruta Rei do Mato, which protects the important showcave Gruta Rei do Mato.

**Data Collection**

The colony was first identified during a visual inspection of the cave on October 16 and 17, 2024. Subsequently, a monitoring station was set up at the cave's main entrance to observe nocturnal activity without disrupting the roosting site, thereby confirming the species' presence. Photographic records were used to confirm the species identification and video recordings of the bat emergence were used to estimate the population size, employing a non-invasive approach that safeguarded the species' natural behaviors and minimized disturbance to their habitat (Figure 2).

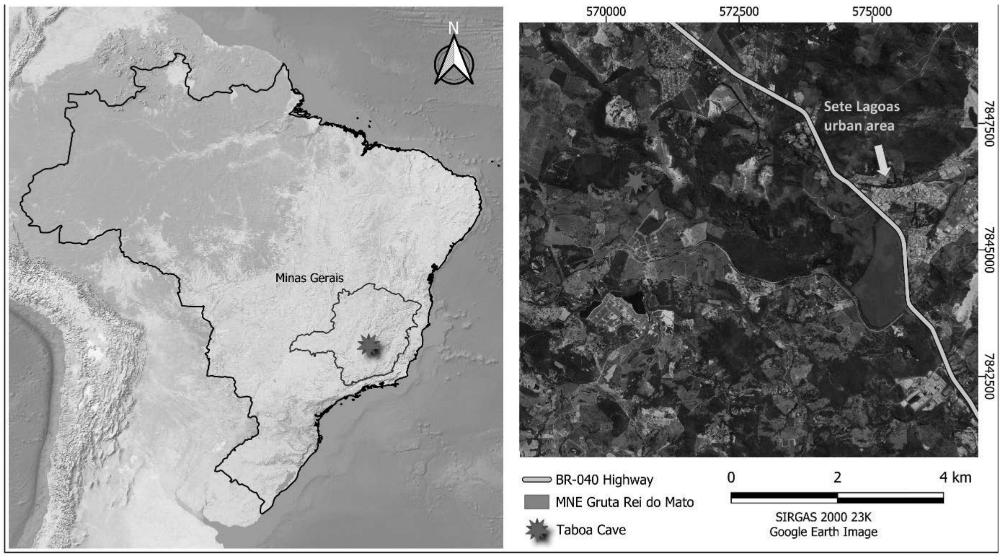

***Figure 1:*** *Map showing the location of Taboa Cave.*

## 3. Results

A noninvasive survey confirmed the presence of a large colony of *N. macrourus* in Taboa Cave, Sete Lagoas, Minas Gerais. The population size was estimated between 100 and 120 individuals, making this the largest known colony of the species in southeastern Brazil. The colony was located at the end of the cave near a watercourse.

In addition to the endangered species, six other bat species were registered: *Desmodus rotundus, Diphylla ecaudata, Mimon bennettii, Carollia perspicillata, Platyrrhinus lineatus*, and *Glossophaga soricina*, all from the Phyllostomidae family. Taboa cave is considered a high-richness cave, according to GUIMARÃES & FERREIRA (2014).

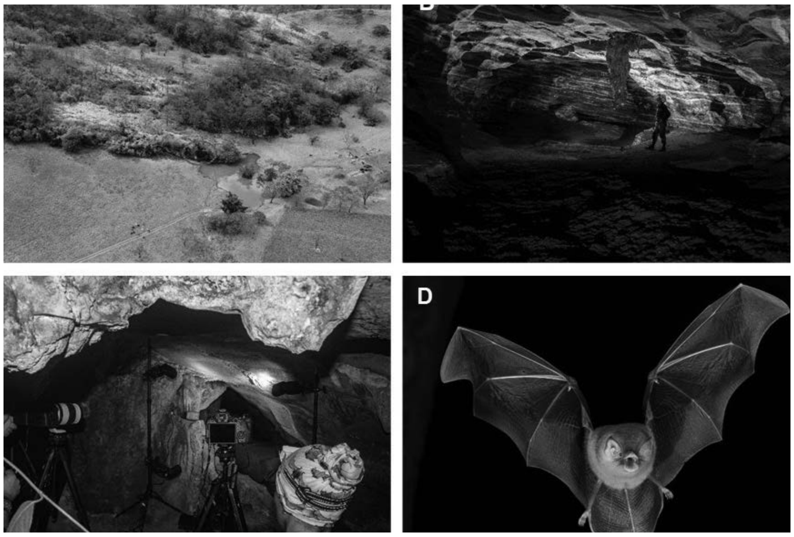

***Figure 2:*** *A) Detail of the location of the main entrance to the Taboa Cave (red circle) in the landscape; B) Detail of the main conduit inside the cave; C) Detail of the passive sampling station for photographic records installed at the main entrance of the cave; D) Photographic record of an individual of N. macrourus in flight.*

## 4. Discussion

The discovery of a significant *N. macrourus* colony in Taboa cave, Sete Lagoas, Minas Gerais, highlights the critical role of this cave in the conservation of the species. With an estimated population of 100 to 120 individuals, this colony represents the largest recorded group of N. macrourus in southeastern Brazil. This finding contrasts with previous records for the region, where large colonies were not documented. Although colonies of *N. macrourus* are known to exist within the Alto Ribeira Tourist State Park (PETAR, SP), the only available documentation currently reports 18 individuals observed (ARNONE 2008 unpublished). While there is potential for larger colonies in the region, as indicated by the cave environments of PETAR, no such larger populations have been formally reported to date. Similarly, 11 individuals have been recorded in the Peruaçu Caves National Park (Itacarambi, MG) (TRAJANO & GIMENEZ 1998), further suggesting that, although these regions may offer suitable habitats, large colonies of *N. macrourus* have yet to be observed. This emphasizes the exceptional conditions of Taboa Cave, which may provide a uniquely favorable habitat for *N. macrourus* in the region.

The different bat species have specific requirements to select their caves as ideal roosts; this can be related to microclimatic conditions, the cave's physical characteristics, and also with the surrounding landscape (BARROS et al. 2020). *Natalus macrourus* is usually associated with the most humid and warm parts of the caves (TORRES-FLORES & LÓPEZ-WILCHIS 2010). The species' presence was also related to the size of caves in the Brazilian Caatinga, where their colonies were registered only in larger caves, bigger than 200 meters (VARGAS-MENA et al. 2020). Larger caves with small entrances, like Taboa cave, present a high stability index, which helps the maintenance of ideal microclimatic conditions (FERREIRA 2004) for species like *N.macrourus* (BARROS & BERNARD 2023b). That way, both the size and the presence of water inside Taboa cave seem to provide the ideal internal requirements for the establishment of the large colony of *N. macrourus*. External habitat quality directly influences the cave's internal conditions, such as temperature and humidity, which are vital for the survival of cave-dependent species. The landscape in the cave surroundings can be a determinant factor in the cave's bat richness, composition, and abundance (BARROS et al. 2020, VARGAS-MENA et al. 2020). Studies comparing cave's bat assemblage and abundance in degraded areas and preserved areas showed that, in general, hematophagous bat species are more associated with agricultural areas, with bigger colonies where the livestock availability is higher, while insectivorous, frugivorous, nectarivorous, and animalivorous bat species are associated with more preserved areas (BARROS et al. 2020, VARGAS-MENA et al. 2020, CAJAIBA et al. 2021). Despite that, in highly degraded landscapes, caves can play an important role as crucial refuges for trogloxenes species, like bats (FRAGA et al. 2023), even allowing the presence and maintenance of large colonies of sensitive species like *N. macrourus*, as seems to be the case in Taboa cave. Moreover, the cohabitation of *N. macrourus* with other bat species underscores the ecological importance of Taboa cave as a multi-species refuge. This cohabitation may reflect the availability of diverse resources within the cave or complementary habitat requirements among species, further emphasizing the need to preserve such critical environments.

The high richness found in Taboa cave and the dependency of *N. macrourus* on stable cave microclimates emphasizes the necessity of protecting both subterranean habitats and their surrounding landscapes in the region. Given the classification of *N. macrourus* as "Vulnerable" in Brazil, primarily due to habitat loss driven by agricultural expansion, mining, and urban development (GUIMARÃES & FERREIRA 2014, DELGADO-JARAMILLO et al. 2017), conserving the Bambuí Group caves becomes even more urgent. These caves likely function as critical refuges for the isolated population, providing shelter and stability amidst a rapidly changing landscape.

Taboa cave's ability to support the largest known *N. macrourus* colony in southeastern Brazil suggests that the Bambuí geological system plays a pivotal role in subterranean biodiversity conservation. This finding highlights the need for further ecological and population studies to understand the complex interactions between N. macrourus and its cave habitats.

## 5. Conclusion

The discovery of a large *N. macrourus* colony in Taboa cave, Sete Lagoas, Minas Gerais, represents a significant contribution to our understanding of this species' distribution and conservation status in southeastern Brazil. With an estimated population of 100 to 120 individuals, this colony is the largest known in the region, underscoring the vital role of caves within the Bambuí Group as critical refuges for this vulnerable species.

This finding emphasizes the urgent need for targeted conservation strategies that not only protect cave ecosystems but also safeguard and recover the surrounding landscapes, which are essential in maintaining the stable microclimates necessary for the species' survival. Given the growing threats posed by habitat degradation—such as agricultural expansion, mining, and urban development—this discovery further highlights the importance of ongoing monitoring and continued ecological research to ensure the long-term protection of *N. macrourus* and its habitat.

Taboa cave emerges as a key conservation site, offering unique environmental conditions that support a thriving population of *N. macrourus*. This study lays the groundwork for future research and conservation efforts, providing critical insights into the species' ecological needs and emphasizing the broader significance of cave ecosystems in preserving biodiversity.

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# Dinâmica sazonal e riqueza de morcegos em cavernas da Floresta NNacional de Carajás, no município de Canaã dos Carajás, Pará, Brasil

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## Resumo

Cavernas são abrigos essenciais para morcegos, suportando o ciclo de vida de muitas espécies e promovendo interações ecológicas importantes. Investigamos a fauna de morcegos em 204 cavernas da Floresta Nacional de Carajás, sudeste do Pará, avaliando: (1) classificação das cavernas quanto à riqueza, (2) como a composição e a riqueza de espécies variam em relação a sazonalidade, e (3) os padrões de ocupação em relação as zonações (eufótica, penumbra e afótica) e a riqueza de espécies de morcegos. Entre junho de 2018 a maio de 2019, foram registradas 19 espécies, sendo *Peropteryx kappleri* a mais frequente. A família Phyllostomidae apresentou a maior diversidade com 12 espécies. Em 27 cavernas, não foram detectadas evidências de ocupação, enquanto a maioria apresentou baixa riqueza, apenas duas cavidades registraram alta riqueza de espécies. A análise PERMANOVA revelou estabilidade na composição de espécies entre estações, corroborada pela ausência de diferenças significativas na riqueza entre a estação seca e chuvosa. Cavernas com áreas afóticas apresentaram maior riqueza média em comparação àquelas com apenas penumbra, ou com incidência solar direta. Esses resultados destacam que cavernas com condições ambientais estáveis em função da ausência permanente de luz favorecem a formação de colônias residentes, sendo fundamentais para a conservação de muitas das espécies dependentes deste hábitat.

## Abstract

Caves are essential habitats for bats, supporting the life cycle of many species and promoting important ecological interactions. We investigated the bat fauna in 204 caves in the Floresta Nacional de Carajás, southeastern Pará, evaluating: (1) classification of caves according to species richness, (2) how species composition and richness vary in relation to seasonality, and (3) occupancy patterns in relation to zonations (euphotic, penumbra and aphotic) and bat species richness. Between June 2018 and May 2019, 19 species were recorded, with Peropteryx kappleri being the most frequent. The family Phyllostomidae showed the highest diversity with 12 species. In 27 caves, no evidence of occupancy was detected, while most caves showed low richness, with only two caves recording high species richness. The PERMANOVA analysis revealed stability in species composition between seasons, corroborated by the absence of significant differences in richness between the dry and rainy seasons. Caves with aphotic areas presented higher average richness compared to those with only shadows or direct sunlight. These results highlight that caves with stable environmental conditions due to the permanent absence of light favor the formation of resident colonies, being essential for the conservation of many of the species dependent on this habitat.

## 1. Introdução

As cavernas desempenham um papel essencial como abrigo para morcegos, fornecendo locais propícios para o desenvolvimento de seu ciclo de vida, desde a reprodução até atividades sociais e de descanso (BARROS et al. 2023). Estes ambientes também promovem interações sociais e ecológicas entre espécies, já que diferentes morcegos podem formar colônias de alta densidade e, em certos casos, compartilhar esses espaços com outras espécies, gerando associações intra e interespecíficas (Kunz 1982). A presença dos morcegos nas cavernas, sendo eles "trogloxenos" - organismos que usam o ambiente subterrâneo, mas dependem da superfície para sobrevivência - introduz nutrientes para o ecossistema cavernícola, essenciais para a cadeia alimentar subterrânea (GUIMARÃES & FERREIRA 2014, BARROS et al. 2023) e para o aumento da diversidade de invertebrados nessas cavernas (JAFFÉ et al. 2016).

Cavernas da Amazônia brasileira abrigam grande diversidade de espécies (TORRES & BICHUETTE 2024), muitas delas com dependência direta desses abrigos (FRAGA et al. 2023). Variações climáticas podem acarretar alterações sobre a dinâmica de ocupação de refúgio dos morcegos em cavernas, bem como mudanças comportamentais e ecológicas (GENELHU et al. 2023).

Este trabalho realizou um inventário da fauna de morcegos em 204 cavernas na Floresta Nacional de Carajás (PA), com o objetivo de avaliar a riqueza e variações sazonais na composição e ocupação.

## 2. Materiais e Métodos

**Área de estudo**

A área de estudo está inserida na região conhecida como Serra Sul, localizada na Floresta Nacional de Carajás, sudeste do estado do Pará. A Unidade de Conservação situa-se a aproximadamente 550 km ao sul de Belém, no Mosaico de Carajás, área de 1.207.000 hectares (MOTA et al. 2018) (Fig. 1). A região apresenta um clima tropical quente e úmido (Am), com precipitação anual de 1600 mm, concentrada principalmente na estação chuvosa, entre os meses de verão (ALVARES et al. 2013). A vegetação predominante é a Floresta Ombrófila Densa, intercalada com campos rupestres e áreas de vegetação de canga, adaptada ao solo rico em ferro (MOTA et al. 2018).

**Coleta de dados**

As 204 cavernas foram amostradas em duas ocasiões, uma na estação chuvosa e outra na seca, entre junho de 2018 e maio de 2019. A captura e manipulação dos morcegos foram realizadas seguindo os protocolos éticos do Guia da American Society of Mammalogists (SIKES et al. 2016). Os animais capturados foram identificados quanto à taxonomia, sexados, avaliados quanto à condição reprodutiva e tiveram suas medidas corporais padrão aferidas. Alguns indivíduos foram marcados com anilhas numeradas. As identificações taxonômicas seguiram DIAZ et al. (2016). Para avaliação do padrão de ocupação no interior das cavidades, foram identificadas a presença e localização das zonas eufótica (incidência direta de luz natural), penumbra (incidência indireta de luz natural), e afótica (sem incidência de luz natural).

**Análise dos dados**

A classificação das cavernas quanto à riqueza de espécies seguiu os critérios de GUIMARÃES & FERREIRA (2014), categorizando-as em baixa (0-3 espécies), média(4-6 espécies), alta (7-9), e elevada (≥ 10) riqueza. Para investigar a influência da sazonalidade na composição de espécies, foi utilizada a análise PERMANOVA (ANDERSON 2001) e o teste PERMDISP2 (ANDERSON et al. 2006), permitindo avaliar diferenças médias e a dispersão na composição entre as estações seca e chuvosa. Para identificar os padrões de ocupação foram testadas a relação entre as zonas das cavernas (eufótica, penumbra e afótica) e a riqueza de espécies por meio de testes não paramétricos de Mann-Whitney (MANN & WHITNEY 1947).

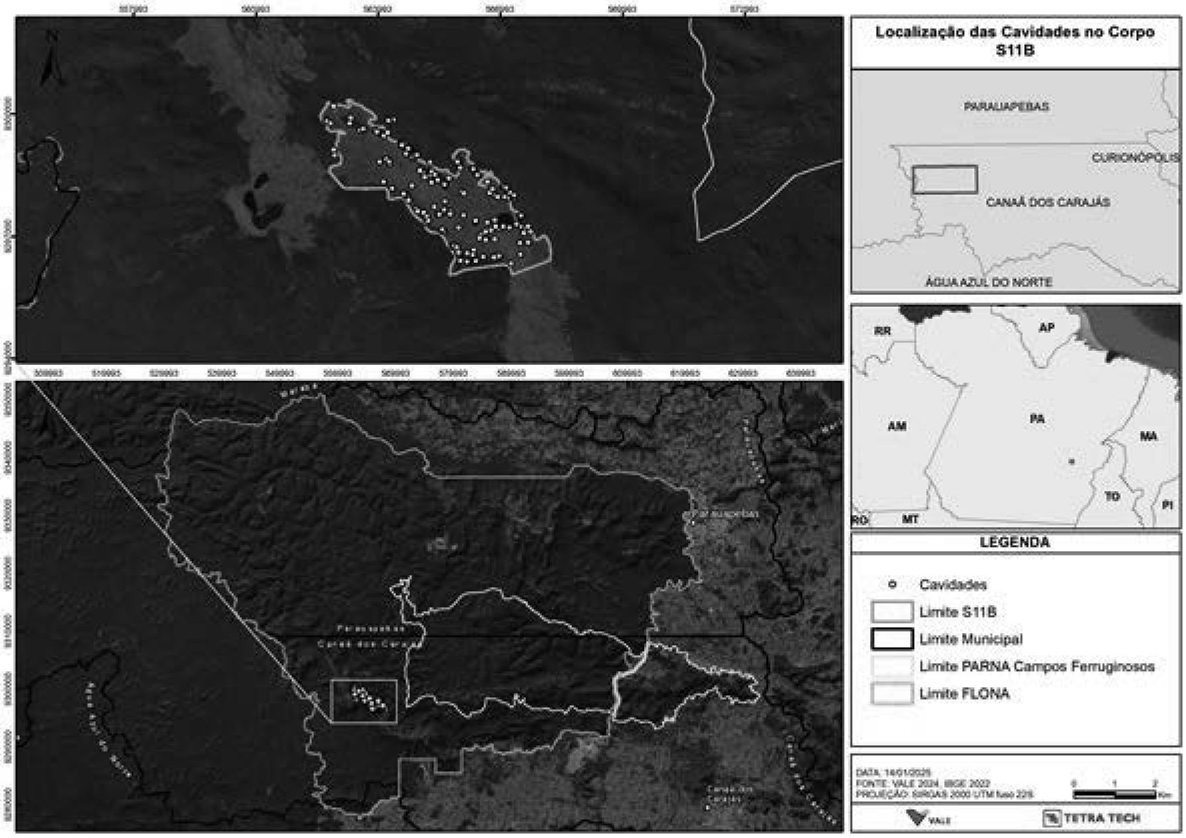
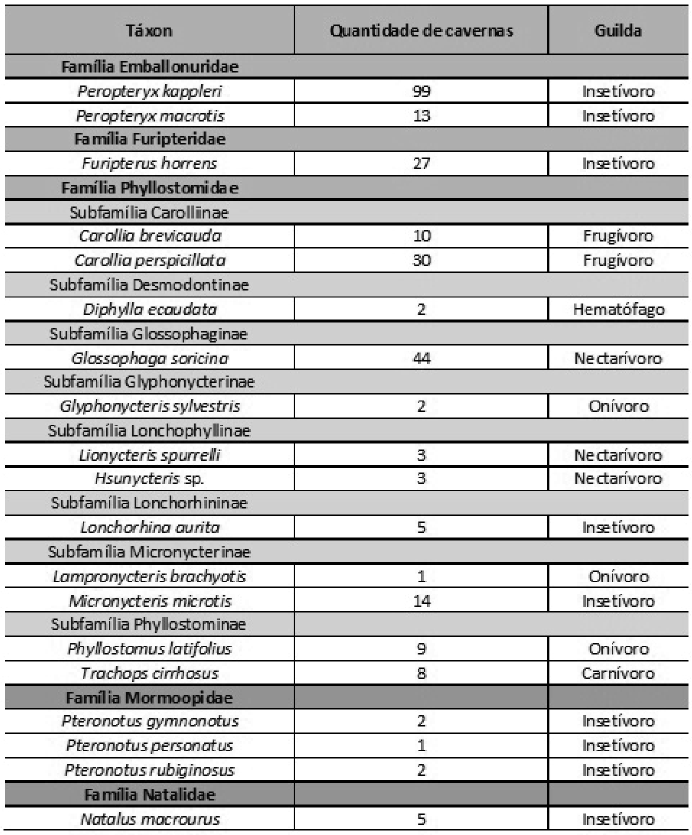


*Figura 1: Mapa de localização regional das cavernas estudadas no corpo B da região de Serra Sul, Floresta Nacional de Carajás, estado do Pará, Brasil.*

## 3. Resultados

Durante o estudo foram identificadas 19 espécies de morcegos (Fig. 2 e Fig. 3). *Peropteryx kappleri* foi a espécie mais frequente. A família Phyllostomidae apresentou a maior diversidade, com 12 espécies. Em 27 cavidades não foram observadas evidências de ocupação por morcegos, ou seja, a presença de indivíduos, ou de vestígios como guano e carcaças. Das cavernas analisadas, 179 apresentaram baixa riqueza, 18 riqueza média, cinco (5) alta riqueza, e duas (2) apresentaram riqueza elevada (Fig. 4).

***Figura 2:*** *Morcegos registrados durante o estudo. A: Carollia brevicauda, B: Diphylla ecaudata, C: Lonchorhina aurita, D: Lampronycteris brachyotis, E: Carollia perspicillata, F: Glossophaga soricina, G: Hsunycteris sp., H: Lionycteris spurrelli, I: Furipterus horrens, J: Glyphonycteris sylvestris, K: Micronycteris microtis, L: Natalus macrourus, M: Pteronotus gymnonotus, N: Pteronotus rubiginosus, O: Peropteryx macrotis, P: Phyllostomus latifolius, Q: Pteronotus personatus, R: Peropteryx kappleri, S: Trachops cirrhosus. Fotos: Ativo Ambiental.*



| Táxon | Quantidade de cavernas | Guilda |
|---|---|---|
| **Família Emballonuridae** | | |
| *Peropteryx kappleri* | 99 | Insetívoro |
| *Peropteryx macrotis* | 13 | Insetívoro |
| **Família Furipteridae** | | |
| *Furipterus horrens* | 27 | Insetívoro |
| **Família Phyllostomidae** | | |
| Subfamília Carolliinae | | |
| *Carollia brevicauda* | 10 | Frugívoro |
| *Carollia perspicillata* | 30 | Frugívoro |
| Subfamília Desmodontinae | | |
| *Diphylla ecaudata* | 2 | Hematófago |
| Subfamília Glossophaginae | | |
| *Glossophaga soricina* | 44 | Nectarívoro |
| Subfamília Glyphonycterinae | | |
| *Glyphonycteris sylvestris* | 2 | Onívoro |
| Subfamília Lonchophyllinae | | |
| *Lionycteris spurrelli* | 3 | Nectarívoro |
| *Hsunycteris* sp. | 3 | Nectarívoro |
| Subfamília Lonchorhininae | | |
| *Lonchorhina aurita* | 5 | Insetívoro |
| Subfamília Micronycterinae | | |
| *Lampronycteris brachyotis* | 1 | Onívoro |
| *Micronycteris microtis* | 14 | Insetívoro |
| Subfamília Phyllostominae | | |
| *Phyllostomus latifolius* | 9 | Onívoro |
| *Trachops cirrhosus* | 8 | Carnívoro |
| **Família Mormoopidae** | | |
| *Pteronotus gymnonotus* | 2 | Insetívoro |
| *Pteronotus personatus* | 1 | Insetívoro |
| *Pteronotus rubiginosus* | 2 | Insetívoro |
| **Família Natalidae** | | |
| *Natalus macrourus* | 5 | Insetívoro |

***Figura 3:*** *Lista de espécies registradas durante diagnóstico de quirópteros em cavidades naturais no Corpo B da Serra Sul, localizado na Floresta Nacional de Carajás, sudeste do Pará.*

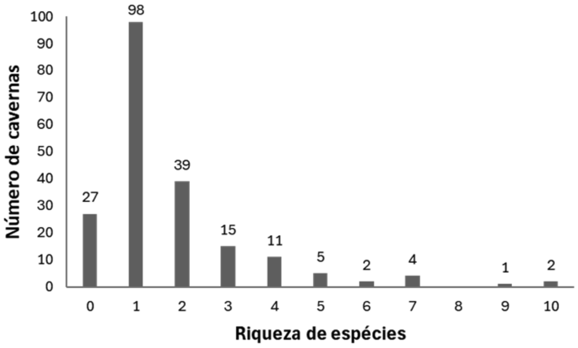


***Figura 4:*** *Número de cavernas para cada riqueza de espécies de morcegos registrada na região de Serra Sul, Floresta Nacional de Carajás, estado do Pará, Brasil.*

A análise PERMANOVA não revelou diferenças significativas na composição de espécies entre as estações (F = 0,45; P = 0,88), indicando estabilidade ao longo do ano. A riqueza também não variou significativamente entre as estações (W = 2053; p = 0,90). A riqueza média considerando as duas estações foi de 1,75 (dp ± 1,73). Considerando apenas a estação seca, a média de riqueza observada foi de 1,33 (dp ± 1,46). Na estação chuvosa a média observada foi de 1,31 (dp ± 1,37).

Em relação à influência da luminosidade nas cavidades, a riqueza média de morcegos nas cavidades com zonação completa foi de 4,06 (dp ± 2,6), enquanto nas cavidades com até zona de penumbra foi de 1,84 (dp ± 1,56) e nas cavidades com apenas zona eufótica foi de 1,12 (dp ± 0,73). As cavidades com zonas afóticas apresentam riqueza média de morcegos superior às cavidades que possuem penumbra (W = 1272; p < 0,01) ou apenas zona eufótica (W = 2430; p < 0,01). As cavidades com zona de penumbra (mas sem zonas afóticas) também apresentaram riqueza média superior às cavidades com apenas zona eufótica (W = 4720; p < 0,01).

## 4. Discussão

Os resultados indicam que *Peropteryx kappleri* é a espéce de maior prevalência em cavernas da região estudada, embora este achado seja distinto de outros estudos em cavernas (GUIMARÃES & FERREIRA 2014, VARGAS-MENA et al. 2018, BARROS et al., 2023, TORRES & BICHUETTE 2024). A alta diversidade da família Phyllostomidae é coerente com padrões relatados para morcegos Neotropicais (VARGAS-MENA et al. 2018, BARROS et al. 2023), refletindo a adaptabilidade dessa família a uma ampla gama de ambientes.

A ausência de mudanças na composição da fauna das cavernas entre as estações pode ser evidência da estabilidade ambiental e climática que a região proporciona para as espécies de morcegos. Apesar de mudanças significativas em termos de pluviosidade entre estações, as cavernas da região de Carajás são rodeadas por uma matriz de floresta ombrófila densa, que pode proporcionar segurança na disponibilidade de recursos alimentares ao longo de todo o ano. Assim, não há uma pressão para que as espécies de morcegos residentes em cavernas se desloquem para outras áreas em busca de recursos alimentares (GENELHÚ et al. 2022). Além disso, a própria presença de muitas cavidades na região (mais de 840 - JAFFÉ et al. 2016) pode minimizar conflitos por abrigo, o que favoreceria o estabelecimento de colônias fixas, refletindo na pouca alteração da composição entre estações.

O fato de cavidades com zonas afóticas terem maior riqueza pode estar associado a dois fatores. O primeiro deles é que cavidades com zonas afóticas tendem a ser mais estáveis climaticamente, o que favorece o estabelecimento de mais espécies, um padrão já identificado na literatura (BARROS et al. 2020; VARGAS-MENA et al. 2020). Além disso, cavernas com zonas afóticas tendem a ser aquelas com maiores dimensões, o que representa maior quantidade de abrigos potenciais em seu interior e assim maior diversidade de espécies que podem ocupá-las (BARROS et al. 2020, BRUNET & MEDELLÍN 2001, PHELPS et al. 2016). Tais características favorecem processos como reprodução e formação de colônias, garantindo ambientes seguros e estáveis para as espécies (KUNZ 1982).

## 5. Conclusão

A alta riqueza de espécies encontradas no conjunto de cavernas de Serra Sul, Floresta Nacional de Carajás, indica como estes hábitats são importantes como abrigos para os morcegos na região. A estabilidade na composição das espécies ao longo do ano reforça a ideia de que as cavernas oferecem condições favoráveis para a ocupação contínua, independentes da sazonalidade. Esses achados contribuem para o entendimento da ecologia dos morcegos em ambientes cavernícolas e enfatizam a necessidade de conservação dessas áreas como habitats críticos para a biodiversidade local.

## Agradecimentos

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# Host discovery and new locality records of cave-dwelling mite *Chiasmanyssus cavernicola* Gomes-Almeida & Pepato, 2021 (Acari: Macronyssidae) from Brazil

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## Abstract

The family Macronyssidae comprises 35 genera that parasitize a wide range of vertebrates. Among them, *Chiasmanyssus* is a recently described monotypic genus in Brazil, known exclusive from specimens collected on cave soil in Minas Gerais, Pará, and Rio de Janeiro states. However, the host for this mite species remains unknown. Here, we report for the first time the occurrence of *C. cavernicola* associated with bat hosts, including *Anoura caudifer, Carollia perspicillata, Chrotopterus auritus, Desmodus rotundus, Diphyla ecaudata, Micronycteris megalotis* and *Mimon bennetti*. Additionally, we present new records within and surrounding caves in the karstic region of Pains and four state conservation units: Parque Serra do Rola Moça, Parque do Sumidouro, Parque da Lapa Grande and Parque Serra Nova e Talhado, expanding the geographical range for this mite from north to south in Minas Gerais state, Brazil.

## Resumen

La familia Macronyssidae comprende 35 géneros que parasitan una amplia gama de vertebrados. Entre ellos, *Chiasmanyssus* es un género monotípico descrito recientemente en Brasil, conocido exclusivamente por especímenes recolectados en el suelo de cuevas en los estados de Minas Gerais, Pará y Río de Janeiro. Sin embargo, el hospedador de esta especie de ácaro sigue siendo desconocido. Aquí, informamos por primera vez la presencia de *C. cavernicola* asociado con hospedadores murciélagos, incluyendo *Anoura caudifer, Carollia perspicillata, Chrotopterus auritus, Desmodus rotundus, Diphylla ecaudata, Micronycteris megalotis* y *Mimon bennettii*. Además, presentamos nuevos registros dentro y alrededor de cuevas en la región kárstica de Pains y en cuatro unidades de conservación estatales: Parque Serra do Rola Moça, Parque do Sumidouro, Parque da Lapa Grande y Parque Serra Nova e Talhado, ampliando el rango geográfico de este ácaro de norte a sur en el estado de Minas Gerais, Brasil.

## 1. Introduction

The family Macronyssidae Oudemans, 1936 comprises 35 genera known worldwide (RADOVSKY 2010; BASSINI-SILVA et al. 2021; GOMES-ALMEIDA & PEPATO 2021). This family includes mainly ectoparasitic mites that are blood-feeding (hematophagous) and infest various vertebrates, such as bats, marsupials, rodents, birds, reptiles and humans (RADOVSKY 1966; RADOVSKY 2010). Some species of this family have medical and economic importance (TEIXEIRA et al. 2020; BASSINI-SILVA et al. 2022). Macronyssid mites have a semi-permanent ectoparasitic life cycle, alternating between periods on host and on substrate, or roost or nest of host. Out of the five life cycle stages: egg, larva, protonymph, deutonymph, and adults (male and female), only the protonymph and adults actively feed on the host (FONSECA 1948; DOWLING 2006; SAUNDERS 1975; RADOVSKY 1966; 1967; 2010).

The genus *Chiasmanyssus* is monotypic and recently described from Brazil, with its type species *Chiasmanyssus cavernicola*. According to GOMES-ALMEIDA & PEPATO (2021), this genus may be distinguished from other Macronyssidae genera by: sternal shield with a distinct X-shaped area, bearing three pairs of setae and two pairs of pores; sternal gland absent; anterior spur on coxa II lacking; adults with dorsal shield entire and j and J series complete; adults and protonymphs with setae J5 minute; males with strong-pores on dorsal shield and peritrema short extending from posterior margin of coxae II to the anterior margin of coxae IV. Despite having known that the species is parasitic, because many individuals collected had been engorged, the host was unknown. Since, the specimens examined and used for describing the species were all collected on cave soil in Minas Gerais, Pará, and Rio de Janeiro states (GOMES-ALMEIDA & PEPATO 2021).

Thus, here, we report for the first time the occurrence of cave-dwelling mites *C. cavernicola* associated with bats host, seven Phyllostomidae bat species. Additionally, we report new records within and surrounding caves in protected and non-protected areas from Minas Gerais, which expands the geographical range of this mite from north to south of the state.

## 2. Materials and methods

Mites were collected from 25 localities on caves, epigean habitats and artificial galleries in Minas Gerais State, Brazil. Most mites were preserved in 70% alcohol at room temperature and kept in 95–100% ethanol at -20°C upon acceptance at the Centro de Coleções Taxonomicas da Universidade Federal de Minas Gerais (CCT-UFMG) acarological collection and in the Collection of Subterranean Invertebrates from Lavras (ISLA-UFLA), Federal University of Lavras, Biology Department, Zoology Sector, Lavras, Minas Gerais, Brazil. Individuals newly collected by authors were preserved directly in 95–100% alcohol, refrigerated in the field, and then stored at -20 °C. The latter was collected under ICMBio, SISBIO 71120–4 license, along with the "Instituto Estadual de Florestas (IEF 009/2020)" of Minas Gerais state, besides following the ethics committee for animal use in research "Comissão de Ética no Uso de Animais (CEUA)" of UFMG, with protocol number 50/2020).

Mites were actively sampled on cave soil and on the body of seven phyllostomid bats, representing seven species distributed in seven genera, which were captured with mist nets. Mites parasitizing hosts were delicately removed using fine pincers and alcohol-soaked brushes, and preserved in absolute ethanol (95–100%) for identification. Hosts were identified in the field following keys proposed by GARDNER (2007), supplemented by LEMOS et al. (2020) and VELAZCO & PATTERSON (2019) for *Carollia* species, and HURTADO-MIRANDA & PACHECO-TORRES (2014) for *Mimon* species. Subsequently, hosts were released at the exact location of capture and, in cases of uncertain identification, up to two voucher specimens being euthanized for detailed laboratory identification and later deposited at the CCT-UFMG Mastozoological collection.

Mites were mounted on permanent microscope slides using Hoyer's medium (WALTER & KRANTZ 2009) for morphology examination. Identification and microphotographs were performed with a Leica DM 750 optical microscope equipped with an ICC50W digital camera. Besides, mites identification followed the keys proposed by RADOVSKY (2010) and GOMES-ALMEIDA & PEPATO (2021). The terminology for dorsal and ventral chaetotaxy, as well as the nomenclature of the dorsum, followed RADOVSKY (2010). Sternal pores terminology is based on EVANS & TILL (1979). The preparation of the map was performed using R Software (v4.1.2; R Core Team 2021). All figures were prepared with Krita v.4.4.2 program (https://krita.org/en/).

## 3. Results

A total of 172 specimens of macronyssid mites, including protonymphs and adults, have been collected parasitizing bats from Phyllostomidae family and on cave soil from 25 hypogean or associated localities in Minas Gerais, Brazil. These mites have been identified morphologically as Chiasmanyssus cavernicola (Fig.1-2).

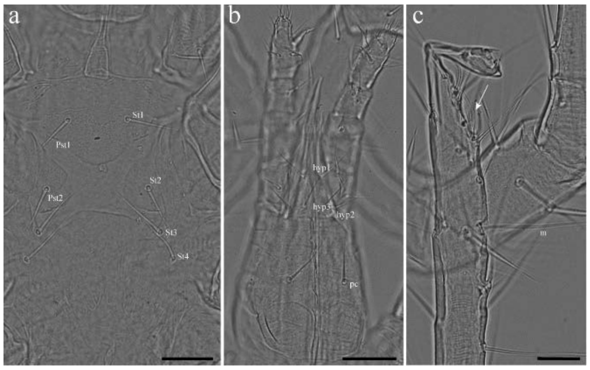

**Figure 1:** *Chiasmanyssus cavernicola female. a – Sternal shield, note the distinct X-shaped sclerotization, b – Gnathosoma, ventral, c – Tarsus I with setae in the sensillary field. Scale Bars: a & b 100 µm, c 50 µm.*

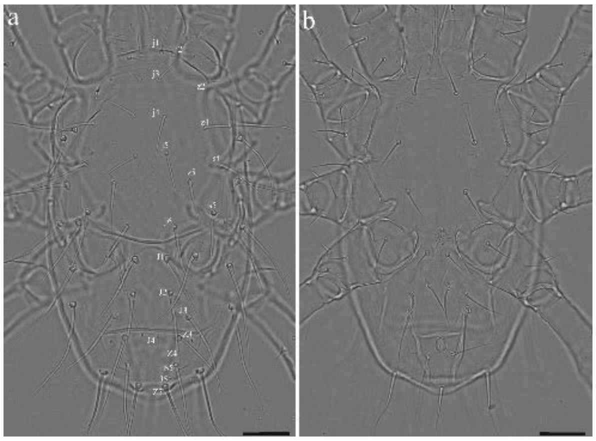

**Figure 2:** *Chiasmanyssus cavernicola protonymph. a – Idiosoma, dorsum, b – Idiosoma, ventral. Scale bars: a, b 50 µm.*

## 4. Discussion

Our findings revealed seven Phyllostomidae species as hosts of *C. cavernicola*. It is noteworthy that even mites collected off-host have been found in caves and others subterranean habitat inhabited by bats, vertebrates that use caves as shelter efficiently and permanently (KUNZ 1982). Of the nine families of the order Chiroptera that occur in Brazil, the Phyllostomidae is the most diverse and abundant, with 94 species distributed in 43 genera (NOGUEIRA et al. 2014; GARBINO et al. 2020; GARBINO et al. 2022). They are widely distributed bats occupying a variety of trophic guilds and habitats in different biomes of Brazil, and provide important ecosystem services (KUNZ et al. 2010). Their wide geographic distribution suggests that geographic distribution of *C. cavernicola* mite may also be extensive due to its association with them (Figure 3). *Anoura caudifer, Diphylla ecaudata, Chrotopterus auritus, Desmodus rotundus, Carollia perspicillata, Micronycteris megalotis* and *Mimon bennettii*, all of which are commonly found in Brazilian caves (GUIMARÃES & FERREIRA 2014), are among the phyllostomid bat species infested by *C. cavernicola*. This association suggests a possible close relationship between this semi-permanent parasitic mite and cave-dwelling habitat. Furthermore, high affinity with Phyllostomidae is observed for *C. cavernicola*, but is not specific to a single bat species, indicating a wide host range, similar to other genera of macronyssidae (RADOVSKY 2010).

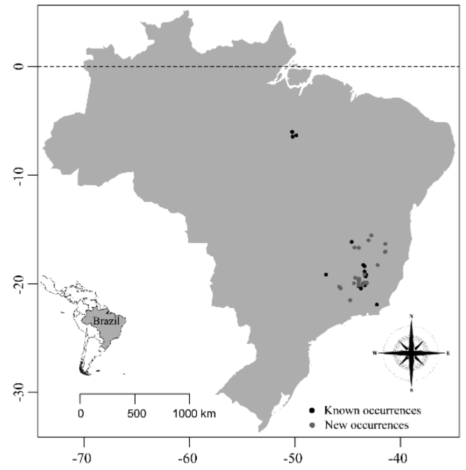

***Figure 3:*** *Distribution of Chiasmanyssus cavernicola from Brazil.*

## 5. Conclusion

This study reveals for a first time that bats are hosts to this mite, *Chiasmanyssus cavernicola*, extending the knowledge of the ecology and geographical distribution for this species. Their association to seven species of cave-dwelling bats from Phyllostomidae suggests a non-specific parasitic relationship but does not rule out a possible affinity with cave habitats. In addition, these new records in protected and non-protected areas of Minas Gerais have expanded its distribution from north to south of Minas Gerais. These findings contribute to the knowledge of underground biodiversity and ecological interactions between mites and bats in Brazil.

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# Ecos invisíveis: integrando a bioacústica ao inventário de morcegos cavernícolas

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## Resumo

Morcegos são organismos trogloxenos que desempenham importantes serviços ecossistêmicos, como polinização, dispersão de sementes e controle de insetos, além de serem um importante elo da cadeia trófica cavernícola por meio da deposição de guano. Devido a diversidade de espécies e importância ecológica, o emprego de métodos eficazes e complementares de levantamento de espécies se faz necessário. Sendo assim, este estudo teve como objetivo realizar o levantamento da quiropterofauna em 80 cavidades localizadas na região de Carajás, estado do Pará, Brasil, utilizando diferentes métodos de amostragem, sendo eles a captura ativa e a bioacústica. Os métodos de captura ativa registram 13 espécies, enquanto a bioacústica permitiu o registro de 23 espécies, se mostrando uma importante ferramenta complementar em estudos de inventário em cavernas. Os resultados reforçam a importância da combinação de métodos para maior abrangência na amostragem e na compreensão do uso das cavernas por diferentes espécies de morcegos. Esses achados têm implicações para estratégias de monitoramento e conservação da quiropterofauna.

## Abstract

Bats are trogloxene organisms that play essential ecological roles, such as pollination, seed dispersal and insect control, in addition to being a crucial link in the cave food chain through guano deposition. Due to the diversity of species and ecological importance, the use of effective and complementary methods for species surveys is necessary. Therefore, this study aimed to survey bats in 80 caves located in Carajás region, Pará State, Brazil, using two different sampling methods, active capture and bioacoustics. The active capture methods recorded 13 species, while bioacoustics allowed the recording of 23 species, proving to be an important complementary tool in cave inventory studies. The results emphasize the importance of combining methods for broader sampling and a better understanding of cave usage by different bat species. These findings have implications for monitoring and conservation strategies for bats.

## 1. Introdução

A quiropterofauna desempenha importantes serviços ecossistêmicos, incluindo a polinização, dispersão de sementes e controle biológico de insetos (COSTA et al., 2018), com 186 espécies atualmente descritas no Brasil (GARBINO et al., 2024). Apesar disso, as assembleias de morcegos na floresta amazônica permanecem pouco compreendidas, carecendo de mais estudos.

De hábitos predominantemente noturnos, os morcegos colonizam as cavernas de forma eficiente e permanente (BREDT et al., 1999). As cavernas oferecem abrigo, proteção a extremos de temperatura e a predadores (ESBÉRARD et al., 2009). São espécies trogloxenas e representam um importante elo da cadeia trófica por meio da deposição de guano em cavernas, que garante o aporte energético aos invertebrados (PELLEGRINI & FERREIRA, 2013).

Na Floresta Nacional (FLONA) de Carajás, uma das unidades espeleológicas mais importantes do país (UE Carajás), com número expressivo de cavidades naturais subterrâneas (PILÓ et al., 2015), são 83 espécies de morcegos registradas (COSTA et al., 2018), além de abrigar grande biodiversidade de espécies cavernícolas (JAFFÉ et al., 2018). Dada a alta diversidade de morcegos e de abrigos, o emprego de técnicas eficientes de amostragem se torna um desafio. Os métodos tradicionais de amostragem de morcegos incluem a instalação de redes de neblina (CHAVES-RAMÍREZ et al., 2021); quando em abrigos, o uso de puçás é considerado mais eficiente (PACHECO, 2004). Atualmente, o uso da bioacústica em programas de inventário e monitoramento vem ganhando cada vez mais espaço. A técnica apresenta grande potencial para superar algumas das limitações encontradas em técnicas convencionais (DEICHMANN et al., 2018; BURIVALOVA et al., 2019), como registrar espécies antes subamostradas (SILVA & BERNARD, 2017), reduzir riscos nas atividades de campo, assim como o uso de recursos humanos e financeiros. No entanto, a técnica deve ser utilizada com cautela e de forma complementar a outros métodos (APPEL et al., 2021), uma vez que a probabilidade de detecção varia de acordo com a espécie, habitat, o posicionamento do detector, hardware e software usados para coletar e analisar os chamados, entre outros (VAN GUNST et al., 2020).

O presente trabalho teve como objetivo realizar o levantamento da quiropterofauna em 80 cavidades localizadas na região de Carajás, estado do Pará, Brasil. Para tanto, foram implementadas duas técnicas distintas: a captura ativa e a bioacústica. Adicionalmente, é proposto um protocolo de validação dos dados levantados pela bioacústica para se atribuir o uso das cavernas como abrigo pelos morcegos.

## 2. Materiais e Métodos

**Área de estudos**

As 80 cavidades alvo do levantamento encontram-se inseridas na Unidade Espeleológica Serra de Carajás, na Unidade Geomorfológica de Serra Norte, no Platô N3, município de Parauapebas, Pará (Fig. 1).

