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Special Issue Reprint

Cave Communities

From the Surface Border to the Deep Darkness

Edited by
Raoul Manenti and Enrico Lunghi

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Contents

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Editorial

Cave Communities: From the Surface Border to the Deep Darkness

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Abstract: The discipline of subterranean biology has provided us incredible information on the diversity, ecology and evolution of species living in different typologies of subterranean habitats. However, a general lack of information on the relationships between cave species still exists, leaving uncertainty regarding the dynamics that hold together cave communities and the roles of specific organisms (from the least to the most adapted species) for the community, as well as the entire ecosystem. This Special Issue aims to stimulate and gather studies which are focusing on cave communities belonging to all different typologies of subterranean habitats, with the overarching goal to corroborate the key role of the subterranean biology in ecological and evolutionary studies.

Keywords: cave biology; subterranean habitats; vertebrates; invertebrates; community dynamics; biospeleology; hypogean; conservation; biodiversity; troglobite; troglophile; trogloxene

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Introduction

The study of subterranean habitats (i.e., all natural and artificial subterranean voids and groundwater suitable for human exploration; [1]) and their related fauna is a discipline that is intriguing scientists from many points of view [2–5], and its broad interest is testified by the large number of published researches, see [6–10]. Subterranean environments are of special importance for diversity as they often host a highly specialized fauna, with unique, unusual and sometimes even bizarre morphological, behavioural and ecological adaptations [11,12].

The peculiar ecological features characterising the subterranean habitats are probably one of the most important causes of the astounding diversity occurring there [12,13]. One of the most evident is the absence of light, as the solar radiation does not go beyond a few meters from the entrance (i.e., the connection with the surface), preventing organisms dependant on light, such as plants, from settling. [8,14]. Consequently, without the presence of these important primary producers, a general paucity of organic matter occurs within subterranean habitats, and the resident species mostly depend on allochthonous inputs [8]. Another consequence of the shelter from the sunlight and climatic fluctuations, is an increase of the stability of the subterranean microclimate, especially in the deepest parts [15,16].

Cave-dwelling species need to cope with the particular environmental conditions occurring in subterranean habitats, and to do that, they show a specific set of behavioural, physiological and morphological features [17–20]. Such features are generally considered as the result of species adaptation to the peculiar local ecological conditions [21–23]; however, several researches are documenting that also other processes, such as neutral mutation or genetic drift, may represent alternative important factors [24–26]. Cave species are generally classified based on both the presence of specific adaptations to subterranean habitats and their ability to complete their life cycle there [12,27], although sometimes such classification may be too strict [10,28]. The most adapted species are the troglobionts: they often show evident adaptations (e.g., reduction of eyes and pigmentation, elongation of appendages) and only reproduce in subterranean habitats. Troglophiles are a species that are able to reproduce in both subterranean and surface habitats, and show some adaptations to cave life. Trogloxenes are occasional visitors in caves and only reproduce in surface habitats. Although the wide contribution on the knowledge of cave-adapted species, researchers often overlooked trogloxenes in their studies [29–31], thus limiting the information on the potential effects that these transient species may have on cave communities and the overall ecosystem [32–34]. Cave animals often occupy specific areas of the subterranean habitats, the less adapted being closer to the connection with surface, and the most adapted in the deepest parts [8,30,35,36]. Consequently, different cave communities can occur [16,37–39], each one characterised by distinct diversity and dynamics, with species holding different ecological roles [33,40–43], and often with blurred borders.

This Special Issue of Diversity aims to explore the relationships among cave-dwelling species, considering not only troglobionts, but all the organisms occurring from the entrance to the deepest sectors, a topic which is still poorly explored. Our goal is to stimulate and collect new research focused on multiple cave species [37,44], or on the ecological role that single species have for the local ecosystem [31,45]. For example, considering the ecological gradient occurring from the cave entrance to the deepest areas (light, microclimatic variability and food availability vs. darkness, microclimatic stability and food scarcity; [27]), species occupy areas according to their preference [46–49], and consequently form different communities characterised by specific intrinsic dynamics [30,43,50,51]. Studying the relationships between species within cave communities will not only let us understand how species interact (e.g., competition, mutualism, prey–predator interactions), but will also allow us to determine their ecological importance for the entire subterranean ecosystem. Indeed, species from the communities inhabiting the areas close to the cave entrance are likely to have a key role in supporting the overall subterranean habitat, as they are able to transfer new organic matter from surface habitats to the subterranean one [42,43,45,52,53]. Consequently, some of the species from deep cave areas (if not entire communities) are strongly dependant on the operations of shallowest communities [42,54].

From a geological point of view, several types of subterranean environments exist (e.g., natural and artificial caves, shallow subterranean environments sensu [7], small fissures and interstices, etc.) and each one can host a unique set of organisms, from bacteria and fungi to invertebrate and vertebrate species, that are often geographically restricted and numerically rare [55–57]. Improving the knowledge on subterranean communities will allow an increase in the effectiveness of conservation plans towards single cave species as well as the entire ecosystem [56,58,59]. Indeed, conservation plans towards key species will have a cascade of positive effects on the entire ecosystem [60,61]. Furthermore, understanding the role of cave communities and the relationships occurring between species with different levels of adaptation can allow us to predict the potential effects due to subterranean biodiversity loss, as cave species (especially stenoendemic ones) are highly sensitive to multiple factors, such as environmental changes, pathogen spread, invasion of alien species and even poaching [62–66].

Since its beginning, subterranean biology has been characterised by two main branches, one related to taxonomic investigations of subterranean organisms [2,8], and the other considering caves as a powerful natural laboratories to perform evolutionary, ecological and behavioural studies on selected model species [27,67,68]. The study of subterranean diversity has the potential to lead the advances of modern science and solve some of the current scientific challenges [27]. We hope that this Special Issue could provide new insights of broad interest, and develop a new hypothesis to test and highlight the role of the subterranean biology as one of the leading disciplines in ecology and evolution.

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Article

A Primer on Spider Assemblages in Levantine Caves: The Neglected Subterranean Habitats of the Levant—A Biodiversity Mine

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Abstract: Caves share unique conditions that have led to convergent adaptations of cave-dwelling animals. In addition, local factors act as filters on regional species-pools to shape the assemblage composition of local caves. Surveys of 35 Levantine caves, distributed along a climate gradient from the mesic in the north of Israel to hyper-arid areas in the south of Israel, were conducted to test the effect of cave characteristics, location, climate, bat presence, and guano level on the spider assemblage. We found 62 spider species and assigned four species as troglobites, 28 as troglophiles, and 30 as accidentals. Precipitation, elevation, latitude, minimum temperature, and guano levels significantly affected the composition of cave-dwelling spider assemblages. Caves situated in the Mediterranean region had higher species richness and abundance, as well as more troglobite and troglophile arachnids. These discoveries contribute to the knowledge of the local arachnofauna and are important for the conservation of cave ecosystems. By comparing spider assemblages of Levantine caves to European caves, we identified gaps in the taxonomic research, focusing our efforts on spider families that may have additional cryptic or yet to be described cave-dwelling spider species. Our faunistic surveys are crucial stages for understanding the evolutionary and ecological mechanisms of arachnid speciation in Levantine caves.

Keywords: accidental cave visitors; Arachnida; Araneae; arid; hypogean; levant; Mediterranean; species diversity; troglobite; troglophile

1. Introduction

Different caves, similar patterns. Subterranean habitats (caves and other hypogean habitats) around the world can be found in different climates, rock formations, and biogeographical regions. Additionally, they can be formed by various means such as volcanic, glacial, mechanical, and erosion/solution processes. However, the majority of these subterranean habitats share unique abiotic conditions such as a limitation of light, stable and narrow range of temperature, and high relative humidity [1–3]. The peculiar abiotic conditions found in subterranean habitats, together with specific regional and local factors, determine species richness and assemblage composition of a particular cave. Processes at the regional scale include geological and climatic events together with historical biogeography, dispersal, extinction, and speciation, which shape the regional species pool [4–6]. At the local scale, ecological interactions and local abiotic conditions act as filters on the regional species pool to shape each cave assemblage composition [7–10].

Depending on the number of cave openings, their size and location, as well as the size and depth of the cave, a gradient of light intensity, climatic conditions and nutrients can be found, creating up to five defined zones: entrance, twilight, transition, deep, and air-stagnant zones [1,11–13]. The abiotic conditions at the deep and air-stagnant zones of caves are less affected by seasonality and daily cycles and therefore are more stable environments in comparison to the cave entrance, twilight, and transition zones [14–16]. There is some evidence that different cave zones are inhabited by species with different environmental requirements and constitute discrete assemblages [14].

Connectivity of epigean and hypogean habitats also affect assemblage composition inside the cave via colonization and dispersal within and between epigean and hyopgean systems [17]. While it is suggested that species richness of troglobites (organisms obligated to life in caves) is explained by historical biogeography, species richness of troglophiles (organisms with strong affinity to caves) and accidental or occasional visitors in caves may be explained by local ecological factors [18]. Regions of the world that have rich regional species pools coupled with diverse cave formations and a range of abiotic conditions are expected to have rich assemblages of cave-dwelling visitors and resident species (both troglophiles and troglobites).

1.1. The Levant and Its Caves

The Levant is a diverse biogeographic unit formed by the northeastern African and northwestern Arabian plates and the eastern Mediterranean Levantine basin [19]. Its geographical location at the junction of three continents (Europe, Asia, and Africa), and diversity of habitats and climate zones (Mediterranean, steppe, and arid) lay the foundation for a diverse regional species pool.

The Levant has a diversity of caves that differ in microclimate, age and the type of their rock substrate [20]. The different rock substrates, with intensive karstification in the north of Israel [21], and a climatic gradient from a mesic alpine climate at the north of Israel (Mt. Hermon) to the arid Negev desert in the south of Israel, resulted in a gradient of Karst features with very few or no karst features in the southern Negev desert and Arava. Furthermore, both epigenic and hypogenic caves (formed above or below the water table, respectively) [22,23] can be found in the Levant in basaltic bedrock [24], sandstone, limestone, dolostone, marl, chalk, chert [25–28], and salt rocks [29]. While many of the caves in the Levant are natural, some are manmade, such as burial caves, and some chambers of natural caves were formed or enlarged as part of a secondary use by man [30].

1.2. Spider and Other Arachnid Assemblages in Caves

It is very common to find troglophile arachnids in the entrance of Levantine caves, as in caves of other regions of the world. Arachnids (and among them spiders) are often numerically dominant in caves and are considered dominant predators in many cave foodwebs [31–34]. Of the 11 extant terrestrial arachnid orders, five orders (i.e., Araneae, Opiliones, Palpigradi, Pseudoscorpiones, and Scorpiones) and the polyphyletic sub-class Acari were reported to have troglobite species around the world [35–38], while troglobite species of three orders (i.e., Amblypygi [39], Ricinulei, and Schizomida) were reported only from subtropical and tropical regions [35].

The arachnofauna of caves in the Levant is poorly known in comparison with European caves. In the present study, we investigated the spider assemblages, and specifically the presence of accidentals, troglophile, and troglobite species in eastern Levantine caves (Israel and Palestine) in relation to geographic location, physical characteristics, and ecological zonation (entrance, twilight, and deep) of the caves, as well as the presence of bats and guano in the caves. The data were collected in field surveys between 2013 to 2015. Several new family records for Israel and species new to science discovered in the course of this field survey were reported elsewhere [38–41], including a distribution model for a common Levantine troglophile pholcid spider: *Artema nephilit* Aharon, Huber, and Gavish-Regev (2017) [17], while some are reported here for the first time. Here, we used the data collected in the same ecological field survey to describe the spider assemblages in 35 caves along a rainfall gradient and analyzed the environmental factors shaping these assemblages.

2. Materials and Methods

2.1. Field Surveys

Arachnids were collected from 35 caves located in Israel and Palestine (West Bank) (Figure 1) from three cave ecological zones: the cave entrance (inside the cave in the vicinity of the entrance); the twilight zone (in the intermediate part of the cave when it was applicable); and the inner dark zone (when it was applicable), as well as outside each cave (near the cave entrance). The caves are distributed along the climatic gradient from the Mediterranean (mesic) climate in the north and center of Israel (12 caves for each region, among them 6 caves in Palestine, Figures 1 and 2A–F), to the arid and hyper-arid climate in the south of Israel (11 caves, Figures 1 and 2G–I). Data on the caves is summarized in Appendix A.

In each cave ecological zone, temperature and illumination were measured. The temperature was measured using PicoLite 16-K and a single-trip USB temperature logger (FOURTEC), with measurements taken once an hour for 74–77 days. The illumination was recorded at the time of each sampling using an ExTech 401025 Lux Light Meter. The light meter was positioned on the ground until the reading stabilized for a minimum of 1 min. Measurements inside caves ranged between 0 to 420 lux, while measurements outside caves ranged between 60 to 70,000 lux (Supplementary Table S1). Temperature and illuminance were also measured outside the cave. Caves were assigned to three cave size categories: large (13 caves, more than 50 m with twilight and dark zones), medium (12 caves, 11 to 50 m with twilight zone, lacking a dark zone), and small (10 caves, 10 m or shorter, lacking twilight and dark zones) (Figure 1). Cave size and length were estimated from cave maps when available (Israel Cave Research Center) or in the field, and representing the distance from the cave opening toward the darkest region of the cave we could reach. Elevation and geological data were provided by the GIS (geographic information system) center at the Hebrew University of Jerusalem. Annual average precipitation data was taken from the Israel Meteorological Service (https://ims.gov.il/en/ClimateAtlas, accessed on 1 February 2021) from 1980–2010, which came from the closest meteorological station to each cave (Appendix A).

Each of the 35 caves was sampled according to a specific protocol twice during 2014 (6 March–6 April 2014 and 22 May–22 June 2014). Due to differences in cave morphology (including microhabitat, fractal shape of the substrates, size, and volume) we standardized our sampling effort by time. Therefore, our protocol included a 20-min thorough visual search by one of three experienced arachnologists in 3 to 10 m long sectors using headlamps and UV lights in each cave ecological zones. In the first visit to each cave, most of the arachnid observed were collected by hand for further identification in the lab. In further visits to each cave, we thought some species populations were more sensitive or common, thus finding it possible to identify a species level in situ. Such data were recorded and not collected.

We visited several additional caves outside of the formal 2014 cave survey and collected additional arachnids. On these occasions, only the locality and date were documented for the arachnids. Therefore, we refer to the arachnid species collected in these caves only in the general paragraph of the results and discussion, but not in the analysis of the survey data.

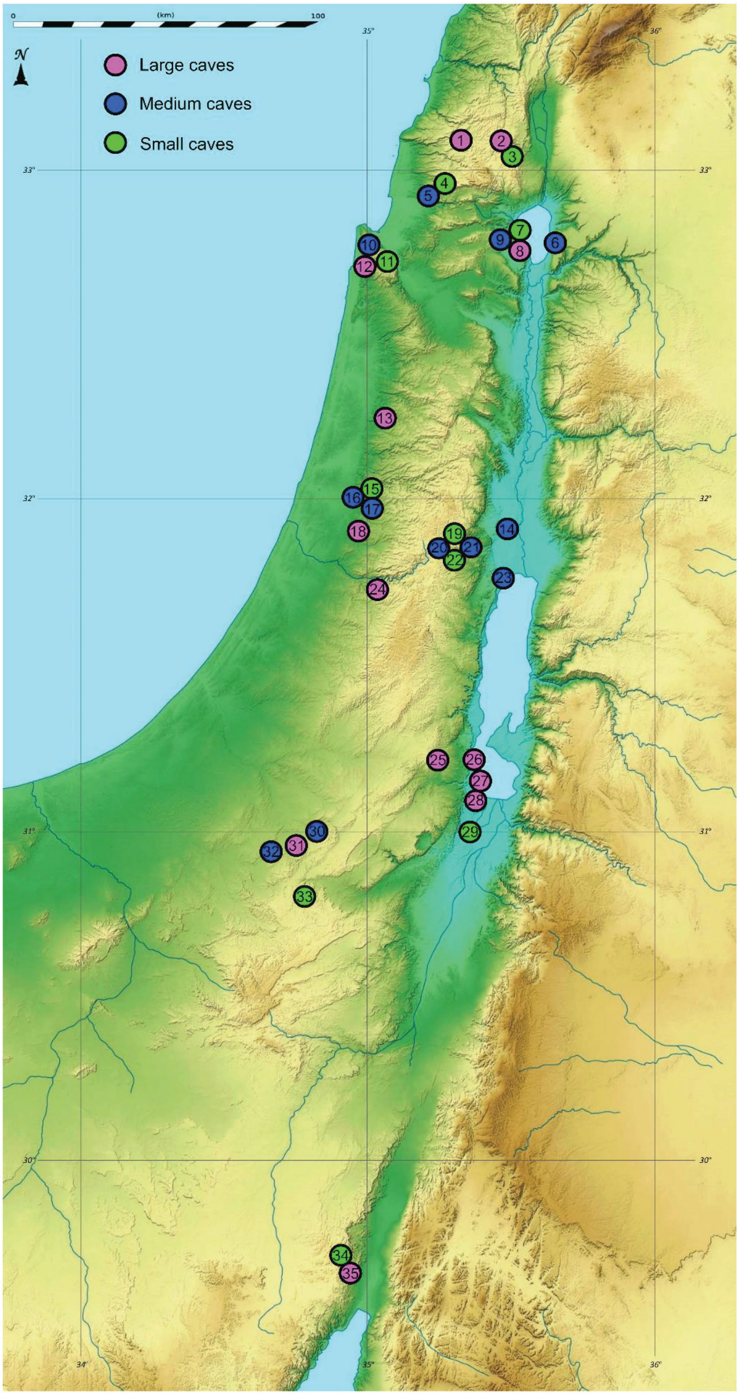

Figure 1. Distribution of the 35 caves. Large caves are represented by pink dots, medium caves by blue dots, and small caves by green dots. Numbers denote a specific cave (see Appendix A).

Figure 2. Caves and habitats from the survey (cave numbers in parentheses). (**A**–**C**): Northern Israel, Mediterranean climate. (**A**): The area near the opening of Yonim cave (upper Galilee, 5). (**B**): The north-facing slope of Oren wadi where Ezba' cave (12) is located (taken from Oren cave, Karmel). (**C**): The south-facing slope of Oren wadi where Oren cave (Karmel, 10) is located. (**D**–**F**): Central Israel and Palestine, Mediterranean and semi-arid climates. (**D**): The area near the opening of Teomim cave (Judean Mountains, 24). (**E**): The area near the opening of Haruva cave (HaShfela, 18). (**F**): The north-facing slope of Perat wadi where 'Perat Inbal cave (cave 1, northern Judean desert, Palestine, 20) is located. (**G**–**I**): Southern Israel, arid climates. (**G**): The east-facing slope where Zavoa cave is located (southern Judean desert, 25). (**H**): The east-facing slope where Arubotayim cave is located (southern Dead-sea region, 26). (**I**): The area where Ammude Amram caves are located (Arava valley, 34–35). Pictures: (**A**–**C**,**F**–**I**) Shlomi Aharon, (**D**,**E**) Igor Armiach Steinpress.

2.2. Statistical Analysis

Generalized linear models (GLMs) were used to test the effect of the geographical region (north, center, and south of Israel) on spider richness (Poisson distribution and log link) and abundance (normal distribution and log link) using the entire dataset of troglobite, troglophile, and accidental spider species.

Using a set of GLMs with binomial distribution (logit link), we tested separately for each spider guild [42] the probability that a spider species observed in a cave belongs to the particular spider guild, and whether the probability changes in relation to the chosen environmental variables. Geographic region can encompass different environmental variables. In Israel, there is a strong north to south gradient of several correlated climatic variables such as precipitation, air humidity, evaporation, and air temperature. We added the presence of bats in caves as an additional explanatory variable as bat activity changes the habitat for other cave dwellers, such as spiders. To further test for such effects, we included an alternative model in which, instead of bat presence, we used an estimation of guano level on the cave floor from our field assessment, i.e., none, low (when less than 15% of the cave floor had guano deposition), medium (when 15% to 40% of the cave floor had guano deposition), or high (when more than 40% of the cave floor had guano deposition).