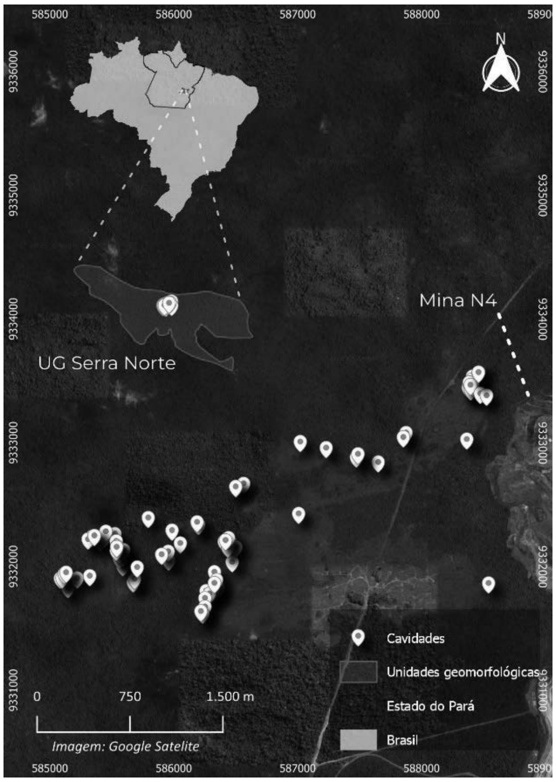


*Figura 1: Localização das cavernas-alvo do presente estudo. Em detalhe são apresentados a UG Serra Norte, estado do Pará e Brasil.*

**Coleta de dados**

Em uma primeira etapa de amostragem, durante o período diurno foi realizada a interceptação de morcegos em voo utilizando dois métodos de busca ativa, sendo eles puçá e redes de neblina. Os morcegos capturados foram identificados em nível de espécie, com o auxílio de chaves de identificação (REIS et al., 2017; DIAZ et al., 2021).

Na segunda etapa, foi empregada no período noturno a amostragem acústica dos morcegos. Os dispositivos *AudioMoth* foram instalados a cerca de dois metros da entrada das cavidades, no ambiente de superfície. A amostragem foi realizada em dois períodos distintos, das 17h30 às 21h e das 3h às 6h, totalizando 6h30 de gravações em cada cavidade. A criação de espectrogramas e a identificação acústica das espécies de morcegos foram realizadas manualmente por meio de software Raven.

**Análises dos dados**

A fim de comparar os dois métodos amostrais empregados, primeiramente foram construídas curvas de acumulação de espécies para cada um dos métodos e considerando-os em conjunto.

Posteriormente, verificamos a existência de diferenças significativas na riqueza média de espécies de acordo com o método amostral, construímos um modelo linear generalizado (GLM) tendo como variável resposta a riqueza de espécies por cavidade e como preditor o método amostral, se por busca ativa ou bioacústica.

Classificamos a quiropterofauna amostrada em N3 de acordo com o tipo de uso da cavidade seguindo a proposta de GUIMARÃES & FERREIRA, 2014 e BARROS & BERNARD, 2023a. As espécies foram então classificadas em essencialmente cavernícolas, cavernícolas oportunistas, e não cavernícolas. Para as espécies não avaliadas por GUIMARÃES E FERREIRA, 2014 e BARROS & BERNARD, 2023a, buscamos informações na literatura sobre o tipo de uso das cavidades (ex. SIMMONS E VOSS, 1998; SIMMONS et al., 2002; BERNARD 2003; REIS et al. 2013)

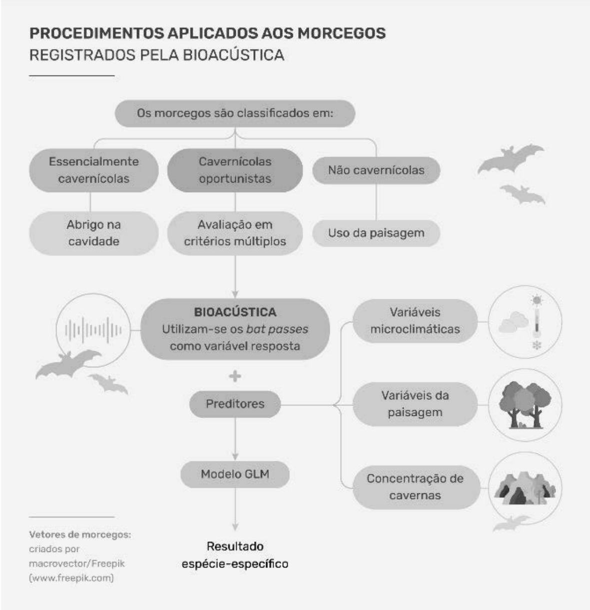


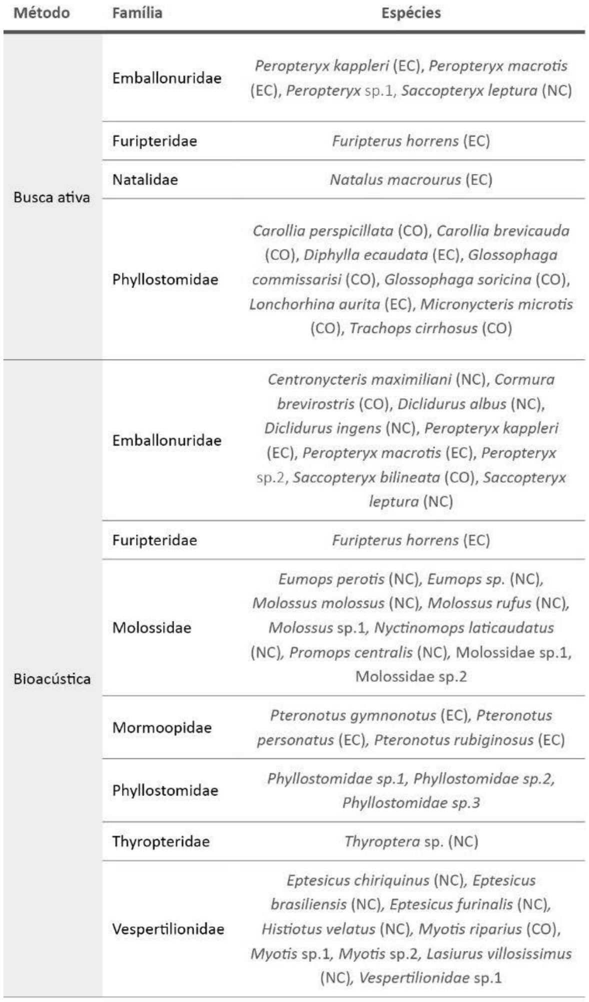
*Figura 2: Representação esquemática dos procedimentos aplicados aos morcegos registrados pela bioacústica.*

Para a validação dos dados obtidos pela bioacústica as espécies classificadas como “essencialmente cavernícolas”, foram consideradas por se abrigarem na cavidade. Para as espécies “não cavernícolas” os registros foram considerados de passagem no ambiente externo, não sendo consideradas por utilizar cavidade. Para as espécies restantes, “cavernícolas oportunistas” ou sem classificação, permaneceu a dúvida se seriam somente registros de passagem no ambiente externo ou se haveria possibilidade de uso da cavidade.

A fim de inferir sobre a relação dessas espécies com as cavidades de N3, realizamos análises multifatoriais. Tais análises nos permitiram verificar se os locais de maior atividade das espécies de morcegos (bat passes), estariam relacionadas aos fatores da paisagem adjacente ao ponto de registro ou se pela ocorrência de um maior adensamento de cavidades no local. Nestes casos, os dados de bat passes de cada espécie foram utilizados separadamente como variável resposta na construção de modelos lineares generalizados (GLMs) (Fig. 2).

## 3. Resultados

A partir da amostragem tradicional, realizada de forma ativa nas 80 cavidades de N3 com redes de neblina e puçás, foram observadas 13 espécies associadas às cavidades (Fig 3). A partir do método da Bioacústica nas 80 cavidades em estudo, foram extraídos 8.324 registros sonoros de morcegos. Esses registros foram identificados em 35 sonótipos diferentes os quais foram atribuídos a 23 espécies distintas, além de 12 sonótipos sem identificação a nível específico (Fig. 4).



| Método | Família | Espécies |
|---|---|---|
| Busca ativa | Emballonuridae | *Peropteryx kappleri* (EC), *Peropteryx macrotis* (EC), *Peropteryx* sp.1, *Saccopteryx leptura* (NC) |
| | Furipteridae | *Furipterus horrens* (EC) |
| | Natalidae | *Natalus macrourus* (EC) |
| | Phyllostomidae | *Carollia perspicillata* (CO), *Carollia brevicauda* (CO), *Diphylla ecaudata* (EC), *Glossophaga commissarisi* (CO), *Glossophaga soricina* (CO), *Lonchorhina aurita* (EC), *Micronycteris microtis* (CO), *Trachops cirrhosus* (CO) |
| Bioacústica | Emballonuridae | *Centronycteris maximiliani* (NC), *Cormura brevirostris* (CO), *Diclidurus albus* (NC), *Diclidurus ingens* (NC), *Peropteryx kappleri* (EC), *Peropteryx macrotis* (EC), *Peropteryx* sp.2, *Saccopteryx bilineata* (CO), *Saccopteryx leptura* (NC) |
| | Furipteridae | *Furipterus horrens* (EC) |
| | Molossidae | *Eumops perotis* (NC), *Eumops sp.* (NC), *Molossus molossus* (NC), *Molossus rufus* (NC), *Molossus* sp.1, *Nyctinomops laticaudatus* (NC), *Promops centralis* (NC), Molossidae sp.1, Molossidae sp.2 |
| | Mormoopidae | *Pteronotus gymnonotus* (EC), *Pteronotus personatus* (EC), *Pteronotus rubiginosus* (EC) |
| | Phyllostomidae | *Phyllostomidae sp.1*, *Phyllostomidae sp.2*, *Phyllostomidae sp.3* |
| | Thyropteridae | *Thyroptera* sp. (NC) |
| | Vespertilionidae | *Eptesicus chiriquinus* (NC), *Eptesicus brasiliensis* (NC), *Eptesicus furinalis* (NC), *Histiotus velatus* (NC), *Myotis riparius* (CO), *Myotis* sp.1, *Myotis* sp.2, *Lasiurus villosissimus* (NC), *Vespertilionidae* sp.1 |

***Figura 3:*** *Espécies capturadas de acordo com o método amostral, busca ativa ou bioacústica. As siglas entre parênteses indicam a classificação de cada espécie quanto ao uso da cavidade: EC = essencialmente cavernícola, CO = cavernícola oportunista, NC = não cavernícola.*

Com a comparação entre os métodos, observamos que a amostragem faunística foi bem implementada, uma vez que foi atingida a assíntota ao se considerar cada um dos métodos empregados, tanto separadamente quanto quando considerados ambos os métodos (Fig. 3). Ambos os métodos amostrais estabilizam a curva de acumulação muito antes de se coletar nas 80 cavidades em estudo. A coleta ativa é capaz de amostrar 90% de sua diversidade com 56 cavidades amostradas, enquanto a Bioacústica amostra 90% de sua diversidade com 60 pontos amostrais. Ao se considerar os dois métodos em conjunto, a estabilização é ainda mais rápida. Com 39 cavidades amostradas atinge-se 90% da diversidade observada na região.

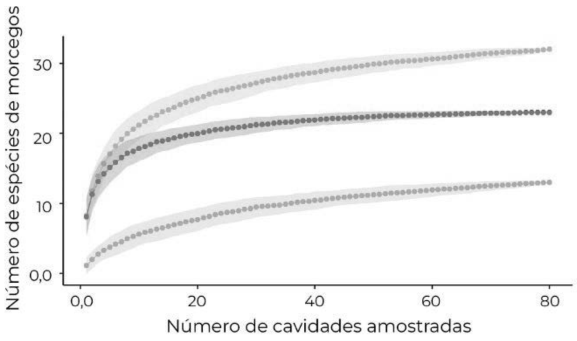


***Figura 4:*** *Curva de acumulação de espécies por cavidade amostral considerando-se todos os métodos amostrais (de forma independente e combinados). As áreas coloridas representam o desvio padrão, para a assembleia geral de Chiroptera. Em amarelos estão representados todos os métodos, em vermelho a bioacústica e verde a coleta ativa.*

Em se tratando apenas dos indivíduos ou/e sonótipos que permitiram a identificação a nível de espécies, a riqueza média de Chiroptera encontrada em cada cavidade foi maior pelo método da Bioacústica (Fig. 5).

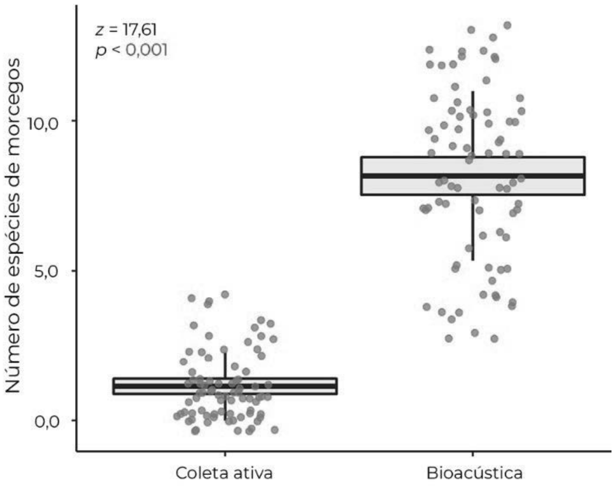


***Figura 5:*** *Média de espécies capturadas por cavidade de acordo com os métodos de coleta. Os testes estatísticos informam o valor de z e as diferenças significativas (p < 0,05).*

A partir das amostragens em N3, foi possível detectarmos nove espécies essencialmente cavernícolas. Dessas, três foram identificadas apenas pelo método de captura ativa, três apenas pela bioacústica e três foram amostrados por ambos os métodos. Outras nove espécies foram consideradas cavernícolas oportunistas, a maioria dessas identificadas pela bioacústica. Por fim, 17 espécies foram classificadas em não cavernícolas, das quais somente uma foi identifica em ambos os métodos. Todas as demais foram amostradas pela bioacústica.

Com relação aos indivíduos e sonótipos identificados a nível de espécie, foi possível verificar maior riqueza média de espécies essencialmente cavernícolas ou cavernícolas oportunistas dentre as amostragens por bioacústica quando comparada com a coleta ativa. Por outro lado, ao se comparar o percentual médio das espécies cavernícolas em relação ao total de espécies registradas nas cavidades do platô N3, a proporção de espécies cavernícolas foi maior na amostragem ativa (Fig. 6).

As doze espécies listadas como cavernícola oportunistas ou sem classificação registradas pelo método da bioacústica tiveram o seu registro validado por meio dos modelos estatísticos construídos. Os bat passes da espécie *C. brevirostris* foram influenciados positivamente pela concentração de cavidades na região e pelo comprimento linear de drenagens na proximidade da cavidade. Com isso, as cavidades nas quais houve o registro acústico de *C. brevirostris* serão consideradas como sítios de uso pela espécie. Phyllostomidae sp.3 foi influenciada significativamente e positivamente pela concentração de cavidades, pelo TWI (índice topográfico de umidade) e pela área de canga no entorno das cavidades. Com isso, as ocorrências da morfoespécie serão consideradas como sítios de uso por Phyllostomidae sp.3. Para as demais espécies analisadas, uma vez que não foi detectada relação com a concentração de cavidades na paisagem, as cavidades não serão consideradas como sítios de uso pela espécie.

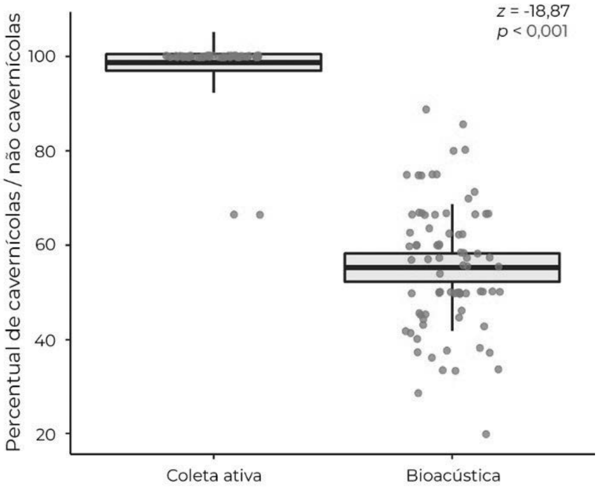


**Figura 6:** *Percentual de espécies consideradas cavernícolas essenciais ou cavernícolas oportunistas em relação ao total de espécies capturadas de acordo com os métodos de coleta. Os testes estatísticos informam o valor de z e as diferenças significativas ($p < 0,05$).*

## 4. Discussão

O presente estudo teve como objetivo principal comparar métodos distintos de amostragem, captura ativa e bioacústica, no levantamento de espécies de morcegos. Os resultados revelaram 13 espécies e dois morfótipos amostrados pelos métodos tradicionais de captura. Essas espécies são majoritariamente pertencentes à família Phyllostomidae. A bioacústica foi capaz de detectar os chamados sonoros de 23 espécies, além de 12 sonótipos identificados a nível de morfótipos. Esse método de captura passiva amostrou preferencialmente espécies de Emballonuridae, Molossidae e Vespertilionidae com nove sonótipos cada, contra três sonótipos de Phyllostomidae e um Thyropteridae.

A utilização da bioacústica no presente estudo permitiu o levantamento de diversos elementos da quiropterofauna que não haviam sido detectados pelos métodos tradicionais, no entanto há limitações da metodologia que devem ser consideradas. Foi notada certa dificuldade em se amostrar algumas espécies ou até mesmo famílias. Um exemplo disso é a espécie *Furipterus horrens*, que acreditamos ter sido subamostrada em função de suas características sonoras de baixa intensidade, curta duração e curto intervalo entre pulsos dos chamados de ecolocalização (FALCÃO et al., 2015), ou até mesmo por características do dispositivo utilizado. Esse déficit de amostragem fica evidente ao se comparar os dois métodos de amostragem utilizados. Pelos métodos ativos de captura foram nove cavidades com ocorrência de *F. horrens*, contra apenas dois pontos de detecção do sonótipo da espécie pela bioacústica.

O registro de espécimes da família Phyllostomidae, segunda família mais rica no mundo e primeira no Brasil dentre os Chiroptera (SIMMONS E CIRRANELLO, 2019), também representa um desafio ao uso da bioacústica (BRIGHAM et al., 2004). Evidências sugerem que membros dessa família não vocalizam em todas as direções o tempo todo, sendo os pulsos direcionados aos pontos de interesse, o que dificulta a detecção pelos dispositivos (SURLYKKE et al., 2013; KUGLER & WIEGREB, 2017). Além disso, os chamados são muito similares entre espécies, mesmo ao se considerar espécies de guildas tróficas distintas (YOH et al., 2020), dificultando sua identificação. Com isso, a grande disparidade ao se comparar as nove espécies de Phyllostomidae amostradas pelos métodos de captura ativa em N3 contra os três morfótipos registrados pelos dispositivos seria esperada. Dada as divergências em composição de espécies, os dois métodos implementados devem ser considerados complementares. Isso pode ser evidenciado pela curva de acumulação de espécies, sendo capaz de atingir 90% da diversidade local com menos da metade das cavidades amostradas.

Por outro lado, estudos em bioacústica têm sido eficientes em detectar a composição de morcegos insetívoros aéreos e seu uso da paisagem (CRUZ et al., 2015; FALCÃO et al., 2021; SILVA-SOUZA et al., 2022). Salvo as três morfoespécies de Phyllostomidae, todos os demais sonótipos foram atribuídos a famílias de espécies insetívoras aéreas em território brasileiro, as quais foram representadas pelas famílias Emballonuridae, Molossidae, Thyropteridae, Vespertilionidae e Mormoopidae (REIS et al., 2017).

Em se tratando das espécies que poderiam de fato estabelecer populações no interior das cavidades, o número de espécies de morcegos considerados essencialmente cavernícolas e cavernícolas oportunistas foi ligeiramente maior na captura ativa (12 spp.) do que pela bioacústica (9 spp.). Ao se considerar o número médio dessas espécies por cavidade, foi encontrada maior riqueza pelo método da bioacústica. Por outro lado, a proporção das espécies cavernícolas em comparação às não cavernícolas é muito maior para o método de captura ativa. Provavelmente esse resultado decorre do local de instalação dos gravadores. Os gravadores foram posicionados na área externa da cavidade, para facilitar o registro das vocalizações no momento de emergência dos morcegos (REVILLA-MARTIN et al., 2021).

## 5. Conclusão

Os resultados do estudo evidenciam que a bioacústica compreende uma importante ferramenta em estudos de inventário da quiropterofauna cavernícola quando usada de modo complementar a técnicas tradicionais. Além disso, o posicionamento do dispositivo na entrada das cavidades deve ser mais apropriado, conforme proposto em estudos anteriores (REVILLA-MARTÍN et al., 2021; BARROS & BERNARD, 2023b). A partir da detecção mais fidedigna da diversidade local da quiropterofauna, é possível oferecer subsídios importantes aos programas de monitoramento e elaborar estratégias de conservação mais assertivas e abrangendo uma maior compreensão da utilização da paisagem cárstica por diferentes espécies de morcegos, assegurando assim, a manutenção da biodiversidade local, bem como dos processos ecológicos que envolvem a quiropterofauna.

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# A new species of *Ceriochernes* Beier, 1937 (Pseudoscorpiones, Chernetidae) from Northeastern Brazil

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## Abstract

A new species of pseudoscorpion from the genus *Ceriochernes* is described from Lapa dos Peixes cave, northeastern Brazil. This study provides a detailed morphological description, diagnostic characteristics, and illustrations for *Ceriochernes* sp1. Additionally, we present a comparative discussion of the genus *Ceriochernes* and include a habitat description. We also emphasize the need for further exploration in uncharted areas, as additional new species are likely to be discovered.

## 1. Introduction

Pseudoscorpions represent an important order of arachnids, with a worldwide distribution except for Antarctica (Benavides 2019). They inhabit a variety of environments, including tree trunks, leaf litter, spaces under rocks, and caves, where they can exhibit significant diversity. The order presents 26 families (and one extinct), 15 of which occur in Brazil, with 10 families already recorded in caves. Among these, Chernetidae stands out as the third-largest pseudoscorpion family, consisting of approximately 731 species (17% of pseudoscorpion diversity) and 120 genera (WAC 2024), including *Ceriochernes*, the focus of this study.

The genus *Ceriochernes* was first described based on specimens found in Phillipines (Beier, 1937), and further expanded by the description of additional species from Sri Lanka, Nepal and Brazil (Beier, 1973; Beier, 974; Mahnert, 1985). This disjunct distribution can be due to vicariance or, alternatively, to the lack of detailed studies along the distributional gap of the genus.

From the 8 valid species of *Ceriochernes*, 3 occur in Brazil: *Ceriochernes brasiliensis Beier*, 1974 from Santa Catarina state, Southern Brazil; *Ceriochernes foliaceosetosus* Beier, 1974, from Amazon state, Northern Brazil and *Ceriochernes foliaceosetosus* Beier, 1974 Ceriochernes amazonicus Mahnert, 1985, the most widespred distributed species in the country, with records from Santa Cataria, Pernambuco and Amazonas states, spanning from Southern to Northern Brazil.

In this study, we describe a new species of *Ceriochernes* found in a cave from Bahia state, northeastern Brazil. We herein provide a complete description of the new species, discuss some potential traits that could indicate adaptations to cave life and also provide some aspects of the species´ habitat and threats.

## 2. Methodology

**Study area**

Specimens of the new species of *Ceriochernes*, herein described were collected in the Lapa dos Peixes cave, which is part of the Água Clara cave system (ACCS), located in the karst region of Serra do Ramalho, municipality of Carinhanha, Bahia state, Brazil (Fig. 1). The ACCS has approximately 24 km, and is composed of four limestone caves trespassed by an intermittent stream, active during the rainy period (October until March). According to Köppen's climate classification system, the local climate is "Aw", with dry winter and an average annual rainfall of 640 mm3 (Alvares et al. 2013). The Serra do Ramalho region is inserted in the Caatinga domain (the only Brazilian semiarid biome), with transitional areas to the Cerrado (Brazilian Savanna) (Cole 1960).

**Field sampling**

The specimens were collected in Lapa dos Peixes cave as part of an extensive invertebrate survey. The holotype was gathered on September 7, 2023, by R.L. Ferreira, while the paratype was collected on June 3, 2024. Both specimens were gathered using fine brushes moistened with ethanol and preserved in vials containing 70% ethanol.

**Analysis and preparation**

The specimens were analyzed using a Zeiss Axio Scope A1 optical microscope, with image analysis conducted through the ZEN 2012 software. Illustrations of the specimens' bodies and appendages were created using a drawing tube attached to an Olympus BX40 optical microscope with phase contrast capabilities. Kaiser's glycerol gelatin was applied as the mounting medium to ensure stability during both observation and illustration processes. Photographs of structures and appendages were taken using a Zeiss Axio Zoom V16, with image capture facilitated by the ZEN 2.1 software. These images were subsequently vectorized using Inkscape 1.1 software (Montesanto 2015; available at inkscape.org). Further examination of the paratypes was performed with a Hitachi TM4000 scanning electron microscope (SEM). The holotypes and paratypes of both species have been deposited in the Coleção de Invertebrados Subterrâneos de Lavras (ISLA).

## 3. Results

**Family Chernetidae Menge, 1855**
**Subfamily Chernetinae Menge, 1855**
**Tribe Chernetini Menge, 1855**
***Ceriochernes* sp1**

**Material Examined.** Holotype male (ISLA 125649), preserved in ethanol: Brazil, Serra do Ramalho, Bahia, Lapa dos Peixes (13° 49' 22.1" S 43° 57' 25.2" W), September 7, 2023, collected by R.L. Ferreira. Paratype female (ISLA 125650), same data as the holotype, except it was collected on June 3, 2024.

**Etymology.**

**Diagnosis.** *Ceriochernes* sp1 differs from other species of the genus by hexagonal reticulate epicuticle; carapace 0.080 mm longer than wide; presence of modified leaf-shaped setae, filamentous setae and other branched setae; cheliceral chaetotaxy with 8 setae; hand and finger of the chela with equivalent proportions. Trichobothria: *st* closer to *t* than to *sb* and *b*; *ist* proximal to *it*; *ist* adjacent to *est*; *esb* slightly distal to *eb*; *et* and *it* adjacent and opposite.

**Description.** Body and Legs, orange-brown; pedipalps darker orange--brown; chelicerae almost translucent orange-brown. Entire body covered by a reticulated epicuticle with a hexagonal shape, resembling a beehive.

**Carapace:** 0.080 mm longer than wide (paratype 0.041mm wide than longer); showing a difference between ocular width and posterior width of 0.197 (0.302) mm; without ocular apparatus; Two transverse grooves, in the medial and posterior position; 48 modified setae, leaf-like, (of these, 9 on lateral margins, 11 between the first and second groove and 4 between the last groove and the posterior margin.)

**Coxas:** Intercoxal tubercle absent, the palpal coxae with reticulate epicuticle, with 17 setae, 7 modified setae closer to the margin, 10 filamentary in the internal portion, has reticulate epicuticle; coxae of the legs, without reticulated epicuticle, with only filamentary setae, chaetotaxy I–IV: 13(14):16(17):20:28(29).

**Tergites:** Divided horizontally except for I and XI. All setae modified. Chaetotaxy I–XI: 4:4:4:5:4:4:6:6:6:6:7.

***Figure 1:*** *Figure 7. New species' habitus.*

**Sternite:** Covered with reticulate epicuticle of hexagonal shape. Granular integument resembles scales. Chaetotaxy II–XII: 44:21:8:13:14:13:12:11:8:3:4 (anal). Modified setae smaller than those on tergites. Segments II, III, and the two central setae of segment V are filamentous. Female genital operculum with (35)38 setae in the anterior region and 20(22) along the posterior margin.

**Chelicera:** Hand with 7 setae and movable finger with 1, sbs, bs, bs1 and bs2 are branched; ls long, es short. Fixed finger with 8 teeth, pointed and facing backwards (the first three distal ones smaller and spaced apart); movable finger without teeth; galea with bifid tip and a subdistal ramus; external serrula with 19 blades, Internal serrula with four distal blades (the first elongated and three rounded), bearing small spine-like projections, followed by a continuous, uniform structure resembling a veil; rallum with 3 blades, the third most distal blade is larger and serrated, the others smooth.

**Palps:** Trochanter 0,17 (0.15) mm times wider than long, with a rounded (ventral) protuberance; femur 0.43 (0.43) mm times longer than wide, with internal margin convex, like an arch; patella 0.31 (0.38) mm times longer than wide, most proximal part thin, and the distal part in oval shape; hand similar size to the fingers, with modified leaf-shaped setae; fingers with filamentous setae, without reticulated epicuticle; chela with some bifid and branched setae; movable finger slightly curved inwards like a arch, with 51(48) teeth, facing backwards, with pointed apex, 2 smaller accessory teeth, located distally in the external lateral region; fixed finger straight with 43(45) homogeneous teeth in the shape of a trapezoid, with pointed apex, and 5 accessory teeth located distally in the external lateral region. Trichobothria: st closer to t than to sb and b, ist proximal to it, ist adjacent to est, esb slightly distal to eb, et and it are adjacent and opposite. Well-developed venom apparatus on the mobile finger.

**Leg I:** Arolium smaller than the claw; tarsus with predominant of modified setae, filamentous setae on the internal part and around the most distal tip; tibia with a single seta on the distal part; femur and patella (not fused), with only modified setae; trochanter with modified setae only on the outer part and completely granulated (thorn-like granules).

**Leg IV:** Arolium smaller than the claw; tarsus with predominant of modified setae, filamentous setae on the internal part and around the most distal tip; tibia with 3 filamentous setae on the internal part, femur and patella (fused) with filamentous and modified setae distributed around the limb; trochanter with filamentous setae on the internal part and two modified setae on the outer part.

**Measurements (mm):** Female holotype (Paratypes in parenthesis): body length 2.23 (2,50); carapace 0.70/0.62(0,764/0,805). Chelicera 0.23/0.10(0.29/0.14). Pedipalp: trochanter 0.39/0.22(0.45/0.30); femur 0.63/0.20(0.68/0.24); patella 0.55/0.23(0.65/0.27); hand (with pedicel) 0.54/0.36(0.62/0.42); chela (with pedicel) 1.13/0.36(1.25/0.42); chela (without pedicel) 1.085/0.36(1.20/0.42); movable finger length 0.60(0.66). Leg I: trochanter 0.16/0.12 (0.17/0.13); femur 0.21/0.14 (0.20/0.14); patella 0.20/0.12 (0.30/13); tibia 0.32/0.10 (0.34/0.10); tarsus 0.35/0.07 (0.40/0.07). Leg IV: trochanter 0.25/0.13 (0.21/0.13); femur + patella 0.53/0.12 (0.60/0.13); tibia 0.50/0.11 (0.51/0.11); tarsus 0.36/0.08 (0.40/0.07)

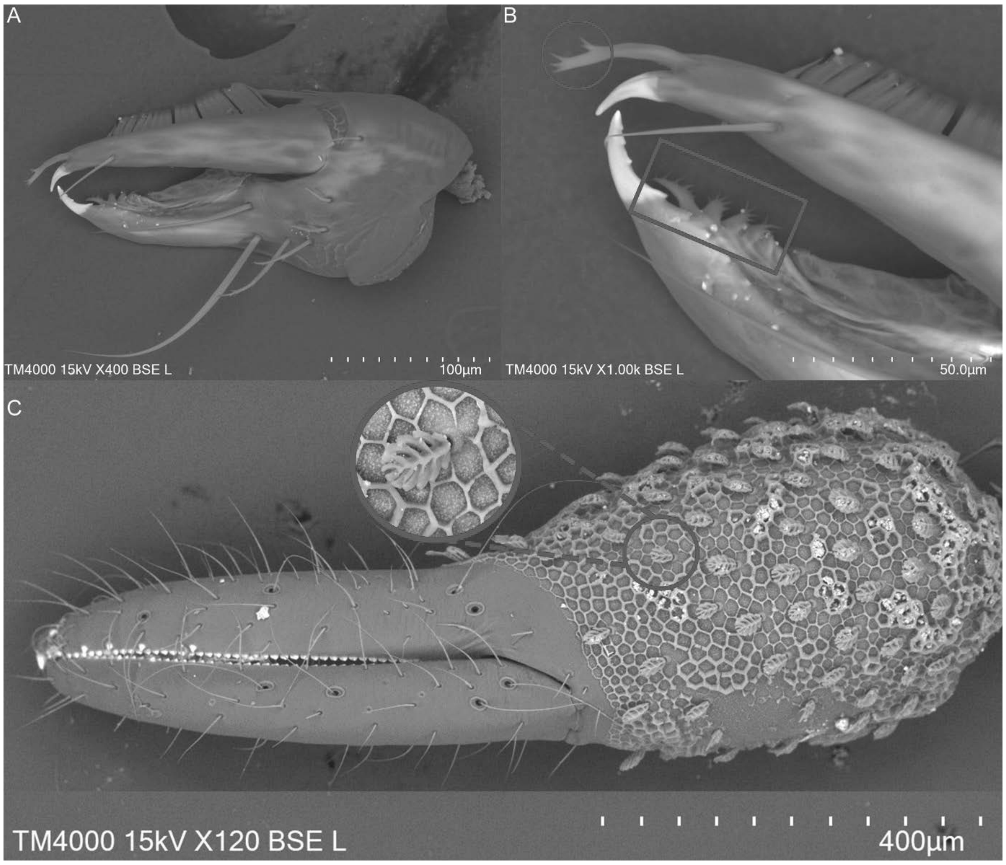

***Figure 2:*** *Ceriochernes sp1 female paratype A) Right chelicera, dorsal view B) Detail of left chela, showing galea branching, and internal serrula C) Left chela, external view, highlighting modified setae and reticulated epicuticle.*

## 4. Discussion

The genus *Ceriochernes* comprises eight known species worldwide, distributed across four countries. *Ceriochernes detritus* Beier, 1937, the first species described for the genus, is found in the Philippines and differs from the newly described species in several characteristics: the setae are not modified into leaf-like shapes, the chelicera has a rallum with three smooth setae (two short and one long), the galea has 3 to 6 lateral branches, the tergite has divided segments except for the last one, and it is further distinguished by the number of teeth on the chelae (48 on the fixed finger and 48 on the movable finger).

*Ceriochernes besucheti* Beier, 1973, from Sri Lanka, differs by the shape of the carapace (0.12 mm longer than wide), the short setae that are not modified into leaf-like shapes, the galea with 6 to 7 lateral branches in the chelicera, the tergite entirely divided, and in the chelae, the fingers are slightly shorter than the palm (without a pedicel).

In Nepal, three species occur: *Ceriochernes martensi* Beier, 1974, distinguished by its reticulated epicuticle (without an hexagonal pattern), almost invisible ocular marks, and two transverse sulci on the carapace, with the sub-basal sulcus close to the posterior margin. The chelicera has 5 setae, and the tergite has divided segments except for the last one. *Ceriochernes nepalensis* Beier, 1974, differs by its reticulated epicuticle (without an hexagonal pattern), short and thick setae, and the chelicera with 5 setae. In the chelae, the fingers are shorter than the palm (without a pedicel). *Ceriochernes vestitus* Beier, 1974, is distinguished by the reticulated epicuticle (without an hexagonal pattern), two transverse sulci on the carapace, with the sub-basal sulcus near the posterior margin, and the shape of the carapace (0.05 mm longer than wide). The chelicera has 5 setae.

In Brazil, three species have already been described: *C. brasiliensis*, which differs in the shape of the carapace (0.12 mm longer than wide), the chelicera with 7 setae, the tergite with divided segments except for the last one, and in the chelae, the fingers are almost the same size as the palm (without a pedicel). C. foliaceosetosus, differs by the shape of the carapace (0.05–0.06 mm longer than wide), the external serrula of the chelicera with 17 lamellae, the cheliceral setae with 6–7 setae, the galea branched with 5 lateral branches, the tergite with divided segments except for the last one, and the number of teeth on the chelae (35 to 40 teeth on both the fixed and movable fingers). The fingers are slightly shorter than the palm (without a pedicel), and there are differences in certain body proportions. *C. amazonicus* Mahnert, 1985, differs in the flattened vestiture setae, the chelicera with 7 setae, the galea branched with 6 apical branches, the female genital operculum with 30 setae, and the tergite entirely divided.

Although the descriptions of these species are older and not very detailed, they exhibit several characteristics that differ significantly from *Ceriochernes* sp1 nov., confirming it as a new species of the genus. A future revision of the genus would be essential to clarify gaps in the current knowledge of the group, along with molecular analyses to better understand the genus' distributio.

## 5. Conclusão

The description of this new species increases the number of species in the genus *Ceriochernes* to nine, four of which are found in Brazil. Furthermore, by incorporating several previously unillustrated characters in the descriptions of other species within the genus, this work serves as a model for future descriptions of new species that may be discovered. Finally, this new species confirms the taxonomic diversity and consequent significance of the Água Clara system, the richest hotspot of tropical subterranean biodiversity.

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# Cave invertebrates similarity driver by microhabitats atributes and "lajeados" distribution

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## Abstract

Geographic distance, habitat heterogeneity in caves, and variables such as distance from the entrance, availability of shelters, and trophic resources directly affect the diversity and composition of subterranean fauna. In this context, the aim of this study was to evaluate the determining factors of similarity and composition of cave invertebrate communities in discrete karst outcrops within the landscape, at different spatial scales (mesoscale and microscale). The study was conducted with sampling performed in 26 sectors (30m²) and 78 quadrants (1m²). The results showed the presence of 1161 individuals, distributed across 126 morphotypes, of which 8 exhibited troglomorphic traits. PERMANOVA revealed significant differences in fauna between karst outcrops and PERMDISP indicated greater homogeneity in similarity within karst outcrops. DistLM analysis showed that geographic distance and the presence of guano deposits influence fauna composition at the mesoscale. At the microscale, distance between caves and shelter availability were significant variables for composition. The availability of shelters and guano deposits are essential for maintaining diverse communities. These factors should be considered in conservation strategies to ensure the preservation of subterranean biodiversity.

## 1. Introduction

The distribution of invertebrate communities is closely related to environmental heterogeneity, which varies across different temporal and spatial scales and is influenced by physical, chemical, trophic, and microclimatic factors (Huston 1994). The diversity of microhabitats and the structural complexity of the environment are key determinants in the organization of biological communities, as they favor the coexistence of species with distinct life histories and ecological habits (MacArthur & MacArthur 1961; Pianka 1966; Magurran 1988; Stein et al. 2014). Thus, understanding species distribution and community organization allows for the development of more effective strategies for the conservation of fauna and their habitats (May 1986; Myers et al. 2000).

Habitat diversity and the availability of trophic resources are essential factors for the colonization and persistence of invertebrates in subterranean environments, where caves offer more stable conditions of temperature, humidity, and resource availability compared to epigean environments (Mammola et al. 2016, 2019a; Poulson & White 1969). The main energy source in these systems comes from allochthonous organic matter, transported by physical processes or introduced by animals in the form of feces, guano, and carcasses, playing a crucial role in the trophic dynamics of cave fauna (Culver & Pipan 2009; Souza-Silva et al. 2013). The distribution of subterranean biodiversity is influenced by spatial factors such as the geographic distance between caves and the rock formation in which they are located, with closer caves tending to share greater faunal similarity, while more distant caves exhibit higher dissimilarity (Mammola et al. 2019, 2020; Souza-Silva et al. 2020a, 2020b; Reis-Venâncio et al. 2022). Moreover, environmental heterogeneity along the cave gradient, from the entrance to deeper areas, influences spatial beta diversity, promoting variations in fauna due to changes in microclimatic conditions and resource availability (Zagmajster et al. 2018; Pacheco et al. 2020a; Souza-Silva et al. 2021; Vaz et al. 2025). The distribution of subterranean fauna may also vary according to the sampling scale (mesoscale and microscale), being influenced by microclimatic factors (temperature, humidity), trophic factors (guano, roots, plant debris), and physical factors (water bodies, rock fragments) (Simões et al. 2015; Pacheco et al. 2020a; Souza-Silva et al. 2021).

The conservation of cavernicolous invertebrate fauna faces significant challenges due to the limited understanding of the main environmental factors that influence its distribution. In Brazil, research on faunal composition, distribution, and similarity across multiple scales remains scarce and concentrated in specific carbonate regions (Souza-Silva et al. 2021; Cardoso et al. 2022; Reis-Venâncio et al. 2022, 2024; Vaz et al. 2025). In the semiarid region of Rio Grande do Norte, studies address taxonomic, geological, phylogeographic, and ecological aspects (Hoch & Ferreira 2013; Ferreira et al. 2010; Cruz et al. 2010; Bento et al. 2016, 2021, 2024). Bento et al. (2021) investigated the similarity of cavernicolous fauna in relation to physical and trophic factors, as well as the environmental influence on species composition and richness. However, there remains a gap in understanding the relationship between faunal structure and physical and climatic factors in caves in the region, using different sampling scales to assess the influence of environmental factors and micro-habitats on these communities.

The present study aimed to evaluate potential variations in the structure of terrestrial invertebrate communities between caves in discrete outcrops in the landscape and determine the main environmental variables of physical, trophic, and microclimatic micro-habitats that determine the composition and richness of cavernicolous invertebrate fauna. We hypothesized that the composition of invertebrate fauna between caves located in spatially distinct geological units will be dissimilar due to geographical distance and the "island" effect of the outcrops, as well as distinct habitat characteristics. Distance between caves will be a significant variable for determining the composition of cavernicolous invertebrate fauna.

## 2. Materials and Methods

The study was conducted in 18 carbonate caves distributed across the municipalities of Baraúna, Caraúbas, Felipe Guerra, Governador Dix-Sept Rosado, Jandaíra, Martins, and Mossoró, in the state of Rio Grande do Norte (RN), northeastern Brazil (Fig. 1).

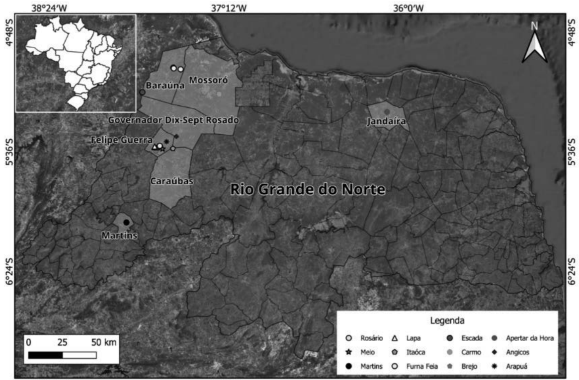

*__Figure 1:__ Location of the study area, caves (geometric shapes), and outcrops (color of geometric shapes) in the municipalities of Baraúna, Mossoró, Governador Dix-Sept Rosado, Felipe Guerra, Caraúbas, Martins, and Jandaíra, state of Rio Grande do Norte, Brazil.*

These caves, with the exception of the Martins cave, are part of the Apodi Group, Jandaíra Formation, which dates back to the Upper Cretaceous and consists of extensive limestone outcrops known as "lajedos" that host cave complexes (Cruz et al. 2010) (Fig. 2).

***Figure 2:** Drone photos of outcrops in Rio Grande do Norte, Brazil.*

The Martins Cave, Casa de Pedra, is situated within the marbles of the Jucuturu Formation (Seridó Group), deposited during the Pre-Cambrian period. Located in the semi-arid region of the Caatinga biome, this formation is characterized by strong seasonal hydric variation and shallow, rocky soils. Subterranean water resources are the primary water source during drought periods, highlighting its ecological and socioeconomic importance (Bento et al. 2021). According to the Köppen classification, the Caatinga region is classified as BSh (hot and dry semi-arid climate with low latitude and altitude areas) (Alvares et al. 2013).

The sampling of biotic data and habitat structure was conducted during field campaigns in 2018, 2019, and 2021. To carry out the sampling, units were distributed along the length of the caves, using two distinct scales: a meso-scale, with 10x3m sectors (30m²), and a micro-scale, with 1x1m quadrants (1m²). Three quadrants were placed within each sector: two at the ends and one in the center (Souza-Silva et al. 2021, Furtado et al. 2022). In total, 26 sectors and 78 quadrants were sampled, distributed across 18 caves.

Invertebrate sampling was conducted separately in the quadrants and sectors through active visual searches and manual collection (Wynne et al. 2019), using tweezers and brushes. The species sampled in the quadrants were also considered for the meso-scale. Invertebrate specimens were preserved in 70% alcohol for identification.

The invertebrates were sorted and identified to the most accessible taxonomic level and grouped into morphotypes with the aid of stereo microscopes, following the methodologies described by Oliver and Beattie (1996). Troglomorphic characteristics, such as the reduction or absence of ocular structures and pigmentation, and the elongation of appendages, were used to identify potentially troglobitic species (Culver & Pipan 2009). The collected invertebrates are deposited in the Collection of Subterranean Invertebrates of Lavras (ISLA), affiliated with the Center for Studies in Subterranean Biology (CEBS) at the Federal University of Lavras (UFLA).

The geographical location of the caves was obtained from latitude and longitude data available in the National Cave Information Register (CANIE/CECAV 2024). Temperature and humidity were measured using a digital hygrometer, positioned at floor level and stabilized for at least 15 minutes (Pellegrini et al. 2016, Souza-Silva et al. 2021). To measure and characterize the substrate structure in the sectors, its extent was divided into ten sections of 1 meter by 3 meters. The characterization and quantification of habitat structure were performed through visual inspection, based on the proportion of each type of substrate present in each section (Souza-Silva et al. 2021). For the microscale, photographs taken with the camera positioned at a 90-degree angle relative to each quadrant documented the structural features of the habitat. These photographs were then analyzed using the ImageJ software (Rasband 1997) to characterize the types of substrate and quantify their coverage area in the quadrants (Pacheco et al. 2020b; Furtado et al. 2022). The environmental substrate variables obtained for the quadrants were: guano, plant detritus, small boulders, blocks, coarse gravel, fine gravel, sand, silt, hardpan, speleothem floor, smooth and rough bedrock.

The richness and abundance of invertebrates were determined by the sum of morphotypes and individuals in the sampling units, respectively. To assess differences in faunal composition between the rock formations, we performed a permutational analysis of variance (PERMANOVA), using 999 permutations and the Bray-Curtis similarity matrix (Anderson et al. 2008). Additionally, a multivariate dispersion analysis (PERMDISP) was conducted to verify the homogeneity of multivariate dispersions between the rock formation groups, evaluating the distances of the samples to the centroid of each group. For graphical visualization of the data and the groups formed, we performed non-metric multidimensional scaling (nMDS) using the Bray-Curtis similarity matrix. To facilitate the PERMANOVA, PERMDISP, and nMDS analyses, only the quadrants were used as the sampling unit.

For the analysis of the influence of environmental variables on faunal composition, we grouped substrate variables into categories of resources and environmental conditions, including guano (GUA), plant resource (RV), shelter (AB), fine substrate (SF), and inorganic substrate (SI). The substrate diversity variable (DS) was obtained by applying the Shannon index (H') using all substrate components (Pellegrini et al. 2016). To obtain the distance between caves (DIST), we used their geographic locations. Latitude and longitude values were input into PRIMER, and we then used the "indicators" function to obtain the DIST variable (Bento et al. 2021). To assess the influence of substrate characteristics on faunal composition in the caves, we performed a distance-based linear model (DistLM) at both sampling scales, using a Bray-Curtis similarity matrix with a forward procedure and adjusted $R^2$ model selection criterion, with 999 permutations (Anderson et al. 2008). The environmental variables tested were DIST, GUA, RV, AB, SF, SI, and DS. For the meso-scale analysis, the variables temperature (TEMP) and humidity (UMI) were also tested. All the analyses listed above were performed using the Plymouth Routine in Multivariate Ecological Research – Primer 7 + Permanova Software (Anderson et al. 2008). The significance level considered was $P \leq 0.05$.

## 3. Results

A total of 1161 invertebrates were recorded, distributed across 126 morphotypes, belonging to at least 47 families. The most diverse taxa were Araneae (30 spp.), Acari (13 spp.), Coleoptera (8 spp.), and Opiliones (8 spp.). Eight species were considered troglobitic, including Araneae (*Oonopidae* sp., *Metagonia* sp.), Scolopendromorpha (*Newportia* sp.), Polyxenida sp., Diplura (*Japygidae* sp.), Hemiptera (*Kinnapotiguara troglobia* (Hoch & Ferreira 2013), Isopoda (*Styloniscidae* sp.) and Pseudoscorpiones sp.

The PERMANOVA analysis showed a significant difference in faunal composition between the lajedos (Pseudo-F = 1.9736, p = 0.001), indicating that the faunal composition is dissimilar. The variance attributed to the analysis revealed that 25.97% of the variation in the data can be explained by the factor "lajedo."

Regarding the homogeneity of multivariate dispersions between lajedo groups, the PERMADISP analysis showed no significant difference (F = 4.4317, p = 0.093), indicating that the variations within communities across lajedos are similar. The nMDS plot showed a good fit quality (stress = 0.09) (Fig. 3).

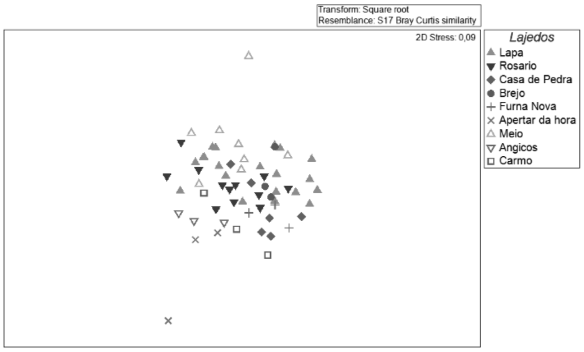

***Figure 3:*** *Multidimensional Scaling (MDS) showing the similarity in faunal composition between quadrants of caves from discrete outcrop areas in 13 caves from the state of Rio Grande do Norte, Brazil.*

For the meso-scale, the variation in invertebrate composition is significantly related to Guano (p = 0.001; Pseudo-F = 2.133; Adj R2 = 0.0426) and Distance between Caves (p = 0.005; Pseudo-F = 1.563; Adj R2 = 0.0429), with the model explaining 14.34% of the variation. For the micro-scale, the variables DIST (p = 0.001; Pseudo-F = 2.056; Adj R2 = 0.028) and Shelter (p = 0.009; Pseudo-F = 1.895; Adj R2 = 0.014) were significant for the variation in invertebrate composition, with the model explaining 4.84% of the variation.

## 4. Discussion

The results confirm the hypothesis of dissimilarity in the faunal communities of caves located in distinct outcrops, attributed to geographical location and local barriers that hinder the dispersion of certain species. In the studied region, factors such as the watershed and the presence of water inside the caves influence faunal similarity, with the most evident differentiation observed in troglobitic organisms (Bento et al. 2021). The relationship between geographical distance and faunal dissimilarity follows a pattern in which caves located closer to one another exhibit higher similarity, while dispersion is limited by physical and climatic barriers, particularly for strictly cavernicolous species (Nekola & White 1999; Mammola et al. 2019b; Souza-Silva et al. 2020b; Culver & Pipan 2009). The Caatinga biome, characterized by its environmental heterogeneity and high invertebrate diversity, includes endemic species whose distribution may be influenced by the regional species pool, with interactions between epigean and hypogean fauna (Ferreira et al. 2010; Mendes-Rabelo et al. 2021; Reis-Venâncio et al. 2022). Some of these species transit between underground and surface environments, particularly near the cave entrances, playing a key role in community structuring (Prous et al. 2004, 2015). Faunal similarity between outcrops may indicate connectivity between caves through micro and mesocavities, functioning as interconnected systems (Mammola et al. 2020). Additionally, these environments can be considered analogous to subterranean islands, where fauna utilize macro and micro spaces for dispersal, as observed in ferruginous caves in Brazil (Ferreira 2005).

The results confirmed our hypothesis, demonstrating that the distance between caves significantly influences faunal composition at both sampling scales. At the mesoscales, faunal composition was determined by the trophic resource guano, while at the microscale, shelter availability was the main factor. The distance between caves plays a central role in the structuring of invertebrate communities, particularly in areas near the entrance (Reis-Venâncio et al. 2022, 2024), and is one of the primary factors explaining variation in species composition in quartzite caves of the Atlantic Forest (Souza-Silva et al. 2020b). The relationship between subterranean fauna and the regional species pool suggests a connection between hypogean and epigean environments, although cave environmental filters result in differences in faunal composition, restricting the occurrence of certain species to areas around the entrance (Prous et al. 2004, 2015; Mendes-Rabelo et al. 2021). Only a small proportion of the sampled organisms were troglobites, indicating a stronger influence of the regional species pool on the recorded fauna.

Guano is an essential nutritional resource for cave fauna in oligotrophic environments, influencing diversity, abundance, and the structure of trophic webs, as well as providing a protected and humid habitat (Ferreira & Martins, 1999; Souza-Silva et al. 2011; Ferreira 2019). Its significance was confirmed in studies using various sampling methodologies, including sectors, quadrants, and direct collections in the Rio Grande do Norte region (Pacheco et al. 2020a, 2020b; Cardoso et al. 2022; Bento et al. 2021). At the microscale, shelter availability, characterized by fissures, blocks, and rocks of various sizes, is associated with subterranean habitat heterogeneity, influencing the structuring of cave fauna (Ferreira & Souza-Silva 2001; Reis-Venâncio et al. 2022). This heterogeneity reduces niche overlap, competition, and predation, favoring the diversification of biological communities (Stein et al. 2014; Pacheco et al. 2020a; Reis-Venâncio et al. 2022; Vaz et al. 2025).

The conservation of cave environments requires an in-depth understanding of the factors that shape their biological communities. Trophic aspects and the physical characteristics of the microhabitat play fundamental roles in maintaining subterranean biodiversity. Furthermore, the geographic location of caves, especially in relation to connectivity and isolation, is a crucial factor in understanding the patterns of similarity and dissimilarity among cave communities.

In the context of Rio Grande do Norte, with areas of significant speleological value and the presence of endemic species, this information is important for supporting conservation strategies. Many speleologically significant regions in the area remain unprotected, and understanding how environmental and spatial factors influence cave communities is essential to develop measures that ensure the preservation of these environments.

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# Does size matter? Cave size driving seasonality effects on cave communities in brazilian savannah

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## Abstract

Cave environments are known to be isolated and climatically stable habitats. However, seasonality can be an important matter when talking about animal communities in these subterranean ecosystems. To understand how seasonality can affect and be affected by cave characteristics, animal species were sampled in 62 caves at the Brazilian Savannah, and species richness analyses were performed aiming to assess differences between dry and rainy seasons, and to determine which factors can act over seasonality impacts. The results showed that during the rainy season the species richness was significantly higher, but in small caves the differences can be more aggressive than when compared with large caves. This reinforces the importance of two sampling events, in different seasons, to better understand the subterranean fauna of Brazil and help make decisions on environmental subjects.

## 1. Introduction

The cave environment is characterized by climatic stability due to its isolation from the external environment, resulting in conditions as the absence of light, a temperature close to the annual external average and high humidity (CULVER et al., 2019; POULSON, WHITE, 1962; MOLDOVAN et al., 2018). Thus, some cave attributes, such as the number and position of entrances associated with the cavity length, may influence the climatic stability, with greater isolation leading to higher stability (SIMÕES et al., 2015).

Despite the stability of the hypogean environment, small environmental variations are enough to alter the dynamics of species inhabiting the cave environment (FERREIRA et al., 2015). Significant differences in subterranean fauna richness have been observed between the rainy, and dry seasons (BENTO et al., 2016), as well as fauna behaviours related to the search for milder environmental conditions, which are provided by the climatic stability of caves (CHELINI et al., 2011; CAMP & JENSEN, 2007). In limestone caves located in the Caatinga biome, it was observed that species richness and abundance were higher during the rainy season. This result is likely corroborated by an increase in organic resources brought by rainfall, a rise in decomposition rates, and an increase in guano quantity (BENTO et al., 2016). On the other hand, there are species that use caves especially during the dry season, such as a population of harvestmen that use these environments as refuges during the dry winter (CHELINI et al., 2011). These variations highlight the complexity of cave communities and its dynamism.

In addition to seasonality, it is well known that the subterranean species richness is positively influenced by the stability of the cave, which includes factors such as cave size and the number of entrances (CARDOSO et al., 2024). Moreover, species richness can also be influenced by factors like the presence of water bodies, such as streams (RABELO et al., 2021; SIMÕES et al., 2015), the amount of organic resources, and cave genesis (JIMÉNEZ-VALVERDE et al., 2017).

Considering the complexity of factors that can influence cave richness and the importance of seasonality in this process, we aim to investigate how seasonality affects the species richness of limestone caves located in the Cerrado biome, specifically in the municipality of Engenheiro Navarro (Minas Gerais, Brazil), in an area known as Fazenda Tirirical, which is designated for cave preservation. Thus, we hypothesize that the larger the cave, the lower the seasonal interference on species richness. This will help provide greater insight into the influence of seasonality on the richness of tropical limestone caves.

## 2. Materials and methods

The study was conducted in 62 caves located on Fazenda Tirirical, in the municipality of Engenheiro Navarro, Minas Gerais, Brazil (Fig. 1). The region falls within the domains of the Cerrado biome, showcasing its varied typologies (Fig. 2). The vegetation includes a series of altitudinal strata from the Cadeia do Espinhaço, where high-altitude areas feature rupestrian fields and cloud forests. Although the area is not part of any Conservation Unit, it is situated within the Buffer Zone of the Serra do Espinhaço Biosphere Reserve and within the São Francisco River basin. For this study, data were collected during two sampling events, one during the rainy season (January and February) and the other during the dry season (May and June).

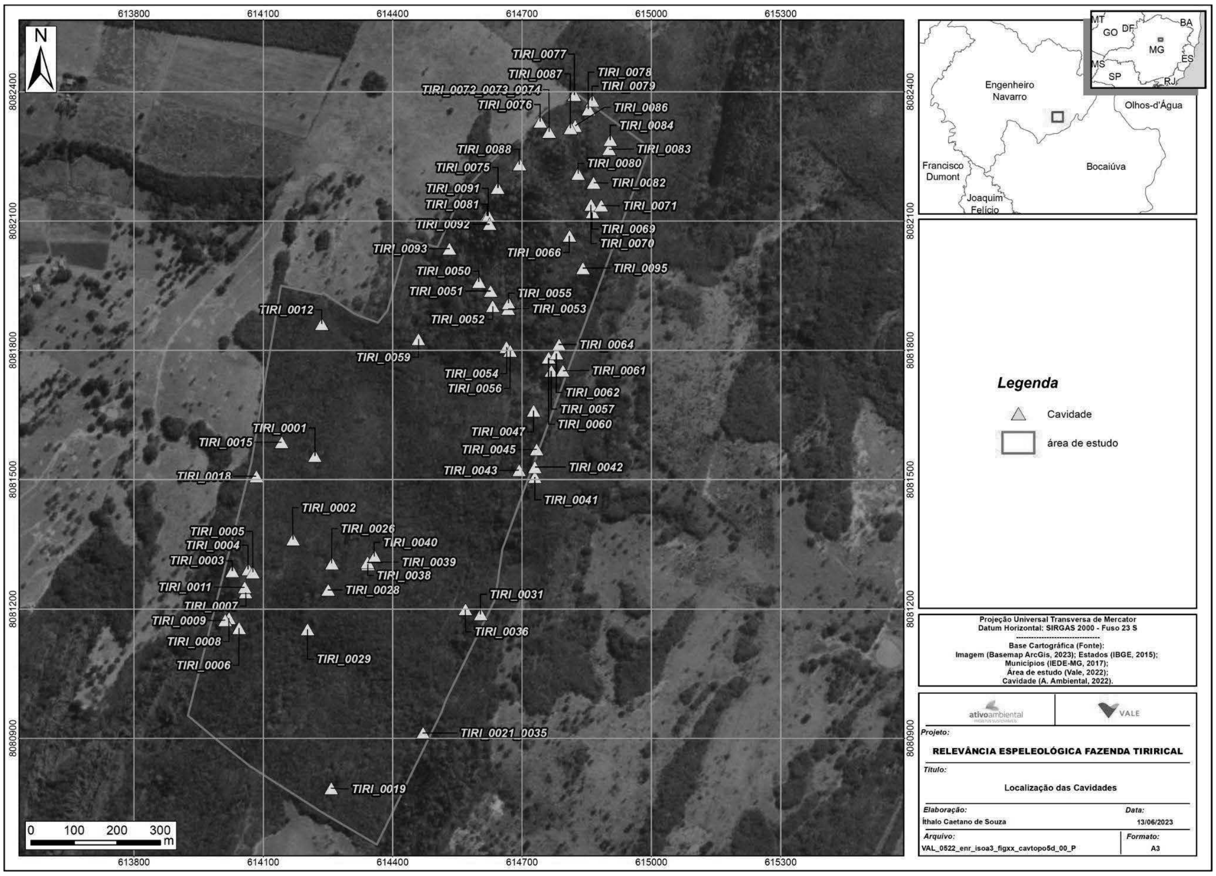

***Figure 1:** Locality of Fazenda Tirirical, at Engenheiro Navarro municipality, Minas Gerais State, and the 62 caves studied.*

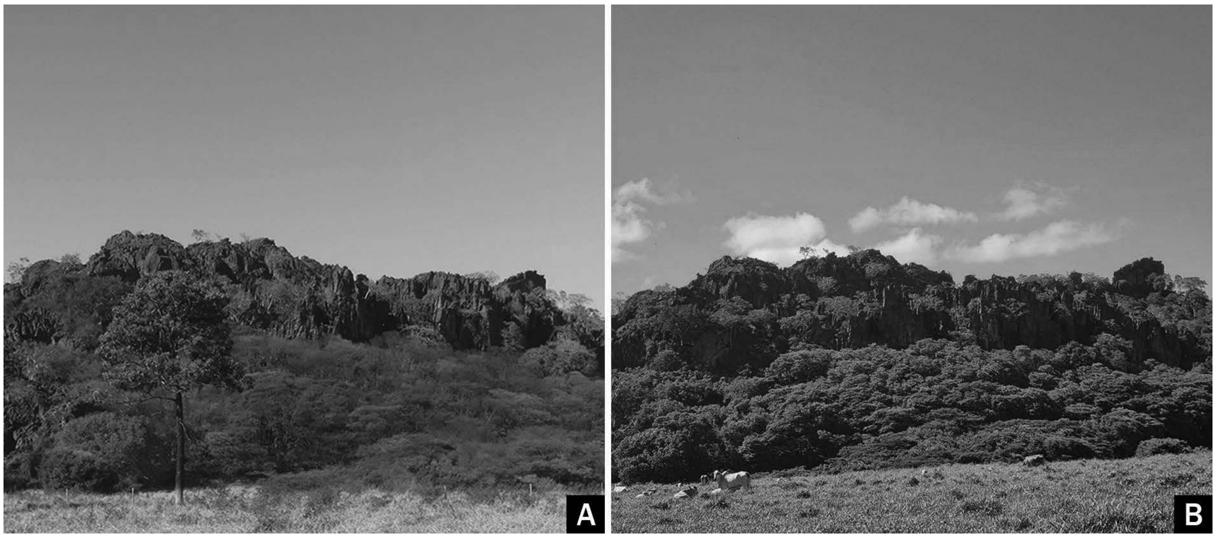

***Figure 2:** Landscape of the study area during the dry (A) and rainy (B) seasons.*

The use of the caves by bats was verified through direct observation of the animals. During daytime, when bats remain in their roosts, each accessible part of the cave was inspected. Bat capture was performed using hand nets. Captured animals were identified through the American Society of Mammalogists protocol (SIKES et al., 2011). After these procedures, the animals were released in the same cave where they were captured.

The recording of the anuran fauna and other non-flying vertebrates was carried out through visual searches for adult individuals. To ensure accurate identification, individuals were photographed in their natural position whenever possible. Species identification was performed by group specialists with the aid of field guides and identification keys.

Invertebrates were sampled using the active search method across all accessible sections of the cave, prioritizing microhabitats and organic deposits. At least one specimen of each observed species was collected using tweezers and brushes. Collected invertebrates were stored in containers with 70% alcohol. The specimens were sorted and identified in the laboratory under a stereomicroscope and stored in microtubes with 70% alcohol. After this process, the biological material was sent to specialists for more detailed identification and analysis of potential troglobitic species. Upon identification, all invertebrates were sent to scientific reference collections for archiving.

To assess the difference of the mean fauna richness between dry and rainy seasons, the Wilcoxon test was performed. Later, a Variance test was used to understand the difference of data variance between the two sample events. Also, to understand the factors that can impact the difference of richness between dry and rainy seasons, a generalized linear mixed model (GLMM) was performed using Poisson distribution. The model used the volume (m³) of the cave as a proxy of its size, the seasons and the relationship between them as explanatory variables. The cave itself was used as a random variable to limit the influence of geographic differences. All the analyses were performed using RStudio (R CORE TEAM, 2023) software and lm4 package.

## 3. Results

Regarding the fauna found in the 62 caves studied, 674 morphospecies were registered. The invertebrate fauna was dominant, with 657 morphospecies, in which Diptera was the most representative, with 110 morphospecies. Also, Araneae and Coleoptera were important groups, with 86 and 68 morphospecies, respectively. Moreover, 17 vertebrates were found (9 Chiroptera; 4 Anura; 2 Squamata; 1 Testudines; 1 Strigiformes) (Fig. 3).

From all animals found in the caves, 16 were considered troglobitic. The strict subterranean fauna was grouped in eight Orders, with Araneae being the most representative, with seven morphospecies. Within the 62 caves in the project, 33 had occurrences of at least one troglobitic species. Most of these animals have at the moment only the caves here analyzed as their known habitat.

Considering both seasons, the mean richness of the caves was 59.23 (± 20.03) morphospecies. In general, the richness was higher in the rainy season, with mean 43.11 (± 15.27), while the dry season had mean 26.11 (± 16.18) (Fig. 4). The Wilcoxon Test demonstrated that the species richness between the two seasons is significantly different (p-value = 3.74e-10; W = 3175). However, the Variance Test did not show significant distinctness between dry and rainy seasons (p-value = 0.08; F = 1.57).

The results of the GLMM showed a significant interaction between season and volume of the caves (Estimate = 0.0926; p-value = 0.0012). This indicates that volume modulates the effect of seasonality on species richness. Specifically, larger caves show a lower difference in richness between rainy and dry seasons (Fig. 4). Additionally, the model corroborates the Wilcoxon test, also presenting that richness was lower during the dry season (Estimate = -0.5141; p-value < 0.001). The volume of the cave isolated was not significant (p-value = 0.1026), but its interaction with seasonality highlights its influence in reducing the seasonal difference in species richness as cave volume increases.

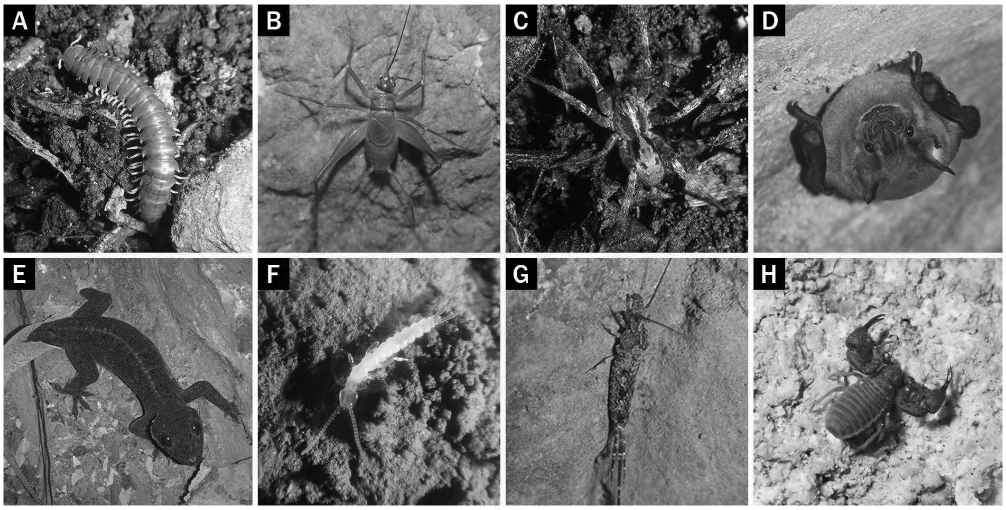

***Figure 3:*** *Some of the fauna observed during the study: Eurydesmus sp.1, rainy season (A); Endecous didymus, rainy season (B); Ctenidae sp., rainy season (C); Artibeus planirostris, rainy season (D); Phyllopezus pollicaris, rainy season (E); Projapygidae sp.1, rainy season (F); Neomachilellus sp.1, rainy season (G); Spelaeichernes sp.1, dry season (H).*

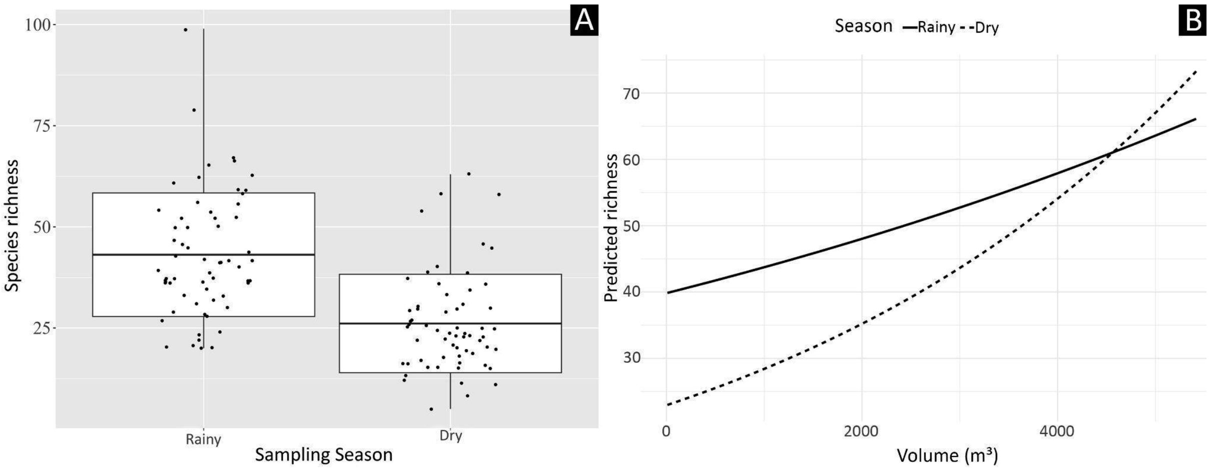

***Figure 4:*** *(A) Boxplot displaying the difference of species richness between rainy and dry season; (B) Prediction of species richness being affected by the volume of the cave, based on the Generalized Linear Mixed Model (GLMM), at dry and rainy season.*

## 4. Discussion

The species richness (674 spp) documented in the 62 caves aligns with what is expected for a region of high biodiversity, such as the Cerrado biome. The substantial number of invertebrates (657 spp) underscores their dominance in hypogean habitats, while the vertebrates recorded (17 spp) highlights the usage of subterranean environments by these animals, primarily as shelters, but also for foraging resources (MATAVELLI et al., 2015; PACHECO et al., 2020a; SOUZA-SILVA et al., 2021; FURTADO-OLIVEIRA et al., 2022).

Fauna richness in cave habitats is often directly related to the epigean species pool (RABELO et al, 2021). In a biodiverse area such as the Cerrado, particularly within the buffer zone of the Serra do Espinhaço Biosphere Reserve, the high richness observed in subterranean environments can be partially attributed to the conservation status of the surrounding epigean habitats (SIMÕES et al., 2014; FRANÇOSO et al., 2015; RABELO et al, 2020).

The troglobitic fauna (16 spp) identified in the study area reflects the strong eco-evolutionary relationship between invertebrates and cave ecosystems in the Brazilian savannah. This is further corroborated by the increased number of caves within the Fazenda Tiririca region where troglobitic species were found. It is important to note that the majority of the troglobitic species have only been recorded in the caves of the study area, emphasizing the critical importance of conserving this region.

As in other karstic landscapes, the higher species richness found in the caves can be related to the dependence of subterranean environments to allochthonous resources (CULVER & WHITE, 2005; BENTO et al., 2016). Based on the idea that vegetations on limestone areas tend to accumulate more litter during dry seasons, is expected that rain, streams, and flood pulses throughout rainy periods may transport these vegetal resources to the subterranean environments (BRINA, 1998; SOUZA-SILVA et al, 2007; SOUZA-SILVA et al, 2011a; BENTO et al., 2016), increasing species richness in caves (SIMÕES et al., 2015). Additionally, the rainy events can also carry animal carcasses and increase percolation processes (SOUZA-SILVA et al., 2012; BENTO et al., 2016). As shown by the GLMM, even though the volume of the cave was not important for the fauna richness, its interaction with the different seasons may be a key factor to understand the effects of seasonality over subterranean environments.

Although caves are often interpreted as isolated and stable ecosystems, they may reflect delayed feedback of epigean climate variations (TOBIN et al., 2013). While larger caves present higher environmental stability, small caves suffer more from the effects of external climate changes. In the majority of the caves, external seasonality alterations can lead to air flow shifts, which cause temperature and moisture alterations, mainly in entrance zones (CIGNA, 2002; BENTO et al., 2016).

Therefore, communities found in small caves experience stronger effects from these climate changes, which may lead to species turnover and differences in species abundance and richness (BENTO et al., 2016). Thus, this reinforces the importance of sampling caves in both seasons, dry and rainy, for a more realistic knowledge of subterranean fauna, as required by Brazilian legislation for activities potentially harmful for natural caves (MMA, 2017).

Another important point to highlight is that climate changes can also represent a vast threat to subterranean environments. Variations in temperature and rainfall, caused by climate change are expected for South America, and even being highly stable habitats, caves in Brazil will probably be affected by these alterations, especially the small ones (MAMMOLA et al., 2019; BENTO et al., 2016). This puts at risk a very sensible ecosystem, that is the home for unique biodiversity in a super biodiverse country as Brazil.

## 5. Conclusion

This study demonstrated a significant difference in the species richness of subterranean fauna across the 62 targeted limestone caves, with higher richness observed during the rainy season compared to the dry season. Furthermore, the results indicate that the volume of the caves modulates the effect of seasonality on biological richness, as evidenced by the interaction whereby larger caves exhibit smaller differences in richness between seasons.

Thus, the findings, combined with the complexity of subterranean fauna communities, support the idea that fauna sampling should be conducted during two events, one in each season, especially in projects encompassing multiple caves that likely present diverse physical characteristics, such as volume.

In this way, the study contributes to a better understanding of the dynamics of subterranean communities in tropical limestone caves, which is essential for improving conservation and management efforts for these caves.

## Acknowledgments

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# A depth matter: divergent responses of troglobite and non-troglobite fauna to habitat traits

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## Abstract

Habitat characteristics are key factors for fauna distribution in caves, so this work aims to understand how environmental traits influence the richness and composition of invertebrate communities in 24 caves in the western Bahia state, in the Brazilian semi-arid. Landscape, physical, trophic, and microclimatic traits were used to understand that. A total of 338 species were found, with 41 considered troglobites. The results show that the distance from the nearest entrance was the most important factor for communities' composition and richness but with a contrast between non-troglobite and troglobite fauna. While the restricted subterranean fauna responds negatively to the increase in distance, the restricted species suffer a positive effect.

## 1. Introduction

Habitat heterogeneity is one of the key factors for the distribution patterns since a higher heterogeneity allows the coexistence of a higher number of species, thus the usage of different microhabitats from the fauna (YANG et al. 2015; STEIN et al. 2015; VARGAS-MENA et al. 2020; PACHECO et al. 2020; SOUZA-SILVA et al. 2021).

This affirmative is also true in subterranean environments once the fauna is influenced by the unique characteristics of these habitats (PACHECO et al. 2020; SOUZA-SILVA et al. 2021).

Even though the subterranean environments are not limited to them, the caves are probably the most studied hypogean habitats until the moment (JUBERTHIE et al., 1980; MAMMOLA et al., 2016; RABELO et al., 2020; PACHECO et al., 2020; SOUZA-SILVA et al. 2021). These natural cavities are known for having peculiar characteristics, such the higher climatic stability when compared to the epigeous zone, with stable temperature all year and humidity next to the saturation (HOWARTH 1980, 1983). These habitats are also characterized by the absence of light and a predisposition to oligotrophy (CULVER & PIPAN 2009).

The distance from entrances stands out between the mechanisms that drive the subterranean biodiversity. Since a majority proportion of cave trophic resources come from carriage from the entrances, a higher distance limits the energy available for the communities (TOBIN et al. 2013; MOSELEY 2008; FICETOLA et al. 2018; MAMMOLA 2019). Moreover, regardless of the general stability, caves present a gradient of conditions from near-to-entrance (usually more unstable) to deep zones (MOSELEY 2009; TOBIN et al. 2013; LUNGHI et al. 2014; PROUS et al. 2015; MAMMOLA & ISAIA 2018; LUNGHI & MANENTI 2020; SOUZA-SILVA et al. 2021). This zonation creates distinct microhabitats for cave species, which vary in terms of substrate types as well as climatic and photic properties (MOSELEY 2008; SOUZA-SILVA et al. 2011b; DU PREEZ et al. 2015; LUNGHI et al. 2017; MAMMOLA & ISAIA 2017; MAMMOLA 2019; LUNGHI & MANENTI 2020; MAMMOLA et al. 2020; SOUZA-SILVA et al. 2021).

The unique environmental traits found in caves only allow animals with pre-adaptations to colonize these habitats. This creates high singular evolutive pressures that may produce restricted subterranean fauna, called troglobitic, which can maintain viable populations exclusively in hypogean environments (RACOVITZA 1907; SKET 2008).

Given the high potential and the limited number of studies in the western region of Bahia state, Brazil, this study aims to investigate how habitat variables influence the richness and composition of cave invertebrates (CECAV, 2022). We hypothesize that environmental factors will affect both these caves' species composition and richness.

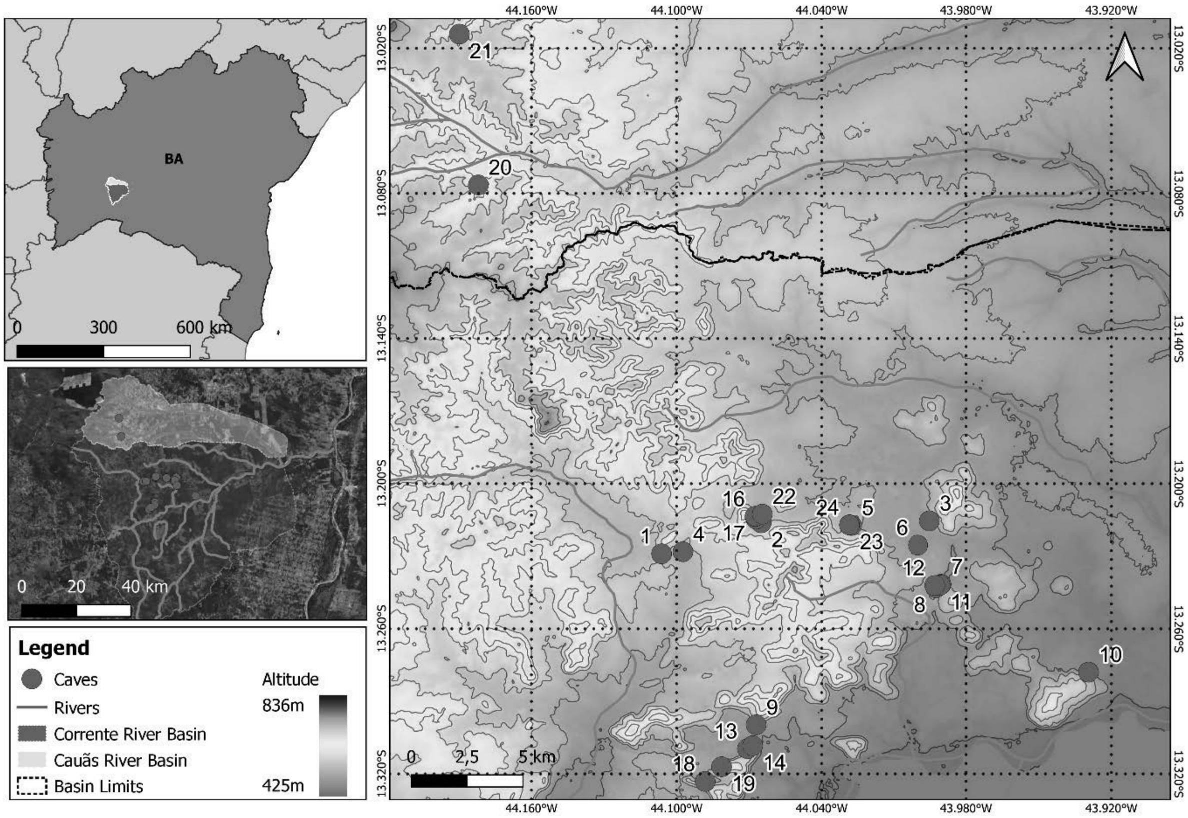

***Figure 1:*** *Locality of the 24 caves studied at Santana, Santa Maria da Vitória and Canápolis municipalities, Bahia state, Brazil.*

## 2. Materials and methods

Two sample campaigns occurred during the dry season in the municipalities of Santana, Santa Maria da Vitória e Canápolis, Bahia State, Brazil. This transition zone between Seasonal Dry Forests and Caatinga is classified by KÖPPEN (1936) as Aw, with dry winters and rainy summers, and has a high potential for endemic species (DINERSTEIN 2017) (Fig. 1).

The composition and richness of cave invertebrates, as well as the habitat structure characteristics, were determined along 122 transects (10 × 3 meters each) distributed on the floor of 24 caves, from the entrances to the deeper regions (SOUZA-SILVA et al. 2021). Invertebrate sampling was executed by visual search along the transects and was only finished when all the invertebrates had been sampled and/or accounted for (SOUZA-SILVA et al. 2021). Invertebrates were preserved in jars with 70% ethanol labeled. In the laboratory, the specimens were sorted with a Stemi 508 (ZEISS) stereomicroscope, identified until the lowest possible taxonomic level, and separated into morphotypes (OLIVER & BEATTIE, 1996). Potential troglobitic species were identified by the presence of troglomorphic characteristics, such as pigmentation and eyes, appendage elongation, among others (CULVER & PIPAN 2009).

The measurement of the habitat structure traits in the transects was carried out according to the methodology used by SOUZA-SILVA et al. (2021). In order to visually quantify the surface area occupied by various organic and inorganic substrates, each transect was divided into 10 parts (1 x 3 m). The distances were obtained by a laser tape measure or by the plot of each transect on the cave map. For the definition of the Micro Drainage Basins, the SAGA Next Generation plugin was used with the aid of a Digital Elevation Model (DEM) in the QGIS 3.22.11 software. The DEM was also used to extract the altitude information for each sector. The sectors with 600m or higher in elevation were classified as Recharge Zones and the ones under this altitude were classified as Discharge Zones.

All the analyses were run in the R Studio software (R CORE TEAM, 2023). Previously the correlation between variables was tested with CHART.CORRELATION function from the 'PerformanceAnalytics' package and the variables with correlation value > 0.70 were excluded from the models. The functions VIF and VIF.CCA from the 'Car' package was used to test the multicollinearity of variables, and the ones with value > 10 were discarded. The distribution of the data was verified using the SHAPIRO.TEST function from the package 'Stats'. A Mantel test was performed to try the spatial autocorrelation between the samples.

All the substrates in each sector were evaluated, classified and grouped into classes. Using a Shannon-Weaver Index (BUTTIGIEG & RAMETTE 2014), were determined the Substrate Diversity, the Shelter Diversity and the Trophic Resources Diversity for each sector. The classes were also used to generate, by sum, the Shelter Availability and the Trophic Resources Availability for each sector.

The abiotic attributes were then divided into Landscape features, such as Micro Drainage Basins, Water Zones, and Caves; Physical features, which comprise the Distance of each transect from the nearest entrance, Substrate Diversity, Shelter Diversity, and Shelter Availability; Trophic Resources grouped Trophic Resources Diversity and Trophic Resources Availability; the Microclimatic Variables considered were Temperature and Moisture.

Trying to understand the correlation between troglobite and non-troglobite invertebrate richness with physical, trophic, and microclimatic traits, Generalized Linear Models (GLM) and Generalized Linear Mixed Models (GLMM) were fulfilled. The model used Microclimatic Variables, Distance from the nearest entrance, Diversities (general substrate, shelter, and trophic resources), and Availabilities (shelter and trophic resources). For those models, the Poisson family was adopted because it better fitted the data. To evaluate the correlation between troglobite and non-troglobite invertebrate composition with landscape, physical, trophic, and microclimatic variables a Distance-Based Redundancy Analysis (dbRDA) was performed (CLARKE ET AL. 2014). The model used Landscape features, Microclimatic Variables, Cave, Distance from the nearest entrance, Diversities (general substrate, shelter, and trophic resources), and Availabilities (shelter and trophic resources).

## 3. Results

A total of 2,754 specimens were collected in the caves, totalizing 338 species. These were separated into 37 orders and 93 families. The 24 sampled caves have at least 41 cave-restricted species belonging to 8 higher taxa and 14 families. from all 41 troglobitic species, 25 are found in Gruta do Padre Cave (Fig. 2).

The Mantel test revealed the existence of spatial autocorrelation for non-troglobite fauna (p = 0.0004), which explain 20.17% of the variation seen in the composition. For the troglobites, the geographic distances were not important.

The dbRDA for non-troglobite communities' composition displayed the Cave (F = 2.530; p = 0.005), Distance from the nearest entrance (F = 3.608; p = 0.005), and Shelter availability (F = 2.625; p = 0.005) as significative variables. For the troglobite communities' composition, Distance from the nearest entrance (F = 8.171; p = 0.005) and Temperature (F = 4,330; p = 0.005) were the important traits.

Since non-troglobite data presented spatial autocorrelation, a GLMM was used for the model with the caves as random variable, while for troglobite species, a GLM was applied. For both faunas, the Distance from the nearest entrance was the only significant feature in the richness analysis. For the non-troglobites model, the fixed attributes alone explained 35.20% of the variation, increasing to 53.19% with the random variable (Estimate = -0.324; p = 1.98e$^{-05}$). For the troglobite model, 13.37% of the variation were explained (Estimate = 0.244; p = 0.003) (Fig. 3).

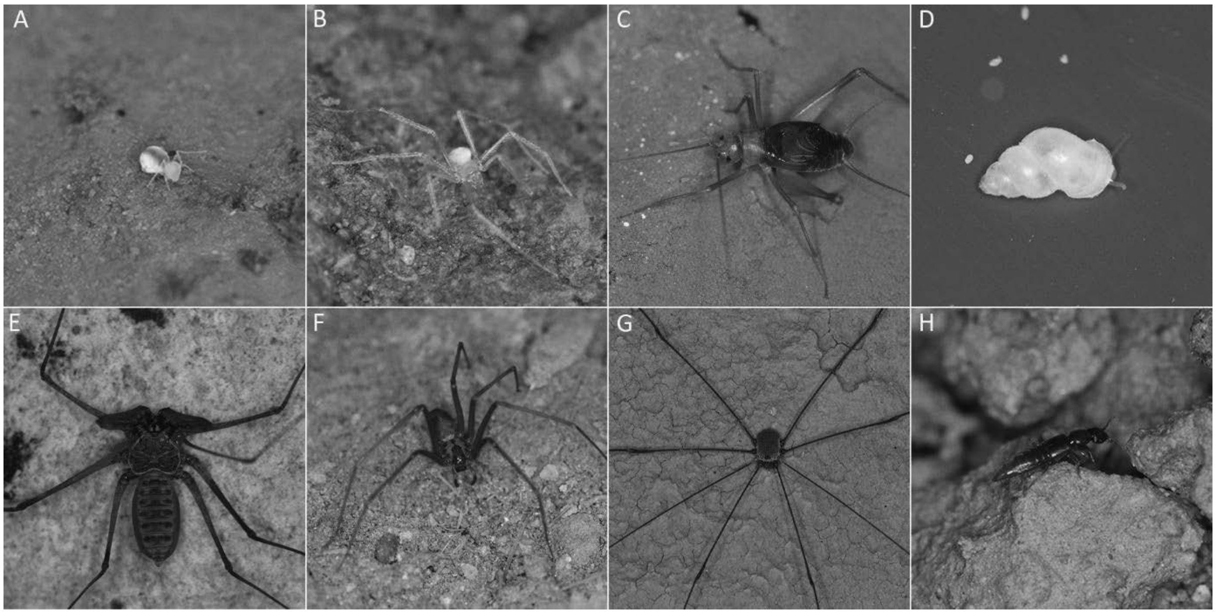

***Figure 2:*** *Some of the fauna observed during the study: (A) Symphypleona sp.; (B) Ochyroceratidae sp.; (C) Endecous sp.; (D) Gastropoda sp.; (E) Thrichodamon sp.; (F) Loxosceles sp.; (G) Gagrellineae sp.; (H) Staphylinidae sp.*

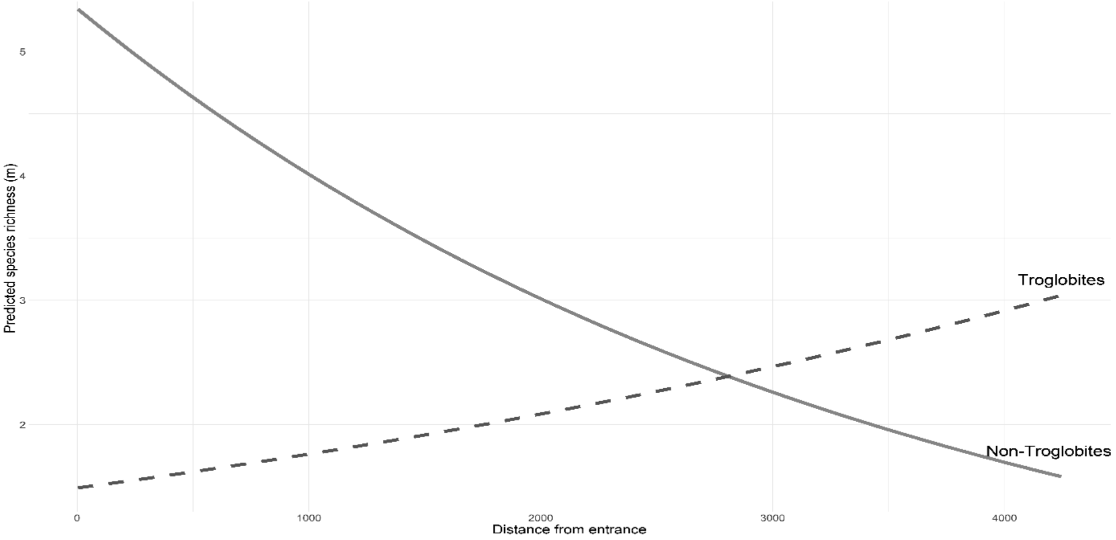

***Figure 3:*** *Prediction of species richness towards deeper zones of the caves for troglobite and non-troglobite fauna.*

## 4. Discussion

The non-troglobite and troglobite fauna respond differently, both in composition and richness, to the habitat traits. Although the Distance from the nearest entrance was an important factor for each group, the type of response may differ.