Two multivariate analyses methods were performed in order to better understand the factors affecting the assemblage composition of troglobite and troglophile species: hierarchical clustering, using R v.4.0.3. [43] and a direct CCA ordination (canonical correspondence analysis), using Canoco [44,45]. For the cluster analysis, pairwise Pearson correlation between the cave communities was determined using the 'cor' function with the 'pairwise.complete.obs' method, and the output hierarchically clustered as a Euclidean distance matrix with an 'average' linkage using the 'hclust' function. The resultant clustering was plotted using the 'heatmap.2′ function of the 'gplots' package [46], with scaling by row. For the CCA ordination, we used the unrestricted Monte Carlo permutation tests (4999 runs) and forward selection, testing 9 explanatory environmental variables: geographic location (latitude and longitude, continuous), elevation (continuous), cave estimated length (continuous), minimum temperature in the cave during the survey (continuous), average annual precipitation (continuous), rock category of the cave (6 categories: basalt, carbonate rocks, chalk, marlstone, salt, and sandstone), minimum age of the rock (continuous), bat inhabitance (three categories: no bats, insectivorous, frugivorous), and guano level (four categories: no guano, low level, medium level, and high level of guano).

For both multivariate analyses, only 34 of the 35 caves were used, as 'Ammude 'Amram (a small cave, 34) had two Solifugae individuals, but no spiders were found in our two visits. We restricted the statistical analyses to troglobite and troglophile species ($N_{species}$ = 32), omitting the accidental species ($N_{species}$ = 30). The latter were epigeic species that occurred in low abundance and were scattered among the different caves.

3. Results

3.1. Cave Arachnofauna from Field Surveys and Additional Visits to Caves

A total of 1132 arachnids were found during the surveys, comprised mainly of species of the order Araneae (1054 individuals). However, representatives of additional arachnids were also found (Figure 3), i.e., Acari (65 individuals, among them two soft-tick species (Argasidae) *Argas vespertilionis* (Latreille, 1796) (Figure 3A) and *Ornithodoros tholozani* Laboulbène and Mégnin, 1882 (Figure 3B), as well as hard-ticks (Ixodidae)); Pseudoscorpiones (four individuals, awaiting identification, Figure 3C,D); Amblypygi (although many individuals were observed, only three were collected: two Charinidae species *Charinus ioanniticus* (Kritscher, 1959) and *Charinus israelensis* Miranda, Aharon, Gavish-Regev, Giupponi, and Wizen, 2016, Figure 3E) [39]; Scorpiones (although more individuals were observed, we were able to catch only three juveniles (Buthidae)); Solifugae (two juveniles; identification was not possible) and one Opiliones of the species *Mediostoma haasi* (Roewer, 1953) (Nemastomatidae).

In other visits to several additional caves outside of the 2014 survey, we found one additional Opiliones species, the troglobitic eyeless *Haasus naasane* Aharon et al., 2019 (Pyramidopidae, Figure 3F) [38], an Opilioacaridae species (in preparation, Figure 3G, [47]), Palpigradi species (in preparation, Figure 3H, [47]), and one spider family that was not reported before from Israel: Nesticidae (the invasive species *Eidmannella pallida* (Emerton, 1875) and an additional unidentified species, both reported here for the first time).

The 1054 spiders found during the 2014 survey in 34 of the 35 caves were identified to 62 species and morphospecies in 38 genera and 22 families (Supplementary Table S2). As in many faunistic surveys, the Levantine cave spider rank-abundance curve was skewed, with nine species that have 33–152 individuals each and account for 72% of the overall abundance (762 individuals out of 1054), 29 singleton, and doubleton species (42 individuals out of 1054). Moreover, 24 species have 3–28 individuals each (249 individuals out of 1054) (Figure 4). Of the 62 species, 46 are either species with valid taxonomic status (26 species), are in the process of description (six species), or need further taxonomic study (14 species: eight linyphiids, two theridiids (*Steatoda* Sundevall, 1833), two pholcids (*Pholcus* Walckenaer, 1805 and *Spermophorides* Wunderlich, 1992), one theraphosid (*Chaetopelma* Ausserer, 1871), and one oonopid (*Megaoonops* Saaristo, 2007)). The rest (16 morphospecies) were not adults and could be identified only to the genus or family level based on morphology (Supplementary Table S2).

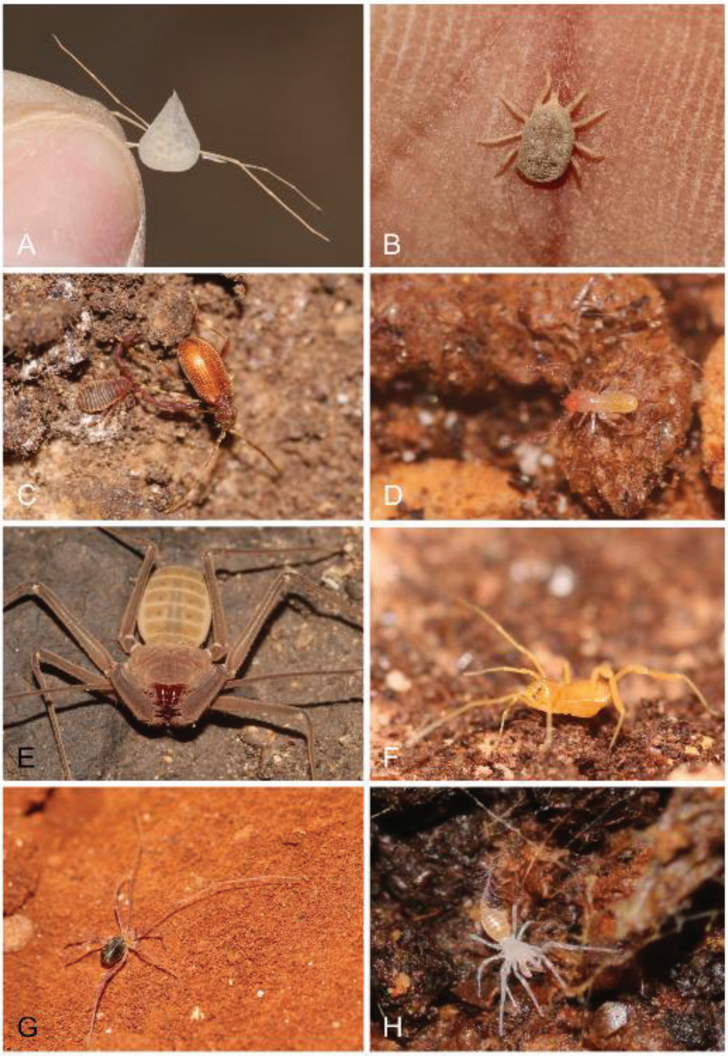

Figure 3. Arachnids found during cave surveys. (**A**): *Argas vespertilionis* (Latreille, 1796), (Acari, Argasidae); (**B**): *Ornithodoros tholozani* Laboulbène and Mégnin, 1882, (Acari, Argasidae); (**C**,**D**): Pseudoscorpiones; (**E**): *Charinus israelensis* Miranda, Aharon, Gavish-Regev, Giupponi, and Wizen, 2016, (Amblypygi, Charinidae); (**F**): *Haasus naasane* Aharon et al., 2019 (Opiliones, Pyramidopidae); (**G**): Opilioacaridae (Acari); (**H**): *Eukoenenia* Borner, 1901 (Palpigradi, Eukoeneniidae). Pictures: Shlomi Aharon.

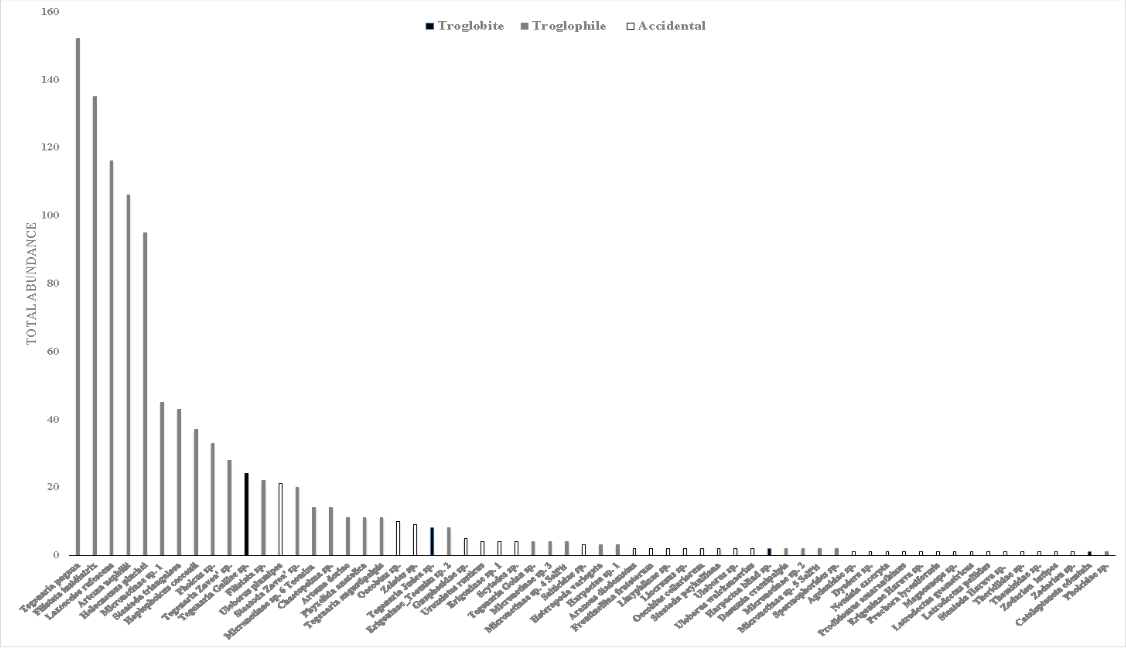

Figure 4. Rank-abundance graph of 62 spider species from 34 Levantine caves (‘Ammude ‘Amram small cave (34) had no observed spiders during our two visits).