Whereas non-troglobite species suffer negative effects with distance increasing, the troglobite richness rises. Thus, for non-specialized fauna, the distance from the cave entrance is a known limiting factor for species distribution. This restrictive nature of the distance from the entrance is associated with the reduction of trophic resources, as well as the habitat heterogeneity, from near-to-entrance to deep zones of the caves (TOBIN et al. 2013; MOSELEY 2008; FICETOLA et al. 2018; MAMMOLA 2019; SOUZA-SILVA et al. 2021).

In this way, higher distances restrict the amount of energy that is accessible for the communities since the bulk of cave trophic supplies are transported from the entrances (TOBIN et al. 2013; MOSELEY 2008; FICETOLA et al. 2018; MAMMOLA 2019; SOUZA-SILVA et al. 2021, FURTADO et al. 2022).

Otherwise, the higher climatic stability found in deeper zones of the caves favors the existence of troglobite fauna of the adaptations in these organisms. Reduced metabolic rates and cuticle thinning, which increases the risk of desiccation, limit the distribution of these species to areas with minor temperature and moisture variations, which are often deeper areas (TOBIN et al. 2013; LUNGHI et al. 2014 and 2017; KOZEL et al. 2019; SOUZA-SILVA et al. 2021).

On the other hand, this preference of troglobitic organisms for stabler habitats can come with a cave characteristic initially thought to be limiting for the communities, the oligotrophy. However, as demonstrated by HÜPPOP (2005), the K strategy life history adopted by these animals added to reduced metabolic rates, allowing them to survive a long time without any food. Thus, restricted fauna is more frequent in areas with fewer trophic resources, avoiding non-troglobite competitors (SKET 1999; DEHARVENG & BEDOS 2000; SOUZA-SILVA et al. 2021). Is important to emphasize that a boost in the organic matter rate in these oligotrophic zones can be dangerous for the troglobites, since it could result in a substitution by the more competitive and energetically needed non-troglobites (SKET 1999; SOUZA-SILVA et al. 2021).

Even though some of the caves in this study have entrances covered by native vegetation, is clear to see that the original biomes are been replaced by pasture cultivation for cattle feeding. Deforestation in cave surroundings can, directly and indirectly, affect the energetic dynamics of subterranean invertebrate communities.

Directly, the loss of vegetation in the cave surroundings can decrease the amount of litter available to be carried to the hypogean environments. This organic matter of vegetal origin is one of the most important food sources for cave invertebrates, and its prejudice may imbalance the whole subterranean trophic web (CROWTHER 1987; BRINA 1998; SOUZA-SILVA et al. 2011b). Indirectly, deforestation can limit bat food availability, which may decrease the apport of guano to the caves. Bat guano is probably one of the crucial energy sources to dry caves permanently and it lack risks entire communities (FARIA 1996; FERREIRA & MARTINS 1999; SOUZA-SILVA et al. 2011b; FERREIRA 2019).

Even though the general population of the Santana region knows about some of the larger caves, such as Gruta do Padre Cave, most of the cavities are neglected. Either by fear or by lack of opportunities, few locals have been inside caves, even in a high-potential speleological area. This low awareness reflects the general Brazilian population, which knows little or nothing about the more than 20,000 caves registered in the country (CECAV 2022).

## 5. Conclusion

This study highlights the contrasting responses of troglobite and non-troglobite fauna to environmental traits. While non-troglobite species experience a decline in richness with increased distance from the entrance due to limited trophic resources and habitat heterogeneity, troglobite species thrive in deeper zones, benefiting from stable climatic conditions. However, the delicate balance of these ecosystems is threatened by external factors such as deforestation. Conservation efforts must consider the direct and indirect impacts of habitat alteration to preserve the unique biodiversity within these subterranean habitats.

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# Aves in Caves: A comprehensive review

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## Abstract

Aves are known to occur in all the known habitats. However, Aves in caves are often overlooked or insufficiently researched unless they have economic value. Considering this gap, we reviewed the literature on birds utilizing cave habitats for essential life functions. We documented 86 bird species across seven orders using caves: Caprimulgiformes (50%), Passeriformes (23.3%), Strigiformes (9.3%), Accipitriformes (9.3%), Falconiformes (5.8%), Columbiformes (1.2%), and Anseriformes (1.1%). All seven orders utilize the entrance zone. Caprimulgiformes, Passeriformes, and Strigiformes exploit the twilight zone, while only Caprimulgiformes with echolocation abilities use the dark zone. Most species utilize caves for roosting (75.6%) and nesting (73.3%). A few (24.4%) bird species visit caves to find prey. The study highlights the significance of caves as essential habitats for birds. Despite their role in supporting bird diversity, caves remain an understudied habitat in avian ecology. This review encourages and underscores the need for detailed research to understand the dynamics of birds in caves. Given the ecological importance of subterranean ecosystems, it is crucial to recognize them as sensitive habitats that require conservation efforts. Protecting cave ecosystems will safeguard the avian diversity using caves and preserve the ecological network they support.

## 1. Introduction

Birds are known to inhabit a diversity of ecosystems (ASMARE ET AL. 2023). These various types of terrestrial habitats provide essential resources, which are crucial for their survival and reproduction. Although different epigean (above-ground) animals inhabit hypogean (subterranean/underground) habitats, this study focuses on birds that utilize caves for various ecological aspects. Caves, typically associated with bats, insects, and other creatures, have been largely overlooked in avian studies, despite their potential to offer unique ecological niches.

This paper aims to fill this gap by examining the role of caves in avian ecology, focusing on how birds interact with these often-neglected environments. Through this review, we also endeavor to highlight the importance of cave habitats for bird conservation.

## 2. Materials and methods

We conducted a systematic literature search and prepared a qualitative synthesis of existing studies on cave use by birds. A comprehensive search was carried out using databases such as Web of Science, Scopus, and Google Scholar. Keywords including “birds and caves,” “bird cave usage,” “cave habitat and birds,” “roosting in caves by birds,” and “breeding in caves by birds” were used in various combinations. We also utilized cross-referencing to identify key articles and additional relevant studies. Key information was systematically extracted from each article, including pertinent data such as the bird species examined, methods employed, and significant findings regarding cave utilization by birds.

The extracted information was qualitatively synthesized to identify the ecological significance of caves as roosting, breeding, and/or foraging habitats for birds. The collected data was categorized into (1) Order, (2) Family, (3) Species, (4) Zone of the cave used by the bird, and (5) Cave used for roosting, breeding, and/or foraging. Based on the descriptive analysis, the findings are discussed.

## 3. Results

A comprehensive review identified 86 bird species across seven orders (Caprimulgiformes, Passeriformes, Columbiformes, Strigiformes, Falconiformes, Accipitriformes, and Anseriformes) that utilize caves (Fig. 1). Among these orders, Caprimulgiformes had the highest number of species (n=43), while both Columbiformes (n=1) and Anseriformes (n=1) had the fewest. The majority of these species use caves for roosting (75.6%) and nesting (73.3%), while a smaller portion (24.4%) visit caves to hunt for prey. Only the members of Order: Caprimulgiformes use all (entrance, twilight, and dark) zones of the caves for nesting and roosting.

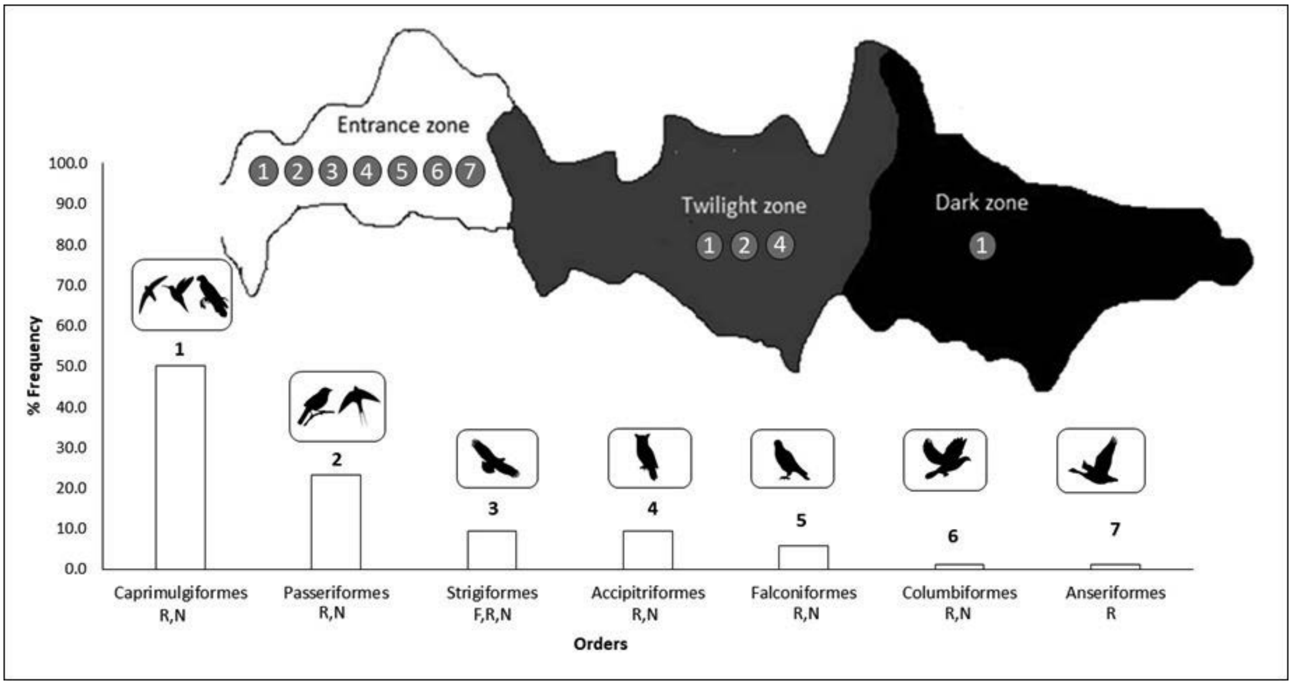

***Figure 1:*** *Aves use caves for various purposes including roosting (R), nesting (N), and foraging (F)*

## 4. Discussion

The present review suggests that birds utilize caves for multiple ecological functions, such as roosting, nesting, and finding prey. Out of the 86 species, Caprimulgiformes (50%) use caves for roosting and nesting. Caprimulgiformes primarily includes birds from the families Apodidae (Swifts and Swiftlets), Caprimulgidae (Nightjars), Nyctibiidae (Potoos), Steatornithidae (Oilbird), and Trochilidae (Hummingbirds) (WINKLER ET AL. 2024). In this family, Apodidae (Swifts and Swiftlets) are known to roost and nest from the entrance to the deep, dark regions of the cave. Most swiftlets from the Oriental region (*Aerodramus* sp.) and Steatornithidae (Oilbird) (HOLLAND ET AL. 2009) in South America have the ability to echolocate for navigation in the dark (BRINKLØV ET AL. 2013). Other families, which include Caprimulgidae (Nightjars) and Nyctibiidae (Potoos), are nocturnal and have not yet been recorded using caves. However, one species, the Andean Hillstar (Trochilidae), is known to rest while clinging to vertical rock surfaces or nesting under overhanging rocks or glued to rock surfaces, sometimes in caves (FJELDSÅ ET AL. 2023). Passeriformes, which include Muscicapidae (Chats) (COLLAR & SHARPE 2020), Hirundinidae (Swallows), Furnariidae (Spinetails), and Tyrannidae (Tyrants), primarily use caves for roosting, particularly in entrance zones. According to PEARSON ET AL. (1953), hummingbirds and other passerines at high altitudes retreat to holes or caves where thermal advantages are present and where they can find safety from predators. The other three families — Strigiformes (Owls), Falconiformes (Falcons), and Accipitriformes (eagles, hawks, vultures) — use caves for food and nesting (MOHAMMAD ET AL. 2017). In the caves, the primary prey base for both diurnal and nocturnal raptors consists of bats and birds (MANCHI & SANKARAN 2009). Furthermore, there are species such as the Andean Goose (Anseriformes) that have been observed feeding their nestlings inside a cave (PEARSON ET AL. 1953).

Beyond these 86 species, there is preliminary evidence that birds inhabiting small holes and crevices, like Colaptes (Flickers), Geositta (Miners), and Sicalis (Ground Finches), might also exploit cave environments. However, detailed investigations are necessary to confirm these behaviors. Overall, this review underscores birds' complex and varied use of caves and calls for more targeted research to substantially elucidate these subterranean habitats' ecological significance.

## 5. Conclusion

In summary, while birds are well-documented for their use of diverse habitats, caves remain an underexplored yet potentially significant environment. The stable microclimates and inherent protective qualities of caves suggest that these spaces may serve as critical sites for roosting and nesting. Although preliminary observations indicate that birds may strategically exploit cave environments during key life stages, the current literature is limited and largely anecdotal. This review advocates the need for further systematic research to quantify the extent of cave use by birds and understand the ecological and conservation implications of this behavior. Recognizing and incorporating caves into broader habitat conservation strategies could be important for long-term bird conservation.

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We are grateful to the late Dr. Ravi Sankaran, who pioneered the conservation of cave birds in India and highlighted the need for the preservation of cave habitats. It is his efforts that have motivated us to undertake this study and explore further.

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# A new species of Attaleachernes Beier, 1937 (Pseudoscorpiones, Chernetidae) from Northeastern Brazil.

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## Resumo

Uma nova espécie de pseudoescorpião, *Attaleachernes* sp. nov.1, é descrita neste estudo, apresentando características morfológicas detalhadas, traços diagnósticos e ilustrações que a distinguem de seu congênere, *Attaleachernes thaleri* Mahnert, 2009. Além disso, fornecemos uma discussão comparativa sobre o gênero e incluímos uma descrição do habitat da espécie. Essa nova espécie representa apenas a segunda ocorrência registrada do gênero *Attaleachernes*. Também propomos recomendações para pesquisas futuras sobre *Attaleachernes* sp. nov.1, enfatizando a necessidade de uma exploração mais aprofundada de suas características e ecologia.

## Resumen

A new species of pseudoscorpion, *Attaleachernes* sp. nov.1, is described in this study, featuring detailed morphological characteristics, diagnostic traits, and illustrations that distinguish it from its congener, *Attaleachernes thaleri* Mahnert, 2009. Additionally, we provide a comparative discussion on the genus and include a description of the species' habitat. This new species represents only the second recorded occurrence of the genus *Attaleachernes*. We also propose recommendations for future research on *Attaleachernes* sp. nov.1, highlighting the need for further exploration of its characteristics and ecology.

## 1. Introduction

Pseudoscorpions are small predatory arachnids belonging to the class Arachnida and the order Pseudoscorpiones. They are widely distributed across terrestrial habitats on six continents, with the exception of Antarctica (Benavides, 2019). The group comprises 26 families (including one extinct) and 493 genera, 19 of which are extinct. In Brazil, 15 species have been recorded, with 10 documented in cave environments.

The family Chernetidae is the most diverse in terms of genera and the third richest in species, with over 730 species distributed across 120 genera, 23 of which occur in Brazil (WORLD PSEUDOSCORPIONES CATALOG, 2024). The genus *Attaleachernes* Mahnert, 2009 has been recorded in only one Brazilian state and exhibits distinct characteristics, such all trichobothria except et/it in basal half of fixed chelal fingerand female spermathecae with a lemon-like shape.

This study describes a new species of *Attaleachernes* based on specimens collected in cave SL_0190, located in Curionópolis, Pará. To date, only one species of the genus has been recorded: *Attaleachernes thaleri* Mahnert 2009, found in the Pantanal region of Mato Grosso, where it is associated with the canopy of the palm tree *Attalea phalerata*. However, knowledge about the behavior of Attaleachernes species remains limited, emphasizing the need for further research.

## 2. Methodology

### Study area

The specimens were collected from a ferruginous cave in the Serra Leste region, Pará, Brazil. This area is part of the Serra dos Carajás Speleological Unit, which encompasses a vast network of over 2,000 ferruginous caves and is known for its rich mineral deposits, including iron, nickel, copper, and gold (Piló et al., 2015).

The region experiences a tropical climate, with temperatures ranging from 23°C to 25°C and an average annual rainfall of approximately 2,400 mm. The dry season spans from June to September, with monthly precipitation varying between 10 and 90 mm, while the rainy season extends from October to April, with rainfall levels ranging from 160 to 340 mm per month (Sahoo et al., 2016).

### Field sampling

Fieldwork was carried out on November 2–15, 2016 Spelayon et al. The specimens were collected during fieldwork conducted as part of environmental licensing procedures by a consultancy company. They were subsequently deposited in the collection of the Center for Subterranean Biology Studies at the Federal University of Lavras, Minas Gerais, Brazil.

### Analysis and preparation

The specimens were examined using a Zeiss Axio Scope A1 optical microscope, with image analysis conducted via ZEN 2012 software. Illustrations of body structures and appendages were created using a camera lucida attached to an Olympus BX40 optical microscope equipped with phase contrast.

Photographs of anatomical structures and appendages were captured using a Zeiss Axio Zoom V16 microscope, with image acquisition facilitated by ZEN 2.1 software. The images were subsequently vectorized using Inkscape 1.1 software (Montesanto, 2015; available at inkscape.org). Additional examinations of the paratypes were performed using a Hitachi TM4000 scanning electron microscope (SEM).

The holotypes and paratypes were deposited in the Subterranean Invertebrate Collection of Lavras (ISLA).

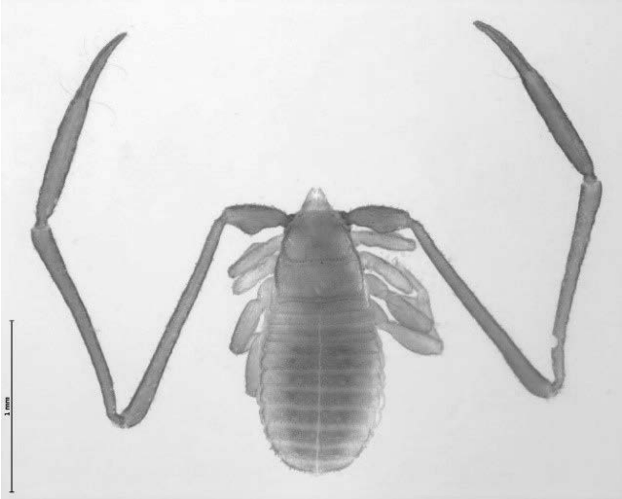

***Figure 1:*** *Attaleachernes sp. nov.1, dorsal habitus. Scale bar: A (1.0 mm.).*

## 3. Results

**Family Chernetidae Menge, 1855**
**Genus Attaleachernes Mahnert, 2009**
***Attaleachernes* sp. nov.1**

**Material Examined.**

Holotype male (ISLA 39675), preserved in ethanol: Brazil, Curionópolis, Pará, Cave SL_0190 (6° 0’ 9.55’’ S, 49° 36’ 58.33’’ W), November 2–15, 2016, collected by Spelayon et al. Paratype: 3 males, 2 tritonymphs, same data as the holotype. The holotype and paratypes are deposited in the Subterranean Invertebrate Collection of Lavras (ISLA), Federal University of Lavras.

**Diagnosis.**

*Attaleachernes* sp. nov.1 differs from *Attaleachernes thaleri* by the following combination of characters: Morphological characteristics: Absence of a median mark on tergite I; absence of sclerotization and darkened zones on the femur, and the presence of five setae on the anterior margin of the carapace (instead of six in *A. thaleri*); all tergites are divided; tergites I–IV with 9–11 setae, and the subsequent ones with 13–16 setae on the posterior margin (*A. thaleri*: I–IV with 5–6 setae, subsequent ones with 7–8); tergite XI with 10–12 setae and the presence of two tactile setae (*A. thaleri*: 13–15 setae, no tactile setae); external serrula with 12–16 blades (*A. thaleri*: 18–22). Pedipalps: 10.4–11.7 times longer than wide and 1.1–1.2 times longer than the patella (*A. thaleri*: 8.5–10.1 times longer than wide and 1.03–1.09 times longer than the patella); pedipalpal patella 9.1–9.8 times longer than wide (*A. thaleri*: 7.0–8.6 times); trochanter with protuberance (*A. thaleri*: absent). Chelicerae without pedicel, 8.5–9.3 times longer than wide (*A. thaleri*: 6.8–7.5); central part of the cheliceral hand without dark or depigmented zones at the base (*A. thaleri*: present); fixed finger with 45–65 teeth (*A. thaleri*: 51–58). Leg I with tarsus 4.5–4.9 times longer than deep (*A. thaleri*: 5.1–6.2); leg IV with femur + patella 4.1–4.6 times longer than wide (*A. thaleri*: 4.7–5.5); tibia (male) 3.4–3.6 times longer than wide (*A. thaleri:* 5.5–5.8); tarsus with two long pseudotactile setae at the central tip, one larger (*A. thaleri*: a single short pseudotactile seta near the lateral tip, 0.10–0.11 mm long). On the fixed finger, all trichobothria, except et/it/est/ist, are located on the basal half of the finger (*A. thaleri*: only et/it); est and it are very close (*A. thaleri*: isb much closer to est than to it); isb and st are distant (*A. thaleri*: *isb* at the level of st); only et is near the tip of the finger, with it slightly further away (*A. thaleri*: et/it are close to the tip of the finger, with it slightly proximal to *et*).

**Description.**

Body pale orange brown, pedipalps darker; chelicerae translucent; pedipalps yellow-reddish and covered with scales. Body granulated and adorned with clavate, simple, and dentate vestitural setae.

**Carapace:** 1.0–1.15 times longer than wide; strongly granulated; presence of ocular spots; lateral sides of the median transverse furrow darker; short and clavate setae; two wide and deep furrows, finely granulated; subbasal furrow clearly closer to the posterior margin than to the median furrow; 5 setae on the anterior margin, 12–16 setae (some in a discal position) on the posterior margin.

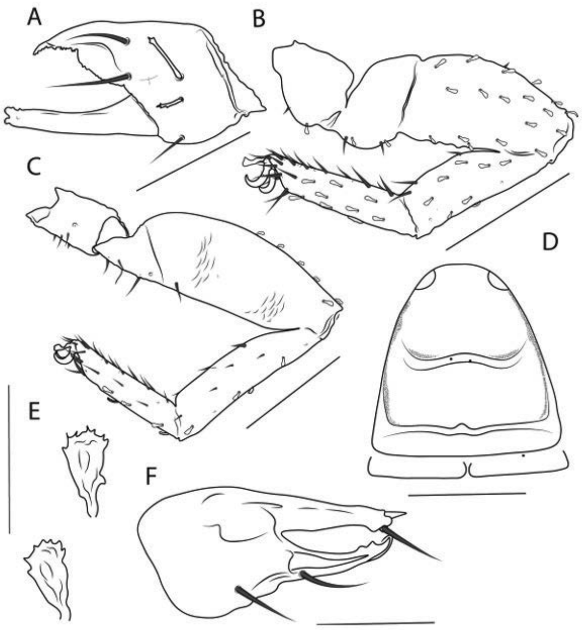

***Figure 2:*** *Attaleachernes sp. nov.1. A) Male Left Chelicera (paratype); B) Leg I of male (paratype); C) Leg IV of male (paratype); D) Carapace of male (holotype); E) abdomen male setae det (paratype); F) Male Left Chelicera (holotype). Scale bars: A 0,1 mm (A, D e F), 0,2 mm (B e C), 0,04 mm (E).*

**Coxae:** Palpal coxal lobe with 3 marginal setae (one microseta) and one discal seta; palpal coxa distinctly granulated on the lateral and anterior sides; coxa I with 10–13 setae, coxa II with 9-14 setae, III with 10–15 setae, IV with approximately 25–30 setae; intercoxal tubercle absent.

**Tergites:** All tergites are divided, starting from tergite II with a central reddish-brown mark. The setae are clavate.

Tergites chaetotaxy: 10: 10: 15: 17-21: 16-21: 20-23: 22-24: 20-24: 21-23: 19-23: 11-12.

**Chelicera:** Hand with 5 setae, two (bs' and bs") apically dentate; fixed finger with 1 larger tooth and 3 small subapical teeth; movable finger with a bicuspid subapical lobe; smooth subgalea seta, reaching beyond the tip of the galea; robust and smooth galea; rallum with 3 blades (the anterior one with 3–5 teeth); external serrula with 12–16 blades.

**Palps:** Strongly granulated, simple and clavate setae, strongly granulated; trochanter with a protuberance in the posterior central region, with simple and clavate setae on the anterior lateral side. It is 2.1–2.5 times longer than wide. Femur slightly clavate, 10.4–11.7 times longer than wide, and 1.1–1.2 times longer than the patella. Patella 9.1–9.8 times longer than wide. Chelae 8.5–9.3 times longer than wide. Chelal movable finger 8.1–9.8 times longer than wide. Chelal fixed finger with 45–65 small, sharp teeth.

**Leg I:** Trochanter: 1.5–1.8 times longer than wide; Femur + patella 2.6–3.1 times longer than wide; tibia 4.0–4.7 times longer than wide; tarsus 4.5–4.9 times longer than wide; a pair of long pseudotactile setae at the central tip of the tarsus; clavate setae present in the antiaxial region and smooth setae in the paraxial region; simple curved terminal seta; smooth claws longer than the undivided arolium.

**Leg IV:** Trochanter: 1.1–1.5 times longer than wide; Femur + patella 2.8–3.0 times longer than wide; Tibia 3.4–3.6 times longer than wide; Tarsus 4.1–4.6 times longer than wide; pair of long pseudotactile setae at the central tip of the tarsus, one of them longer; clavate setae present in the antiaxial region and smooth setae in the paraxial region; one subterminal simple curved seta; claws smooth and longer than the undivided arolium.

Measurements (mm): (length/width or depth in mm; ratios in parentheses, calculated with three significant digits): Range for the holotype and male paratypes.

Body length 0.369–0.407/0.175–0.163 [2.1–2.0]. Carapace 0.600–0.621/0.591–0.541 (2.0–2.3). Palps: Trochanter 0.16–0.20/0.12–0.13 (2.1–2.5), femur 1.217–1.348/0.117–0.115 (10.4–11.7), patella 1.104–1.256/0.121–0.128 (9.1–9.9), chela 1.142–1.203/0.1–0.1 (8.5–9.3), movable finger length 0.488–0.518/0.060–0.053 (8.1–9.8). Leg I: Trochanter 0.1–0.2/0.1–0.1 (1.1–1.5), femur 0.34–0.35/0.07 (5.1–5.4), patella 0.18–0.19/0.05 (3.3–3.7), femur/patella 0.4/0.1 (2.8–3.0), tibia 0.2–0.3/0.1 (3.4–3.6), tarsus 0.2/0.1 (4.1–4.6). Leg IV: Trochanter 0.1–0.2/0.1 (1.5–1.8), femur + patella 0.4–0.5/0.2 (2.6–3.1), tibia 0.3–0.4/0.1 (4.0–4.7), basitarsus 0.18–0.19/0.06 (3.1–3.3), telotarsus 0.34/0.04 (9.1–9.6).

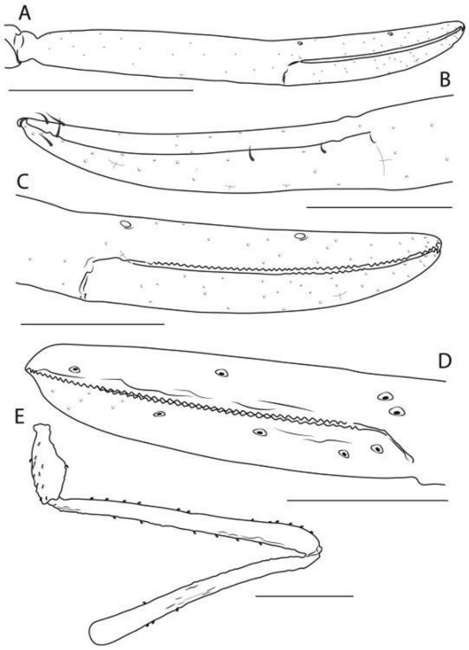

***Figure 3:*** *Attaleachernes sp. nov.1. A) right chela paraxial distal male (paratype); B) chela male ventral view (paratype); C) right chela paraxial distal male (paratype); D) left chela antiaxial distal male (paratype); E) pedipalps of male (paratype). Scale bars: 0,2 (A, B, C e D) 0,5 (E).*

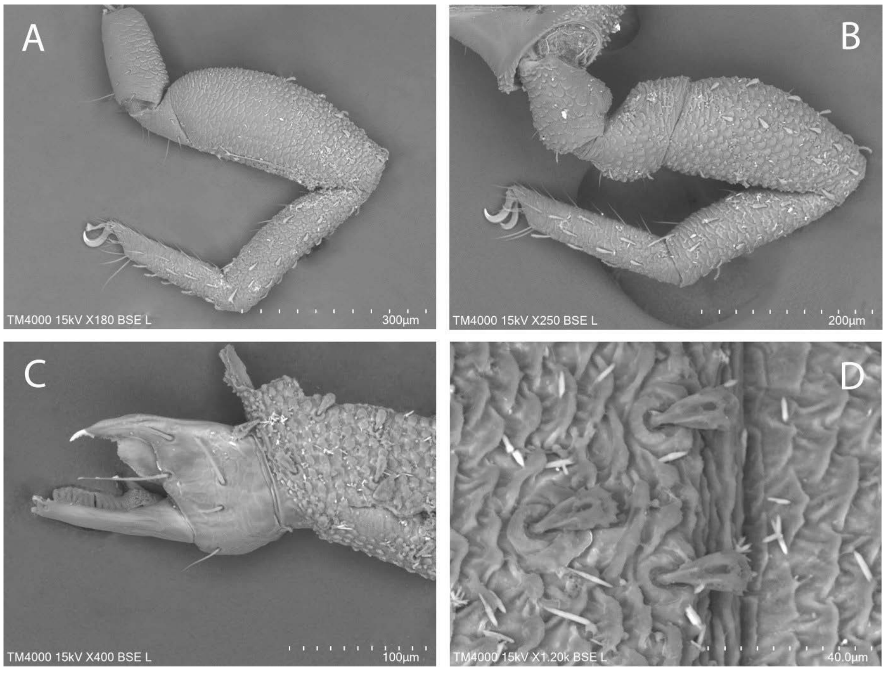

***Figure 4:*** *Attaleachernes Sp. nov. 1. A Leg IV of male (paratype); B Leg I of male (Paratype); C Male Left Chelicera (paratype); D abdomen male setae detail (paratype).*

## 4. Discussion

The discovery of *Attaleachernes* sp. nov.1 in this study challenges the previously held assumption that the genus is restricted to the Brazilian Pantanal. While studies such as Battirola et al. (2017) have recorded 1.197 individuals of 16 pseudoscorpion species associated with the soil, leaf litter, and canopy of *Attalea phalerata*, particularly emphasizing the family *Chernetidae*, the new species was identified in a cave in Curionópolis, Pará. This finding broadens our understanding of the genus' distribution and suggests that some species may inhabit distinct environments, including caves, rather than being strictly arboreal.

The presence of *Attaleachernes* sp. nov.1 in a subterranean habitat highlights the importance of exploring understudied ecosystems to better understand the diversity and biogeographical patterns of pseudoscorpions in Brazil. However, Brazilian cave fauna is increasingly threatened, particularly due to recent legislative changes that weaken cave protection laws. Ferreira et al. (2022) have warned about the risks these changes pose to subterranean habitats, which are already under pressure from mining and infrastructure development. A striking example is the Serra Leste 10 Mtpa Project in Curionópolis, Pará, which is expected to extract 107 million tons of iron ore, potentially causing significant damage to cave ecosystems and their specialized fauna.

Taxonomic knowledge of *Attaleachernes* remains incomplete, particularly regarding the morphological characters used to differentiate species. The newly described *Attaleachernes* sp. nov.1. presents distinct characteristics that set it apart from *A. thaleri*, including the number of lamellae on the external serrula (12–16 in sp. nov.1. vs. 18–22 in *A. thaleri*), the presence of two tactile setae on tergite XI (*Attaleachernes* sp. nov.1), which are absent in *A. thaleri*, and a distinct trichobothrial pattern on the fixed chelal finger. Additionally, *Attaleachernes* sp. nov.1. exhibits larger pedipalps (10.4–11.7 times longer than wide) compared to *A. thaleri* (8.5–10.1 times longer than wide) and possesses two long pseudotactile setae at the apical position of tarsus IV, whereas *A. thaleri* has only one short pseudotactile seta in the subapical position.

Subterranean ecosystems, such as caves, remain among the least explored environments on Earth, yet they harbor a high diversity of specialized organisms. These habitats represent a unique fraction of global biodiversity, both taxonomically and functionally. As emphasized by Mammola et al. (2019), the conservation of subterranean biodiversity is crucial to preserving these fragile ecosystems and their endemic species. The restricted distribution of *Attaleachernes* sp. *nov*. 1 underscores the urgent need for conservation measures to protect these habitats, which often serve as refugees for rare and vulnerable species.

## 5. Conclusion

The description of *Attaleachernes* sp. nov. 1 expands our understanding of pseudoscorpion biodiversity and represents only the second recorded occurrence of the genus *Attaleachernes* in both Brazil and globally. This new species is distinguished by its unique morphological traits, including notable differences in trichobothrial patterns, pedipalp proportions, and the number of tactile and pseudotactile setae compared to its congener, *A. thaleri*.

This discovery highlights the critical role of taxonomic research in underexplored subterranean environments, such as caves, where biodiversity remains largely underestimated. It also reinforces the significance of Brazil as a hotspot of biological diversity, particularly for lesser-known arthropod groups like pseudoscorpions. Future studies should focus on exploring the ecological, behavioral, and evolutionary aspects of *Attaleachernes sp. nov. 1*. *Attaleachernes sp. nov. 1*, contributing to a deeper understanding of its role within subterranean ecosystems and informing conservation efforts for these fragile habitats.

## Acknowledgements

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# Uncovering the Mycobiota of the Teto de Seixos Cave

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## Abstract

Caves are unique environments with a great diversity that is still unknown, especially when it comes to tropical caves. In Brazil, recent studies have shown a great potential for discovering new cave species. Thus, the objective of this study was to identify the cultivable filamentous fungi of the Teto de Seixos cave, located in the Serra do Espinhaço Meridional of Minas Gerais. Samples of airborne particles, sediments, organic litter and animal dung were collected. Total genomic DNA was extracted from the isolates and the ITS, LSU, TUB and CAL regions were amplified and sequenced for fungal identification. A total of 480 isolates were obtained, of which 164 isolates have been identified at the genus level. Of these, 28 isolates were obtained from airborne particle samples, 28 from animal dung, 44 from litter samples and 68 from sediments. The 164 isolates were grouped into 45 genera, 36 families, 20 orders, 7 classes, and 3 phyla. Aspergillus, Penicillium and Cladosporium were the genera with the highest number of isolates found. Preliminary results indicate that there are potential new fungal taxa in the cave.

## Resumo

As cavernas são ambientes únicos com uma grande diversidade ainda desconhecida, principalmente quando se refere a cavernas tropicais. No Brasil, estudos recentes demostram um grande potencial de descobertas de novas espécies de fungos cavernícolas. Assim, o objetivo desse trabalho foi investigar os fungos filamentosos cultiváveis da caverna Teto de Seixos, situada na Serra do Espinhaço Meridional de Minas Gerais. Amostras de partículas suspensas no ar, de sedimento, serapilheira e fezes de animais foram coletados. Para identificação a nível de gênero, foi extraído o DNA genômico total dos isolados e as regiões ITS, LSU, TUB e CAL foram amplificadas e sequenciadas. Ao todo, 480 isolados foram obtidos, dos quais 164 isolados foram identificados a nível de gênero, sendo 28 isolados provenientes de amostras de partículas suspensas no ar, 26 de fezes de animais, 44 de amostras de serapilheira e 66 de sedimento. Os 164 isolados foram agrupados em 45 gêneros, 36 famílias, 20 ordens, 7 classes e 3 filos.  Os gêneros Aspergillus, Penicillium e Cladosporium foram os gêneros com maiores números de isolados encontrados. Os resultados preliminares indicam que existem potenciais novos táxons fúngicos na caverna.

## 1. Introduction

Caves are unique ecosystems with spatial delimitation, partial or total absence of direct sunlight, little variation in temperature and limited amount of available nutrients (ALVES et al., 2022; POLI et al., 2024; ZHANG et al., 2021). These characteristics classify the caves as extreme environments for life development, but various studies demonstrate the great occurrence of fungal in caves, including the discovery of new taxa (ALVES et al., 2022; CONDÉ et al., 2023; DUTRA et al., 2023; LEÃO et al., 2024; POLI et al., 2024; ZHANG et al., 2021).

In Brazil, more than 23 thousand caves are registered in the Centro Nacional de Pesquisa e Conservação de Cavernas – ICMBIO/CECAV (MINISTÉRIO DO MEIO AMBIENTE, 2022), and nearly 50% of these are in the state of Minas Gerais. The mycobiota of caves in Minas Gerais are little studied, recently some studies showed a great potential for fungal discoveries. Forty-seven species distributed in 15 genera, mainly in the genera Aspergillus and Penicillium, were isolated from bat guano and air samples from the dolomitic cave Lapa Nova (TAYLOR et al., 2013). In 2014, TAYLOR et al., (2014) identified 32 fungal species in sediment samples collected in the RM3 cave located in the Iron Quadrangle. Recent studies in caves in the Serra do Espinhaço Meridional (SEM) resulted in the description of one new fungal genus and seven new species of filamentous fungi (CONDÉ et al., 2023; DUTRA et al., 2023; LEÃO et al., 2024). However, there are still many to discover on the mycobiota of caves in Minas Gerais. Thus, these study aimed  was to report the cultivable filamentous fungi present in samples collected in the Teto de Seixos marble cave, located in the SEM of Minas Gerais.

## 2. Material and methods

### 2.1. Study area and collections

Teto de Seixos cave is constituted of marble rocks which is located in Santana do Riacho, in the part of the SEM known as Serra do Cipó. This cave is located within a conservation unit named APA Morro da Pedreira. The cave is approximately 500 meters long and has three entrances (SOUZA et al., 2011; 2019).

Samples of airborne particles, sediments, organic litter, and animal dung were collected at three different points in the cave. The collection of airborne particles was performed using the Koch sedimentation method (KUZMINA et al., 2012), using a one-meter-high support to elevate the Petri plates. At each point, two plates containing culture media Malt Extract Agar added with chloramphenicol (2 mg/L) (MEA+), Dichloran-Rose Bengal-Chloramphenicol Agar (DRBC), and National Botanical Research Institute's Phosphate growth medium (NBRIP) were exposed to the cave environment for 15 minutes. Sediment samples were collected at a depth of 1–5 cm after removing 1 cm of the surface layer. When present, organic litter samples were collected and stored in paper bags (LEÃO-FERREIRA et al., 2013), while animal dung samples were collected and stored in falcon tubes. All samples were stored under refrigeration and transported to the Laboratório de Micologia e Etiologia de Doenças Fúngicas da Universidade Federal de Viçosa.

## 2.2. Fungal isolation

For animal dung and sediment samples, the serial dilution method was used until reaching a dilution factor of 10-5 (ZHANG et al., 2017). One hundred microliters of each dilution were spread on the surface of the MEA+, DRBC and NBRIP culture media in duplicates. All plates were incubated at 25 °C for 30 days, the fungal growth was observed daily and the new colonies were periodically transferred to plates coitanning potato dextrose agar (PDA).

The organic litter samples were washed in running tap water, followed by storage in a humid chamber (CASTAÑEDA-RUIZ et al., 2005). The samples were periodically observed under a stereoscopic microscope for 30 days, and reproductive fungal structures were transferred to PDA plates, and incubated at 25 °C.

Pure cultures were obtained using the hyphal tip method (TUITE, 1969) and then stored in 2 mL microtubes containing a 10% glycerol solution at –20 °C. All isolates are stored in the cave fungi collection of the Laboratório de Micologia e Etiologia de Doenças Fúngicas da Universidade Federal de Viçosa.

## 2.3. DNA extraction, amplification and sequencing

Total genomic DNA was extracted by collecting mycelia grown in PDA medium for 7 days, using the commercial Wizard Genomic DNA Purification kit (Promega®) and the protocol described by PINHO et al., (2013). The DNA amplification of the internal transcribed spacer regions 1 and 2, together with the 5.8S subunit (ITS), 28S rDNA nuclear region (LSU), partial beta-tubulin gene region (TUB), and calmodulin gene region (CAL) were used for fungal identification. The PCR results were visualized by 0.8% agarose gel electrophoresis. The PCR products were purified and sequencing at Macrogen Inc., South Korea (http://www.macrogen.com).

## 2.4. Fungal identification

Isolates were identified by comparing sequences in the NCBI database using the megablast tool within the BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST searches were limited to sequences from type material to enhance accuracy. Isolates with sequence identity greater than 95% were considered within the related genus. Those sequences that were below 95% were assigned only into family.

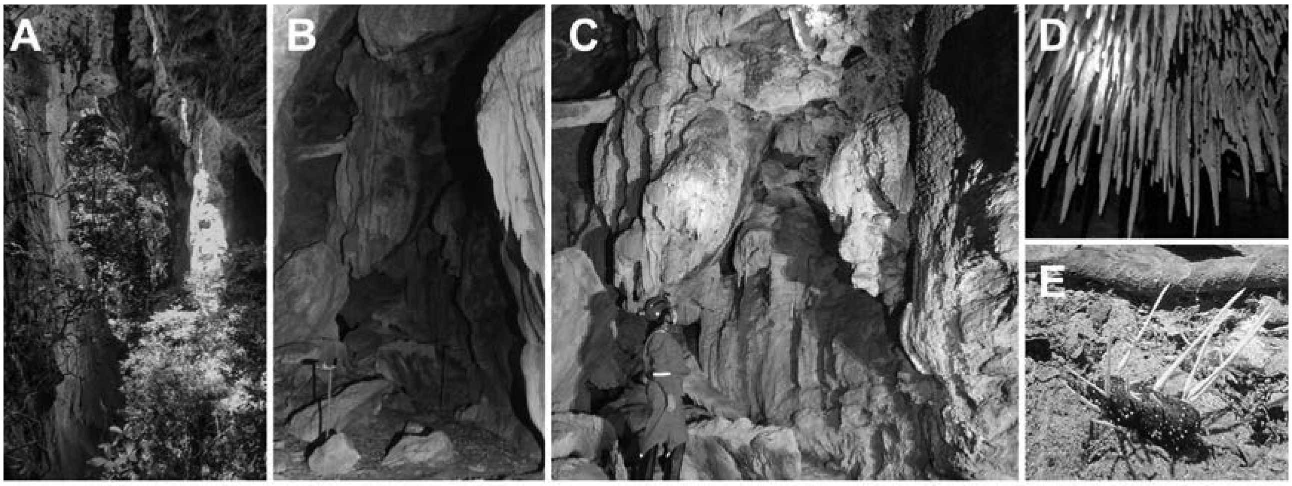

***Figure 1:*** *Teto de Seixos cave. A: Entrance to the cave; B: Airborne particles sample collection; C–D: Speleothems; E: Animal dung colonized by Amphichorda sp. and other fungi.*

# 3. Results

A total of 480 fungal isolates were obtained from Teto de Seixos cave, of which 164 were identified at the genus level. Of these, 28 isolates were recovered from airborne particle samples, 26 from animal dung, 44 from organic litter, and 66 from sediment (Fig. 2A). The 164 isolates were grouped into 45 genera, 36 families, 20 orders, and seven classes distributed in three phyla. The genera identified in this study are listed in Table 1.

The *phylum Ascomycota* presented the largest number of representatives (157 isolates and 42 genera), followed by *Mucoromycota* (5 isolates and 2 genera), and *Basidiomycota* (2 isolates and one genus) (Fig. 2B). The most representative ascomycetous genera were *Aspergillus*, *Penicillium*, *Cladosporium*, *Amphichorda*, and *Wardomycopis*, with 35, 18, 10, 7, and 6 isolates, respectively (Fig. 2C). *Mucoromycota* was the second most abundant phylum, with two isolates of *Absidia*, two of Mucor, and one unidentified isolate from the family *Lichtheimiaceae*. In *Basidiomycota*, only two isolates were found, one belonged to the genus *Minimedusa* and one unidentified genus within the family *Polyporaceae*.

Fourteen genera were found in airborne particle samples (Fig. 2A), with *Cladosporium* (six isolates), *Aspergillus* (3), *Mycosphaerella* (2), and *Microascus* (2) being the most found. Eight genera (*Amycosphaerella, Annulohypoxylon*, *Botryotinia*, *Cercospora*, *Epicoccum*, *Exutisphaerella*, *Mycosphaerella,* and *Neokalmusia*) were found exclusively from this sample (Fig. 2D). Eleven genera were identified in animal dung (Fig. 2A), of which *Aspergillus* (8) and *Amphichorda* (5) were the most abundant. Four genera (*Furcasterigmium*, *Gibellulopsis*, *Marquandomyces*, and *Nigrograna*) were found only in animal dung samples (Fig. 2D).

In organic litter, the isolates were distributed in 21 genera (Fig. 2A), among which *Aspergillus* (10) was the most abundant. Nine genera were exclusively found in this substrate (Fig. 2D), namely, *Alternaria*, *Botryosphaeria*, *Lecanicillium*, *Minimedusa*, *Orbilia*, *Scolecobasidium*, *Striatibotrys*, and *Xepicula*. Sediment samples presented the highest number of identified genera (23), of which we observed a predominance of isolates within the order *Eurotiales*, accounting for almost 50% of the identified isolates (32 of the 66). *Aspergillus* (14) and *Penicillium* (13) were the most abundant, whereas nine genera were exclusive to this substrate (*Collariella*, *Humicola*, *Malbranchea*, *Metarhizium*, *Mucor*, *Paracamarosporium*, *Staphylotrichum*, *Talaromyces*, and *Trichocladium*) (Fig. 2D). Only *Aspergillus* and *Penicillium* were found in all analyzed substrates (Figure 2D).

In total, 22 isolates could not be identified in any of the known genera (Fig. 2C). These isolates were included in 14 families, 11 orders and three phyla.

***Table 1:*** *List of identified genera and number of isolates obtained from each substrate in Teto de Seixos cave.*

| | **Cave substrates** | | | |
|---|---|---|---|---|
| **Genus** | **Airborne particles** | **Animal dung** | **Organic litter** | **Sediment** |
| *Absidia* | | 1 | | 1 |
| *Alternaria* | | | 1 | |
| *Amphichorda* | | 5 | 1 | 1 |
| *Amycosphaerella* | 1 | | | |
| *Annulohypoxylon* | 1 | | | |
| *Aspergillus* | 3 | 8 | 10 | 14 |
| *Botryosphaeria* | | | 1 | |
| *Botryotinia* | 1 | | | |
| *Cercospora* | 1 | | | |
| *Cladosporium* | 6 | | 3 | 1 |
| *Clonostachys* | | | 2 | 1 |
| *Collariella* | | | | 1 |
| *Epicoccum* | 1 | | | |
| *Exutisphaerella* | 1 | | | |
| *Furcasterigmium* | | 1 | | |
| *Fusarium* | | 1 | 1 | 1 |
| *Gibellulopsis* | | 1 | | |
| *Gliomastix* | | | 1 | 1 |
| *Humicola* | | | | 1 |
| *Lecanicillium* | | | 1 | |
| *Malbranchea* | | | | 3 |
| *Marquandomyces* | | 1 | | |
| *Metarhizium* | | | | 1 |
| *Microascus* | 2 | | | 3 |
| *Minimedusa* | | | 1 | |
| *Monocillium* | | | 1 | 1 |
| *Mucor* | | | | 2 |
| *Mycosphaerella* | 1 | | | |
| *Neokalmusia* | 1 | | | |
| *Nigrograna* | | 1 | | |
| *Orbilia* | | | 1 | |
| *Paracamarosporium* | | | | 1 |
| *Penicillium* | 1 | 1 | 3 | 13 |
| *Purpureocillium* | | | 1 | 2 |
| *Scolecobasidium* | | | 1 | |
| *Simplicillium* | 1 | | 1 | |
| *Sirastachys* | | 1 | 2 | |
| *Staphylotrichum* | | | | 1 |
| *Striatibotrys* | | | 1 | |
| *Talaromyces* | | | | 4 |
| *Trichocladium* | | | | 1 |
| *Wardomycopsis* | | 1 | | 5 |
| *Xepicula* | | | 2 | |
| *Xylaria* | 1 | | | 2 |

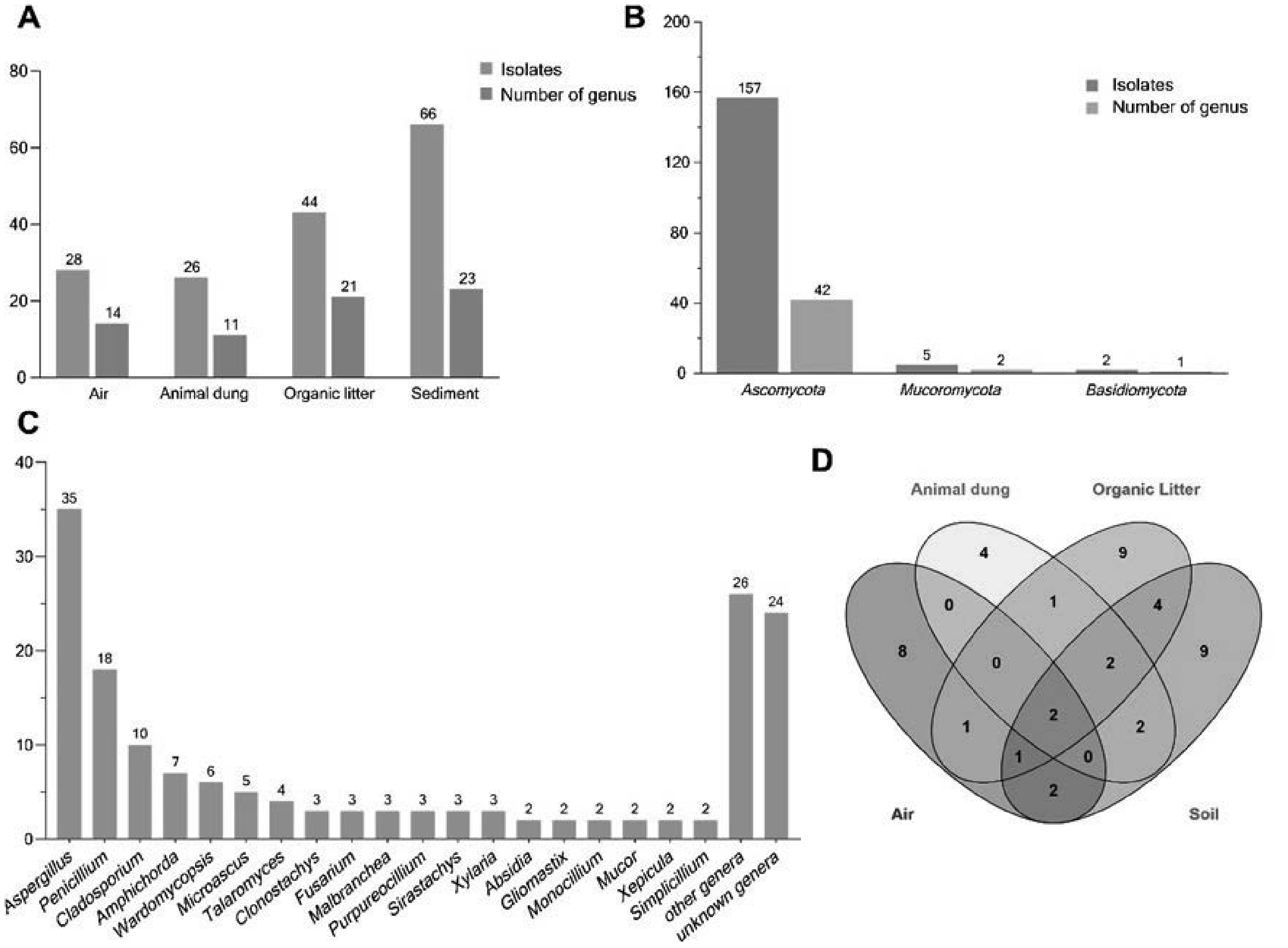

***Figure 2:*** *Statistics of fungi isolated from Teto de Seixos cave. A: number of isolates and fungal genera obtained from different substrates. B: number of isolates and genus found in each fungal phylum. C: most common genera found in this study. D: Venn diagram of fungal genera found in this study.*

## 4. Discussion

Airborne particles are one of the main sources of fungal dispersal into the cave environment, and the genera *Aspergillus*, *Penicillium*, and *Cladosporium* are among the genera with the highest number of reports in cave studies (CONDÉ et al., 2023; CUNHA et al. 2020; PEREIRA et al., 2022; ZHANG et al., 2021). In this study, *Aspergillus* and *Cladosporium* isolates were identified in air particle samples in large quantities; however, only one isolate of *Penicillium* was identified.

Samples of animal dung in caves, especially bat dung, are often associated with pathogenic fungi such as *Histoplasma capsulatum* (CUNHA et al., 2020; SILVA et al., 2013). Conversely, it is also a substrate that can be a source for new fungal discoveries (CONDÉ et al., 2023; ZHANG et al., 2017; 2021;). CONDÉ et al. (2023) described the genus *Parahumicola*, and species *P. guana*, from bat dung in the quartzite cave Gruta Monte Cristo in the SEM. In the animal dung samples collected from Teto de Seixos cave, we identified isolates belonging to the genus *Amphichorda*. This genus has some species described as coprophilous (ZHANG et al., 2017; 2021) and has potential for biotechnological use (BELOUSOVA et al., 2023; JIANG et al., 2021; 2023; XU et al., 2018; YUAN et al., 2022). In Brazil, one species of this genus, *Amphichorda monjolensis*, has been described in the carbonate cave Gruta Velha Nova in Monjolos, Minas Gerais (LEÃO et al., 2024).

Approximately 45% of the fungal isolates obtained in the present study were retrieved from the sediment samples. Sediment samples are generally among the richest in organic matter content when compared to other samples studied in caves, which provides greater fungal growth and consequently greater numbers of isolates (ALVES et al., 2022; CUNHA et al. 2020; ZHANG et al., 2021).

The genera *Aspergillus*, *Penicillium* and *Cladosporium* have been widely reported in caves (CONDÉ et al., 2022; CUNHA et al., 2020; DUTRA et al., 2024; POLI et al., 2024; VANDERWOLF et al., 2013; ZHANG et al., 2017; 2021). In this study, these genera were the most abundant, representing more than 40% of the isolates, and were found in all the samples analyzed. These genera present several characteristics that allow for great adaptation to different substrates, including spore dispersal over long distances, low substrate selectivity, and the ability to colonize animals and plants (POLI et al., 2024; VANDERWOLF et al., 2013; ZHANG et al., 2021).

Twenty-two isolates could not be assigned to any known genera. Further analysis based on a taxonomic polyphasic approach, using molecular phylogeny and morphological characterization of reproductive structures, will allow the identification of possible new genera and species from Teto de Seixos cave.

## 5. Conclusion

Forty-five genera were identified in the Teto do Seixos cave, demonstrating that caves is harbor of a great fungal richness, with potential for identifying new taxa. Until this work, Teto de Seixos cave was still unknown from a mycological point of view, which demonstrates a potential for new fungal discoveries and reinforces the importance of studying the mycology of caves.

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# Bat Mycobiota: a Preliminary Assessment in a Cave in Northeast Brazil

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## Resumo

O presente estudo buscou isolar e identificar os fungos presentes no corpo de morcegos da caverna Urubu, em Sergipe, dada a escassez de informações sobre esses fungos no Brasil. Foram coletadas amostras de *Pteronotus personatus* e *Pteronotus gymnonotus*, e obtidas cerca de 251 e 225 UFCs, respectivamente. No total, 14 isolados fúngicos foram obtidos, sendo a maior riqueza encontrada na região do corpo, seguida pela cavidade oral e asas. Até o momento, quatro gêneros foram identificados como *Aspergillus*, *Blastobotrys*, *Fusarium* e *Penicillium*, sendo *Aspergillus* o mais predominante.

## Abstract

This study aimed to isolate and identify fungi present on the bodies of bats from Urubu Cave in Sergipe, due to the scarcity of information about these fungi in Brazil. Samples were collected from *Pteronotus personatus* and *Pteronotus gymnonotus*, yielding approximately 251 and 225 CFUs, respectively. In total, 14 fungal isolates were obtained, with the highest diversity found in the body region, followed by the oral cavity and wings. So far, four genera have been identified: *Aspergillus*, *Blastobotrys*, *Fusarium*, and *Penicillium*, with *Aspergillus* being the most predominant.

## 1. Introdução

A região Nordeste do Brasil é conhecida por ser uma área propícia à descoberta de novos ambientes subterrâneos (AULER E ZOGBI, 2005), sendo esta região, o local da maioria das cavernas habitadas por morcegos, as bat caves ou hot caves (LADLE et al., 2012; OTÁLORA-ARDILA et al., 2019). Esses ambientes desempenham um papel crucial como locais de reprodução para diversas espécies de morcegos, além de serem fundamentais para a conservação de espécies ameaçadas de extinção (DELGADO-JARAMILLO et al., 2017). As bat caves são cavernas que se destacam por abrigar populações excepcionais, estáveis ou temporárias de morcegos, com características únicas (LADLE et al., 2012; OGÓREK et al., 2016; PIMENTEL et al., 2022). Esses locais são abrigos peculiares, com temperaturas frequentemente acima de 35°C e umidade relativa do ar superior a 90% (RODRÍGUEZ-DURÁN, 1998). No Brasil, essas cavernas são predominantemente encontradas no Nordeste, abrangendo os estados de Sergipe, Pernambuco, Rio Grande do Norte e Ceará (OTÁLORA-ARDILA, 2018), além da região amazônica, nos estados do Amazonas e Pará (BERNARD, 2018).

De acordo com VANDERWOLF et al. (2013), os morcegos são importantes dispersores de esporos de diversas espécies de fungos, transportando-os para dentro das cavernas e, posteriormente, para o ambiente externo. Nesse contexto, KOKUREWICZ et al. (2016) destacam os morcegos como principais vetores de fungos anemófilos em cavernas. A emergência da síndrome do nariz branco (WNS), uma doença fúngica que causa grandes mortalidades entre os morcegos no leste da América do Norte, revelou uma lacuna significativa no conhecimento sobre as comunidades fúngicas associadas a esses animais e seus hibernáculos (LORCH et al., 2012). Apesar disso, os estudos micológicos relacionados aos morcegos no Brasil ainda são limitados.

Diante dessa lacuna de informações sobre a micobiota associada aos morcegos, torna-se fundamental expandir a pesquisa nessa área, contribuindo para o conhecimento da diversidade de fungos presentes, o que servirá de base para estudos futuros e para o desenvolvimento de estratégias de conservação desses microrganismos e animais. Nesse contexto, o presente estudo tem como objetivo caracterizar, de forma preliminar, a micobiota associada aos morcegos da Caverna Urubu, localizada em Sergipe, Brasil.

## 2. Materiais e Métodos

Localizada em Sergipe, fora de Unidades de Conservação, a bat cave do Urubu encontra-se inserida em rochas calcárias da bacia sedimentar Sergipe-Alagoas. Com aproximadamente 150 metros de extensão, esta cavidade natural apresenta mudanças graduais nos fatores abióticos e bióticos ao longo do percurso. Caracterizada como uma gruta permanentemente seca, a caverna do Urubu apresenta três zonas distintas, com salões de diversos tamanhos, e registra elevadas temperaturas e umidade (SILVA et al., 2007) (Fig. 1).

***Figura 1:*** *Entrada da caverna do Urubu. Foto: Éder Barbier.*

Para a coleta dos fungos foram realizados esfregaços na cavidade oral, asas e corpo dos animais utilizando Swabs estéreis imersos em uma solução de água destilada com antibiótico (CUNHA et al., 2020) (Fig. 2). Foram coletadas amostras em diferentes indivíduos de duas espécies distintas de morcegos (*Pteronotus personatus* e *Pteronotus gymnonotus* (SISBIO 68992–3)(CEUA 114/2019). Em laboratório, os Swabs foram passados na superfície do meio de cultura Ágar Sabouraud (SAB) contido em placas de Petri e incubadas por 7 dias, no escuro, a 28 °C para posterior contagem das Unidades Formadoras de Colônias (UFC).

Para identificação morfológica dos fungos, foram observadas as características macroscópicas e microscópicas das estruturas somáticas e reprodutivas conforme metodologias específicas para cada grupo. Os fungos que representaram novidades científicas estão em processo de descrição, e fotografias e/ou ilustrações estão sendo realizadas para documentar as características morfológicas e auxiliar na identificação e na posterior publicação dos resultados.

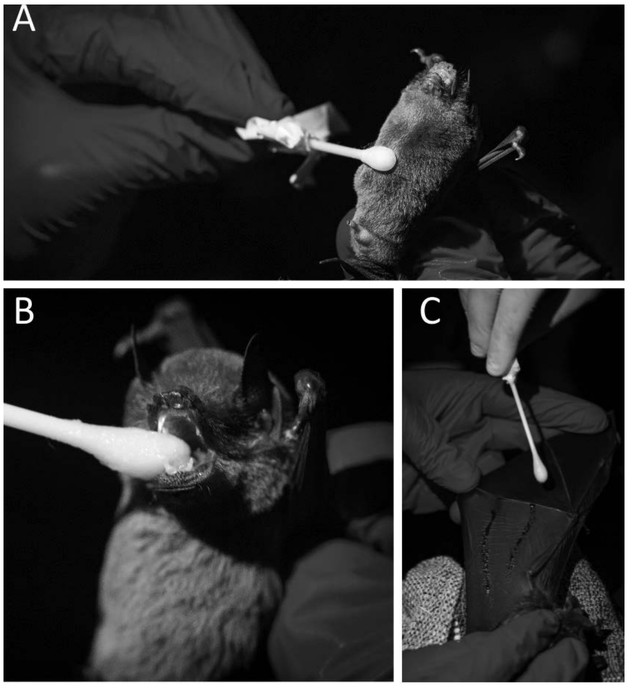
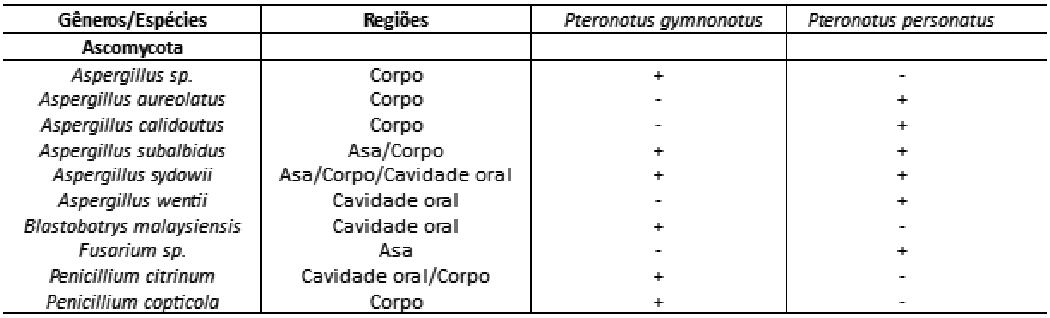


***Figura 2:*** *Método de coleta dos fungos em morcegos. A- Esfregaços no corpo, B- Esfregaços na cavidade oral, C- Esfregaços nas asas. Foto: Diego Bento.*

Além da identificação morfológica, os fungos foram caracterizados por meio de técnicas de sequenciamento de genes e análises filogenéticas. O DNA genômico foi extraído com o Kit de extração de DNA genômico seguindo recomendações do fabricante. Foram utilizados os primers ITS1/ITS4 e LR0R/LR5 para amplificar as regiões ITS (Internal Transcribed Spacer) e LSU (Partial Large Subunit), respectivamente. Outros genes como tef1, RPB1, RPB2, CmD e BenA estão sendo amplificados conforme o gênero ou grupo taxonômico dos fungos identificados. As sequências foram analisadas e posteriormente serão depositadas no NCBI (BARBOSA et al., 2018), usando o GenBank, com cada sequência acompanhada de um voucher da Micoteca URM para rastreabilidade. Esse procedimento viabilizará futuras consultas sobre a diversidade genética de fungos tropicais, facilitando o acesso a dados essenciais para pesquisas científicas globais.

## 3. Resultados

Das espécies de morcegos coletadas, o maior quantitativo de UFC foi encontrado em amostras de *Pteronotus personatus*, com 251 UFCs, enquanto para *Pteronotus gymnonotus* foram obtidas 225 UFCs. Ao todo, 14 isolados fúngicos foram obtidos, sendo a região do corpo a maior detentora em número de espécies, seguida da cavidade oral e asas. Até o momento, quatro gêneros foram identificados: *Aspergillus*, *Blastobotrys*, *Fusarium* e *Penicillium*, sendo a maioria das espécies isoladas pertencentes a *Aspergillus*. Cerca de dez espécies foram identificadas (Fig. 3), sendo *Aspergillus subalbidus* e *Aspergillus sydowii* comuns para as duas espécies de morcegos analisadas. Uma espécie pertencente ao gênero *Aspergillus* está sendo considerada como possível novidade taxonômica.



| **Gêneros/Espécies** | **Regiões** | *Pteronotus gymnonotus* | *Pteronotus personatus* |
|---|---|---|---|
| **Ascomycota** | | | |
| *Aspergillus sp.* | Corpo | + | - |
| *Aspergillus aureolatus* | Corpo | - | + |
| *Aspergillus calidoutus* | Corpo | - | + |
| *Aspergillus subalbidus* | Asa/Corpo | + | + |
| *Aspergillus sydowii* | Asa/Corpo/Cavidade oral | + | + |
| *Aspergillus wentii* | Cavidade oral | - | + |
| *Blastobotrys malaysiensis* | Cavidade oral | + | - |
| *Fusarium sp.* | Asa | - | + |
| *Penicillium citrinum* | Cavidade oral/Corpo | + | - |
| *Penicillium copticola* | Corpo | + | - |

***Figura 2:*** *Relação das espécies de fungos e sua ocorrência em morcegos da Caverna Urubu, Sergipe, Brasil.*

## 4. Discussão

Ambientes cavernícolas pouco explorados no Nordeste brasileiro possuem grande relevância para o conhecimento da micobiota presente no corpo dos morcegos. Ainda existe um grande déficit de estudos relacionados a esses organismos, dificultando então, a elaboração de planos de manejo para melhor conservação desses ambientes e sua biota (VANDERWOLF et al., 2013). Desta forma, este estudo é um dos pioneiros na elucidação da micobiota de morcegos em uma *bat cave* do Nordeste Brasileiro (LIMA et al., 2024).

Todos os indivíduos de morcegos coletados da caverna Urubu apresentaram associação fúngica, sendo a espécie *Pteronotus personatus* a maior detentora da abundância fúngica. As três microrregiões estudadas a partir das duas espécies de morcegos, apresentaram composição e ocorrência de espécies comuns e distintas entre elas (Fig. 3), contudo, o corpo demonstrou maior riqueza de espécies que as demais, o que pode estar associado à presença de dípteros ectoparasitas nos morcegos (CARVALHO et al., 2022). Ao todo foram obtidos 14 isolados, dentre eles os gêneros fúngicos identificados até o presente momento foram *Aspergillus*, *Blastobotrys*, *Fusarium* e *Penicillium* o que corrobora com o estudo da micobiota de morcegos realizado na Bat Cave Meu Rei, no Vale do Catimbau, Pernambuco (CUNHA et al., 2020).

Em um estudo sobre fungos psicrofílicos e psicrotolerantes em morcegos de Illinois e Indiana, JONHSON et al. (2013), isolaram fungos dos gêneros *Cladosporium* e *Penicillium* das asas dos morcegos. Gêneros estes relatados em diversos estudos como habitantes de ambientes cavernícolas (VANDERWOLF et al., 2013; ALVES et al., 2022; LIMA et al., 2024). Outro gênero identificado foi *Fusarium*, cujas espécies são frequentemente descritas como patógenos de plantas, sapróbios ou patógenos oportunistas (CUNHA et al., 2020).

Não foram isolados *Histoplasma capsulatum* e *Pseudogymnoascus destructans* das amostras da cavidade oral, corpo e asas dos morcegos, o que corrobora com o estudo da micobiota de morcegos realizado na Bat Cave Meu Rei, no Vale do Catimbau, Pernambuco (CUNHA et al., 2020). Contudo, das espécies encontradas, algumas são consideradas como potenciais patógenos ao homem, ou com capacidade de causar micoses oportunistas, principalmente a depender do estado imunológico do indivíduo. Destas, *Aspergillus sydowii* e *Penicillium citrinum* merecem destaque por apresentarem risco à saúde humana.

## 5. Conclusão

A análise dos fungos associados a morcegos em caverna revelou resultados importantes para compreensão da biodiversidade micológica e dos riscos potenciais à saúde humana. O gênero *Aspergillus* é um gênero cosmopolita e comumente relatado em ambientes cavernícolas, foi o mais abundante, presente em todas as regiões coletadas das espécies de morcegos da caverna Gruta do Farias, incluindo asa, boca e corpo. Esse estudo é o primeiro relato do gênero *Hortaea* isolado do corpo de morcegos, ampliando o conhecimento sobre a diversidade micológica nessas cavernas.

As espécies de morcegos *Carollia perspicillata* e *Pteronotus gymnonotus* apresentaram isolados fúngicos em todas as regiões analisadas, o que evidencia a importância de uma controle rigoroso nas áreas de visitação. Destacamos a importância de pesquisas que avaliem a complexidade da microbiota associada aos morcegos e suas interações. Esses dados fornecem informações cruciais para a elaboração e/ou melhoramento de planos de manejos dos ambientes cavernícolas e proteção dos visitantes.

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# Caves of the Potiguar Karst: the role of mycological studies in management plans

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## Resumo

Este trabalho investiga a biodiversidade micológica em cavernas do Carste Potiguar, situado no estado do Rio Grande do Norte, região semiárida do Nordeste do Brasil. Foram analisadas três cavernas: Catedral, Crotes e Furna Nova, com foco na importância da pesquisa para a conservação e segurança dos ecossistemas subterrâneos. A coleta de amostras de ar e sedimentos/guano resultou na identificação de gêneros fúngicos como *Alternaria, Aspergillus, Cladosporium, Fusarium, Microascus, Penicillium*, entre outros, sendo alguns desses fungos com potencial patogênico, especialmente para indivíduos imunocomprometidos. Os resultados obtidos foram incorporados a planos de manejo, como o da Caverna Furna Nova/Plano de Manejo Espeleológico - Portaria ICMBio Nº 1074 e da Caverna dos Crotes/Licença Simplificada - Nº 2023-197885/TEC/LS-0317. Embora as cavernas abrigam fungos oportunistas, a visitação pode ser realizada com segurança, desde que sejam seguidas as diretrizes de manejo adequadas. O estudo reforça a necessidade de monitoramento constante para proteger tanto a saúde humana quanto a biodiversidade e as formações geológicas desses ecossistemas frágeis, garantindo a conservação do patrimônio natural subterrâneo.

## Abstract

This study investigates the fungal biodiversity in caves of the Potiguar Karst, located in the state of Rio Grande do Norte, in the semi-arid region of northeastern Brazil. Three caves were analyzed: Catedral, Crotes, and Furna Nova, with an emphasis on the importance of research for the conservation and safety of subterranean ecosystems. Air and sediment/guano samples were collected, resulting in the identification of fungal genera such as *Alternaria, Aspergillus, Cladosporium, Fusarium, Microascus, Penicillium*, among others, some of which are potentially pathogenic, especially to immunocompromised individuals. The results were incorporated into management plans, such as the Furna Nova Cave/Espeleological Management Plan - ICMBio Ordinance No. 1074 and Crotes Cave/Simplified License - No. 2023-197885/TEC/LS-0317. Although the caves harbor opportunistic fungi, visitation can occur safely if proper management guidelines are followed. The study emphasizes the need for constant monitoring to protect both human health and biodiversity, as well as the geological formations of these fragile ecosystems, ensuring the conservation of subterranean natural heritage.

## 1. Introdução

O estado do Rio Grande do Norte, com 1.362 cavernas registradas até o momento, é a quarta maior região de cavernas do Brasil (ICMBIO, 2022). As cavernas do Carste Potiguar são formadas principalmente pelos calcários do grupo Apodi e pela formação Jandaíra, constituindo um importante patrimônio natural devido aos processos de dissolução das rochas calcárias (CRUZ et al., 2010). Ecologicamente, esses ambientes cavernícolas abrigam uma rica biodiversidade, com espécies de fauna e flora adaptadas (OGÓREK et al., 2013), além de formarem paisagens fascinantes, com espeleotemas, rios subterrâneos, fósseis e pinturas rupestres (NOVÁKOVÁ et al., 2012). Tais características não só despertam interesse científico, mas também têm grande importância econômica, especialmente no espeleoturismo, que gera fontes de renda para muitas comunidades locais (TRAJANO et al., 2024).

No entanto, as cavidades naturais são ecossistemas extremamente frágeis, e qualquer alteração ambiental pode comprometer sua biodiversidade e suas formações geológicas (TRAJANO et al., 2024). Por isso, um manejo adequado é essencial para garantir a conservação desses ambientes. O manejo eficaz envolve monitoramento constante das cavernas, limitação do acesso de visitantes, controle sobre as atividades desenvolvidas, educação ambiental para a comunidade local e a criação de áreas protegidas, como monumentos naturais, parques ou reservas (ICMBIO, 2021).

O estudo micológico em cavernas é crucial para entender a biodiversidade desses ecossistemas e suas interações ecológicas. Os fungos desempenham papeis essenciais, como decompositores de matéria orgânica, contribuindo para a ciclagem de nutrientes, o biointemperismo de rochas e minerais, além de servirem como alimento para outros organismos (SOCIETY OF MYCOLOGICAL SCIENCES, 2023). No entanto, alguns fungos, como espécies dos gêneros Aspergillus, Candida e Histoplasma, possuem potencial patogênico para os seres humanos, sendo frequentemente encontrados em ambientes subterrâneos, especialmente em locais com presença de morcegos (VANDERWOLF et al., 2013; CUNHA et al., 2020; LIMA et al., 2024). Nesse contexto, estudos micológicos em cavernas não só são fundamentais para compreender a biodiversidade local, mas também para a saúde pública, pois auxiliam na avaliação de riscos associados à visitação e contribuem para o desenvolvimento de medidas de segurança e protocolos de manejo adequados. O objetivo deste estudo é discutir a relevância dos estudos micológicos na identificação de fungos patogênicos nas cavernas do Carste Potiguar e a importância da incorporação dessas informações nos planos de manejo, especialmente para garantir práticas seguras no turismo espeleológico. A pesquisa visa ressaltar os cuidados necessários para o acesso seguro a essas cavernas, a fim de proteger tanto os visitantes quanto a integridade dos ecossistemas cavernícolas.

## 2. Materiais e Métodos

Três cavernas localizadas no estado do Rio Grande do Norte, no Carste Potiguar, foram selecionadas para realização de um levantamento micológico e observação da ocorrência de fungos patogênicos. A caverna dos Crotes (05° 33' 37,92'' S; 37° 39' 30,89'' W), e a caverna Catedral (05°33'50,49'' S; 37°39'57,37'' W) estão situadas na zona rural do município de Felipe Guerra, enquanto a caverna Furna Nova (5°2'3» S, 37°34'16» W) está localizada no Parque Nacional de Furna Feia, no município de Baraúna (Fig. 1). Todas as cavernas estão localizadas na região semiárida do Bioma Caatinga. As coletas de amostras do ar, sedimento/guano foram realizadas entre agosto de 2019 e março de 2020.

***Figura 1:*** *A - Caverna Furna Nova; B - Caverna do Crotes; C - Caverna Catedral. Foto: Diego Bento.*

Os pontos coletados foram distribuídos de acordo com as áreas de importante acesso aos turistas e com diferentes condições ambientais. Resumidamente, para a coleta dos fungos do ar, placas de Petri 90mm, contendo meio de cultura Ágar Sabouraud (SAB), foram abertas por 20 minutos a 1 m do chão, sobre suportes de PVC (CUNHA et al., 2020). Após a exposição, foram transportadas para o laboratório e incubadas a 25°C por até 14 dias no escuro. Posteriormente foram contabilizadas as Unidades Formadoras de Colônias (UFC) e purificadas para análises posteriores.

A amostragem de sedimento foi realizada em triplicata, com cerca de 10g coletadas de cada ponto do ambiente cavernícola, sendo acondicionadas em recipientes esterilizados para processamento em laboratório. O isolamento fúngico foi realizado por meio de diluições seriadas até $10^{-4}$, onde 1g de sedimento foi suspenso em 9mL de água destilada esterilizada. A partir dessa suspensão, foi feito o plaqueamento direto nas superfícies de placas de Petri de 90mm, contendo meio de cultura SAB e Ágar Brain Heart Infusion (BHI). As placas foram incubadas a 25°C, por até 14 dias no escuro para crescimento de colônias. Após o período de incubação, o número de UFCs por grama foi calculado com base na média das réplicas, e as colônias foram purificadas (OGOREK et al., 2016).

Para identificação das espécies, foram realizadas análises morfológicas com base em características macroscópicas e microscópicas de acordo com literatura taxonômica especializada. Posteriormente, análises moleculares foram realizadas por meio da extração de DNA fúngico utilizando o Kit de extração de DNA Genômico da Promega (Wizard Genomic DNA Purification Kit). As regiões alvo foram amplificadas por PCR utilizando primers específicos conforme os gêneros e as recomendações da literatura. Os produtos de PCR foram purificados com as enzimas EXO+SAP e sequenciados na Plataforma Multiusuária de Sequenciamento de DNA do Centro de Biociências/UFPE.

Para a filogenia, as sequências de DNA foram editadas e comparadas com dados do GenBank. As análises filogenéticas utilizaram sequências do GenBank e do presente estudo, com base em artigos taxonômicos específicos para cada gênero.

Após identificação, os táxons encontrados nas cavernas Catedral, Crotes e Furna Nova foram listados e comparados com o Atlas of Clinical fungi, bem como com outras fontes, incluindo artigos científicos e catálogos de espécies de risco (DE HOOG et al., 2020; BRASIL et al., 2022; THAMBUGALA et al., 2024). Com base nessa análise, foi elaborada uma lista de inventário, e os dados foram fornecidos para os órgãos competentes para elaboração dos respectivos planos de manejo.

## 3. Resultados

As análises revelaram variações no número de táxons entre as três cavernas estudadas. Na Caverna dos Crotes, foram identificadas cerca de 15 espécies, pertencentes a pelo menos 10 gêneros. Na caverna Catedral, foram encontradas pelo menos 35 espécies, distribuídas em 15 gêneros. Por fim, na caverna da Furna Nova, catalogaram-se 16 espécies pertencentes a 10 gêneros. Todas incluindo as amostras de fungos do ar e sedimento/guano. A lista com os dados de todas as espécies/gêneros de fungos relatados em cada caverna se encontram nos Planos de Manejo da Caverna Furna Nova/Plano de Manejo Espeleológico - Portaria ICMBio Nº 1074, da Caverna dos Crotes/Licença Simplificada - Nº 2023-197885/TEC/LS-0317 e da Caverna Catedral. Estes são os primeiros Planos de Manejo do Nordeste que contém informações sobre fungos.

Após as análises e comparativos, cerca de oito espécies isoladas e identificadas nas cavidades estudadas apresentam alguma patogenicidade ao homem (Fig. 2).

| Fungos | Cavernas | Referências |
|---|---|---|
| *Aspergillus niger* | Catedral / Crotes / Furna Nova | Gniadek et al. (2017) |
| *Aspergillus ochraceus* | Crotes | Gniadek et al. (2017) |
| *Aspergillus sydowii* | Catedral | Nagashima et al. (2023) |
| *Aspergillus tamarii* | Catedral | Homa et al. (2019) |
| *Cladosporium tenuissimum* | Catedral / Furna Nova | Nasiri-Jahrodi et al. (2023) |
| *Meyerozyma guilliermondii* | Furna Nova | Thambugala et al. (2024) |
| *Microascus trigonosporus* | Catedral | Schoeppler et al. (2015) |
| *Penicillium citrinum* | Catedral / Crotes | HC Nguyen (2023) |

***Figura 2:*** *Espécies relatadas como patogênicas na literatura encontradas nas cavernas Catedral, Crotes e Furna Nova.*

## 4. Discussão

A identificação e o monitoramento dos fungos presentes em cavernas têm se mostrado fundamentais para garantir a segurança de seus visitantes e para a conservação dos ecossistemas subterrâneos. No Carste Potiguar, o estudo de fungos isolados do ar e de sedimentos/guano das cavernas revelou a predominância de gêneros do filo Ascomycota, como *Alternaria, Aspergillus* e *Penicillium*. As espécies pertencentes a esses grupos são conhecidas por sua grande capacidade de adaptação, o que facilita sua colonização em ambientes cavernícolas ( CUNHA et al., 202; ALVES et al., 2022; LIMA et al., 2024). O gênero *Aspergillus*, em particular, foi identificado em todas as cavernas estudadas, sendo a espécie *Aspergillus niger* comum entre elas. Esses fungos são reconhecidos pela sua capacidade de adaptação. Vários desses gêneros incluem espécies com potencial patogênico, como *Aspergillus sydowii, A. tamarii, A. ochraceus* e *Penicillium citrinum*, isoladas nesse estudo (VANDERWOLF et al., 2013), as quais são responsáveis por infecções pulmonares, aspergilose e doenças cutâneas, principalmente em indivíduos imunocomprometidos (GNIADEK et al., 2017; HOMA et al., 2019; NAGASHIMA et al., 2023).Outras espécies, como *Cladosporium tenuissimum* e *Meyerozyma guilliermondii*, também foram relatadas como causadoras de doenças, podendo induzir sinusite fúngica e micoses oportunistas (NASIRI-JAHRODI et al., 2023; THAMBUGALA et al., 2024).

A distribuição dos esporos fúngicos no ar e seu potencial patogênico podem variar conforme a região geográfica, condições climáticas e ambientais. Estudos realizados no Brasil identificaram os gêneros *Aspergillus, Penicillium, Cladosporium, Curvularia* e *Fusarium* como os mais prevalentes no ar atmosférico (SUEHARA et al., 2023). Tais gêneros foram isolados nas cavernas estudadas, assim como todos possuem espécies com potencial patogênico para humanos, o que destaca a relevância dos estudos micológicos para a segurança dos visitantes das cavernas(VANDERWOLF et al., 2013; MARIN-FELIX et al. 2017a, b; LIMA et al., 2024).

Os fungos identificados no estudo, são comumente encontrados em outros ambientes, e para desenvolverem doenças precisam de outros fatores para a sua manifestação (VANDERWOLF et al., 2013). Determinando dessa maneira que, não foram encontrados fungos verdadeiramente patogênicos, mas sim fungos oportunistas que necessitam da fragilidade imunológica para ocasionar uma doença (HOMA et al., 2019; NAGASHIMA et al., 2023). Essa descoberta ressalta a importância de adotar medidas preventivas no turismo espeleológico, como a implementação de práticas de segurança como o uso de Equipamentos de Proteção Individual (EPIs). É de extrema importância que os turistas e guias sigam protocolos específicos, como o uso de roupas e calçados fechados, capacetes e máscaras, além de evitar o contato com o guano de morcegos ou áreas onde a concentração de esporos possa ser maior, como sob colônias de morcegos (ICMBio, 2023).

Integrar os dados micológicos nos planos de manejo das cavernas torna-se essencial para a gestão sustentável desses ecossistemas e para a mitigação dos riscos à saúde pública. Com a inclusão dessas informações nos planos, é possível adotar estratégias de monitoramento contínuo, controle de acessos e aplicação de medidas de prevenção, garantindo que o turismo nas cavernas seja seguro para os visitantes e minimizando o impacto sobre a biodiversidade.

Os resultados obtidos sobre os fungos encontrados nas cavernas Catedral, Crotes e Furna Nova foram incorporados a planos de manejo, como da Caverna Furna Nova/Plano de Manejo Espeleológico - Portaria ICMBio Nº 1074 e Caverna dos Crotes/Licença Simplificada - Nº 2023-197885/TEC/LS-0317. Orientações básicas de visitação das cavernas garantem que a experiência seja segura e tranquila para visitantes e guias. Nesse contexto, é evidente a importância do monitoramento de espécies fúngicas oportunistas, uma vez que a compreensão dos riscos envolvidos na exposição a esses organismos é crucial para a segurança da saúde humana. Além disso, esse estudo contribui para o avanço do conhecimento da micobiota cavernícola e  auxilia na conservação do ecossistema subterrâneo.

## 5. Conclusão

O estudo micológico em cavernas é essencial para a conservação da biodiversidade e para o desenvolvimento de planos de manejo eficazes. No Nordeste, o primeiro plano de manejo contendo informações sobre fungos foi o da Caverna Furna Nova/Plano de Manejo Espeleológico - Portaria ICMBio Nº 1074. O conhecimento da microbiota de cavernas permite a identificação de fungos potencialmente patogênicos, garantindo assim, uma visitação turística mais segura nesses ambientes. As cavernas do Carste Potiguar, como Furna Nova, Catedral e Crotes, possuem grande relevância ecológica e científica. A implementação dos Planos de Manejo também visa reduzir a exploração desordenada, preservar a vegetação nativa e fomentar a pesquisa científica, promovendo a educação ambiental e o turismo consciente.

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# First microbiological analyses of gypsum caves in Emilia-Romagna (Italy)

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## Abstract

The gypsum subterranean environments of Emilia-Romagna (Italy) are unique and fragile ecosystems, recently designated as a UNESCO World Heritage Site. These environments harbor a largely unexplored microbial diversity that plays a significant role in rock substrate alteration and ecosystem functioning. Moreover, microorganisms serve as bioindicators of cave health, with certain bacterial groups acting as potential proxies for human impact and environmental contamination. In this study, we present microbiological analyses of two gypsum caves—Buless and La Tanaccia. DNA-based techniques were used to characterize microbial communities in various biofilm samples collected from water bodies, cave walls, and ceilings. Sequencing data revealed that Buless is predominantly inhabited by sulfur-oxidizing and sulfur-reducing bacteria, while La Tanaccia hosts microorganisms typically associated with oligotrophic (nutrient-poor) cave environments. Ongoing research is expanding this analysis to additional gypsum caves in the region, with the aim of investigating the relationships among microbial diversity, ecosystem function, and human influence.

## 1. Introduction

Caves are aphotic environments generally categorized as oligotrophic due to the presence of low amount of surface-derived organic carbon. Nevertheless, caves host diverse microbial communities that include various microbial taxa characterized by different metabolic capapcities. Primary producers in caves are chemolithotrophic microbes that can utilize inorganic compounds as carbon and energy sources. Examples of chemolithotrophic activities include the oxidation of inorganic compounds like sulfur, sulfides, nitrites, hydrogen, or ferrous ions (D'ANGELI et al., 2019). Other survival mechanisms can rely on sporadic organic matter that enters the cave from the surface via transportation by water, wind, and/or animals. Cave-dwelling microorganisms provide fundamental ecosystems functions in subterranean environments including their key role in biogeochemical cycles and in the food networks (NAYELI LUIS-VARGAS et al. 2024).

Gypsum karst is widespread in many areas of the world including Italy, US, Eastern Europe, Germany, Great Britain, Spain, the Ural area, and the Baltic States. In these areas, gypsum karst aquifers are important as water resources for the local communities, especially if located in arid climate (PISANI et al., 2019). As compared to limestone and volcanic caves, gypsum caves are much less known and explored, and this knowledge gap also includes microbiology. Nevertheless, recent studies have indicated that gypsum caves are environments with peculiar, often exclusive minerogenetic mechanisms, which host extremely rare or even novel cave minerals (FORTI 2017). The study of cave microbiology might provide new information on the interaction between microbes and rock substrate, that can lead to its modification and specific structure formation. Indeed, microbial activities can contribute to speleogenesis and speleothem formation (SAURO et al., 2018), since microbial metabolisms can directly or indirectly influence the solubilization and precipitation processes. While the role of microorganisms in the formation of limestone speleothems is extensively studied, their role in the dissolution and precipitation of gypsum remains still understudied. Only a few studies have investigated microbial diversity in gypsum caves in Italy and Spain (D'ANGELI et al., 2017, JURADO et al. 2024) and they mainly focused on the effect that bat guano and season have on coliforms and fungi in these caves.

The aim of the present study is to provide first insights into the microbiology of two gypsum caves: "Buless Cave" and "La Tanaccia". These caves, located in Emilia Romagna (Italy), have been recently become a UNESCO World Heritage site (https://whc.unesco.org/en/list/1692/). La Tanaccia is located in the "Parco Regionale della Vena del Gesso Romagnola" and is representative of typical caves in Emilia-Romagna gypsum area. Buless is located in the Eastern Romagna gypsum area and is characterized by the presence of a sulfur spring in the deepest part of the cave.

## 2. Materials and methods

### 2.1. Sampling procedures

In September 2023, fifteen samples representative of different biofilms, biodeposits and colored patinas present on water bodies and walls/ceiling, were collected from Buless and La Tanaccia by applying sterile procedures (Fig. 1). The samples were stored at -80°C before processing.

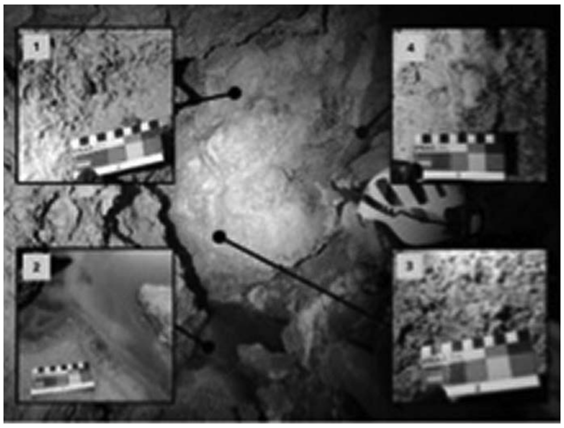

***Figure 1:*** *Pictures of samples collected from Buless cave.*

## 2.2. DNA extraction and 16S rRNA gene sequencing

Total DNA was extracted from each sample using the PowerLyzer PowerSoil kit (QIAGEN) according to the manufacturer's instructions and used as template to PCR-amplify the hypervariable regions V3-V4 by using high fidelity ExTaq Polymerase (TaKaRa) and primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). PCR reactions were performed in a final volume of 50 µL containing 10 ng of total DNA, primers 500 nM, 1x Takara Ex Taq buffer with $MgCl_2$, dNTPs mix 200 µM, Takara Ex Taq Polymerase 0.5 U. The thermocycling program included 1 cycle at 95 °C for 10 s, 30 cycles at 95 °C for 10 s, 58 °C for 30 s, 72 °C for 30 sec, and a final extension at 72 °C for 2 min. Amplicons were submitted to the sequencing service BMR Genomics SRL (Padova, Italy) for library preparation and pair-end sequencing through Illumina MiSeq platform. Raw sequences were trimmed based on their quality, denoised, merged and checked for chimeras by using Qiime2 (version 2024.5.0). Amplicon Sequence Variants (ASVs) were generated by using DADA2 and their taxonomical assignation was performed on SILVA database

# 3. Results

## 3.1. Cave description and geological setting

The gypsum caves of eastern Romagna, unlike other karst areas in Emilia-Romagna, are rich in sulfidic water and native sulfur deposits, the presence of which is evidenced by the various (abandoned) mines in the area.