3.2. Cave Geographic Region, Bat Presence and Spider Richness and Abundance

The geographical region of the caves significantly affected spider richness ($N_{species}$ = 62; $N_{individuals}$ = 1054, $\chi_2^2 = 27.41,\ p < 0.001$). Specifically, spider richness in the southern region was significantly lower than in the other geographical regions monitored (Figure 5A, Table 1). Bat presence did not significantly affect spider richness $\left(\chi_1^2 = 0.002,\ p = 0.968\right)$. Similarly, a non-significant effect was found when the estimate of guano level was used (guano: $\chi_2^2 = 3.845,\ p = 0.279$). Spider abundance followed a similar trend as richness, showing a significant effect of geographic region on abundance ($\chi_2^2 = 7.127,\ p = 0.028$), with fewer individuals detected in southern caves (Figure 5B, Table 1), and no significant effect of bat presence $\left(\chi_1^2 = 1.884,\ p = 0.170\right)$). The similar model with bat guano level was also not significant (geographical region: $\chi_2^2 = 6.328,\ p = 0.487$; guano level: $\chi_2^2 = 2.438$, $p = 0.487$).

Table 1. Spider species richness and abundance (troglobite, troglophile, and accidentals) in caves by region (35 caves). Numbers denoted a specific cave (see Appendix A).

	South (11 Caves)	Center (12 Caves)	North (12 Caves)
Spider species richness (minimum, median, maximum; mean)	0 [1], 2, 8 [2]; 2.27	4 [3], 7, 10 [4]; 7.08	5 [5], 6, 14 [6]; 6.91
Spider abundance (minimum, median, maximum; mean)	0 [1], 9, 56 [2]; 14.73	17 [7], 34, 51 [8]; 33.75	8 [5], 40, 86 [9]; 40.58

[1] ‘Ammude ‘Amram (small cave, 34); [2] Zavoa’ (25); [3] Qumeran (23); [4] Andartat HaBiqa’ (14); [5] Raqqit (11); [6] Yonim (5); [7] Perat cave # 4 (19); [8] Tinshemet (17); [9] Berniki (medium cave, 9).

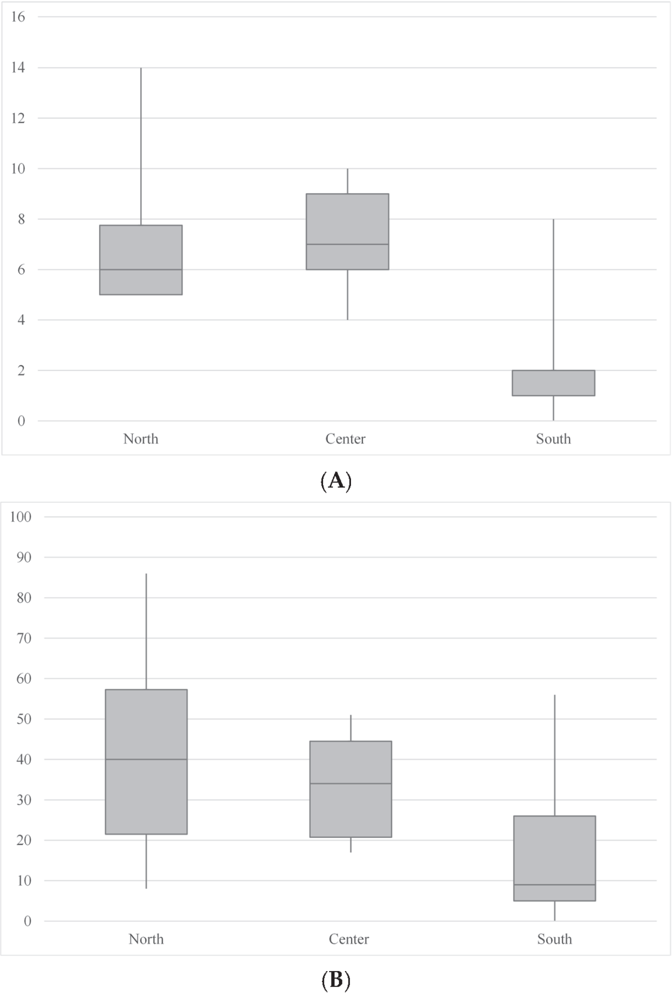

Figure 5. Box plot of (**A**) species richness and (**B**) abundance of troglobite, troglophile, and accidental spiders in 35 caves at the north, center, and south of Israel (minimum, first quartile, median, third quartile, and maximum).

3.3. Troglobites and Troglophiles

We assigned each of the 62 spider species in our survey to one of three categories: troglobite, troglophile, and accidental, according to their distribution and the known use of caves as a habitat in Israel and Europe [33]. We also categorized them based on troglomorphic phenotype. This resulted in a list of 32 troglophile and troglobite spider species inhabiting caves in our region and 30 accidental spider species (Supplementary Table S2). Only three spider families included troglobite species: Agelenidae, with two eyeless species found during visits to several additional caves outside of the 2014 survey, and two eye-reduced species in the genus *Tegenaria* Latreille, 1804; Dysderidae with one eyeless species in the genus *Harpactea* Bristowe, 1939, which may be a species complex based on our preliminary morphological study; and Leptonetidae, with one species *Cataleptoneta edentula* Denis, 1955 (Figures 6 and 7) [41].

Figure 6. Troglobite spiders found during cave surveys (cave numbers in parentheses). (**A**,**B**): *Tegenaria* Latreille, 1804 (Agelenidae). (**A**): *Tegenaria* from Yir'on large cave (upper Galilee, 2); (**B**): *Tegenaria* from Teomim large cave (Judean mountains, 24); (**C**) *Harpactea* Bristowe, 1939 (Dysderidae) eyeless species from Shetula large cave (upper Galilee, 1); (**D**): *Cataleptoneta edentula* Denis, 1955 (Leptonetidae) from Ezba' large cave (Karmel ridge, 12). Pictures: Shlomi Aharon.

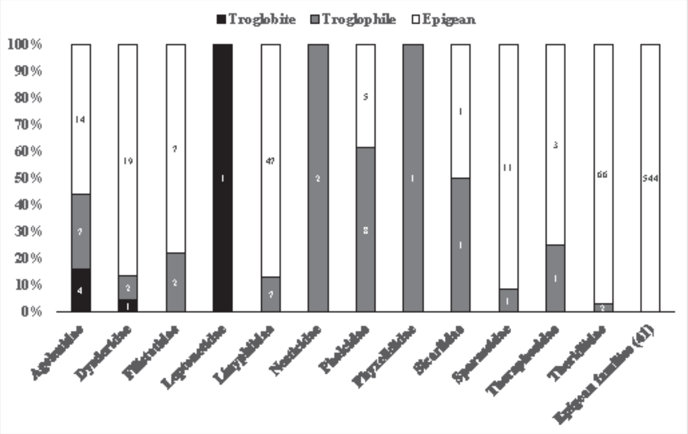

Figure 7. Proportion and number of troglobite, troglophile, and epigean spider species per family in Israel and Palestine (53 families and 758 species including undescribed species, description in progress). Families that have only epigean species were pooled in one column.

In total, 11 spider families in Israel and Palestine included troglophile species (Figures 8 and 9). Pholcidae has 8 troglophile species and is family with the largest number of troglophile species in Israel and Palestine (Figure 7). It includes some abundant species found in caves along the north–south climatic gradient (Figure 8): *Artema nephilit* Aharon, Huber and Gavish-Regev 2017 (Figure 8A) [9,40], *Hoplopholcus cecconii* Kulczyński, 1908 (Figure 8B), *Holocnemus pluchei* Scopoli, 1763 (Figure 8C), and a species in the genus *Pholcus* Walckenaer, 1805 (Figure 8D). However, the three most abundant troglophile species are *Tegenaria pagana* C. L. Koch, 1840 (Agelenidae, Figure 8E), *Filistata insidiatrix* (Forskål, 1775) (Filistatidae, Figure 8F), and *Loxosceles rufescens* (Dufour, 1820) (Sicaridae, Figure 8G), respectively (Figures 4 and 8). We found additional troglophile species (Figures 4 and 7–9) in the families Theridiidae (*Steatoda triangulosa* (Walckenaer, 1802), Figure 8H), Linyphiidae (six or seven species of the sub-family Micronetinae, Figure 9A–D), Theraphosidae (*Chaetopelma* Ausserer, 1871 species, Figure 9E), Phyxelididae (*Phyxelida anatolica* Griswold, 1990, Figure 9F, [41]), Sparassidae (*Heteropoda variegata* (Simon, 1874), Figure 9G), Dysderidae (*Harpactea* Bristowe, 1939, Figure 9H), and Nesticidae. Supplementary Table S2 includes the full list of the spider species with their localities and distribution.