The terminal portion of Buless Cave contains one of the few underground sulfur springs in the gypsum caves of Emilia-Romagna (the other being Befana Cave, near Borgo Tossignano) (Fig. 2). The presence of hydrogen sulfide has been associated with the formation of the prismatic gypsum crystals that cover some of the walls and speleothems inside the cave (FORTI & LUCCI, 2016). Although microbial diversity present in sulfur-rich karst environments has been studied before (e.g. Frasassi caves in Marche, a sulfuric acid cave formed in Jurassic limestones), studies aimed at assessing how hypogean sulfur-rich springs influence microbial communities in the Emilia-Romagna gypsum caves have never been carried out.

The Tanaccia cave is located within the "Parco Regionale della Vena del Gesso Romagnola" and is one of the best known and most studied caves in Romagna (Fig. 3). It is currently used as a show cave, for adventurous caving trips (about 3000 visitors/year). The cavity consists of a big horizontal passage carved by a stream that now flows about ten meters below (this part is active only during floods) (GRUPPO SPELEOLOGICO FAENTINO, SPELEO GAM MEZZANO, 2015), and, unlike the Buless Cave, it is not affected by subterranean sulfur springs (there is an external sulfidic spring some tens of meters downstream of the cave entrance). Given its characteristics, La Tanaccia can be considered representative of many caves found in the gypsum area of the Emilia-Romagna Apennines.

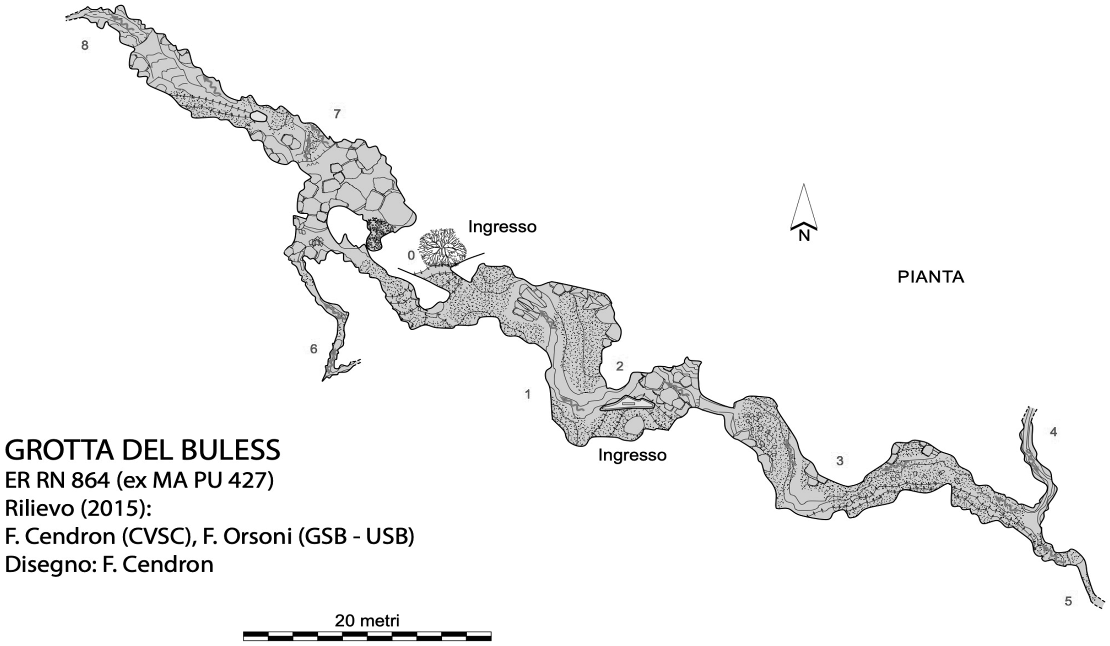

***Figure 2:*** *Map of Buless cave.*

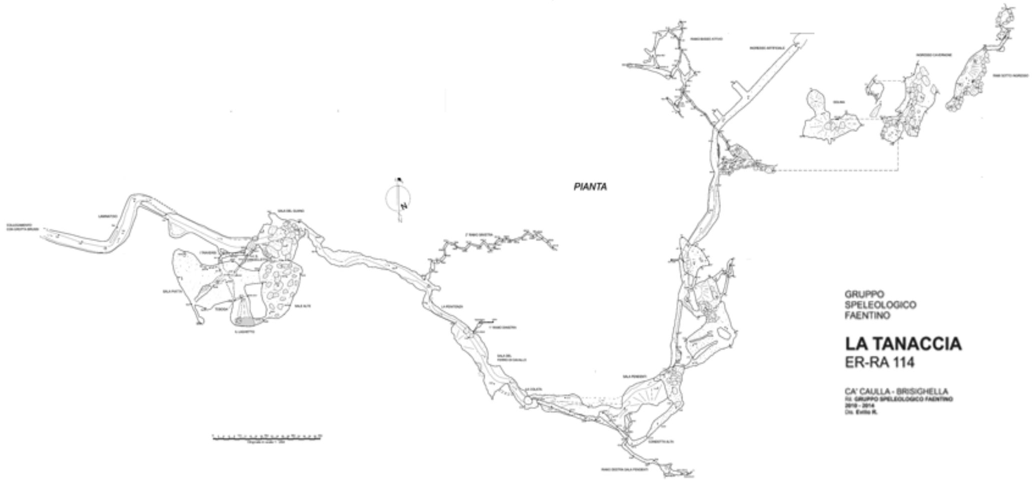

***Figure 3:*** *Map of La Tanaccia cave.*

## 3.2. Microbiological diversity in the two caves

Samples from colored patinas, vermiculations, prismatic gypsum crystals, possible bacterial colonies, and water samples were collected from Buless and La Tanaccia.

Alpha- and-Beta diversity analyses were conducted to analyze the species' heterogeneity inside each sample and to compare the different samples in terms of microbial community composition. Principal Coordinate Analysis (PCoA) showed that the samples clustered separately depending on the cave. The Buless samples further grouped into two sub-clusters, one including the waters and the other including the biofilms on the cave wall (Fig. 3).

## 3.3. Microbial community composition

The microbial community in the Buless cave was mainly composed of bacteria that reduce and oxidize sulfur compounds, including *Sulfurimonadaceae*, *Desulfocapsaceae*, *Sulfurovaceae* and *Geobacteraceae*. This is in line with the high amount of inorganic sulfur compounds in the gypsum substrate and sulfidic water of the spring present in the cave. In contrast, La Tanaccia Cave harbors a microbial community predominantly composed of bacteria involved in different steps of the nitrogen cycle including nitrogen-fixation, like *Nitrosomonadaceae*, *Nitrosococcaceae*, *Nitrospiraceae*, and *Beijerinckiaceae*. Additionally, bacteria highly abundant in the colored patinas from the deepest parts of the cave are known to be able to oxidize methane and/or methanol, such as members of the families Methyloligellaceae and Methylomirabilaceae (Fig. 4). Moreover, biofilms from the cave walls of La Tanaccia exhibited a high abundance of Pseudonocardiaceae, which might be involved in CO$_2$ fixation and carbonate mineralization processes

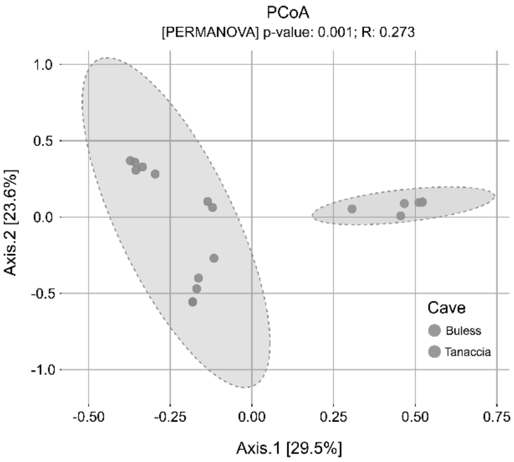

***Figure 4:*** *PCoA analysis of the samples collected from Buless and Tanaccia highlights the presence of distinct microbial communities in the two caves.*

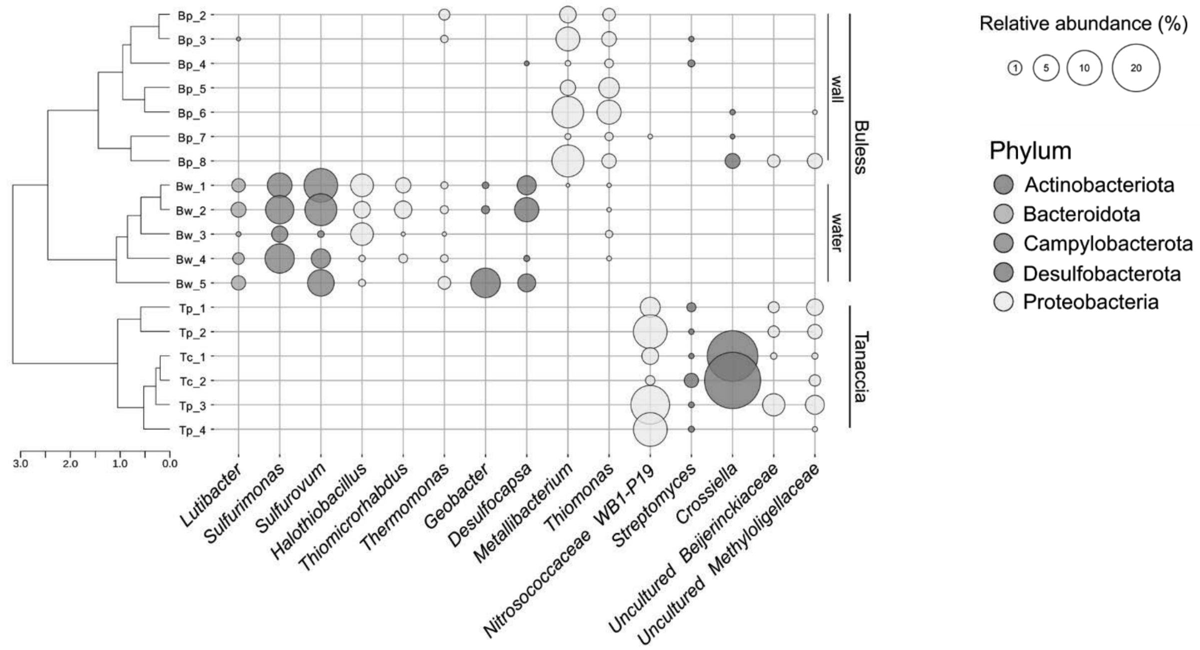

*Figure 5: Composition and clustering analysis of microbial communities from samples collected in the Buless and Tanaccia caves.*

## 4. Discussion

In this work we provide first insights into the microbiology of two gypsum caves that are localized in Emilia-Romagna Italy. In 2024, part of this subterranean area became a UNESCO world heritage site highlighting the uniqueness of this karst area, which requires protection.

The results we obtained in this work indicated that the microorganisms inhabiting the two caves are distinct despite their proximity and the identical main composition of Messinian gypsum rock substrate. Indeed, the microbial community composition inside the two caves seems to be driven by different factors, i.e. the extreme oligotrophy in La Tanaccia, and the presence of $H_2S$-rich rising water in Buless. Indeed, the Buless cave is dominated by bacteria involved in sulfate reduction and the oxidation of reduced sulfur compounds, that are absent in La Tanaccia. Bacteria taxonomically affiliated with those abundant in this cave were also previously found in sulfuric acid speleogenetic (SAS) caves in carbonate substrate (D'ANGELI et al. 2019), indicating how the presence of H2S is a key environmental factor shaping microbial communities' composition. On the other hand, La Tanaccia cave was dominated by members of *Pseudomonadota* and *Actinomycetota* that are known to be involved in nitrogen cycle, carbon fixation and methane oxidation. These findings suggest that inorganic nitrogen compounds and C1 compounds might serve as important energy sources for microorganisms inhabiting La Tanaccia (NAYELI LUIS-VARGAS et al. 2024).

## 5. Conclusion

Although this study has investigated only two gypsum caves, it provides some first insights into the microbial diversity present in gypsum subterranean ecosystems in the UNESCO protected area in Emilia Romagna, providing also hints on the main metabolic strategies that microbes can use to survive in these caves. Future studies will focus on metagenomic analyses of microbial communities and the analysis of culturable microbial strains. Ongoing studies are extending microbiological research to other gypsum caves in the area and are exploring the correlation between microbial diversity/functions, ecosystem functions and possible human impact.

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# Analyses of the peculiar biofilm patterns present in the hydrothermal cave system of Monte Kronio (Sicily)

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## Abstract

Caves are dark and oligotrophic environments where microbial life evolved peculiar metabolisms to cope with the absence of sunlight and oligotrophic conditions. The study of cave microbiology has recently gained significative attention for investigating metabolisms and genetic functions that allow microbial survival in subterranean environments. Here (in the framework of PNRR-DM118/2023), we investigate the biodiversity and metabolic potential of microbial communities exposed to cold and hot air flows present in the hydrothermal Cucchiara cave (Monte Kronio, Sicily) through Nanopore and Illumina 16S rRNA gene metabarcoding and by carrying out hydrolytic and antimicrobial assays. Furthermore, the elemental composition of representative samples was investigated through ICP and CHNS, while the organic matter composition was investigated through analytical pyrolysis. As a result, most of the analyzed microbial communities were found to be represented by a high percentage of members pf Actinobacteria belonging to the uncharacterized order 0319-7L14 (up to 70%), whereas other low abundant taxa appeared to be differently represented depending on the biofilm color. Most of the isolated strains showed enzymatic activities involved in the hydrolysis of different compounds and produced antimicrobial compounds able to inhibit the growth of different pathogens indicating their biotechnological potential.

## 1. Introduction

Caves are dark and oligotrophic environments where microbial life evolved peculiar metabolisms to cope with the absence of sunlight and oligotrophic conditions. These include the production of a wide range of enzymes and secondary metabolites such as antimicrobial compounds, siderophores and quorum sensing molecules (GHEZZI et al. 2024). The study of cave microbiology has recently gained significative attention for investigating metabolisms and genetic functions that allow microbial survival in subterranean environments, as well as for discovering potentially new microbes and natural products with biotechnological applications in medical and industrial fields. Although several studies have investigated the biodiversity and metabolic potential of cave microbial communities, knowledge about cave microbiology remains still limited, and more studies should be carried out. Here we present the first results of an extensive study on the metabolic potential and bacterial diversity within the hydrotermal Cucchiara cave in Monte Kronio (Sicily, Italy). Located in southern Sicily, on the Mediterranean coast, the Cucchiara Cave opens at an altitude of 280 m on the southern slope of Mount Kronio. It is part of an extensive karst system carved into Mesozoic limestone and consists of several levels of subhorizontal galleries connected by steep passages that reach a length of about 500 m and a large shaft roughly 100 m deep. This peculiar hypogean system is characterized by high relative humidity (close to 100%), high level of $CO_2$, and presence of warm and cold airflows. Warm airflows arise from the deepest area of the hypogean system, while the cold ones enter the system from the external environment. In some areas of the cave, warm and cold airflows enter in contact resulting in the formation of strong temperature gradient from the floor (16-20°C) to the ceiling (up to 38°C). Within these areas, cave walls and coralloids are covered by hydrophobic microbial biofilms, whose color seems to match the temperature gradient (Fig. 1). Calcium carbonate coralloids located on the floor (16-20°C) are mostly covered by pinkish colonies, whereas the cave walls are colonized by yellow (temperature around 20-25°C) and white biofilms (from 25 to 35°C). The massive presence of microbial mats, together with the peculiar environmental conditions present in the hydrothermal cave system of Kronio mountain, make it a promising site to carry out microbiological analysis aimed at discovering novel species and metabolisms. To date, only one microbiological study focusing on the characterization of fungal isolates has been carried out (DI PIAZZA et al. 2017). In our work, we are combining isolation strategies and molecular and sequencing approaches to provide the first systematic description of the peculiar biofilms present in Cucchiara cave.

## 2. Materials and methods

### 2.1. Cave description and sampling

Sampling activity was carried out in January and December 2023 within Cucchiara cave, part of the hydrothermal system of Kronio Mountain (Sciacca, Italy). During these two sampling campaigns, several colored microbial biofilms (i.e. pink, yellow and white microbial mats) exposed to different temperatures have been collected using sterile tools and wearing surgical masks to avoid contamination (Fig. 1). All the samples were collected in sterile tubes and divided into two aliquots. These were brought to Molecular Environmental Microbiology lab (Bologna, Italy, https://site.unibo.it/molecular-environmental-microbiology-lab/it) and stored at 4°C (for culture-based analysis) and -20°C (for DNA-based analysis).

### 2.2. DNA extraction and Illumina and ONT sequencing

Microbial diversity within Cucchiara cave was analyzed through 16S rRNA gene Illumina sequencing. To this purpose, DNA was extracted from each sample using the PowerLyzer PowerSoil kit (QIAGEN) according to the manufacturer's instructions and used as template to PCR-amplify the hypervariable regions V3-V4 by using high fidelity ExTaq Polymerase (TaKaRa) and primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). Resulting amplicons were submitted to the sequencing service BMR Genomics SRL (Padova, Italy) for library preparation and pair-end sequencing through Illumina MiSeq platform. Raw sequences were trimmed based on their quality, denoised, merged and checked for chimeras by using Qiime2 (version 2024.5.0). Amplicon Sequence Variants (ASVs) were generated by using DADA2 and their taxonomical assignation was performed on SILVA database. To determine the taxonomy of the most abundant taxa in greater detail, some samples were further analyzed through full-length 16S rRNA gene sequencing by using Oxford Nanopore sequencing Technology. To this purpose, extracted DNA was used as template for PCR amplification and library preparation by using 16S Barcoding Kit SQK-RBK004 (Oxford Nanopore Technologies), Phanta Mix Super-Fidelity DNA Polymerase (Vazyme) and universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). Amplicons were purified using AMPure® XP beads (Beckman Coulter Diagnostics, USA, CA) and quantified by QuBitTM (Thermofisher). The sequencing library was loaded on a flowcell with R9.4.1 chemistry (FLO-MIN106, Oxford Nanopore Technologies) and sequenced with a MinION Mk1C sequencing device. The raw sequencing data were basecalled with Guppy v6.3.7 using the super-accurate model. The resulting reads were filtered by length and quality score with Nanofilt (https://github.com/wdecoster/nanofilt) and finally processed in Emu for taxonomic classification against the NCBI 16S rRNA database and calculation of taxa abundances.

### 2.3. Isolation of cave strains and metabolic analysis

Isolation of cave strains was carried out by resuspending 0.1 g of sample in 1 mL of phosphate buffer and plating serial dilutions on R2A, ISP2, CSA and AIA agar plates, with and without antibiotics (nalidixic acids 30 μg/mL and cycloheximide 100 mg/mL) (GHEZZI et al. 2024). Plates were incubated at the isolation temperature and monitored daily over time. Microbial colonies showing different colors and shapes were purified on fresh agar plates and stocked at -80°C. The whole strains collection was screened for hydrolytic and antimicrobial activities (Fig. 2). Proteoytic, amylolyitc, lipolyitc, ureolyitc and cellulolytic activities were assessed by carrying out plate-assays respectively on skim milk agar, starch agar, tween 80 agar, Christenson agar and CMC agar plates, as indicated by GHEZZI et al. (2024). Antimicrobial activities were carried out through top agar assays using four model pathogens as targets (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). Positivity to the test was determined by observing growth-inhibition halos of pathogens around the cave strains (Fig. 2F). Strains active against at least one of the tested pathogens were taxonomically characterized by amplifying and sequencing the 16S rRNA genes. PCR was performed by using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and BioTaq polymerase (Meridian Biosciences Inc). PCR products were purified using EasyPure PCR Purification kit (TransGen Biotech) and sequenced by Eurofins Genomics (Padova, Italy) through Sanger sequencing. The 16S rRNA gene sequences were individually further analyzed using BLASTn to determine affiliation with reference strains present in GenBank database.

### 2.4. Inorganic and organic geochemistry

To study the elemental composition of white, yellow and pink biofilms, representative samples were mineralized through three steps methodology and analyzed through ICP OES (Agilent 5800). The first step involved deionized water to determine the soluble contribution; the second step include $H_2O_2$ for the biological contribution and the last was done with concentrated $HNO_3$ for the residual. Operatively, samples were dried at 40°C, resuspended in different solvents and incubated at room temperature overnight under shaking (100 rpm). After the incubation, the suspensions were centrifuged and the supernatants were filtered, acidified with 0.2% $HNO_3$. And stored in fridge. The solid remains were dried again at 40° and the cycle was repeated with the next solvent. The organic composition was investigated by analytical pyrolysis (Py) and GC-MS analysis. Py-GC-MS was performed using an EGA/PY-3030D micro-furnace pyrolyser (Frontier Laboratories Ltd., Japan) coupled with a 7890 Agilent HP gas chromatograph (GC) connected to a 5977 Agilent HP quadrupole mass spectrometer (MS) (Agilent Technologies, USA). Representative samples (1±0.1mg) were placed in small crucible capsules and introduced into the furnace for 1 min using helium as carrier gas (1mL min−1), setting the pyrolysis and the interface temperatures at 600 °C and 280 °C, respectively. Pyrolyzed samples were online transferred into the GC-MS for analysis. The GC injector was operated in split mode with a 10:1 ratio at 280 °C. Pyrolysis products were separated by a GC column fused silica capillary column (HP-5MS, Agilent Technologies, USA) with the following temperature program: starting at 50 °C, held for 2 min, then ramping up at 10 °C $min^{-1}$ to 310 °C, where it was held for 5 min, using helium as carrier gas (1mL $min^{-1}$). The MS was operated in EI positive mode (70eV, scanning 30-600 m/z) with transfer line temperature 250 °C, ion source temperature 230 °C and quadrupole temperature 150°C.

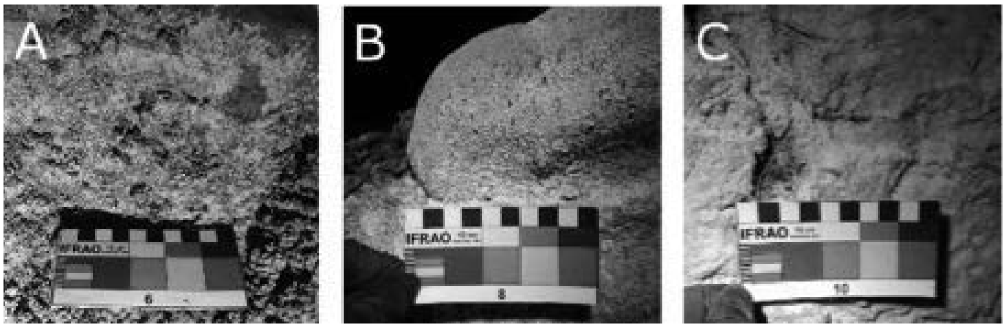

***Figure 1:*** *example of pinkish (A), whitish (B) and yellow (C) biofilms collected in Cucchiara cave from areas exposed to different temperatures (Photo by Ettore Lopo/UNIBO).*

## 3. Results

### 3.1. DNA-based analyses of microorganisms inhibiting Cucchiara cave

Microbial diversity and community composition analysis within Cucchiara cave were carried out through Illumina 16S rRNA gene sequencing. All the microbial biofilms analyzed, regardless of their color and location within the cave, were dominated by the phylum *Actinomycetota* (with a relative abundance ranging from 30 to 90%, depending on the sample), mostly represented by *Euzebyaceae* and other bacteria unclassified at class level. Other abundant phyla were *Pseudomonadota*, *Acidobacteriota*, *Methylomirabilota Nitrospira* and *Plancomycetota* (Fig. 3). Due to the high abundance of unclassified microorganisms, we sequenced full-length rRNA community genes of representative microbial communities to better characterize the taxonomical affiliation of the unclassified *Microorganisms*. Nanopore sequencing data confirmed the high abundance of *Actinomycetota* and revealed that the unclassified bacteria belonged to the order of Actinobacteira 0319-7L14, an uncharacterized taxon previously reported to be associated mainly to soils (PERSHINA et al. 2018).

Alpha- and beta-diversity analysis (based on Illumina sequencing data) highlighted some differences among the microbial communities based on the biofilms color and the temperature to which they were exposed in the cave. PCoA analysis showed that pinkish biofilms formed a distinct cluster, whereas white and yellow biofilms were more similar to each other. Microbial diversity within pinkish biofilms was also higher than that observed in white and yellow patinas, with Shannon indexes ranging from 3 to 5 compared to 2-3.5 for the other samples (Fig. 4).

### 3.2. Microbial isolation and metabolic screening of the cave strains

According to the shape and color of the colonies isolated from yellow, white and pinkish biofilms, we purified and stocked 35 different microbial strains. The whole strain collection was then screened for hydrolytic (protease, lipase, amylase, urease and cellulase) and antimicrobial activities against human model pathogens (strains of *E. coli, S. aureus, K. pneumoniae, P. aeruginosa*) (Fig. 2). Enzymatic assays indicated that protease activity was the most represented, with 12 isolates positive to proteolytic assay. Cellulase, urease and lipolytic activities were represented respectively by 9, 8 and 7 strains, whereas amylolytic activity, was present in 5 strains. Antimicrobial assays showed that 8 strains were capable of inhibiting at least one of the tested pathogens. These included genera belonging to the phyla *Actinomycetota*, (i.e. *Streptomyces, Micrococcus, Allokutzneria, Pseudonocardia*), *Pseudomonadota*, (i.e. *Agrobacterium* and *Sphingosinicella*) and *Bacillota* (i.e. *Brevibacillus*).

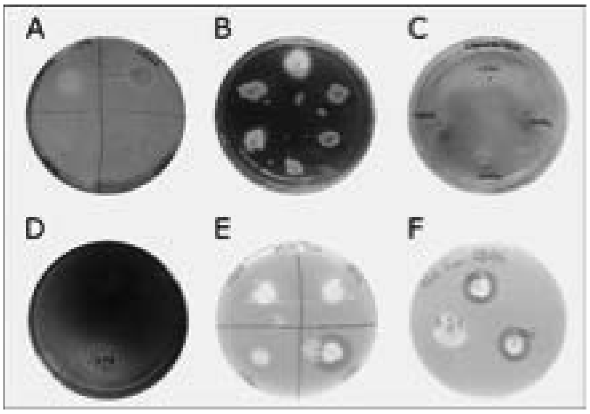

***Figure 2:*** *examples of cellulase (A), lipase (B), urease (C), amylase (D), protease (E) and antimicrobial (F) assays.*

### 3.3. Inorganic and organic geochemistry of representative cave samples

Preliminary results for inorganic geochemistry showed that some elements were differentially enriched in the white, yellow and pink biofilms. Considering the $H_2O_2$ treatment, which represents the biological contribution, white biofilms exhibited the highest concentrations of elements. Some of these were enriched following the order Mn >> Al >> Fe > Zn >> Ba. Yellow biofilms displayed approximately half the concentrations observed in white biofilms, with enrichments in the order Mn ≃ Al ≃ Fe >> Zn. Pink biofilms had the lowest concentrations, with enrichment patterns following Al ≃ Fe ≃ Ba > Zn > Mn. (Figure 5).

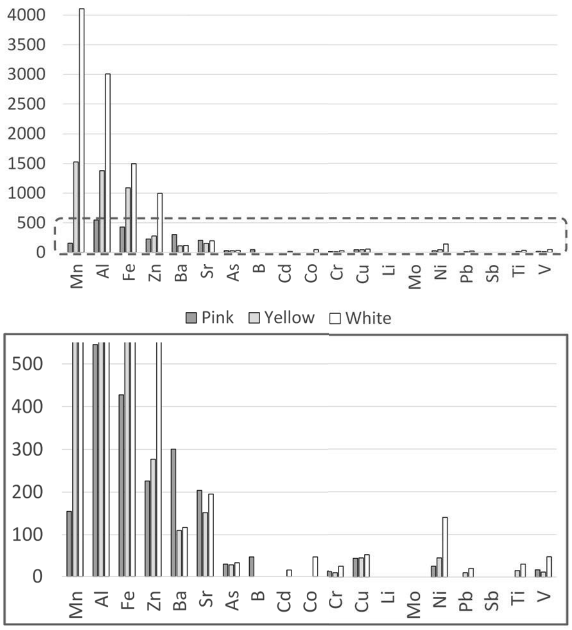

***Figure 5:*** *Elemental concentrations (mg/kg) in pink, yellow and white biofilms after $H_2O_2$ extraction.*

The results of organic matter characterization by analytical pyrolysis showed a complex organic fraction in microbial biofilms, mainly consisting of alkylbenzenes, nitrogen-containing compounds, and a few phenols and carbohydrate products.

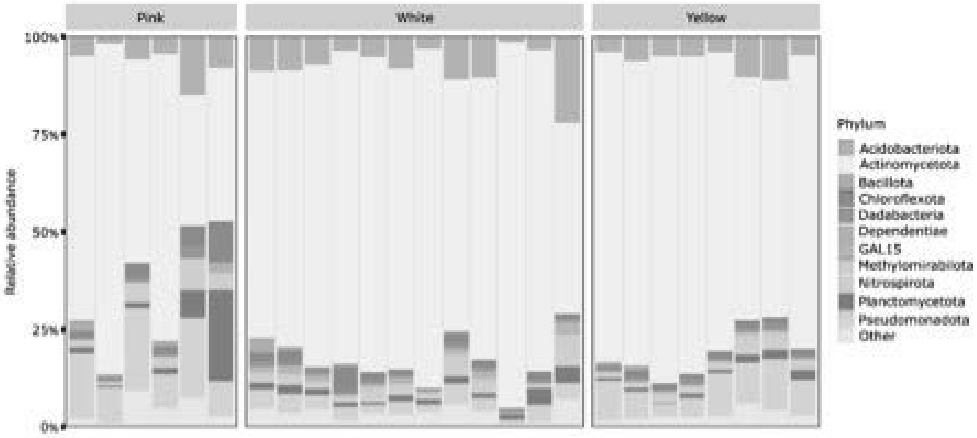

***Figure 3:*** *Barplot showing the microbial community composition within pink, white and yellow biofilms at phylum level.*

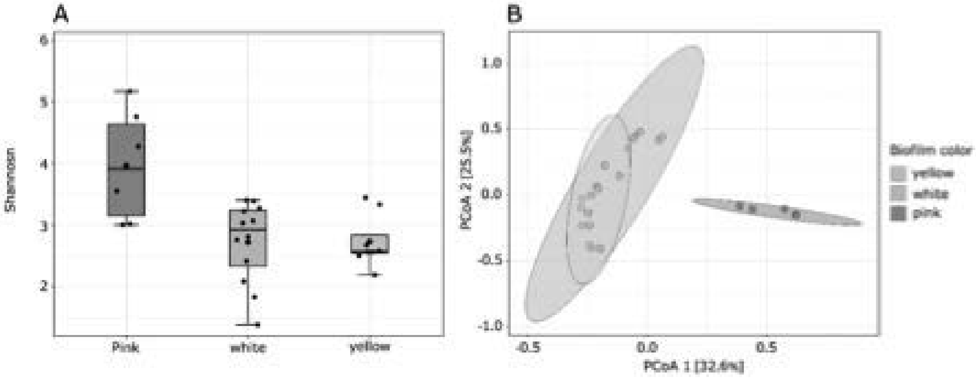

***Figure 4:*** *A) Alpha-diversity (Shannon index) at feature level within pink, white and yellow biofilms. B) PCoA at feature level (based on Bray-Curtis Index) showing the beta diversity among microbial communities. [PERMANOVA] F-value: 7.9163; R-squared: 0.40771; p-value: 0.001.*

## 4. Discussion

Colored biofilms can be found in different caves, including limestone, volcanic and gypsum caves, where they can be present on both the rock substrate and speleothems. In this work we investigated the microbial diversity within peculiar coloured biofilms that are present on the walls of Cucchiara cave (part of the hydrothermal cave system of Monte Kronio). In this cave, the distribution of pink, white and yellow colonies seems to correlate, at least in part, with the gradient temperature present in the cave. Microbial community analyses indicated that all the samples, regardless of the biofilm color, were dominated by the phylum *Actinomycetota* (Fig. 3). These were mostly represented by the family *Euzebyaceae* and the uncharacterized order 0319-7L14 (class *Actinobacteria*), depending on the sample. Despite the high abundance of *Actinomycetota* within cave biofilms, knowledge about their ecological roles and metabolisms is still limited. To date, few works have investigated the microbial diversity of various cave biofilms (especially yellow colonies), suggesting that members of *Actinomyceotota*, such as *Crossiella* and *Euzebyaceae*, might be directly involved in their formation and structure (MARTIN-POZAS et al. 2023, 2024). The order 0319-7L14 has been mainly found in different types of soil (PERSHINA et al. 2018), mine areas and rhizospheres, but also in caves (MARTIN-POZAS et al. 2024,). Nevertheless, it has never been reported to be as abundant as in Cucchiara cave, where it constitutes up to 80% of microbial communities.

In addition to the dominance of actinomycetes in all the samples, however, preliminary statistical analyses also revealed differences in microbial communities' structure depending on the biofilm color (Fig. 4). These differences are due to the presence of low-abundance taxa differentially enriched within the various biofilms. Some authors suggested that the color of cave biofilms might be related to the presence of microorganisms involved in the synthesis of pigments, such as members of the orders Xanthomonadales and *Chromatiales* (PORTILLO et al. 2008). In line with this hypothesis, pink and yellow colonies within Cucchiara cave showed the presence of different bacteria belonging to the families *Pyrinomonadaceae*, *Pirellulaceae*, which have been reported to include bacteria forming colored colonies (WÜST et al 2016). In this regard, the temperature gradient existing in Cucchiara could regulate specific metabolic pathways involved in pigment production (TAKAICHI et al. 1993). Finally, the inorganic characterization revealed varying abundances of trace elements in the investigated biofilms. The total concentrations of Fe, Mn, Al, Zn, and Ba differ among white, yellow, and pink biofilms (SPILDA et al., 2005). Additionally, the relative proportions of these elements appear to have a correlation with the biofilm color and the associated microbial communities, with an emphasis on Ba in pink biofilms. Organic matter characterization by Py-GC-MS seems to show no correspondence between the organic composition and color of the biofilm samples. Py-GC-MS analyses indicated that all the samples, regardless of the biofilm color, were dominated by nitrogen-containing compounds and alkylbenzenes. Alkylbenzenes are commonly observed in the pyrolysis products of natural macromolecular organic matter. Nitrogen-containing compounds such as pyridine, pyrrole, and benzonitrile are mainly of microbial origin. The detection of indole and benzyl nitrile indicates the presence of proteinaceous material in the biofilm samples.

The experimental work on the isolated strains from the different biofilm indicated that the cave strains can hydrolyze several organic substrates and some of them are also able to produce antimicrobial compounds. The production by single strains of extracellular hydrolytic enzymes and secondary metabolites can support synergistic and competitive relationships between microbes in complex microbial communities (GHEZZI et al. 2024) and represent an indication of the biotechnological and metabolic potential of these strains.

## 5. Conclusion

This work provides first results about the high presence of unclassified taxa and bacterial isolates with hydrolytic and antimicrobial activities in different colored biofilms within the hydrothermal cave system of Monte Kronio (Sicily, Italy). Future studies will integrate different omics approaches to delve into the microbial activities involved in biogeochemical cycles and rock interaction in Cucchiara cave and further investigate the association between microbes and the peculiar vertical temperature gradient present in this cave.

## Acknowledgments

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# Poluição luminosa e ruído antrópico: possíveis ameaças negligenciadas à quirópterofauna em cavidades subterrâneas de Minas Gerais

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## Resumo

Minas Gerais, detentor do maior número de cavernas registradas no Brasil, enfrenta o desafio de conciliar as atividades minerárias com a conservação dos ambientes subterrâneos. O estado, caracterizado por um vasto patrimônio espeleológico, tem visto um aumento significativo no número de cavidades cadastradas, especialmente devido aos processos de licenciamento ambiental exigidos. As cavernas oferecem habitat essencial para diversas espécies, incluindo morcegos, que desempenham funções ecológicas fundamentais, como controle de insetos e dispersão de sementes. No entanto, os impactos da mineração, como a poluição luminosa noturna e o ruído antropogênico, representam ameaças pouco exploradas ao ecossistema subterrâneo. A luz artificial pode desorientar morcegos, interferindo em seus padrões de atividade e migração, enquanto o ruído pode comprometer a ecolocalização, dificultando a navegação e a caça. Este estudo visa compreender esses impactos, particularmente em Minas Gerais, propondo a elaboração de um modelo metodológico para calcular a proximidade de fontes de impacto, identificar variáveis relevantes e projetar cenários de risco para a fauna cavernícola. Ao abordar essas questões, o trabalho contribui para a conservação da biodiversidade subterrânea e sugere melhorias nas práticas de licenciamento ambiental e manejo sustentável das atividades minerárias na região.

## Abstract

Minas Gerais, home to the largest number of registered caves in Brazil, faces the challenge of balancing mining activities with the conservation of subterranean environments. The state, known for its vast speleological heritage, has seen a significant increase in the number of registered caves, especially due to the environmental licensing processes required. Caves provide essential habitats for various species, including bats, which play key ecological roles such as insect control and seed dispersal. However, the impacts of mining, such as nighttime light pollution and anthropogenic noise, pose underexplored threats to the subterranean ecosystem. Artificial light can disorient bats, interfering with their activity patterns and migration, while noise can disrupt echolocation, hindering navigation and hunting. This study aims to understand these impacts, particularly in Minas Gerais, proposing the development of a methodological model to calculate the proximity of impact sources, identify relevant variables, and design risk scenarios for cave-dwelling fauna. By addressing these issues, the work contributes to the conservation of subterranean biodiversity and suggests improvements in environmental licensing practices and sustainable management of mining activities in the region.

## Resumen

Minas Gerais, que posee el mayor número de cavernas registradas en Brasil, enfrenta el desafío de conciliar las actividades mineras con la conservación de los ambientes subterráneos. El estado, caracterizado por un vasto patrimonio espeleológico, ha visto un aumento significativo en el número de cavidades registradas, especialmente debido a los procesos de licenciamiento ambiental requeridos. Las cavernas ofrecen hábitats esenciales para diversas especies, incluidos los murciélagos, que desempeñan funciones ecológicas clave, como el control de insectos y la dispersión de semillas. Sin embargo, los impactos de la minería, como la contaminación lumínica nocturna y el ruido antropogénico, representan amenazas poco exploradas para el ecosistema subterráneo. La luz artificial puede desorientar a los murciélagos, interfiriendo en sus patrones de actividad y migración, mientras que el ruido puede afectar la ecolocalización, dificultando la navegación y la caza. Este estudio tiene como objetivo comprender estos impactos, particularmente en Minas Gerais, proponiendo el desarrollo de un modelo metodológico para calcular la proximidad de fuentes de impacto, identificar variables relevantes y diseñar escenarios de riesgo para la fauna cavernícola. Al abordar estas cuestiones, el trabajo contribuye a la conservación de la biodiversidad subterránea y sugiere mejoras en las prácticas de licenciamiento ambiental y manejo sostenible de las actividades mineras en la región.

## 1. Introdução

Minas Gerais é o estado brasileiro com o maior número de cavidades cadastradas no banco de dados do Centro Nacional de Pesquisa e Conservação de Cavernas - ICMBIO/CECAV (CANIE/CECAV, 2025). O registro dessas cavidades é, em grande parte, impulsionado por expedições, pesquisas científicas e, especialmente, pelos processos de licenciamento ambiental. A Resolução CONAMA nº 347/2004 estabeleceu o Cadastro Nacional de Informações Espeleológicas (CANIE), determinando que os órgãos ambientais incluam dados espeleológicos nos processos de licenciamento. Isso resultou no aumento significativo de registros, especialmente, em áreas de mineração (CECAV, 2010). Assim, há uma relação direta entre a atividade minerária em Minas Gerais e o crescente registro e conhecimento sobre as cavidades subterrâneas, particularmente devido às exigências de estudos espeleológicos.

Os ambientes subterrâneos apresentam características geomorfológicas únicas, com variações químicas, físicas e biológicas que podem favorecer a existência de espécies endêmicas, interações ecológicas complexas e a formação de habitats essenciais para a alimentação, reprodução e repouso de diversas espécies (TRAJANO, 2000; CULVER & PIPAN, 2019; LOBO, 2012). Os morcegos, mamíferos voadores altamente diversificados, desempenham funções ecológicas fundamentais e utilizam as cavidades tanto de maneira oportunista quanto essencial. As cavernas, em particular, servem como refúgios para proteção contra predadores, manutenção da temperatura e umidade, e locais para reprodução e cuidado parental (KUNZ, 1982; SILVA, 2013; FERREIRA, 2016, BARROS et al., 2020).

Contudo, o uso antrópico, especialmente a atividade minerária, pode ter efeitos negativos significativos sobre esses ambientes subterrâneos e as interações ecológicas neles presentes (JONES et al., 2011; LOPES & REIS, 2018; GIBSON & BARKER, 2016; SANTOS & LIMA, 2015). Cavernas localizadas em áreas de mineração enfrentam ameaças ainda pouco conhecidas, como a poluição luminosa noturna (LAN) e o ruído antropogênico, ambos derivados dessas atividades, que podem afetar diretamente os padrões de atividade dos morcegos. A LAN pode interferir na busca por alimento, desorientar espécies migratórias e alterar hábitos de forrageamento (VILLARREAL ET AL., 2021; SHEPHERD et al., 2022). Além disso, tanto a LAN quanto o ruído podem prejudicar os sinais de ecolocalização, dificultando a navegação e a captura de presas, além de afetar os padrões de movimento dos morcegos. Esses impactos podem reduzir a disponibilidade de abrigos e fontes alimentares, especialmente para espécies cavernícolas (GIBSON & BARKER, 2017).

A interferência da luz e som nos ciclos naturais, com a consequente alteração dos ritmos biológicos dos morcegos ressaltam a necessidade de entender os limites dessas influências na convivência entre morcegos e atividades minerárias. Este estudo busca explorar esses impactos, especialmente no contexto das cavernas de Minas Gerais, visando embasar uma importante discussão sobre a compatibilidade da conservação e do manejo sustentável com as atividades minerárias. Para isso, ambiciona-se

verificar a viabilidade de uma proposta metodológica com a construção de um modelo avalie a correção das variáveis.

## 2. Materiais e Métodos

A metodologia desta pesquisa visa investigar possíveis correlações entre a distribuição de cavernas, a presença de empreendimentos minerários e a interferência na distribuição de morcegos no estado de Minas Gerais. Para isso, serão utilizados dados geoespaciais provenientes de fontes públicas, como o ICMBio, CECAV e IDE-Sisema. A base de dados de processos minerários do IDE-Sisema (2025) provem da Agência Nacional de Mineração (ANM) e possuem atualizações até o ano de 2024; A base das cavidades registradas em Minas Gerais, são do dia 28/01/2025 e os dados foram baixados na plataforma CANIE (Cadastro Nacional de Informações Espeleológicas); Já a fonte de dados de poluição luminosa é o Atlas Mundial de Poluição Luminosa (The New World Atlas of Artificial Night Sky Brightness. GFZ Data Services). Além disso, os dados utilizados de riqueza potencial de morcegos para o estado são provenientes do mapeamento do ICMBIO e do Laboratório de Ciência Aplicado à Conservação da Biodiversidade- UFPE, em 2022.

Em relação aos dados dos empreendimentos, realizou-se um filtro buscando nas diferentes fases dos processos minerários, por conceção de lavra, visto que o objetivo seria avaliar áreas com potencial de gerar fontes significativas de poluição luminosa e ruído. Além de avaliar a possível sobreposição espacial entre as áreas de mineração e as zonas de maior riqueza de morcegos. Após o levantamento, será realizado o processamento geoespacial utilizando plataforma *ArcGIS 10.7.1*, sobrepondo as camadas de dados de morcegos, cavernas, poluição luminosa e empreendimentos. A visualização das distâncias entre as cavernas e as áreas de maior riqueza de morcegos, além da proximidade de tais cavernas com os empreendimentos minerários podem responder perguntas ecológicas. A proximidade das cavernas e dos empreendimentos minerários em relação à distribuição de morcegos poderá ser testada posteriormente. Além disso, os resultados parciais serão interpretados para entender como a mineração pode afetar as espécies cavernícolas, considerando a alteração de habitats e a diminuição das fontes alimentares, e serão discutidas as implicações para o manejo ambiental e a conservação da fauna subterrânea em Minas Gerais. As limitações dessa abordagem, como a precisão espacial dos dados e a necessidade de incluir variáveis ambientais adicionais, também serão consideradas em análises futuras, mais refinadas. Além disso, serão levados em consideração dados secundários sobre poluição luminosa e ruído antropogênico em ambientes minerários, os quais podem afetar, negativamente, a fauna.