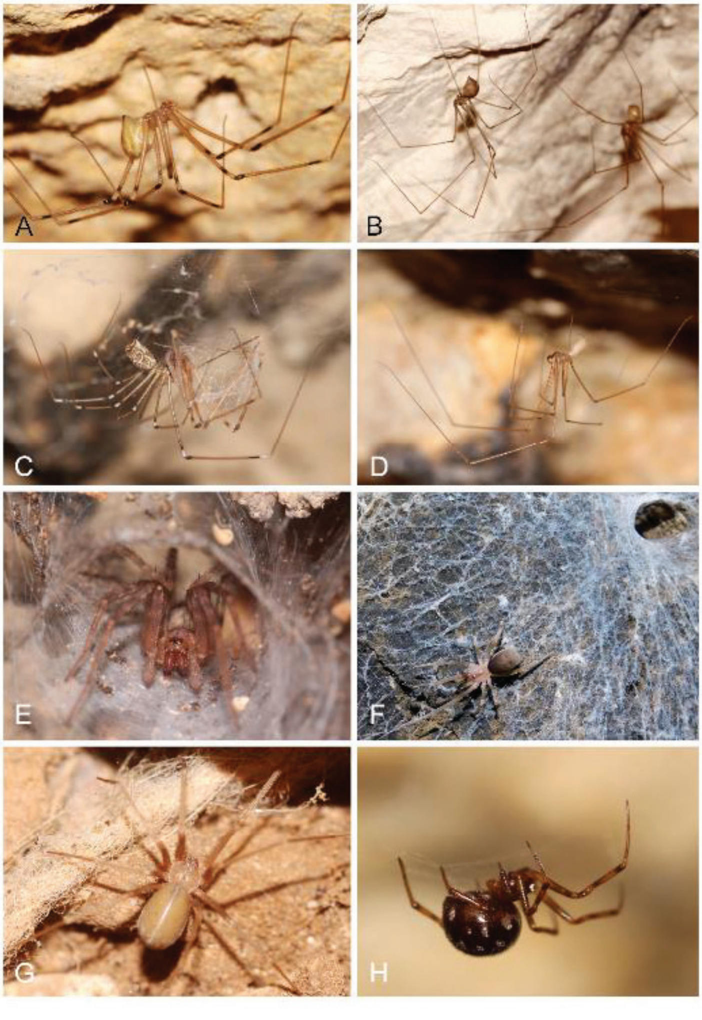

Figure 8. Eight of the nine most abundant troglophile spiders found during cave surveys (cave numbers in parentheses). (**A**–**D**): Pholcidae: (**A**): *Artema nephilit* Aharon, Huber and Gavish-Regev 2017 from Oren medium cave (Karmel ridge, 10); (**B**): *Hoplopholcus cecconii* Kulczyński, 1908 from Yir'on large cave (upper Galilee, 2); (**C**)*: Holocnemus pluchei* Scopoli, 1763 from a cave in Perat wadi (northern Judean desert, 19–22); (**D**): *Pholcus* Walckenaer, 1805 from 'Inbal cave 1 in Perat wadi (northern Judean desert); (**E**): *Tegenaria pagana* C. L. Koch, 1840 (Agelenidae) from Teomim cave (Judean mountains); (**F**): *Filistata insidiatrix* (Forskål, 1775) from 'Inbal cave 1 in Perat wadi (northern Judean desert, 20); (**G**): *Loxosceles rufescens* (Dufour, 1820) (Sicaridae) from Berniki caves (lower Galilee, 7–9); (**H**): *Steatoda triangulosa* (Walckenaer, 1802) (Theridiidae) from Tinshemet cave (western Samaria, 17). Pictures: (**A**–**E**,**G**,**H**): Shlomi Aharon, (**F**): Igor Armiach Steinpress.

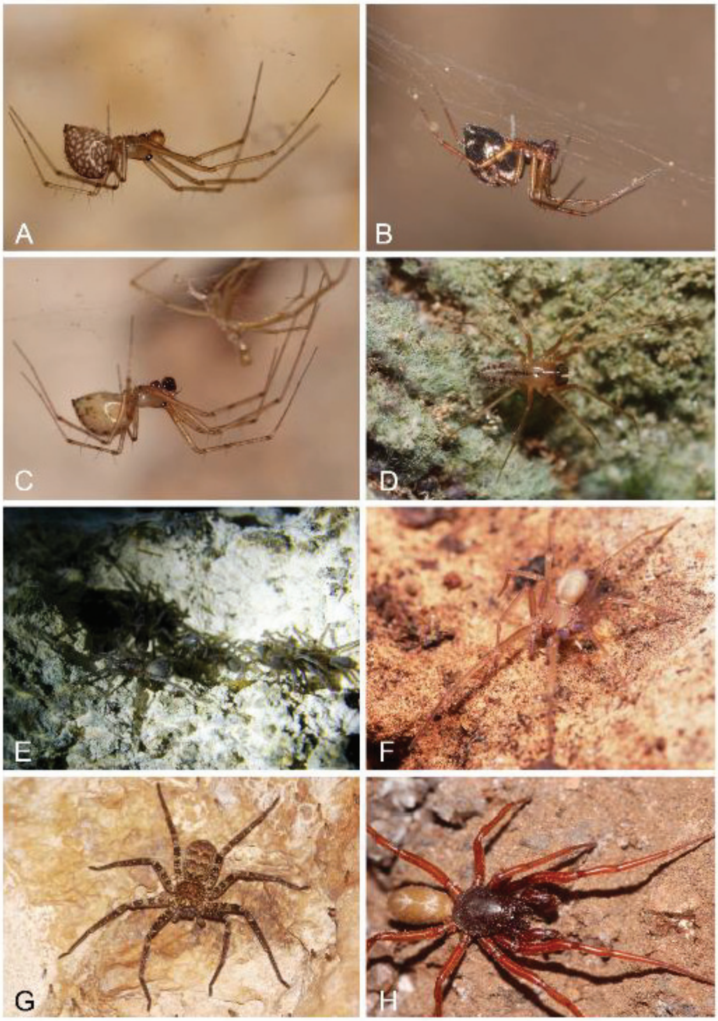

Figure 9. Additional troglophile spiders found during cave surveys. (**A**–**D**): Linyphiidae, Micronetinae: (**A**): Male micronetine sp. 1 form Besor medium cave (Negev desert, 32); (**B**): Male micronetine sp. 6 from Teomim large cave (Judean mountains, 24); (**C**): Male micronetine sp. 1? from Bet 'Arif medium cave (western Samaria, 16); (**D**): Male micronetine from 'Avedat cave (Negev desert, 33); (**E**): *Chaetopelma* Ausserer, 1871 (Theraphosidae) from Modi'in cave (Judea, not part of the survey); (**F**): *Phyxelida anatolica* Griswold, 1990 (Phyxelididae) from Haruva cave (HaShfela, 18); (**G**): *Heteropoda variegata* (Simon, 1874) (Sparassidae) from Yir'on large cave (upper Galilee, 2); (**H**): *Harpactea* Bristowe, 1939 (Dysderidae) from Ezba' large cave (Karmel ridge, 12). Pictures: (**A**–**D**,**F**–**H**): Shlomi Aharon, (**E**): Igor Armiach Steinptress.

3.4. Spider Foraging Guilds

The spiders found in the cave survey represent six foraging guilds [42]: sheet-web weavers, sensing-web weavers, space-web weavers, orb-web weavers, ambush hunters, and other hunters (Supplementary Table S2). Three out of four troglobite species and 24 out of the 28 troglophile species are web-builders (sheet-web: Agelenidae, Linyphiidae, Phyxelididae; space-web: Leptonetidae, Pholcidae, Theridiidae; sensing-web: Filistatidae, Theraphosidae), and only one troglobite and four troglophiles are hunters (Dysderidae, Sicariidae, Sparassidae).

GLM analyses of the geographic region and bat presence or guano level showed that neither factor in the two alternative models was significant (Table 2).

Table 2. GLM analyses of the effect of environmental variables on the probability to detect specific spider guilds in the caves. Wald χ^2 and p values are shown for the two models. Neither geographic region nor bat presence (or guano level) was a significant explanatory variable for any of the guilds.

			Ambush Hunters		**Specialists and Other Hunters**		**Sensing Web**		**Sheet or Space Web**		**Orb Web**	
		Df	**Wald χ^2**	***p*-Value**	**Wald χ^2**	***p*-Value**	**Wald χ^2**	***p*-Value**	**Wald χ^2**	***p*-Value**	**Wald χ^2**	***p*-Value**
Model 1	Geographic region	2	2.144	0.342	2.963	0.227	0.706	0.703	4.440	0.109	1.733	0.420
	Bat presence	1	0.024	0.877	0.394	0.530	0.018	0.892	0.065	0.798	0.001	0.979
Model 2	Geographic region	2	2.047	0.359	4.146	0.126	0.314	0.855	3.776	0.151	1.729	0.421
	Guano amount	3	0.586	0.900	3.021	0.388	0.609	0.894	0.277	0.964	0.339	0.953

3.5. Levantine Cave Spider Assemblages: Similarities and Environmental Variables from Field Survey

We used only the 32 troglobite and troglophile spider species to further analyze the spider assemblages in this cave survey (34 caves). We identified five groups of caves that cluster together based on the correlation between their species assemblages (Figure 10). The most noticeable clusters are based each on one of five dominant (*T. pagana*, *F. insidiatrix*, and *A. nephilit*) and sub-dominant (*S. triangulosa* and *H. cecconii*) troglophile species. One cluster was distinctive from the others by most sharing a single species, *A. nephilit*. This cluster includes caves in arid climates, all of the Dead Sea caves (caves 23, 26–29), one cave from the Negev desert (cave 33), and one cave from the Arava (cave 35). Another cluster was based on *F. insidiatrix* (with either *L. rufescens* or *H. pluchei*), with three upper Galilee caves (caves 3, 5, 8) and four caves from the Judean desert, Galilee, and Karmel (caves 9–10, 20, 22), respectively. All caves in another cluster (which could be divided to three sub-clusters) had *T. pagana* in them (caves 7, 12–13, 15–18, 21). The fourth cluster was based on *S. triangulosa* and includes two caves from the Negev desert (caves 30–31) and a cave from the Karmel (cave 11). Two caves from the upper Galilee (caves 1–2) form the fifth cluster based on *H. cecconii*.

A CCA ordination showed five significant variables explaining 91.4% cumulative percentage variance of the species–environment relation: precipitation (F-ratio = 2.52, $p = 0.0002$), elevation (F-ratio = 2.37, $p = 0.0002$), latitude (F-ratio = 2.03, $p = 0.0032$), minimum temperature (F-ratio = 1.79, $p = 0.0302$), and high guano level (F-ratio = 1.87, $p = 0.0266$) (Figure 11, Table 3). Additional CCA ordination analyses were done using all 62 species (including the accidental species): (1) with 34 caves and the three different ecological zones within the caves (67 samples all together), and (2) with 34 caves and without cave ecological zones, and found similar results. In analysis (1), precipitation, elevation, latitude, minimum temperature bats, and guano level were significant, as were some of the rock categories. Moreover, in analysis (2), precipitation, latitude, and guano level were significant, but not elevation and minimum temperature (see supporting information Figure S1, Tables S3 and S4).