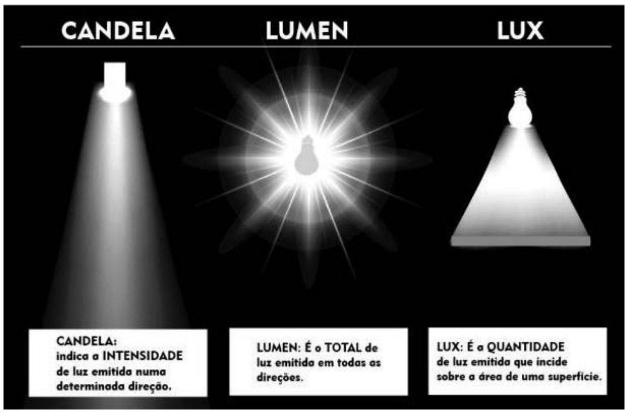
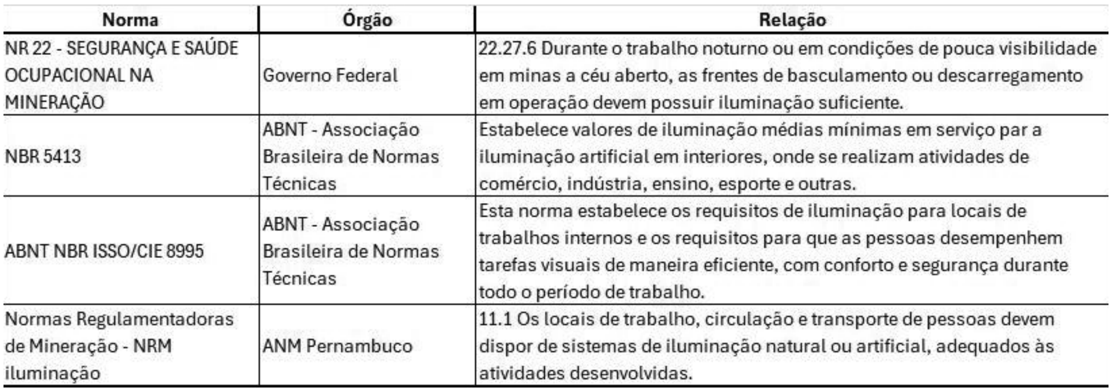


***Figura 1:*** *Esquema Darksky utilizado para relacionar teoricamente a poluição artificial e suas implicações além da área de inserção direta da iluminação.*

## 3. Resultados Parciais

Os empreendimentos estão distribuídos por uma porção significativa do território de Minas Gerais e, frequentemente, estão associados ao registro de cavidades no estado. De acordo com o anuário do CECAV (2022) e a base de dados da mesma plataforma, existem cerca de 13.036 cavernas cadastradas em Minas Gerais, representando mais de 46% do total nacional. As legislações relacionadas à poluição luminosa, no entanto, focam principalmente na segurança do trabalho e na qualidade do ambiente laboral, sem considerar seus impactos ambientais. Os dados dos empreendimentos mineráros abrangem todas fases do processo desde o reconhecimento geológico até a lavra ativa. Este fator dificulta a espacialização precisa e a compreensão das áreas onde ocorrem efetivamente atividades relacionadas ao ruído antrópico e a luz artificial noturna. A legislação ambiental brasileira não estabelece um limite específico de ruído voltado exclusivamente para a proteção da fauna, incluindo a fauna cavernícola. No entanto, o licenciamento ambiental e espeleológico exige que o impacto do ruído seja avaliado em estudos de impacto ambiental (EIA/RIMA) e planos de controle ambiental (PCA). Isso significa que há precedentes para o refinamento metodológico dessas avaliações com foco na conservação das dinâmicas da fauna. Em relação aos morcegos, os dados sobre a riqueza de espécies nos auxiliam a compreender a importância de desenvolver estratégias para manutenção da fauna. Todavia, evidenciam a necessidade de ampliação da coleta de dados e estudos, a fim de refletir a realidade atual da distribuição dos indivíduos. As normas existentes sobre a poluição luminosa, restringem-se, em sua maioria, à proteção do ser humano (Figura 2), e não abarcam a necessidade de aprofundamento das análises em relação aos impactos sobre a fauna (Figura 3).



| Norma | Órgão | Relação |
|---|---|---|
| NR 22 - SEGURANÇA E SAÚDE OCUPACIONAL NA MINERAÇÃO | Governo Federal | 22.27.6 Durante o trabalho noturno ou em condições de pouca visibilidade em minas a céu aberto, as frentes de basculamento ou descarregamento em operação devem possuir iluminação suficiente. |
| NBR 5413 | ABNT - Associação Brasileira de Normas Técnicas | Estabelece valores de iluminação médias mínimas em serviço par a iluminação artificial em interiores, onde se realizam atividades de comércio, indústria, ensino, esporte e outras. |
| ABNT NBR ISSO/CIE 8995 | ABNT - Associação Brasileira de Normas Técnicas | Esta norma estabelece os requisitos de iluminação para locais de trabalhos internos e os requisitos para que as pessoas desempenhem tarefas visuais de maneira eficiente, com conforto e segurança durante todo o período de trabalho. |
| Normas Regulamentadoras de Mineração - NRM iluminação | ANM Pernambuco | 11.1 Os locais de trabalho, circulação e transporte de pessoas devem dispor de sistemas de iluminação natural ou artificial, adequados às atividades desenvolvidas. |

***Figura 2:****Exemplares de normativas referentes à iluminação e atividades minerárias.*

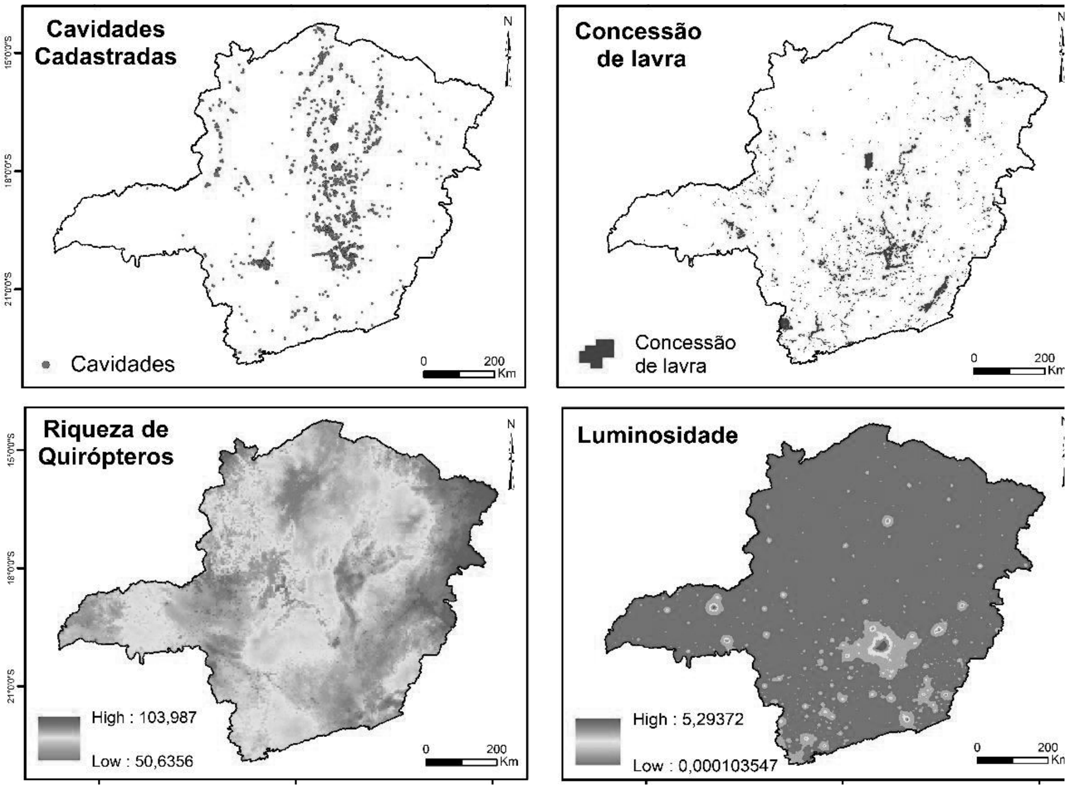


**Figura 3:** *Mapa de cavidades registradas no CECAV/CANIE; Concessão de lavra ANM; Riqueza da quirópterofauna no estado de Minas Gerais; Incidência de poluição luminosa do estado de Minas Gerais-brilho do céu noturno (mcd/m 2).*

## 4. Discussão

O elevado número de cavidades registradas em Minas Gerais e a expansão dos empreendimentos minerários reforçam a necessidade de aprofundar a discussão sobre os impactos ambientais na fauna cavernícola. Entre os potenciais impactos, temos a poluição luminosa e o ruído antrópico que podem comprometer a biodiversidade de morcegos, influenciando seu comportamento e distribuição.

A poluição luminosa, caracterizada pelo excesso de luz artificial noturna, afeta diretamente morcegos, interferindo em seus padrões de atividade e navegação (RUSSO et al., 2019). Além disso, a alteração na disponibilidade de presas, causada pela atração de insetos à iluminação artificial, pode levar esses animais a áreas de maior risco (ROWSE et al., 2016). Além disso, o ruído antrópico, pode mascarar os sinais acústicos essenciais para ecolocalização e comunicação, resultando em maior esforço energético, estresse e redução do sucesso alimentar (SCHAUB et al., 2008). Estudos de bioacústica sugerem que a poluição sonora afeta o comportamento dos morcegos tanto em áreas urbanas quanto naturais, ressaltando a necessidade de pesquisas específicas para ambientes subterrâneos (LIMA; MAGRINI, 2020).

Apesar da exigência de avaliação de ruído nos processos de EIA/RIMA e PCA, não há diretrizes específicas voltadas à fauna cavernícola, o que evidencia a necessidade de revisões normativas. Os autores acreditam que estudos mais refinados visando quantificar os impactos do ruído e da luz artificial sobre os morcegos são essenciais. Estabelecendo parâmetros que pode subsidiar estratégias de mitigação e conservação dentro do licenciamento ambiental. Dessa forma, o desenvolvimento de diretrizes para a emissão de luz e som em áreas com presença de morcegos torna-se essencial para a proteção dessas espécies e a manutenção dos serviços ecossistêmicos que desempenham.

## 5. Conclusão

Apesar de preliminares, as hipóteses levantadas fornecem importantes questionamentos sobre a relação entre cavidades, morcegos e empreendimentos minerários, o que evidencia a relevância do estudo. Essa relação é crucial para a conservação da fauna cavernícola, especialmente os morcegos, que desempenham papéis ecológicos essenciais. A pesquisa destaca uma lacuna na correlação dessas variáveis, sendo um ponto de partida para o desenvolvimento de novas investigações sobre os impactos ambientais na biodiversidade subterrânea.

## Agradecimentos

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# Description of a new troglobitic species of *Litoblatta* Hebard, 1921 (Blattodea: *Blattellidae* ) from southeastern Brazil

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## Abstract

Cave environments often harbor species that have evolved under extreme conditions, such as limited food availability and absence of light. Strictly cave-dwelling species, known as troglobites, typically exhibit specialized adaptations (referred to as troglomorphisms) including depigmented integument, reduced eyes, and elongated appendages. The genus *Litoblatta* Hebard, 1921 (*Blattellidae*) comprises 15 known species, among them *Litoblatta camargoi* Gutiérrez, 2005, the only troglobitic cockroach previously described in Brazil. This study describes a new troglobitic *Litoblatta* species collected from Gruta do Éden cave in Pains, Minas Gerais. The newly identified species exhibits pronounced troglomorphic traits, including reduced and depigmented ocular structures, wing reduction, depigmented integument, and elongated appendages. This discovery expands the known diversity of Brazilian *Blattodea* and enhances our understanding of cave ecosystems and the adaptations of subterranean fauna.

## Resumo

Ambientes de caverna frequentemente abrigam espécies que evoluíram em condições extremas, como recursos alimentares limitados e ausência de luz. Espécies estritamente cavernícolas, chamadas troglóbias, geralmente apresentam adaptações, ou troglomorfismos, incluindo tegumento despigmentado, olhos reduzidos e apêndices alongados. O gênero *Litoblatta* Hebard, 1921 (*Blattellidae*) inclui 15 espécies, entre elas *Litoblatta camargoi* Gutiérrez, 2005, a única barata troglóbia descrita no Brasil. Este estudo descreve uma nova espécie troglóbia de *Litoblatta* coletada na caverna Gruta do Éden, Pains, Minas Gerais. A nova espécie apresenta troglomorfismos acentuados, como estruturas oculares reduzidas e despigmentadas, redução das asas anteriores, tegumento despigmentado e apêndices alongados. Esta descoberta amplia a diversidade conhecida de *Blattodea* no Brasil e aprimora nossa compreensão dos ecossistemas de cavernas.

## 1. Introduction

Cockroaches (*Blattodea*) are a highly diverse group within Insecta, with a predominantly tropical and subtropical distribution, though a few species also occur in temperate regions (GRANDCOLAS et al., 2012). These insects inhabit a wide range of environments, including forests, urban areas, and caves (ROTH & WILLIS, 1960; Bell, 1981).

Caves, natural rock cavities formed by water erosion (GILLIESON, 2021), are characterized by climatic stability and extreme environmental conditions, such as the absence of light and limited resource availability (POULSON & WHITE, 1969). Species that evolve in subterranean habitats often develop specialized adaptations, known as troglomorphisms, to survive under these conditions (CHRISTIANSEN, 1962). These adaptations can be behavioral, physiological, or morphological, including the reduction or loss of eyes and wings, elongation of locomotor and sensory appendages, and depigmentation of the integument (CHRISTIANSEN, 2012).

One notable example of such adaptations is *Litoblatta camargoi* Gutiérrez, 2005, the only troglobitic cockroach species described in Brazil to date. This species exhibits complete loss of ocular structures, depigmented integument, and elongated legs and antennae (GUTIÉRREZ, 2005).

The primary objective of this study is to describe a new troglobitic species of *Litoblatta* Hebard, 1921, discovered in a limestone cave. Additionally, we analyze its key troglomorphic traits and discuss both its conservation status and that of the cave where it was found.

## 2. Materials and methods

**Collection**

Adult individuals of the new *Litoblatta* species were collected through active searching in Gruta do Éden cave (20°23’04.5”S, 45°40’00.5”W), located in the municipality of Pains, Minas Gerais, Brazil (Fig. 1). Specimens were preserved in 70% ethanol and deposited in the Collection of Subterranean Invertebrates of Lavras (ISLA) at the Federal University of Lavras (UFLA).

**Study Area**

Éden Cave (20.384577°S, 45.666798°W; 712 m a.s.l.) is situated within limestone outcrops of the Bambuí Geological Group, near Pains, Minas Gerais, Brazil. It is part of the Arcos-Pains-Doresópolis (APD) speleological province, one of the most significant karst areas in Brazil, with over 2,500 documented caves.

The region experiences a climate characterized by warm, humid summers and dry winters, with average temperatures ranging from 23.3°C in January (the hottest month) to 16.3°C in July (the coldest month). The annual mean temperature is 20.7°C, with an average annual precipitation of 1,344 mm$^3$. Extensive deforestation has transformed much of the native vegetation into monoculture plantations (primarily *Eucalyptus*) and pastures. The original deciduous seasonal forest is now restricted to fragmented patches on limestone outcrops.

Éden Cave is one of the largest known caves in the region, with a linear development of approximately 1,931 meters. It consists of a wide dissolution gallery following North-South structural lineaments, exhibiting a linear morphology. The cave features two distinct levels: a lower level traversed by a perennial stream and an upper level devoid of water flow. It has a single natural entrance, a small skylight providing access to the lower level, as well as an artificial opening created by past mining activities, which has since been sealed with an iron gate.

**Morphological Analysis and Species Description**

Adult males were dissected, and their genitalia were treated with a pancreatin solution (ÁLVAREZ-PAADILLA & HORMIGA, 2007) for at least 24 hours to dissolve muscle tissue and improve visualization of diagnostic structures. The genitalia were examined using a Stemi 2000 (Zeiss) stereomicroscope. The external morphology of both adult males and females was also analyzed under the same microscope.

Descriptions of the head, thorax, abdomen, locomotor and sensory appendages, and genitalia were based on Roth (2003) and Wipfler et al. (2016). Photographs of external morphology and male genitalia were captured using a camera attached to an Axio Zoom V16 stereomicroscope (Zeiss). These images were organized into slides to enhance the visualization of key taxonomic characters.

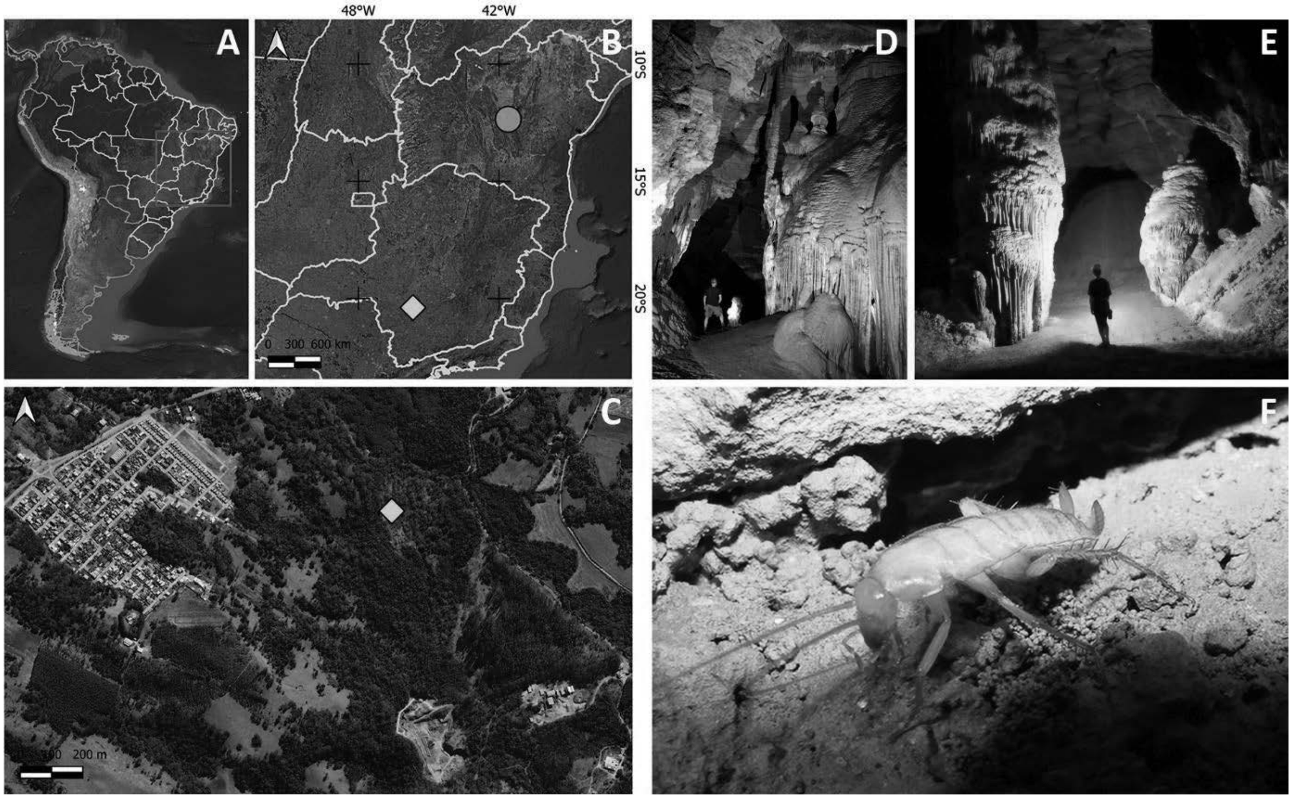

***Figure 1:*** *A) South America, red square highlights the region where the two Brazilian species of troglobitic cockroaches are found; B) Brazil, states of Bahia (north) and Minas Gerais (south), orange circle marks Gruta da Torrinha cave,type locality of Litoblatta camargoi, yellow diamond marks Gruta do Éden cave, type locality of Litoblatta n. sp.; C) Surroundings of Gruta do Éden cave; D-E) Inner chambers of Gruta do Éden cave; F) Adult specimen of Litoblatta n. sp.*

## 3. Results

**Family *Blattellidae*.**

**Genus *Litoblatta*.**

***Litoblatta* n. sp.**

**Description – Male:** Depigmented, light orange-beige in coloration. **Head**. Subtriangular, dorsoventrally elongated, ocelli absent, compound eyes reduced and completely depigmented; antennae pubescent and filiform, longer than the body. **Thorax**. Pronotum subtrapezoidal with rounded latero-basal ends; hindwings absent; forewings reduced, rounded at the apex and wider in the anterior part, with the apex reaching only the anterior margin of the metanotum; veins parallel, with the same starting point on the anterior part of the wing; mesonotum with a concave anterior margin; metanotum covered with elongated bristles; posterior margin arched and with a short medial triangular projection; anterior margin arched in the opposite direction to the posterior margin, with a short medial triangular projection, similar to the posterior margin. **First pair of legs**. Inner ventral margin of femora with thick proximal spines, followed by thinner and shorter distal spines; outer ventral margin with spines of similar length and with greater spacing between them; femora with three apical spines on the inner surface. **Second and third pair of legs**. Spines along the femur similar in length, present on both outer and inner margins, with an inner apical spine; one pair of tarsal claws on each tarsus, arolium reduced. **Abdomen**. Supra-anal plate non-bilobated, posterior margin rounded, with a small medial dent; cerci short and pubescent on the ventral side, with 10 segments, which decrease in size and thickness along the structure; subgenital plate asymmetrical, with rounded apices, right side larger than the left one, which has a small indentation where the left style is inserted; styles pubescent, right style smaller than the left one.

**Female**: Depigmented, light orange-beige in coloration. **Thorax**. Hindwings absent; forewings reduced, rounded at the apex and wider anteriorly, with the tip reaching only the anterior margin of the metanotum; supra-anal plate non-bilobed, posterior margin rounded; subgenital plate symmetrical, rounded at the apex.

**Male genitalia:** Left paraproct sickle-shaped, curved, crossing beneath the genital hook, apex pointed; right paraproct rounded, pubescent, posterior margin covered with elongated and thick setae, medial margin curved inward and covered with short and thick spines, resembling a rounded saw, ventral surface covered with sparsed short and thick spines; left phallomere, genital hook (L3) wider at the base, curved outward, apex concave and shortly bilobated, apical margins rounded; median phallomere (L2vm) thin, sinuous, roughly S-shaped in lateral view, apex bending abruptly, forming a perpendicular short projection (Fig 2).

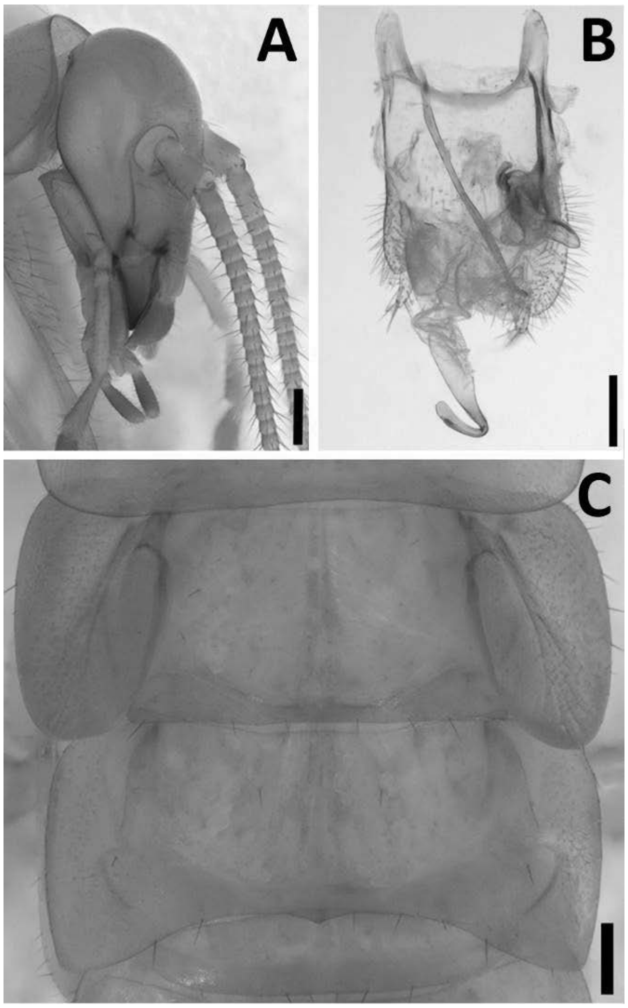

***Figure 2:*** *Adult male of n. sp. A) Head, lateral view ; B) Male genitalia and subgenital plate, dorsal view ; C) Reduced forewings and metanotum ; scale bar 0.5mm .*

## 4. Discussion

**Comparative Morphology and Conservation Status of *Litoblatta* n. sp.**

The genus *Litoblatta* currently comprises 15 species (BECCALONI, 2025), with *L. camargoi* being the only species known to exhibit troglomorphic traits (GUTIÉRREZ, 2005). Consequently, the new troglobitic species described here is compared exclusively with *L. camargoi*.

Significant morphological differences distinguish the two species, particularly in forewing development. Adult males of *L. camargoi* possess fully developed forewings, whereas *Litoblatta* n. sp. exhibits forewing reduction. Both species share depigmented tegument and reduced ocular structures, yet they differ in other morphological features. The supra-anal plate of *Litoblatta* n. sp. is longer than that of *L. camargoi*, and their subgenital plates differ in shape.

Genital morphology further supports their taxonomic distinction. In *Litoblatta* n. sp., the apex of the phallomere hook (L3) exhibits a distinct shape and curvature compared to *L. camargoi*. Additionally, the median phallomere (L2mv) in *Litoblatta* n. sp. is thinner, with a shorter and less developed distal portion than in *L. camargoi*.

The observed morphological differences reinforce the hypothesis that *Litoblatta* n. sp. and *L. camargoi* are distinct species. The use of male genitalia as a diagnostic character in species differentiation is based on the premise that modifications in genital structures contribute to reproductive isolation, ultimately driving speciation (Shapiro & Porter, 1989; Masly, 2011). Such isolation may also be influenced by sensory lock-and-key mechanisms (Masly, 2011). Further studies are needed to investigate the role of chemical and behavioral factors in reproductive isolation and species delimitation.

**Conservation Considerations**

To date, *Litoblatta* n. sp. has been found exclusively in Gruta do Éden cave. Although a formal environmental impact assessment was not conducted, visible signs of human-induced damage were evident. Mining activities have significantly altered the cave's structure, including the creation of an artificial entrance and the installation of a gate by mining companies. The upper level of the cave bears clear evidence of past quarrying operations, with collapsed speleothems and large rock fragments resulting from historical explosive use.

The condition of the subterranean stream is particularly concerning. A substantial accumulation of fine sediment along the streambed indicates severe siltation throughout its course, likely driven by ongoing environmental disturbances. The cave's surroundings have been extensively modified by human activities, including agriculture, livestock grazing, urban expansion, and historical mining operations.

Gruta do Éden is located within an active mining zone adjacent to the city of Pains. A stream enters the cave's lower level through an upwelling and flows for approximately 700 meters before disappearing into a sinkhole. However, before reaching the cave, the stream traverses a highly impacted watershed that has undergone significant anthropogenic pressures. The original vegetation cover has been almost entirely removed, replaced by pastures and agricultural fields. Additionally, domestic sewage and other pollutants further degrade the water quality, compounding the environmental challenges faced by this subterranean ecosystem.

The discovery of a new troglobitic species in Gruta do Éden reinforces the cave's ecological significance and secures its classification as a site of maximum relevance under Brazilian cave conservation legislation (Brasil, 2008). This designation highlights the urgent need for conservation efforts to protect this fragile subterranean habitat.

## 5. Conclusion

The description of *Litoblatta* n. sp. enhances our understanding of *Blattodea* diversity in Brazil and represents a significant step forward in the study of this group's biology. Moreover, the presence of this species in cave ecosystems provides valuable insights into the composition and dynamics of subterranean communities. The discovery of a new troglobitic species further underscores the importance of cave conservation efforts, highlighting the need to protect these fragile ecosystems and their specialized fauna.

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# Invertebrate community in the bird (Aves) and bat (Mammalia) guano from the limestone caves of the Andaman Islands, India

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## Abstract

Bird (Aves) and bat (Chiroptera) guano provide essential organic energy to cave ecosystems, supporting the survival of various invertebrates. Guano-dependent invertebrate studies are limited globally and rare in India. We explored the cave complex of Baratang Island, Andaman and Nicobar Islands, India, to document the invertebrate fauna in bat (Microchiroptera) and bird (swiftlets) guano. Based on availability, between March and June 2020, 18 bird and five bat guano piles were surveyed in 14 caves for invertebrates using the quadrats (30 cm × 30 cm). A total of 5606 invertebrates from 13 orders were identified. Lepidoptera accounted for the highest proportion of individuals (40.13%), while Orthoptera represented the lowest (0.03%). Eight of the 13 recorded orders were found in bat guano, with Lepidoptera (72.80%) dominating. Twelve orders were found in bird guano, with Ixodida being the most abundant (54.13%), followed by Lepidoptera (36.91%), and Orthoptera was the least common (0.07%). The cluster analysis of invertebrates in bird and bat guano revealed distinct ecological groupings based on their similarities. These reflect shared roles, nutrient cycling, decomposition, and prey-predator relationships. This study provides baseline information on guano-dependent invertebrates, highlighting cave-dwelling swiftlets as a potential umbrella species for the cave ecosystem of Baratang Island.

## 1. Introduction

Caves are considered natural laboratories due to their stable microclimatic conditions (POULSON & WHITE 1969, BADINO 2010). These habitats are divided into five environmental zones: entrance, twilight, transition, dark, and stagnant air, each defined by light availability, moisture, airflow, and gas concentration (HOWARTH, 1993). However, three primary zones—entrance, twilight, and dark—are widely recognized, as organisms are classified based on this zonation (MOSELEY 2009). The entrance zone (EZ), or euphotic zone, located near the cave opening, hosts the highest species diversity due to the interaction of epigean and hypogean fauna. The twilight zone (TZ), or disphotic zone, characterized by dim light, remains relatively insulated from external influences. Beyond this lies the dark zone (DZ), or aphotic zone, where stable microclimatic conditions support obligate cave fauna, although occasional stagnation can result in stressful gas concentrations, particularly carbon dioxide (HOWARTH 1993).

In caves, guano—the droppings of troglophilic animals such as bats, birds, rats, and crickets—serves as a vital resource, providing food and shelter for various organisms (PELLEGRINI & FERREIRA 2012). Microchiropteran bats, essential contributors to cave ecosystems, produce guano that sustains a diverse array of cave-dwelling organisms (FERREIRA ET AL. 2000). Similarly, echolocating swiftlets roost and nest in dark cave regions, where their guano is crucial for tropical cave invertebrates (GNASPINI 2012). Guano piles create diverse microhabitats, supporting invertebrate assemblages at different successional stages as physicochemical conditions evolve (EMERSON & ROARK 2007). These assemblages include bacteria, fungi, nematodes, coleopterans, dipterans, lepidopterans, and arachnids (HARRIS 1970). According to DECU (1986) and GNASPINI & TRAJANO (2000), the Guanophages—organisms dwelling in guano (GNASPINI 2012)—can be categorized based on their reliance on guano into three groups: Guanobites (exclusive guano dwellers), Guanophiles (opportunistic dwellers), and Guanoxenes (temporary dwellers) (GNASPINI 2012).

Arthropod communities in bat guano have been extensively studied in various regions of the tropics and temperate zones (RICHARDS 1971, BRAACK 1989, DEHARVENG & LECLERC 1989, GNASPINI & TRAJANO 1994, MOULDS 2005), although research on guano invertebrates in Indian caves remains limited (BISWAS 2009, 2010). Therefore, this study aims to address this gap by documenting arthropod diversity in the bird and bat guano found in the caves of Baratang Island, Andaman & Nicobar Islands.

## 2. Materials and methods

The present study was conducted on the cave complex of Baratang Island in the Andaman and Nicobar Islands (6°45’ to 13°41’N and 92°12’ to 93° 57’E). Baratang Island (12°05’ N, 92° 45’E) is situated lies between Middle Andaman and South Andaman Islands (Fig. 1). It is the largest known cave complex in the archipelago, comprising over 175 caves within an area of 0.77 km$^2$ between Wraffter’s Creek and Naya Dera (GURJARPADHYE ET AL. 2021). We surveyed 14 caves based on accessibility and the presence of birds and bats, sampling 18 swiftlet guano piles and five bat guano piles. In each cave, we examined all three zones (Entrance, Twilight, and Dark) to locate guano deposits.

Given the small size of swiftlet guano piles (<60 cm²), we used a 30 cm × 30 cm quadrat for sampling. During quadrat sampling, we recorded the number of invertebrates within each quadrat. We employed MS Excel (Ver. 2013) to perform descriptive statistics for the preliminary analyses of the invertebrate encounter data. Additionally, we calculated species abundance (the number of individuals of a species divided by the total invertebrate population in the guano). To understand the ecological relationship between orders in different guano types, we conducted a cluster analysis to produce a dendrogram using XLSTAT (Ver. 2020).

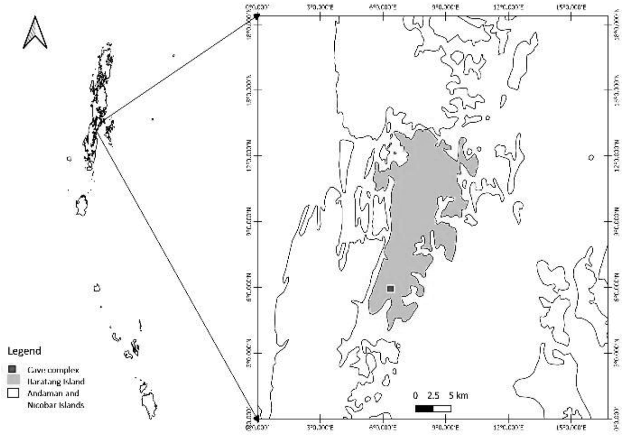

***Figure 1:*** *Geographical location of Baratang Island in Andaman Islands.*

## 3. Results

In the present study, a total of 5,606 invertebrates belonging to 13 orders were recorded from both bat and bird guano: *Blattodea*, Archeognatha, Lepidoptera, Ixodida, Araneae, Pseudoscorpiones, Coleoptera, Diptera, Hymenoptera, Orthoptera, Scolopendromorpha, Isopoda, and Hemiptera. Among these, the order Lepidoptera had the highest number of individuals (2,250), whilst the order Orthoptera had the least (2). We identified 5,606 invertebrates from 13 orders, with Lepidoptera being the most abundant (40.13%) and Orthoptera the least (0.03%). Bat guano hosted eight orders, where Lepidoptera was predominant (72.80%). In contrast, bird guano contained 12 orders, with Ixodida being the most abundant (54.13%), followed by Lepidoptera (36.91%) and Orthoptera the least (0.07%).

Cluster analysis of invertebrate communities in bird and bat guano reveals both similarities and differences in species associations, possibly influenced by guano composition, nutrient availability, and microhabitat conditions. In bird guano, most invertebrates (*Blattodea*, Scolopendromorpha, Araneae, Orthoptera, Hemiptera, Archeognatha, and Diptera) formed a single cluster, closely related to Pseudoscorpiones and Hymenoptera, with Coleoptera displaying some similarity. This clustering likely arises from the relatively drier nature of swiftlet guano, which supports a diverse assemblage of scavengers, detritivores, and predators.

Lepidoptera (larvae), which feed on organic matter, and Ixodida (ticks), which are ectoparasites of birds, formed a separate cluster, possibly due to their specialized roles. In bat guano, Pseudoscorpiones, Diptera, *Blattodea*, Hymenoptera, Ixodida, and Coleoptera grouped together, showing similarity to a distinct Isopod cluster, while Lepidoptera remained isolated. The likely high content of nitrogen and moisture in bat guano supports decomposers such as Diptera and detritivores like Isopods. The distinct clustering of Lepidoptera suggests that their presence in bat guano may be limited to opportunistic species rather than a dominant functional group.

***Figure 2:*** *Swiftlets are energy producers in the cave ecosystem supporting diversity of invertebrates.*

## 4. Discussion

This study highlights the ecological importance of guano in cave ecosystems, particularly in the caves of Baratang Island, where both bat and swiftlet guano serve as primary energy sources, supporting diverse invertebrate communities (Figure 2). Similar to the Oilbird guano ecosystems in South America and Caribbean caves, as well as the swiftlet guano ecosystems in Southeast Asia, the guano-based food web in Baratang exhibits a high level of biodiversity, with moths (Lepidoptera), ticks (Ixodida), and beetles (Coleoptera) being the most dominant taxa

(DEHARVENG & BEDOS 2000, GNASPINI & TRAJANO 2000). These findings are consistent with previous studies, which indicate that guano-rich caves, especially in tropical regions, support a variety of scavengers, detritivores, and predators (GNASPINI & TRAJANO 2000).

Interestingly, this study presents the first-ever documentation of invertebrate orders from swiftlet guano in Indian caves. Similar to bat guano, swiftlet guano supports scavengers such as crickets (Orthoptera), pseudoscorpions (Pseudoscorpiones), centipedes (Scolopendromorpha), and beetles (Coleoptera), along with predators such as spiders (Araneae). The presence of Lepidoptera larvae (bagworms) is significant, as moths are generally not adapted to caves but are known to complete their life cycles in guano-rich habitats (SKALSKI 1972). Furthermore, ticks and mites (Ixodida) are frequently found in both bird and bat guano, likely due to their parasitic relationships with cave-dwelling vertebrates (CORPUZ-RAROS & LIT 2015). While flies (Diptera) are often abundant in subterranean environments, they were not prevalent in these caves, possibly due to variations in microclimatic conditions or competitive interactions with other guanophagous species (GUNN 2004). Cave ants (Hymenoptera) as trogloxenes indicate seasonal foraging behaviour, consistent with previous reports of ants exploiting cave resources for moisture and sustenance (PAPE 2016).

Swiftlet guano harbors a diversity of prey species for predators such as pseudoscorpions and serves as an energy source. Fallen chicks, nests, or birds frequently provide food for various scavenging guanophages. During the study, beetles and ants were observed feeding on the deceased chicks of swiftlets (Fig. 2). In addition to the invertebrate fauna, swiftlets also support various vertebrate fauna (MANCHI & SANKARAN 2009). MANCHI & SANKARAN (2009) identified snakes, raptors (owls, besra, shikra), and rats as predators of swiftlets. Furthermore, the lesser false vampire bat (Megaderma spasma) has been observed preying on swiftlet populations in *ex-situ* conditions (pers. observations). Overall, this study reinforces the role of guano as a keystone nutrient source, shaping cave biodiversity and supporting complex ecological interactions across trophic levels.

| Order | Dependence on guano | | | | | |
|---|---|---|---|---|---|---|
| | Surface dweller | Burrowing | Breeds in guano | Feeds on guano | Prey | Predator |
| Blattodea | Yes | Yes | Yes | Yes | Yes | No |
| Archeognatha | No | Yes | Yes | Yes | Yes | No |
| Lepidoptera | Yes | Yes | Yes | Yes | Yes | No |
| Ixodida | Yes | No | No | No | Yes | No |
| Araneae | Yes | No | No | No | No | Yes |
| Pseudoscorpiones | Yes | No | Yes | No | No | Yes |
| Coleoptera | Yes | Yes | Yes | Yes | Yes | Yes |
| Diptera | Yes | No | Yes | Yes | Yes | No |
| Hymenoptera | Yes | Yes | No | Yes | No | Yes |
| Orthoptera | Yes | No | No | No | Yes | No |
| Scolopendromorpha | Yes | Yes | Yes | No | Yes | Yes |
| Isopods | Yes | No | Yes | Yes | Yes | No |
| Hemiptera | Yes | Yes | Yes | No | Yes | Yes |

***Figure 2:*** *Common habits of the invertebrate guanophages in the caves of Baratang Island.*

## 5. Conclusion

This study highlights the complex relationships within cave ecosystems, where various invertebrate species rely on guano deposits as a primary resource. The diversity of invertebrates found in both bird and bat guano illustrates the critical role these deposits play in supporting a wide range of invertebrates. The findings underscore the importance of maintaining these ecosystems, as their stability is closely linked to the presence of key species and the availability of guano. Effective conservation strategies should focus on protecting the entire cave ecosystem to ensure the survival of both primary producers and the numerous species that depend on them. The study provided baseline information on the guano arthropods and demonstrated that swiftlets could be regarded as an umbrella species for the cave ecosystem of Baratang.

## 6. Acknowledgments

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# Metagenome-Assembled Genomes of Microbial Communities Associated with Plant Roots from Ferruginous Caves in the Amazon

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## Resumo

A Floresta Nacional de Carajás abriga o maior número de cavernas ferruginosas conhecidas no Brasil. Nestas cavernas há raízes provenientes dos ambientes epígeos adjacentes e que sustentam comunidades microbianas e são essenciais nos ciclos biogeoquímicos. No entanto, o microbioma das raízes das cavernas é pouco explorado em função da dificuldade de acesso e no isolamento desses microrganismos subterrâneos. Este estudo utilizou abordagens metagenômicas para investigar as comunidades microbianas associadas às raízes de plantas em cavernas ferruginosas durante as estações seca e chuvosa, além de explorar seu potencial funcional. As amostras de raízes foram coletadas em seis cavernas localizadas na Floresta Nacional de Carajás (Amazônia Oriental, Brasil). O DNA total foi extraído e bibliotecas de sequenciamento *shotgun* foram processadas em uma plataforma Illumina NextSeq 2000. A análise revelou 26 genomas microbianos montados a partir dos metagenomas (MAGs), evidenciando variações sazonais na composição microbiana. Burkholderiales predominaram na estação seca, enquanto Acidoferrales foram mais abundantes na estação chuvosa. A análise funcional mostrou diferenças no metabolismo microbiano, com vias aeróbicas prevalecendo na estação seca e vias anaeróbicas na estação chuvosa. Esses resultados ampliam nossa compreensão da dinâmica microbiana em ecossistemas subterrâneos, destacando o papel dessas comunidades no ciclo de nutrientes na adaptação a ambientes subterrâneos.

## Abstract

The Carajás National Forest is home to Brazil's largest number of known ferruginous caves, which are intricately connected to plant roots of the adjacent epigeal environments. These roots harbor microbial communities uniquely adapted to extreme environmental conditions, playing a vital role in biogeochemical cycles and supporting diverse cave-dwelling fauna. However, the microbiome of cave roots is poorly known due to challenges in accessing and isolating subterranean microorganisms. Thus, we used metagenomics to analyze microbial communities in plant roots from ferruginous caves during the dry and rainy seasons, while also exploring their functional potential. Root samples were collected from six caves in the Serra dos Carajás (Eastern Amazon, Brazil), total DNA was extracted, and *shotgun* sequencing libraries were processed in an Illumina NextSeq 2000 platform. The analysis identified 26 *metagenome-assembled genomes* (MAGs), revealing notable seasonal variations in microbial composition. Burkholderiales dominated in the dry season, while Acidoferrales were more abundant in the rainy season. Functional analysis showed differences in microbial metabolism, with aerobic pathways prevailing in the dry season and anaerobic pathways in the rainy season. These results enhance our understanding of microbial dynamics in cave ecosystems, highlighting their role in nutrient cycling and adaptation to underground environments.

## 1. Introdução

A Floresta Nacional de Carajás (FLONA) abriga o maior conjunto de cavernas ferruginosas conhecidas no Brasil, representando cerca de 9,7% do total nacional (PILÓ & AULER, 2019). Esses ambientes subterrâneos sustentam uma microbiota diversificada e desempenham um papel crucial na manutenção dos ciclos biogeoquímicos. As raízes que se desenvolvem acima e ao redor dessas cavernas são essenciais na dinâmica trófica, sustentando comunidades microbianas e promovendo interações ecológicas com a fauna cavernícola (MARTIN-POZA et al 2020, PAULA 2023). No entanto, o microbioma associado às raízes cavernícolas ainda é pouco compreendido, principalmente devido ao ambiente de difícil acesso e à dificuldade de isolar e cultivar tais microrganismos. Nesse contexto, os microrganismos apresentam grande versatilidade metabólica, sendo fundamentais para processos como decomposição, espeleogênese, transferência de energia e ciclagem de nutrientes (FERREIRA et al. 2019, PAULA 2023). Além disso, eles constituem a base da teia trófica subterrânea e são vitais para a manutenção da biodiversidade desses ecossistemas. A ausência de luz e a baixa disponibilidade de nutrientes favorecem a seleção de microrganismos com perfis específicos, muitos dos quais possuem um potencial biotecnológico inexplorado (ZADA et al. 2022). Dada a importância ecológica das cavernas de Carajás, a pesquisa sobre esses ambientes se torna ainda mais relevante, especialmente diante das ameaças causadas pela degradação das florestas tropicais e pelas mudanças climáticas, que comprometem a integridade desses ecossistemas. Além disso, o microambiente cavernícola pode influenciar o estabelecimento de espécies raras e ameaçadas da flora (ICMbio, 2021), ressaltando a importância dos microorganismo na ecologia subterrânea (KATO et al. 2024). Os recentes avanços nas ciências ômicas, particularmente na abordagem metagenômica *shotgun*, têm permitido a identificação microbiana em nível de espécie e a caracterização dos genes funcionais (QUINCE et al. 2017). Considerando que as cavernas são altamente dependentes de seu entorno, o estudo das raízes de plantas associadas a esses ambientes é fundamental para entender os efeitos das alterações na paisagem circundante sobre o microbioma subterrâneo. Este estudo tem como objetivo utilizar ferramentas metagenômicas para reconstruir genomas microbianos a partir dos metagenomas (*Metagenome-Assembled Genomes* – MAGs) com o proposito de caracterizar as comunidades microbianas associadas às raízes de plantas em cavernas ferruginosas durante as estações chuvosa e seca. Os MAGs representam conjuntos de sequências genéticas de organismos individuais no microbioma, podendo corresponder a uma espécie ou a um táxon próximo ao nível de espécie. Essa abordagem contribui para o entendimento das interações microbianas, da descoberta de novos táxons e da diversidade funcional dessas comunidades. Dessa forma, busca-se ampliar o conhecimento sobre o microbioma das cavernas na Amazônia.