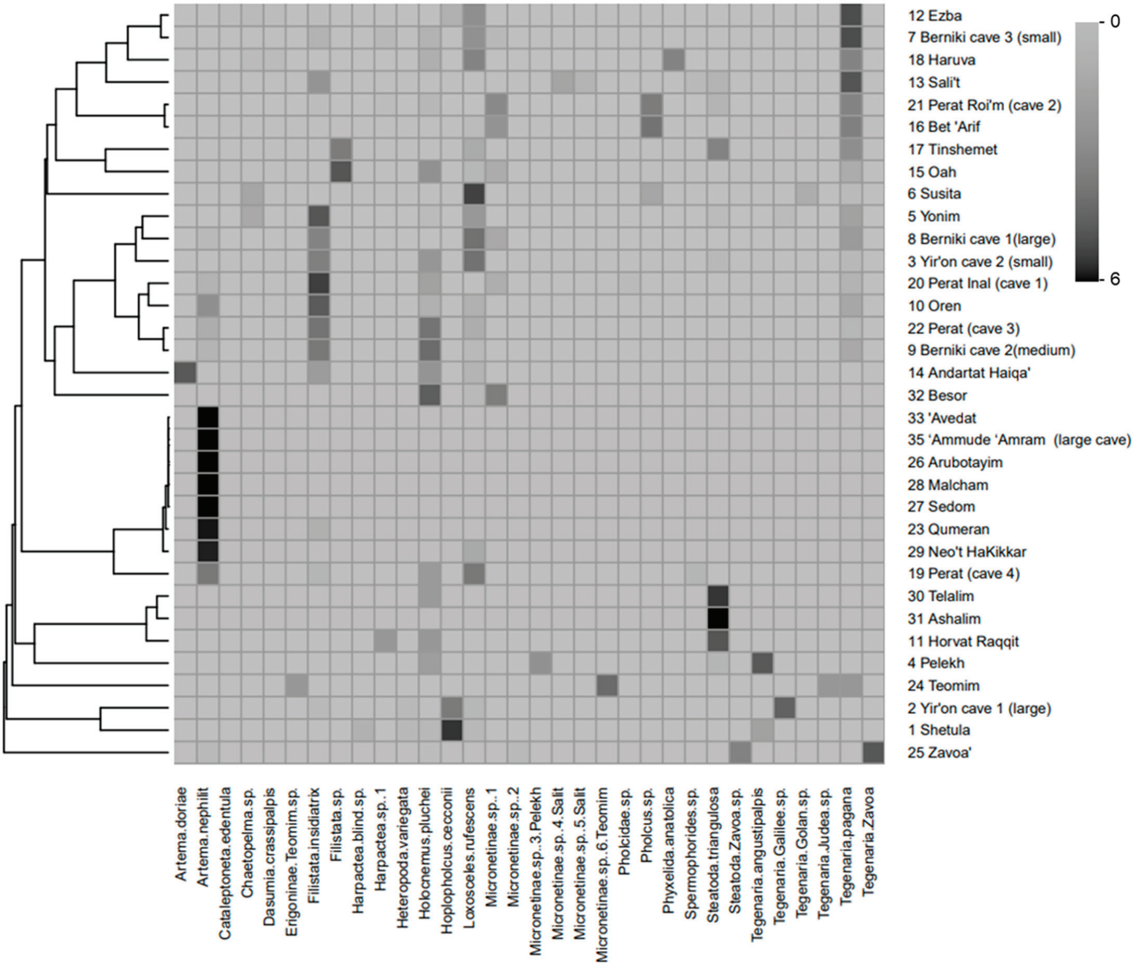

Figure 10. Cluster heatmap for 34 caves (rows) and 32 troglobite and troglophile species (columns) ('Ammude' Amram (cave 34) had no observed spiders during our two visits). Caves are clustered using hierarchical clustering from pairwise Pearson correlation. Darker colors signify higher correlation. Numbers before cave names as in Figure 1 and Appendix A.

Table 3. CCA ordination results.

	Eigenvalues	Species-Environment Correlations	Cumulative Percentage Variance of Species Data	Cumulative Percentage Variance of Species—Environment Relation	Sum of All Eigenvalues	Sum of All Canonical Eigenvalues
Axis 1	0.642	0.917	8.9	31	7.195	2.070
Axis 2	0.589	0.831	17.1	59.5		
Axis 3	0.448	0.839	23.3	81.1		
Axis 4	0.214	0.725	26.3	91.4		

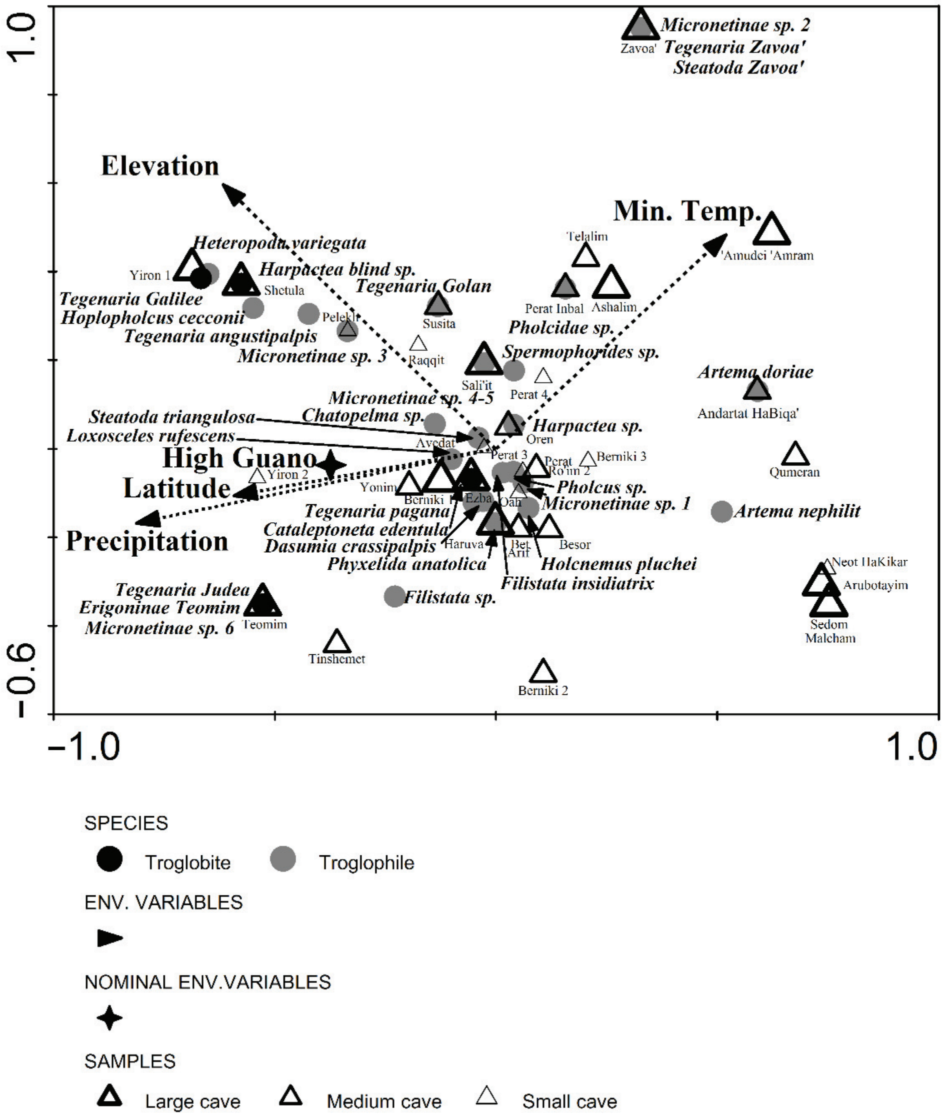

Figure 11. CCA ordination graph of the first and second axes testing 34 caves and 32 troglobite/troglophile species. The significant explanatory variables (precipitation, elevation, latitude, minimum temperature, and high guano level) are plotted on the graph, as well as the cave and species names. Cave sizes are represented by large, medium, and small triangles, respectively, troglobite species by black points, troglophile species by grey points, nominal explanatory variable by a black star, continuous explanatory variables by arrows with the dashed line. The non-dashed line arrows are used to connect between species name and its centroid.

4. Discussion

4.1. Levantine Cave Arachnofauna

We discovered many unique arthropods, including troglobite [38,39] and troglophile [40,41] arachnids and species endemic to our region. Representatives of the orders Amblypygi, Palpigradi, and Pseudoscorpiones were found only in mesic caves in the Mediterranean region, while Opilioacaridae were found in caves in the semi-desert region. Spiders were the most abundant and diverse arachnid order in the caves studied. Currently, 53 spider families, 297 genera, and 758 species are known from Israel ([48], unpublished data). Of them six troglobite species in three families (Agelenidae (two from the survey and two from caves that were not included in the 2014 survey), Dysderidae, and Leptonetidae), and 34 troglophile species in 11 spider families (Figure 7).

4.2. Troglobites and Troglophiles

Based on our results, 20% of the families known from Israel and Palestine include troglobite and/or troglophile species, compared to 34% in Europe (22 out of 64 families reported from Europe [33,49]). From our current data, only about 5% of the spider species known from Israel and Palestine are cave-dwellers (40 species) with approximately 2% endemics. In 2018, Mammola et al. reported a total of 486 cave-dwelling spider species in Europe (195 troglobite and 291 troglophile species) [33], with 90% of them considered endemics of single countries. In the well-studied cave arachnofauna of Slovenia [50,51], for example, 30% of families (out of 43 families) include troglobite or troglophile species, representing about 11% of species known in Slovenia (N = 753). Some of the families with troglobite and troglophile species in Slovenia [33,50] also occurred in our cave survey in Israel and Palestine (Ageleindae, Dysderidae, Leptonetidae, Lyniphiidae, Nesticidae, Pholcidae, and Theridiidae). Linyphiidae had the greatest number of cave-dwelling spider species in Slovenia, with 48 cave-dwelling species out of 221 linyphiid species in total, followed by Dysderidae with seven cave-dwelling species (22 species in total), Agelenidae with seven cave-dwelling species (25 species in total), and Pholcidae with five cave-dwelling species (five species in total). Our survey uncovered a potentially high species richness of troglophiles and troglobites in the same four families as in Slovenia, but with a different order of richness: Agelenidae, Pholcidae, Linyphiidae, and Dysderidae.

Preference of shaded habitats is a common feature of the four most abundant troglophile spider species in our caves. Placing those four species on a cave-affinity continuum provides a better understanding of their distribution in caves. *Loxosceles rufescens* is an opportunistic synanthropic species common in the Mediterranean basin in houses and other manmade habitats (and introduced to other areas around the world) [52]. Although it can be found in shaded natural habitats such as caves and under stones [33], and is very common in caves as reported above, it is not a cave specialist. *Artema nephilit* and *F. insidiatrix* have a higher affinity to caves than to other habitats (Supplementary Table S2, personal observation). Although *A. nephilit* can be found also under boulders, in crevices, and in basements, *F. insidiatrix* can be found in other natural shaded habitats and they each have preference for cave entrances [9,33,49]. Of these four troglophile species, *T. pagana* has the highest affinity to caves and can be found in cave entrance and twilight zones in large numbers. Similar to some other troglophiles, *T. pagana* could also be found in other suitable natural shaded habitats, but it is much more common in caves than in any other shaded habitat in Israel and Palestine (Supplementary Table S2, personal observation, Aharon et al., in preparation).

4.3. Foraging Guilds

Among the cave-dwelling spiders we found, web-builders are more species-rich than hunters (27 vs. five species, respectively; Supplementary Table S2). The four most abundant troglophile spider species in our caves represent four out of the six foraging guilds found: sheet-web weavers (*T. pagana*), sensing-web weavers (*F. insidiatrix*), ambush hunters (*L. rufescens*), and space-web weavers (*A. nephilit*). Higher species richness of web-builders vs. hunters was also found in caves in the Iberian peninsula [53], while ambush hunters and sensing-web weavers were absent from these caves [32,53]. A single species of each of these guilds was abundant in the Levantine caves (*L. rufescens* and *F. insidiatrix*, respectively). The troglobite species of the Levantine caves are represented mainly by sheet-web weavers (Agelenidae), one space-web weaver (Leptonetidae), and one hunter species (or species complex) (Dysderidae). Although the spider family Dysderidae has species that specialize on isopods [42], the diet preferences of the dysderids in caves is unknown [53]. Specifically, we lack diet information on the eyeless and eye-bearing *Harpactea*, eye-bearing *Dysdera, and Dasumia crassipalpis* (Simon, 1882) found in the Levantine caves, and therefore we assigned them to the guild of 'other hunters'.

4.4. Species-Pool, Regions, and Bat Inhabitance

The cave-dwelling spiders of the Levant represent a subset of the regional species-pool, with a maximum of 58 species (the 62 that were found in caves without the four troglobite species) that are known or potentially can be found in epigean habitats. Only two spider families, Leptonetidae and Nesticidae, were found only in hypogean habitats in Israel, while 29 spider families were found only in epigean habitats in Israel, and 22 spider families were found both in hypogean and epigean habitats in Israel (including troglobites, troglophiles and accidentals). This distribution is similar to in agreement with findings in other places of the world [32,53].

Caves in the southern region of Israel had relatively low species richness, with no troglobite arachnids and few troglophile spider species. This desert region, covering about 55% of Israel, harbors many epigean spider species that are adapted to the desert climate (at least 246 according to Zonstein and Marusik [48]) and are rarely found in caves. By contrast, all troglobite arachnids and most troglophile spiders were found in caves located in the Mediterranean mesic climate zone, mainly in the north of Israel but also in central Israel and Palestine (Figures 1, 2 and 7A,B). This could be explained by both the higher precipitation (see results and Figure 11) and intensive karstification in the north of Israel [21], i.e., more caves with regional mesic climate may support higher pre-adapted cave-dwelling species. Our analysis found that geographic region is a significant factor effecting both species richness and abundance in caves, and that precipitation and latitude correlated and had a significant effect on the assemblages (Figure 11).

Our preliminary taxonomic study revealed phenotypic variation between populations of different caves in *Tegenaria*, *Harpactea,* and the micronetine spiders (Linyphiidae). For some of these spiders, morphological variation was found to be lower within cave populations in comparison to between different caves, and these unique morphological characters are sufficient for describing new species. For other spiders, molecular methods were used to assess cryptic species and possible species complexes. Therefore, our future taxonomic efforts will focus on describing the troglobitic and troglophilic species new to science that have mainly been found in caves in the Mediterranean climate region.

One cave in the arid region, Zavoa cave, hosted an exceptional number of spider species (eight species) and individuals (56 individuals) (Figures 1, 2G and 11, Appendix A). Zavoa cave is situated in the southern Judean desert, with an average of 100 mm rainfall a year, and harbors several cave-dwelling species that may be endemics. One additional cave that was not part of our cave survey, A'rak Na'asane, in the northern Judean desert, is home to several troglobite and troglophile endemic arachnids [38,54]. Both Zavoa and A'rak Na'asane are large caves located in a region that experienced aridification during the last glacial interval [55]. Both caves are inhabited by bat colonies. A'rak Na'asane has a high level of guano, while the Zavoa cave has less guano due to the currently lower numbers of bats. We visited several additional caves in the Judean desert, outside the 2014 survey, and found opilioacriformes and other troglophile arachnids as well as evidence of former use of the caves by bats (dry old guano), but it seems that the bats no longer occupy these caves (unpublished data).

Trajano and de Carvalho (2017) suggested that troglophile species richness could be explained by local ecological factors [18]. Guano is probably an important energy source for cave arthropods and may have a bottom-up effect on the cave food-web, as was shown in caves in Brazil [56,57]. One of our hypotheses was that the level of guano in the cave will positively affect spider richness and abundance, but we did not find this to be significant, but we did find a significant effect of the guano level on the spider assemblage composition in caves (Figure 11, Figure S1, Tables S2 and S3). To test the effect of guano on the Levantine cave spider assemblages in more depth, more information is needed on the ecology of spiders in these caves.

4.5. Levantine Cave Spider Assemblages

We showed that Levantine cave spider assemblages are diverse and are affected by the specific geographic location and its climatic characteristics, as well as by the presence of bats and guano level. While dry caves in the desert climate are dominated by pholcid and theridiid spiders, humid (but not wet) caves in the mesic Mediterranean climate region are dominated by agelenid, filistatid, sicariid, and linyphiid spiders. Dysderid, leptonetid, nesticid, and theraphosid spiders were found only in the mesic Mediterranean climate caves. We could not find effects of the estimated age of the cave, its estimated size, nor the cave ecological zone on spider assemblages. In one analysis (see supporting information), we found a significant effect of the surrounding rock type on the spider assemblage, but most of our caves are located in carbonate rocks (27 of 35) and more samples from basaltic, chalk, marlstone, salt, and sandstone caves are needed in order to have a more balanced analysis (see Appendix A). Additionally, we cannot separate between the geographic location of the cave and its surrounding rock, as some rocks are found solely in one region.

5. Conclusions

To conclude, Levantine cave spider assemblages are diverse, with higher species richness and abundance in caves located in the north of Israel. They tend to have high levels of bat guano and high humidity. We suggest that future research on Levantine cave arachnids focus on caves in the mesic Mediterranean climate region, caves with bats, and caves in arid climate regions that have chambers with high humidity. Our faunistic cave survey is the first and crucial stage in understanding the evolutionary and ecological mechanisms of speciation of arachnids in Levantine caves. Our discoveries contribute to the knowledge of the local arachnofauna in general and are important for conservation of these cave ecosystems. By comparing spider assemblages of Levantine caves to European caves, we identified gaps in the taxonomic research, and expect to focus our efforts on spider families that may have additional cryptic or undescribed cave-dwelling spider species: Agelenidae, Dysderidae, Linyphiidae, and Pholcidae.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13050179/s1, Figure S1: CCA ordination graph of the first and second axes testing 34 caves with ecological zones (total 67 samples), and 62 spider species. The significant explanatory variables (precipitation, elevation, latitude, no bats, frugivorous bats and insectivorous bats, low, medium and high guano levels, carbonate and salt rocks) are plotted on the graph, as well as the cave, ecological zone, and species names. Cave sizes are represented by large, medium, and small triangles, respectively; troglobite species by black points; troglophile species by grey points; accidental by empty points; nominal explanatory variable by a black star; continuous explanatory variables by arrows with dashed line, while non-dashed line arrows are used to connect between species name and its centroid; Table S1: LUX values for caves and caves ecological sectors (first visit; second visit). “No“ denote that the ecological sector was missing due to cave size, “Missing” denote a measurement that was not taken. * measurement was taken just before sunset; Table S2: Full list of the spider species with their localities, distribution and guilds; Table S3: CCA ordination results (67 samples), and 62 spider species); Table S4: CCA values for significant environmental variables