## 2. Materiais e métodos

Amostras de raízes foram coletadas em seis cavernas na Serra Norte da FLONA de Carajás, conforme a Fig. 1, durante as estações chuvosa e seca. O DNA foi extraído seguindo os protocolos de DOYLE & DOYLE et al. (1990) e INGLIS et al. (2018), com modificações, incluindo uma precipitação seletiva de polissacarídeos conforme MICHAELS et al. (1994). As bibliotecas de DNA *shotgun* foram preparadas utilizando o protocolo Illumina DNA Prep e sequenciadas na plataforma Illumina NextSeq 2000 (Illumina Inc., San Diego, CA), com leituras pareadas (2 × 150 pb).

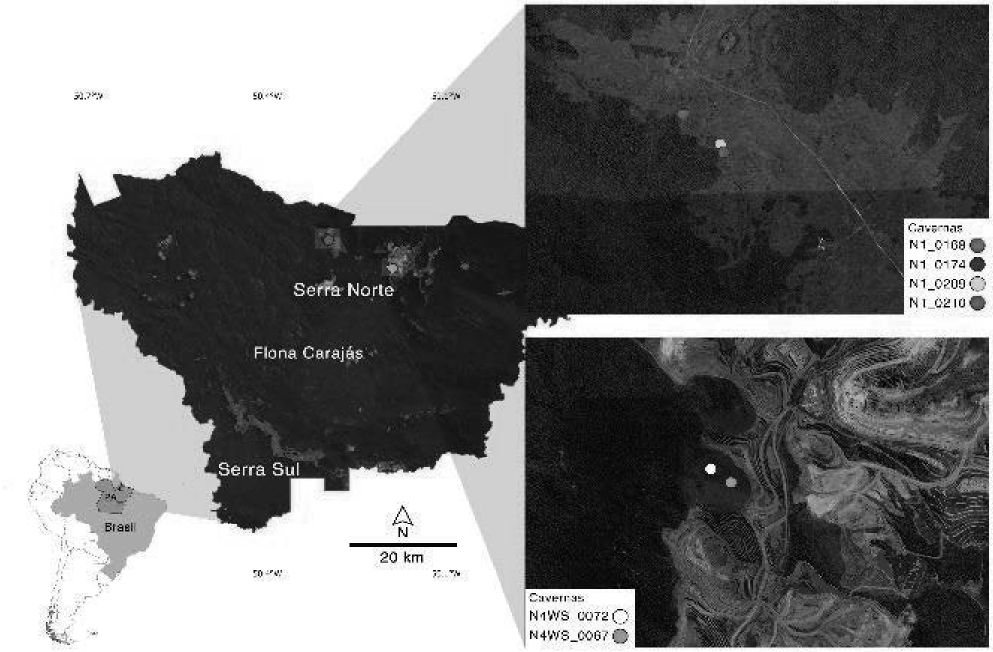
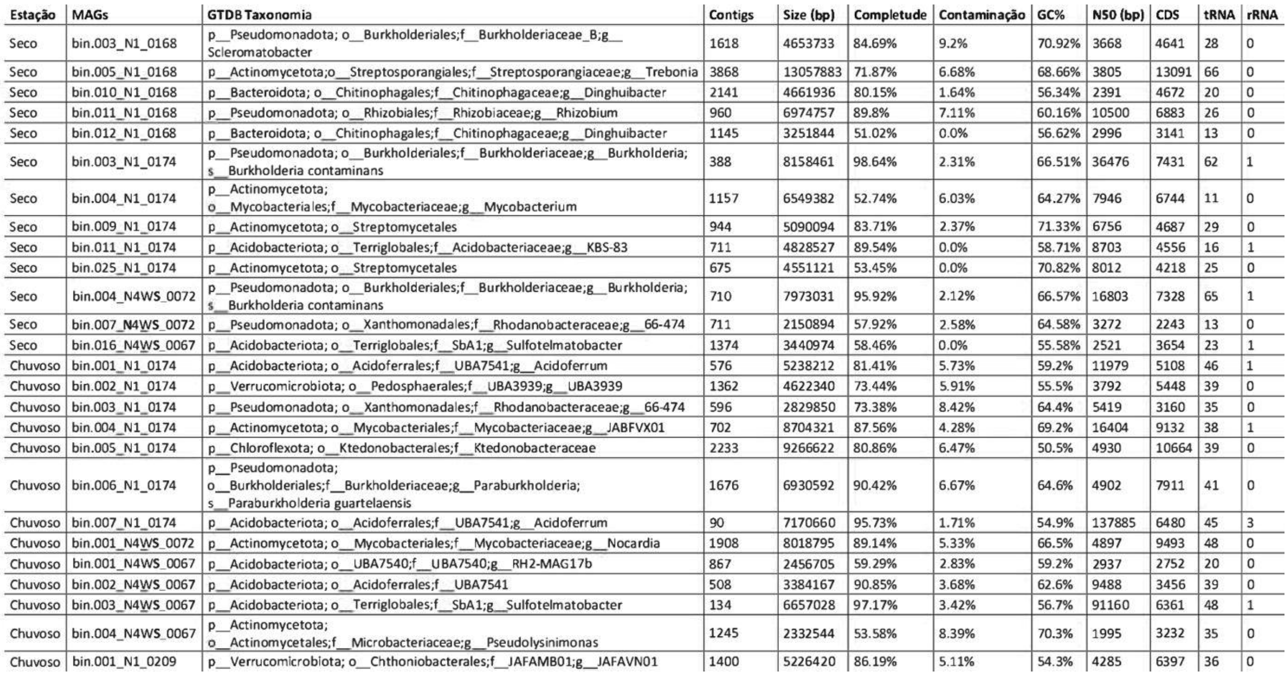


**Figure 1:** *Mapa da área de estudo, destacando a localização das seis cavernas na Serra Norte da FLONA de Carajás.*

A qualidade do sequenciamento foi avaliada utilizando FastQC v0.11.9 (ANDREWS et al. 2012), e as sequências de baixa qualidade e adaptadores remanescentes, removidas com Trimmomatic v0.36 (BOLGER et al. 2014). Os filtros aplicados foram qualidade PHRED >30 e leituras com comprimento superior a 50 pb. A cobertura do metagenoma foi estimada utilizando Nonpareil v3.5.5 (RODRIGUEZ-R et al. 2018). A montagem das sequências foi realizada com MEGAHIT v1.1.2 (LI et al. 2016) e MetaSpades v3.15.5 (NURK et al. 2017). Ambas as ferramentas foram utilizadas para comparar a qualidade e a completude dos *contigs* gerados. A qualidade das montagens foi avaliada com QUAST v5.2.0 (GUREVICH et al. 2013) para garantir que fossem adequados para análises posteriores. As sequencias foram mapeadas contra os *contigs* montados utilizando BBmap v35.85 (BUSHNELL, 2014) para avaliar a integridade das montagens e calcular a cobertura dos *contigs*. Para recuperação de MAGs foram utilizados o METABAT2 (KANG et al. 2019), MAXBIN2 v2.2.4 (WU et al. 2016) e CONCOCT v1.1 (ALNEBERG et al. 2014). Os *bins* gerados foram refinados e derreplicados com DASTool v1.1.2 (SIEBER et al. 2018), visando eliminar redundâncias e obter *bins* de melhor qualidade, a qual foi avaliada com CheckM v1.0.18 (PARKS et al. 2015), utilizando métricas de completude (≥ 50%) e contaminação (≤10%) (BOWERS et al. 2017). A predição de genes foi realizada nos *bins* selecionados utilizando RAST-tK v1.073 (BRETTIN et al. 2015). A classificação taxonômica dos MAGs foi conduzida com GTDB-TK v2.3.2 (CHAUMEIL et al. 2019), enquanto a anotação funcional foi feita com foi feita com Prokka v1.14.5 (SEEMANN et al. 2014) e DRAM v0.1.2 (SHAFFER et al. 2020), permitindo a identificação de vias metabólicas.

## 3. Resultados

No total, recuperamos 26 MAGs bacterianos, evidenciando diferenças na diversidade microbiana entre as estações seca e chuvosa, mas microrganismos não recuperados podem estar presentes em menor abundância no metagenoma. Na estação seca, observamos 13 MAGs, com uma maior diversidade de MAGs atribuída à Burkholderiales (3 MAGs), Chitinophagales (2 MAG) e Terriglobales (2 MAGs), sendo esta última representada pelo gênero *Sulfotelmatobacter* (Fig. 2). Além dessas, a estação seca revelou MAGs das ordens Streptomycetales (2 MAGs) que não puderam ser identificados até o nível de família e gênero, enquanto a ordem Streptosporangiales foi representada pelo gênero Trebonia (Fig.2). Em contraste, na estação chuvosa, observamos 13 MAGs, com um maior número da ordem Acidoferrales (3 MAGs) e Mycobacteriales (2 MAGs). A estação chuvosa também apresentou maior diversidade de ordens exclusivas, incluindo Pedosphaerales, Ktedonobacterales, Actinomycetales e Chthoniobacterales (Fig.2). Algumas MAGs foram provisoriamente classificadas pelo GTDB, sugerindo que essas linhagens ainda não possuem uma designação formalmente descrita na taxonomia, como é o caso da família SbA1, entre outras linhagens. Dos 26 genomas microbianos recuperados (MAGs), seis apresentaram alta qualidade, enquanto os demais foram classificados como de qualidade média (≥ 50% de completude, ≤ 10% de contaminação), abrangendo ambas as estações (Fig.2). Observou-se diferenças no conteúdo de bases nitrogenadas (GC), que pode influenciar a estabilidade do DNA. Também houve variação no número de genes codificadores de proteínas (CDSs), e nos genes de RNA ribossomal (rRNA) e transportador (tRNA), fundamentais para a síntese proteica e a replicação celular. O genoma bin.007, da caverna N1_0174, destacou-se por apresentar um perfil ribossomal robusto (rRNA), sugerindo capacidade de síntese proteica (Fig.2). Esses resultados evidenciam a adaptabilidade das comunidades microbianas às variações sazonais, refletindo diferenças significativas nos perfis taxonômicos, na qualidade genômica e na diversidade funcional ao longo dos períodos seco e chuvoso.



| Estação | MAGs | GTDB Taxonomia | Contigs | Size (bp) | Completude | Contaminação | GC% | N50 (bp) | CDS | tRNA | rRNA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Seco | bin.003_N1_0168 | p__Pseudomonadota; o__Burkholderiales;f__Burkholderiaceae_B;g__ Scleromatobacter | 1618 | 4653733 | 84.69% | 9.2% | 70.92% | 3668 | 4641 | 28 | 0 |
| Seco | bin.005_N1_0168 | p__Actinomycetota;o__Streptosporangiales;f__Streptosporangiaceae;g__Trebonia | 3868 | 13057883 | 71.87% | 6.68% | 68.66% | 3805 | 13091 | 66 | 0 |
| Seco | bin.010_N1_0168 | p__Bacteroidota; o__Chitinophagales;f__Chitinophagaceae;g__Dinghuibacter | 2141 | 4661936 | 80.15% | 1.64% | 56.34% | 2391 | 4672 | 20 | 0 |
| Seco | bin.011_N1_0168 | p__Pseudomonadota; o__Rhizobiales;f__Rhizobiaceae;g__Rhizobium | 960 | 6974757 | 89.8% | 7.11% | 60.16% | 10500 | 6883 | 26 | 0 |
| Seco | bin.012_N1_0168 | p__Bacteroidota; o__Chitinophagales;f__Chitinophagaceae;g__Dinghuibacter | 1145 | 3251844 | 51.02% | 0.0% | 56.62% | 2996 | 3141 | 13 | 0 |
| Seco | bin.003_N1_0174 | p__Pseudomonadota; o__Burkholderiales;f__Burkholderiaceae;g__Burkholderia; s__Burkholderia contaminans | 388 | 8158461 | 98.64% | 2.31% | 66.51% | 36476 | 7431 | 62 | 1 |
| Seco | bin.004_N1_0174 | p__Actinomycetota; o__Mycobacteriales;f__Mycobacteriaceae;g__Mycobacterium | 1157 | 6549382 | 52.74% | 6.03% | 64.27% | 7946 | 6744 | 11 | 0 |
| Seco | bin.009_N1_0174 | p__Actinomycetota; o__Streptomycetales | 944 | 5090094 | 83.71% | 2.37% | 71.33% | 6756 | 4687 | 29 | 0 |
| Seco | bin.011_N1_0174 | p__Acidobacteriota; o__Terriglobales;f__Acidobacteriaceae;g__KBS-83 | 711 | 4828527 | 89.54% | 0.0% | 58.71% | 8703 | 4556 | 16 | 1 |
| Seco | bin.025_N1_0174 | p__Actinomycetota; o__Streptomycetales | 675 | 4551121 | 53.45% | 0.0% | 70.82% | 8012 | 4218 | 25 | 0 |
| Seco | bin.004_N4WS_0072 | p__Pseudomonadota; o__Burkholderiales;f__Burkholderiaceae;g__Burkholderia; s__Burkholderia contaminans | 710 | 7973031 | 95.92% | 2.12% | 66.57% | 16803 | 7328 | 65 | 1 |
| Seco | bin.007_N4WS_0072 | p__Pseudomonadota; o__Xanthomonadales;f__Rhodanobacteraceae;g__66-474 | 711 | 2150894 | 57.92% | 2.58% | 64.58% | 3272 | 2243 | 13 | 0 |
| Seco | bin.016_N4WS_0067 | p__Acidobacteriota; o__Terriglobales;f__SbA1;g__Sulfotelmatobacter | 1374 | 3440974 | 58.46% | 0.0% | 55.58% | 2521 | 3654 | 23 | 1 |
| Chuvoso | bin.001_N1_0174 | p__Acidobacteriota; o__Acidoferrales;f__UBA7541;g__Acidoferrum | 576 | 5238212 | 81.41% | 5.73% | 59.2% | 11979 | 5108 | 46 | 1 |
| Chuvoso | bin.002_N1_0174 | p__Verrucomicrobiota; o__Pedosphaerales;f__UBA3939;g__UBA3939 | 1362 | 4622340 | 73.44% | 5.91% | 55.5% | 3792 | 5448 | 39 | 0 |
| Chuvoso | bin.003_N1_0174 | p__Pseudomonadota; o__Xanthomonadales;f__Rhodanobacteraceae;g__66-474 | 596 | 2829850 | 73.38% | 8.42% | 64.4% | 5419 | 3160 | 35 | 0 |
| Chuvoso | bin.004_N1_0174 | p__Actinomycetota; o__Mycobacteriales;f__Mycobacteriaceae;g__JABFVX01 | 702 | 8704321 | 87.56% | 4.28% | 69.2% | 16404 | 9132 | 38 | 1 |
| Chuvoso | bin.005_N1_0174 | p__Chloroflexota; o__Ktedonobacterales;f__Ktedonobacteraceae | 2233 | 9266622 | 80.86% | 6.47% | 50.5% | 4930 | 10664 | 39 | 0 |
| Chuvoso | bin.006_N1_0174 | p__Pseudomonadota; o__Burkholderiales;f__Burkholderiaceae;g__Paraburkholderia; s__Paraburkholderia guartelaensis | 1676 | 6930592 | 90.42% | 6.67% | 64.6% | 4902 | 7911 | 41 | 0 |
| Chuvoso | bin.007_N1_0174 | p__Acidobacteriota; o__Acidoferrales;f__UBA7541;g__Acidoferrum | 90 | 7170660 | 95.73% | 1.71% | 54.9% | 137885 | 6480 | 45 | 3 |
| Chuvoso | bin.001_N4WS_0072 | p__Actinomycetota; o__Mycobacteriales;f__Mycobacteriaceae;g__Nocardia | 1908 | 8018795 | 89.14% | 5.33% | 66.5% | 4897 | 9493 | 48 | 0 |
| Chuvoso | bin.001_N4WS_0067 | p__Acidobacteriota; o__UBA7540;f__UBA7540;g__RH2-MAG17b | 867 | 2456705 | 59.29% | 2.83% | 59.2% | 2937 | 2752 | 20 | 0 |
| Chuvoso | bin.002_N4WS_0067 | p__Acidobacteriota; o__Acidoferrales;f__UBA7541 | 508 | 3384167 | 90.85% | 3.68% | 62.6% | 9488 | 3456 | 39 | 0 |
| Chuvoso | bin.003_N4WS_0067 | p__Acidobacteriota; o__Terriglobales;f__SbA1;g__Sulfotelmatobacter | 134 | 6657028 | 97.17% | 3.42% | 56.7% | 91160 | 6361 | 48 | 1 |
| Chuvoso | bin.004_N4WS_0067 | p__Actinomycetota; o__Actinomycetales;f__Microbacteriaceae;g__Pseudolysinimonas | 1245 | 2332544 | 53.58% | 8.39% | 70.3% | 1995 | 3232 | 35 | 0 |
| Chuvoso | bin.001_N1_0209 | p__Verrucomicrobiota; o__Chthoniobacterales;f__JAFAMB01;g__JAFAVN01 | 1400 | 5226420 | 86.19% | 5.11% | 54.3% | 4285 | 6397 | 36 | 0 |

***Figure 2:*** *Descrição dos 26 genomas microbianos montados a partir de metagenomas (MAGs) obtidos de amostras de raízes de plantas associadas a cavernas feruginosas da FLONA de Carajás, na Amazônia, durante as estações seca e chuvosa.*

A análise funcional revelou que, durante a estação seca, predominam bactérias com vias metabólicas aeróbicas, como o ciclo de Krebs, glicólise e os complexos da cadeia de transporte de elétrons (ETC), especialmente os complexos de tipo III e IV (Fig.4). Na mesma estação, observou-se uma maior diversidade de microrganismos com funções associadas ao metabolismo de metano e enxofre, incluindo genes envolvidos na oxidação de tiossulfato, metanogênese e na conversão de trimetilamina (Fig.3). Em contraste, na estação chuvosa, o perfil microbiano apresentou um aumento significativo de bactérias com vias anaeróbicas. Além disso, foi observada a presença de genes relacionados ao metabolismo de nitrogênio e à redução de mercúrio (Fig.3). Em ambas as estações, as enzimas CAZymes apresentaram maior frequência em algumas amostras específicas, o que sugere a importância de algumas MAGs no metabolismo de carboidratos e na degradação de polímeros orgânicos em ambientes de caverna (Fig.3). Notavelmente, na estação chuvosa, foi identificado um MAG com alta frequência de genes associados à degradação de compostos fenólicos, indicando uma adaptação a compostos complexos derivados da decomposição de matéria orgânica. Além disso, genes relacionados à conversão de ácidos graxos de cadeia curta (SCFA) e álcool foram observados em diversas amostras nas duas estações, com padrões distintos (Fig.3).

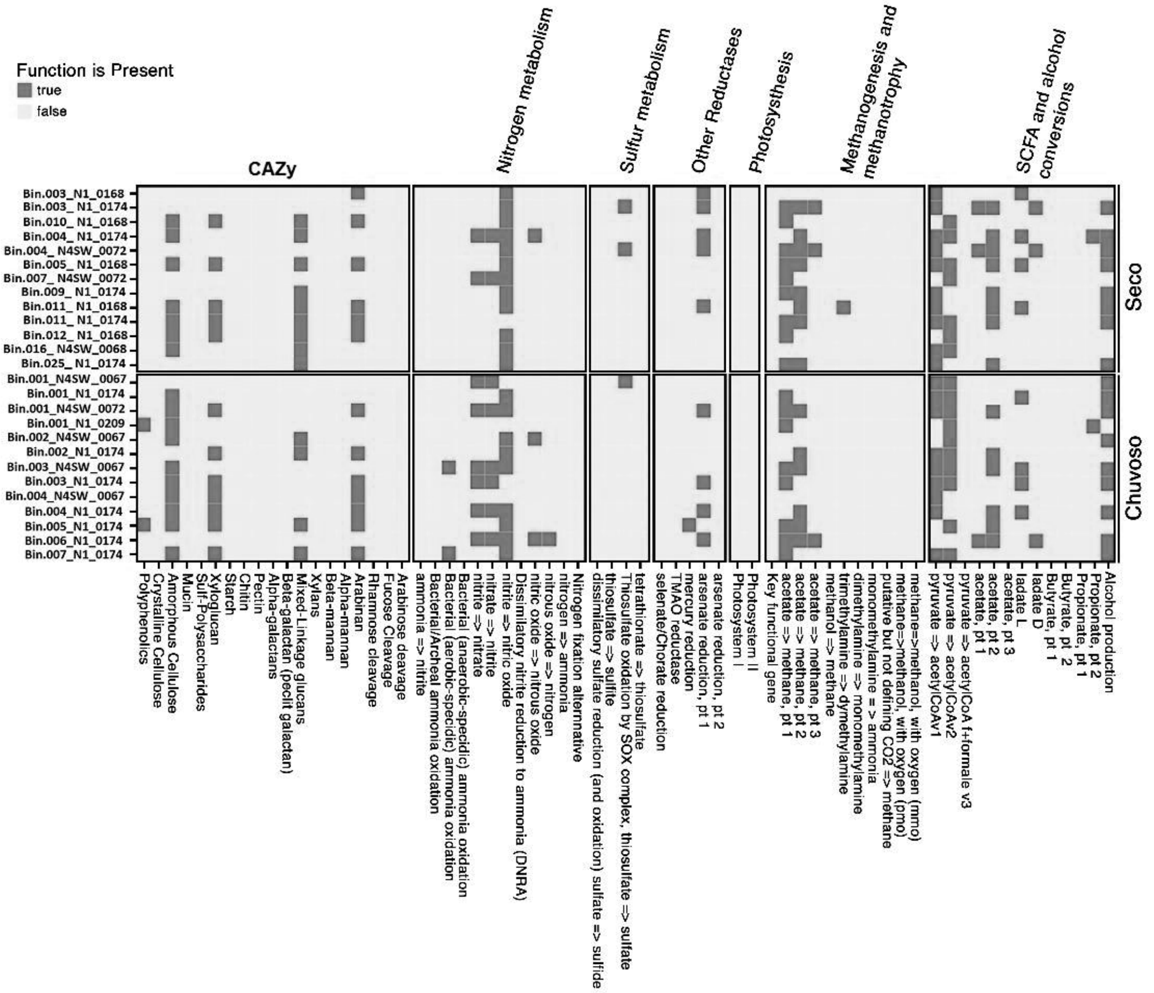


*Figure 3:* Anotações funcionais de vias metabólicas e processos bioquímicos dos 26 MAGs de raízes de plantas associadas a cavernas ferruginosas da FLONA de Carajás, Amazônia, durante as estações seca e chuvosa.

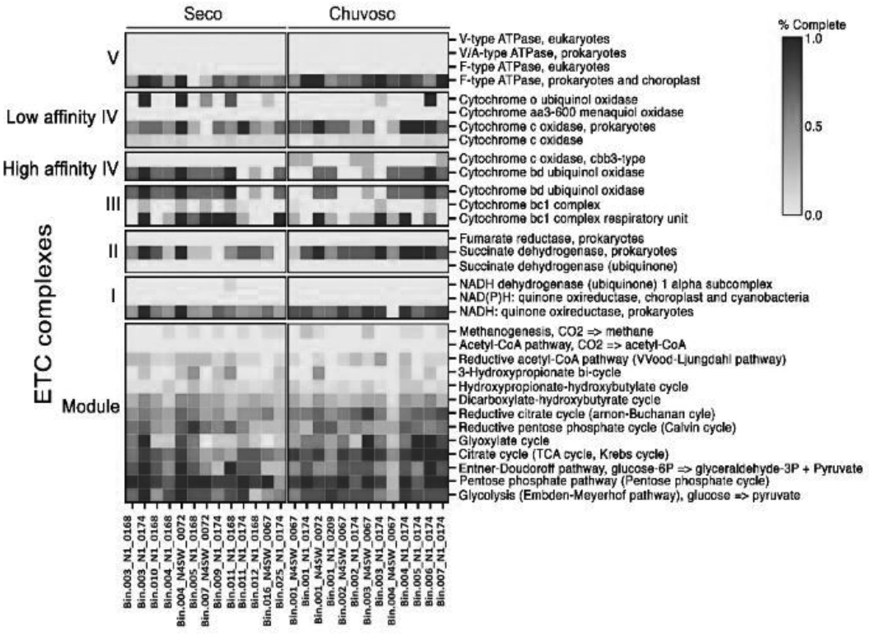


*Figure 4: Complexos da cadeia de transporte de elétrons (ETC), vias e ciclos metabólicos em 26 MAGs de raízes de plantas de cavernas ferruginosas da FLONA de Carajás, nas estações seca e chuvosa.*

## 4. Discussão

A vegetação associada às áreas de canga, onde a maioria das cavidades estão localizadas na FLONA de Carajás, estão sujeitas a condições peculiares típicas de campos rupestres, como solo raso e ácido, com baixa concentração de nutrientes e alta riqueza de ferro e manganês (SCHAEFER et al. 2015). Estas condições específicas do solo e do ambiente circundante influenciam a composição da microbiota associada às raízes das cavernas (FELESTRINO et al. 2018; PAULA, 2023). Os resultados apresentados aqui evidenciam essa relação direta entre as condições das cangas, a vegetação local e a microbiota identificada nas raízes presentes nas cavernas. Durante a estação seca, a abundância de Burkholderiales pode estar relacionada à sua atuação na promoção do crescimento da flora epígea nas cavernas e na ciclagem de nutrientes (MANDRO et al. 2022). A presença de Terriglobales sugere adaptação a ambientes com menor disponibilidade de nutrientes (VENTURINI et al. 2022), enquanto Chitinophagales, provavelmente interagem com fungos nas raízes (MANDRO et al. 2022). Na estação chuvosa, a presença de *Proteobacteria* indica seu papel no ciclo do carbono, especialmente na oxidação de metano ou promoção da mineralização de compostos orgânicos (KUMAR et al. 2021). A detecção de *Acidoferrum*, grupo associado à ciclagem de metais, particularmente ferro, reflete a relevância desse elemento em cavernas ferruginosas (PARKER et al. 2022). A predominância de membros do filo Actinobacteria durante esse período, incluindo o *Nocardia* (Mycobacteriaceae), merece destaque devido ao seu metabolismo secundário, capaz de produzir compostos bioativos de interesse biotecnológico (RANGSEEKAEW et al. 2019). Adicionalmente, a formação de microambientes anóxicos durante a estação chuvosa favorece a produção de metano, como observado por KUMARESAN et al. (2018). A diversidade de genes relacionados ao metabolismo de nitrogênio e à redução de mercúrio sugere a relevância de processos como desnitrificação na manutenção dos ciclos biogeoquímicos (FREITAS et al. 2024). Além disso, a presença de enzimas ativas em carboidratos, indica uma adaptação metabólica das comunidades bacterianas às condições oligotróficas das cavernas (PAULA et al. 2023). A detecção de genes associados à degradação de compostos fenólicos e à conversão de ácidos graxos e álcool reforça a adaptação das comunidades bacterianas às variações sazonais. Além das adaptações metabólicas, a sinergia observada entre microrganismos autotróficos, como *Acidoferrum*, e heterotróficos, como Burkholderiales e Mycobacteriales, sugere uma colaboração metabólica essencial à sobrevivência em ambientes subterrâneos com recursos limitados (KELUSKAR et al. 2013; MA et al. 2021). Essas interações complexas refletem a flexibilidade metabólica das comunidades bacterianas, assegurando funções ecológicas vitais, como oxidação de metano e fixação de nitrogênio, que mantêm a estabilidade dos ecossistemas subterrâneos mesmo sob variações sazonais extremas.

## 5. Conclusão

O estudo do microbioma das raízes de plantas associadas às cavernas revelou uma grande diversidade de grupos bacterianos autotróficos e heterotróficos. Diferentes grupos se destacaram conforme as variações ambientais sazonais, especialmente em condições de estresse hídrico e disponibilidade de nutrientes. Os MAGs recuperados apresentaram qualidade satisfatória, expandindo o conhecimento sobre esses ambientes subterraneos. Em termos funcionais, na estação seca, predominou o metabolismo aeróbico e funções associadas ao metabolismo de metano. Por outro lado, na estação chuvosa, o metabolismo anaeróbico ganhou maior relevância, com um aumento nas funções relacionadas ao metabolismo de nitrogênio e à redução de mercúrio. A presença de enzimas ativas em carboidratos em diversas amostras sugeriu um papel importante no metabolismo de carboidratos e na degradação de compostos complexos nas cavernas. Adicionalmente, a identificação de genes associados à degradação de compostos fenólicos, especialmente na estação chuvosa, revela um potencial ainda inexplorado para a biotecnologia, destacando a importância de compreender mais profundamente as adaptações microbianas nesses ecossistemas. Portanto, este estudo não apenas amplia o conhecimento sobre a dinâmica microbiana nas cavernas e sua relação com as condições ambientais sazonais, mas também ressalta o potencial biotecnológico desses microbiomas, reforçando a importância de investigações futuras em ecossistemas subterrâneos pouco explorados.

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# Landscape influence on *Carollia perspicillata* morphological traits: functional adaptations to anthropogenic disturbances

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## Resumo

Investigamos a influência de características da paisagem sobre os atributos funcionais de *Carollia perspicillata*, na Área de Influência Direta do Projeto Serra Leste 10Mtpa, Parauapebas-PA, Brasil. Avaliamos atributos morfológicos relacionados à performance de voo, como massa corporal, comprimento do antebraço e do calcâneo, em resposta a variáveis da paisagem. Para tal, utilizamos Modelos Generalizados Mistos (GLMM), com os quais identificamos que o comprimento do antebraço aumenta com a distância da mineração, enquanto o calcâneo é menor em áreas próximas a corpos hídricos. Os resultados sugerem que a paisagem atua como filtro ambiental, moldando fenótipos relacionados à locomoção e disponibilidade de recursos. O presente estudo integra ecologia funcional e morfologia em um contexto de paisagem alterada e oferece informações sobre a resposta funcional de *C. perspicillata* frente às alterações antrópicas. Sendo assim, tais resultados podem subsidiar estratégias de manejo que visam mitigar os efeitos de atividades minerárias sobre a quiropterofauna.

## Abstract

We examined the influence of landscape attributes on the functional traits of *Carollia perspicillata* within the Direct Influence Area of the Serra Leste 10Mtpa Project, Parauapebas-PA, Brazil. We evaluated flight-related morphological traits, including body mass, forearm length, and calcaneus length, in relation to landscape variables. To this end, we employed Generalized Linear Mixed Models (GLMM), which revealed that forearm length increases with increasing distance from mining areas, whereas calcaneus length is smaller in proximity to water bodies. These findings suggest that the landscape serves as an environmental filter, shaping phenotypic traits associated with locomotion and resource availability. This study bridges functional ecology and morphology within the context of landscape disturbance, offering insights into functional response of *C. perspicillata* to anthropogenic modifications. Consequently, these results may support management strategies aimed at mitigating the impacts of mining activities on chiropteran populations.

## 1. Introdução

Os morcegos incluem mais de 1300 espécies noturnas, formando a segunda ordem mais rica dentre os mamíferos, dos quais 186 ocorrem no Brasil (GARBINO et al. 2024). A diversidade de espécies se reflete também em uma ampla gama de hábitos, nichos ocupados e diversificação e especialização trófica (POTTER ET AL. 2021). Desempenham, portanto, funções e serviços de relevância ecológica e econômica (BOYLES et al. 2013, KUNZ et al. 2011).

A inclusão de aspectos funcionais em monitoramentos permite compreender, de maneira holística, a influência das alterações ambientais, mesmo que estas não reflitam na riqueza ou abundância das espécies. Dado que a alteração da paisagem por atividades antrópicas atua sob a fauna, alterando sistematicamente a frequência e manifestação de fenótipos (ALBERTI et al. 2017). Desse modo, ambientes antropizados promovem o direcionamento de processos de seleção natural, condicionando mudanças nos atributos funcionais de espécies de morcegos (TOMASSINI et al. 2014, RAMÍREZ-MEJÍA et al. 2021).

O presente estudo tem como objetivo testar a hipótese de que mudanças antrópicas na paisagem de Serra Leste alteram atributos funcionais, relacionados ao fitness, de *Carollia perspiscillata*.

# 2. Materiais e Métodos

## 2.1. Área de estudo:

O levantamento dos atributos funcionais de morcegos teve como alvo 93 cavidades inseridas na Área de Influência Direta (AID) do Projeto Serra Leste 10Mtpa (Fig. 1). Os quais incluíram atividades de campo do período chuvoso (março a maio de 2023) e seco (setembro e outubro de 2023).

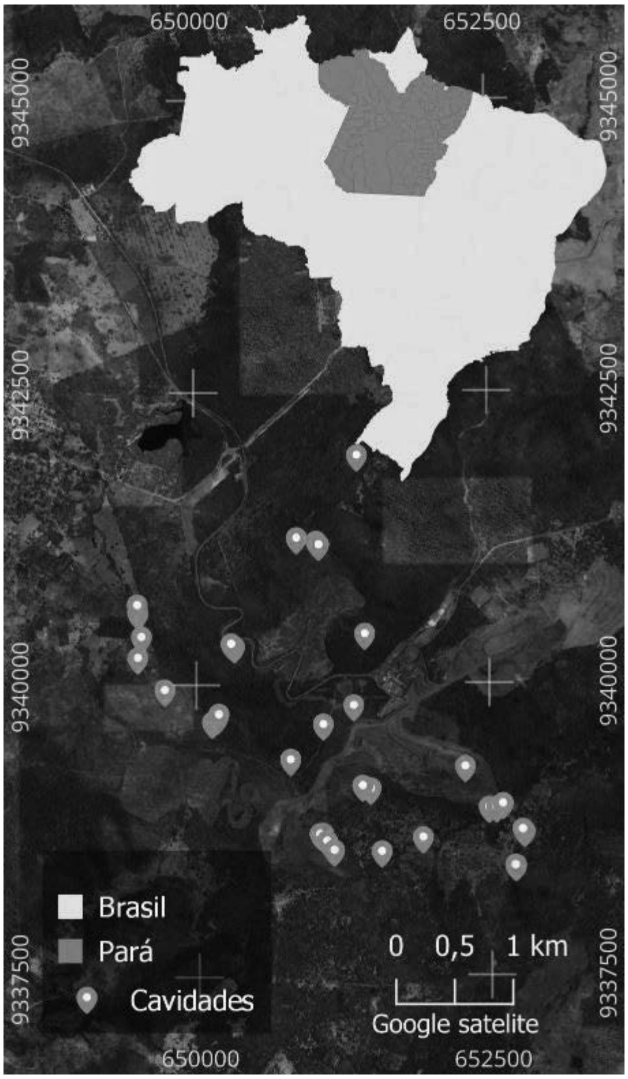

*Figura 1:* *Localização das cavidades na área de influência direta (AID) do Projeto Serra Leste 10 Mtpa, com destaque para as 15 cavidades que atualmente fazem parte do plano de monitoramento da Quiropterofauna.*

## 2.2. Procedimentos:

Primeiramente, realizamos uma vistoria nas cavidades a fim e registrar as colônias e identificar o melhor método para a captura dos morcegos. A interceptação em voo com o auxílio da rede de neblina, a qual foi instalada em locais de difícil acesso ao pesquisador. Para diminuir a perturbação da colônia empregamos o puçá, o qual posicionamos sob a colônia alvo e realizamos a captura dos espécimes. Os indivíduos capturados foram transferidos para sacos individuais de algodão. Realizamos a medição dos atributos funcionais e a identificação das espécies segundo (Reis et al. 2017; Diaz et al. 2021. Os atributos funcionais avaliados foram massa corporal, realizada com Pesola (100g e 300g), comprimento do antebraço e o comprimento do calcâneo com o auxílio de um paquímetro analógico (Fig. 2). Posteriormente, os animais foram marcados com anilha de alumínio numeradas no antebraço e soltos na mesma cavidade de captura.

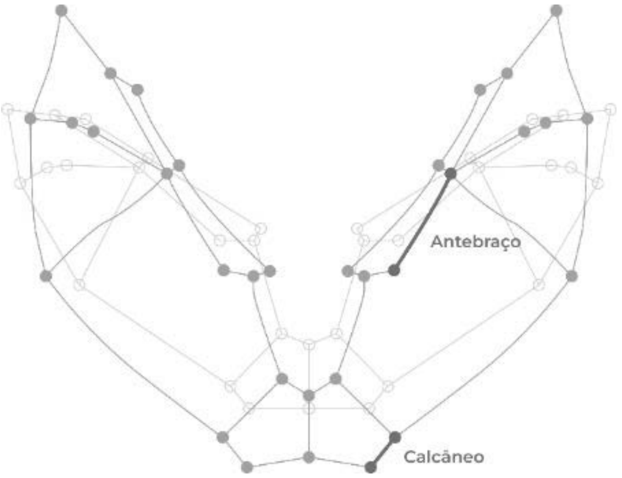

*Figura 2:* *Medidas morfométricas avaliadas nos machos de Carollia perspicillata, com destaque para as medidas que respondem às variáveis do empreendimento.*

Avaliamos a influência da paisagem no *fitness* de *Carollia perspiscillata*, por ser a espécie mais abundante e espacialmente distribuída na AID do Projeto Serra Leste 10Mtpa. Para tal, selecionamos os dados de organismos adultos e machos de *C. perspiscillata*, para evitar variações morfológicas, por dimorfismo sexual ou etarismo, e excluímos dados de recaptura. Selecionamos os atributos funcionais relacionados à performance de voo dos morcegos. A massa corporal, por exemplo, tem relação com a velocidade de voo (MORRISON 1980, WINTER 1999). O comprimento do antebraço é um *porxy* confiável para o tamanho corporal (THIAGAVEL et al. 2017), e no caso dos filostomídeos o aumento no comprimento desse atributo está relacionado à disponibilidade de alimentos e locais de descanso (BALLESTEROS-CORREA 2015, CASTILLO-FIGUEROA & PÉREZ-TORRES 2018). O comprimento do calcâneo confere rigidez e flexibilidade ao uropatágio, sendo importante na aerodinâmica do voo ao evitar resistência do vento, conferindo maior agilidade aos morcegos (WEBSTER & GRIFFIN 1962).

Verificamos a relação entre os preditores da paisagem e atributos funcionais por meio de Modelos Generalizados Mistos (GLMM). Para tal, utilizamos cada atributo funcional como variável resposta na construção dos modelos e inserimos os eventos amostrais como variável aleatória. Além disso, verificamos a colinearidade dos preditores, por meio da função *chart.correlation* do pacote Performance Analytics. Realizamos a construção dos modelos a família Gausiana, função *lmer* do pacote *lme4*, definimos o melhor modelo após a retirada de variáveis não significativas. Todas as análises descritas foram realizadas por meio do programa R (R Core Team 2023).

# 3. Resultados

Após a análise de correlação, variáveis colineares foram excluídas, sendo mantidas para a construção dos modelos: distância para feições hídricas, distância para a mineração, e a cobertura da paisagem por feições hídricas, floresta e canga (Fig. 3).

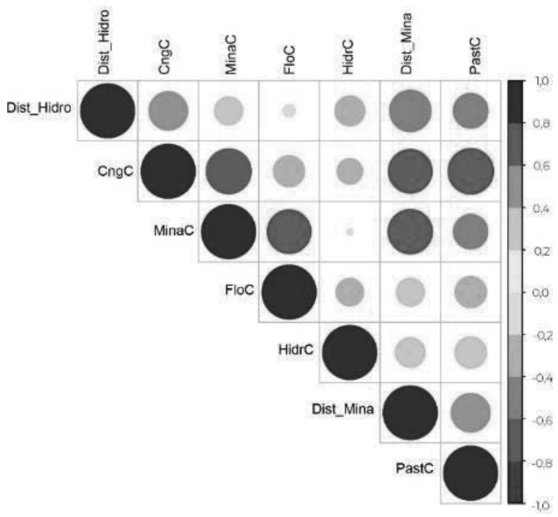

*Figura 3: Correlação dos preditores inseridos no modelo explicativo de composição da quiropterofauna. Dist_Hidro: Distância para a feição hídrica mais próxima; CngC: Cobertura da paisagem por vegetação savânica em um buffer de 500 m; MinaC: Cobertura da paisagem por mineração em um buffer de 500 m; FloC: Cobertura da paisagem por floresta ombrófila em um buffer de 500 m; HidrC: Cobertura da paisagem por feições hídricas em um buffer de 500 m; PastC: Cobertura da paisagem por área de pastagem em um buffer de 500 m; Dist_Mina: Distância para áreas alteradas pelo empreendimento, incluindo, instalações operacionais, estruturas e acessos.*

Nenhum dos preditores foi capaz de explicar significativamente variações na massa corporal dos indivíduos de *C. perspicillata*. Por outro lado, o comprimento do antebraço foi positivamente relacionado à distância da mineração. Por fim, o calcâneo foi negativamente relacionado à cobertura do entorno das cavidades por corpos hídricos (Fig. 4).

| Variável resposta | Preditores | Estimativa | Erro | Valor de t | p |
|---|---|---|---|---|---|
| ANT | Distância da mineração (m) | <0,001 | <0,001 | 122,63 | <0,001 |
| CC | Corpo hídrico (ha) | -0,766 | 0,322 | -2,38 | 0,018 |

*Figura 4: Relação entre os atributos funcionais de Carollia perspicillata amostrada nas cavidades e as características da paisagem. É apresentada a estimativa, o erro padrão, valor de t e nível de significância (p), em vermelho estão representados valores de p < 0,05. ANT: Comprimento do antebraço; CC: comprimento do calcâneo. Variável corpo hídrico em buffer de 500 m a partir da cavidade.*

## 4. Discussão

Nossos resultados evidenciam que, salvo a massa corporal, variações morfológicas intraespecíficas de *Carollia perspicillata* ocorrem de acordo com características do ambiente. Desse modo, as características da paisagem atuam como filtros ambientais na seleção de fenótipos relacionados à performance de voo (RAMÍREZ-MEJÍA et al. 2021) e disponibilidade de alimentos (CASTILLO-FIGUEROA & PÉREZ-TORRES, 2021). CONENNA et al. (2021), por exemplo, demonstrou que a variação de atributos funcionais relacionados ao voo muda frente aos gradientes de aridez.

No presente estudo, os maiores comprimentos do antebraço estão relacionados às maiores distâncias da mineração. Uma vez que este atributo funcional é considerado um proxy do tamanho corporal (THIAGAVEL et al. 2017), há uma diminuição dos indivíduos quanto mais próximos estão do empreendimento. De maneira geral, espécies menores e com asas mais curtas são mais adaptadas à voos rápidos e de longa distância (FERNEDA et al. 2015). Nossos resultados indicam que asas menores conferem vantagens adaptativas em áreas próximas à mineração, uma vez que esses organismos precisam percorrer distâncias maiores para obter recursos tróficos.

Além disso, verificamos que os menores comprimentos do calcâneo estão associados a paisagens com maior proximidade à corpos hídricos. O calcâneo, localiza-se na região do tornozelo dos morcegos, e desempenha papel importante na função do uropatágio (membrana que conecta as pernas e a cauda). Dessa forma, juntamente com o uropatágio ele relaciona-se com a estabilidade, manobrabilidade e eficiência do voo (SWARTZ et al. 2003). É provável que locais com maior cobertura por corpos hídricos demandem voos mais curtos e menos complexos, visto que calcâneos e uropatágio menores diminuem a manobrabilidade dos voos (VAUGHAN 1996). Desse modo, a facilidade de acesso à água modela a morfologia dos organismos, especialmente ao consideramos que a água é um recurso valioso aos morcegos (KORINE et al. 2016),

Segundo Díaz et al. (2023), é importante considerar aspectos funcionais em estratégias de conservação, uma vez que os atributos refletem a capacidade das espécies em persistir em paisagens alteradas. Além disso, permite prever respostas frente às mudanças na paisagem (Ferneda et al. 2015).

## 5. Conclusão

Nossos resultados trouxeram à luz informações valiosas sobre a influência das alterações na paisagem nos atributos funcionais de *Carollia perspicillata*, uma vez que integramos aspectos ecológicos e morfológicos. Demonstramos que alterações antrópicas podem atuar como filtros seletivos, moldando fenótipos relacionados à performance de voo e à disponibilidade de recursos.

A importância deste estudo reside não apenas no avanço do conhecimento sobre a ecologia funcional de morcegos, mas também na aplicação prática desses resultados para a conservação e o manejo de espécies em áreas impactadas por atividades humanas. Ao identificar como características da paisagem afetam a morfologia e, consequentemente, a capacidade de voo e forrageamento de *C. perspicillata*, este trabalho fornece subsídios para a elaboração de estratégias de mitigação de impactos ambientais, visando à manutenção da biodiversidade e dos serviços ecossistêmicos prestados por esses animais.

Em síntese, este estudo contribui para a compreensão dos mecanismos que regem a adaptação de morcegos a paisagens alteradas, reforçando a necessidade de integrar aspectos funcionais em monitoramentos ecológicos. Dentre as aplicações desses dados destaca-se seu uso como ferramenta para a gestão ambiental em cenários de crescente pressão antrópica.

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