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Appendix A

District	Geographic Region	Cave	N	E	Elevation	Climate Type	Precipitation (mm)	Cave Minimum Temp. (E, T, D)	Lithology Cate-gory	Lithology Geological Age	Cave Size	Length Estimate	Bat	Guano Level	Spider Abundance, Richness
North	Upper Galilee	1 Shetula (P)	33.0873	35.3169	690	Mediterenaean	840.4	11.5, 14.5, 15.5	Carbonate rocks	Cenomanian	Large	150	Insectivorous	Low	21, 6
		2 Yir'on (cave 1) (P)	33.0679	35.4665	528		716.4	16.5, 13, 20		Eocene	Large	150	Insectivorous	High	49, 5
		3 Yir'on (cave 2) (P)	33.0672	35.4672	541		716.4	8			Small	10	without	Zero	26, 8
		4 Pelekh (P)	32.9324	35.238	488		648.5	12.5		Turonian	Small	5	without	Zero	16, 5
		5 Yonim (P)	32.9236	35.2168	216		648.5	12.5, 12.5			Medium	50	without	Zero	61, 14
	Golan	6 Susita (P)	32.7793	35.6577	70		382.5	21, 21	Basalt	Pliocene	Medium	50	Insectivorous	Low	31, 5
	Lower Galilee	7 Berniki (cave 3) (P)	32.7775	35.5401	−102		455	17	Carbonate rocks	Turonian	Small	5	without	Zero	23, 6
		8 Berniki (cave 1) (P)	32.7775	35.5401	−102		455	19.5, 17, 20.5			Large	480	Insectivorous	High	53, 6
		9 Berniki (cave 2) (P)	32.7768	35.5413	−166		455	12.5, 12.5			Medium	15	without	Zero	86, 11
	Karmel	10 Oren (P)	32.7144	34.9749	73		611.2	16.5, 15.5			Medium	36	without	Zero	55, 5
		11 Horvat Raqqit (P)	32.7128	35.0123	355		611.2	14.5		Cenomanian	Small	3	without	Zero	8, 5
		12 Ezba' (P)	32.7118	34.9747	120		611.2	14.5, 15.5, 18		Turonian	Large	52	Frugivorous	High	58, 7
Central Israel and Palestine	Northern Samaria	13 Sal'it (P)	32.2454	35.0456	254		620.4	17, 18, 19.5		Cenomanian	Large	108	without	Zero	26, 6
	Central Jordan Valley	14 Andartat HaBiqa' * (E)	32.0524	35.4589	−184	Hyper-arid	19.9	19.5, 25.5		Turonian	Medium	23.5	Insectivorous	Medium	31, 10
	Western Samaria	15 Oah (P)	32.0053	34.9722	123	Mediterenaean	569	16			Small	10	without	Zero	19, 7
		16 Bet 'A'rif (P)	32.0026	34.9642	95		569	15.5, 18, 18			Medium	45	without	Zero	42, 7
		17 Tin-shemet (P)	31.9994	34.9681	100		569	15.5			Medium	43	Frugivorous	High	51, 6
	HaShfela	18 Haruva (P)	31.9133	34.9607	180		537.8	14.5, 16, 18	Chalk	ConiacianCampanian	Large	100	without	Zero	43, 9

	Northern Judean Desert	19 Perat Southern Slope (cave 4) * (PE)	31.8334	35.3054	295	Semi-arid	250	15	Carbonate rocks	Turonian	Small	10	without	Zero	17, 6
		20 Perat ʻInbal (cave 1) * (PE)	31.8332	35.3019	314		250	17, 16.5			Medium	40	without	Zero	37, 9
		21 Perat Ro'im (cave 2) * (PE)	31.8325	35.313	238		250	13.5, 16.5			Medium	25	Insectivorous	Low	45, 9
		22 Perat (cave 3) * (PE)	31.8321	35.3083	268		250	13			Small	10	without	Zero	19, 7
	Nortern Dead-Sea Area	23 Qumeran * (E)	31.7556	35.459	−308	Arid	95.8	21, 21		Cenomanian	Medium	20	without	Zero	30, 4
	Judean Mountain	24 Te'omim (P)	31.7262	35.0217	375	Mediterenaean	509	13, 13.5, 14.5			Large	134	Frugivorous	High	45, 5
South	Southern Judean Desert	25 Zavoa' (PE)	31.2086	35.2311	495	Arid	100	22, 23, 23.5	Carbonate rocks	Turonian	Large	600	Unknown	Low	56, 8
	Southern Dead-Sea Area	26 Arubotayim (E)	31.1016	35.39	−348	Hyper-arid	41.1	20, 19.5, 19.5	Salt	Pliocene	Large	60	without	Zero	5, 1
		27 Sedom (E)	31.0872	35.3958	−381		41.1	20, 19.5, 19.5			Large	1800	without	Zero	28, 1
		28 Malcham (E)	31.0765	35.3971	−380		41.1	20, 19.5, 19.5			Large	10,000	without	Zero	11, 1
		29 Ne'ot HaKikkar (Nezirim Burial cave) (E)	30.9911	35.3465	−333		41.1	20.5	Marlstone	Quaternary	Small	4	without	Zero	5, 2

	Negev Desert	30 Telalim (PE)	30.9734	34.7929	482	Arid	91.4	17, 15	Carbonate rocks	Turonian	Medium	27	without	Zero	7, 2
		31 Ashalim (PE)	30.9434	34.7391	404		91.4	17.5, 19.5, 17			Large	540	Insectivorous	Low	13, 1
		32 Besor (PE)	30.9415	34.6961	356		91.4	12, 16.5			Medium	35	without	Zero	26, 5
		33 ‘Avedat (Nezirim cave) (PE)	30.7941	34.772	601		93.3	11		Eocene	Small	5	without	Zero	9, 2
	‘Arava D esert	34 ‘Ammude ‘Amram (cave 2) (PE)	29.6518	34.9337	288	Hyper-arid	22.5	21	Sandstone	Cambrian	Small	5	without	Zero	0, 0
		35 ‘Ammude ‘Amram (cave 1) (PE)	29.6515	34.9336	293		22.5	21, 24, 24			Large	80	Insectivorous	Low	2, 2

List of the 35 caves sampled in this study (north to south) and their environmental variables. Localities in Israel and Palestine (West Bank) and transliterated names of the localities follow the “Israel Touring Map” (1:250,000) and “List of Settlements”, published by the Israel Survey, Ministry of Labor. Geographic coordinates are given in WGS84 (decimal degrees). Cave size estimates were corrected from Mammola et al., 2019 [9]: Bet ‘Arif was changed from large to medium category, Arubotayim was changed from medium to large category, Tinshemet was changed from small to medium, and Andartat HaBiqa’ was added to medium in the current analysis.

(1) Localities in Palestine (West Bank) are marked by asterisk (*). Letters in parentheses after cave name indicate the zoogeographical region: (P)—Palaearctic; (E)—Ethiopian; (PE)—Palaeoeremic (after Por, 1975).

(2) Precipitation data is taken from Israel Meteorological Service (https://ims.gov.il/en/ClimateAtlas, 1 February 2021) for the average annual mean for 1980–2010, from the closest meteorological station for each cave.

(3) Minimum Temperature is the average of the minimum temperature measured by us for each cave for two months (between the first and second visit to each cave) during the survey.

(4) Length was estimated from cave maps when available, or in field, and represent the distance from the cave opening toward the darker region of the cave that we could reach.

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Updated Distribution of the Mysid *Antromysis cenotensis* (Crustacea: Peracarida), a Protected Key Species in Yucatan Peninsula Cenotes

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Abstract: We present 52 new geographic location records for the peracarid crustacean *Antromysis cenotensis* Creaser, 1936, endemic in cenotes of the Yucatan Peninsula, Mexico. This species is currently considered threatened and, therefore, is protected by Mexican law. These results arise from several expeditions carried out between 2017 and 2020 in 75 locations within the cenote-ring, the interior, and coastal plains of the peninsula. A comprehensive literature review provided 84 geographic location records since the species was described in 1936. A map with 136 geographic location records that better describe the current species distribution is also included. With this information, plus some notes on the ecology of the species, a comprehensive literature and data review, and a brief analysis regarding the possible factors associated with the confirmed absence of the species in some locations in the state of Yucatan, we provide a brief and condensed summary of the actual knowledge on this particular species. The data in Darwin Core format can be retrieved in Zenodo.

Keywords: environmental science; biogeography; distribution records; groundwater; cave biology; underwater caves; cenote; mysid; stygobiont; bioindicator

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1. Introduction

The order Mysida (Crustacea: Malacostraca: Peracarida) contains 1184 species grouped into 179 genera that belong to two families and inhabits a vast diversity of aquatic habitats throughout the world [1]. Over 90% of the species are exclusively marine, while the remaining species inhabit subterranean freshwater and estuarine habitats. Mysids range in size from 3 to 22 mm and are often referred to as opossum shrimps due to the presence of oostegites forming a ventral female marsupium [2]. A statocyst in the proximal part of the endopods of the uropods characterizes the order and facilitates the distinction of these organisms from other crustaceans [3–5].

Mysids are considered omnivores, capable of both filter-feeding and raptorial feeding, exhibiting a variety of diets associated with seasonal changes, diel cycles, ontogenetic development, or food availability, and serve as an important food source for fish and crustaceans [6–9]. These organisms are usually positioned in the intermediate trophic levels of the communities they belong to, and some species play a key role in transferring energy between benthic and pelagic environments [9], and between upper and lower trophic levels [10]. They can be very abundant in certain habitats, reaching >1000 ind. m^{-2} [5,11], which added to the effect of vertical or horizontal migrations in most species, can have a significant impact on food-web dynamics, capable of attenuating the selective pressure generated by oligotrophy in some scenarios [6,8,12]. In addition to their ecological importance, mysids are also used as bioindicators or models for environmental screening in aquatic ecosystems, owing to their sensitivity to changes in water quality and the toxicity of chemical agents such as repellents, pesticides, and fertilizers [13–15].

Stygobionts are highly specialized aquatic organisms that inhabit hypogeal ecosystems [16]. In Mexico stygobiont mysids include representatives within the *Antromysis* and *Spelaeomysis* genera: *A. cenotensis* Creaser 1936 and *A. reddelli* Bowman, 1977, endemic to the Yucatan Peninsula and Oaxaca respectively, and *S. quinterensis* Villalobos, 1951 and *S. villalobosi* García-Garza, Rodríguez-Almaraz & Bowman, 1996, from Tamaulipas and Monterrey. *A. cenotensis*.

Figure 1 was discovered during the first investigations carried out in 1932 by the Carnegie Institution of Washington, focused on the systematic study of stygobitic fauna inhabiting caves and sinkholes, locally called cenotes, found throughout the karstic terrain of the Yucatan Peninsula which connect subterranean passages that can range from several meters to >350 km long [17]. Creaser [18] formally described the species, and Reddell [19] elaborated an extensive compilation of records by several authors which allowed for the first formal study of the species distribution. Said distribution delimited the species reach to freshwater cenotes found on the coastal plain north of the Sierra de Ticul and the Tulum-Coba corridor. Thanks to the development of specialized cave diving techniques in the mid-1980s, more profound explorations and complex studies on groundwater ecosystems with different approaches have been possible [20]. One particular study by Pohlman, et al. [21] shed light on the species ecology through stable isotope analysis, which established *A. cenotensis* as a primary consumer at the middle levels of the trophic chain. Therefore, the species appears to be an important component of groundwater biological communities, given its abundance and position in the food-web [20,21].

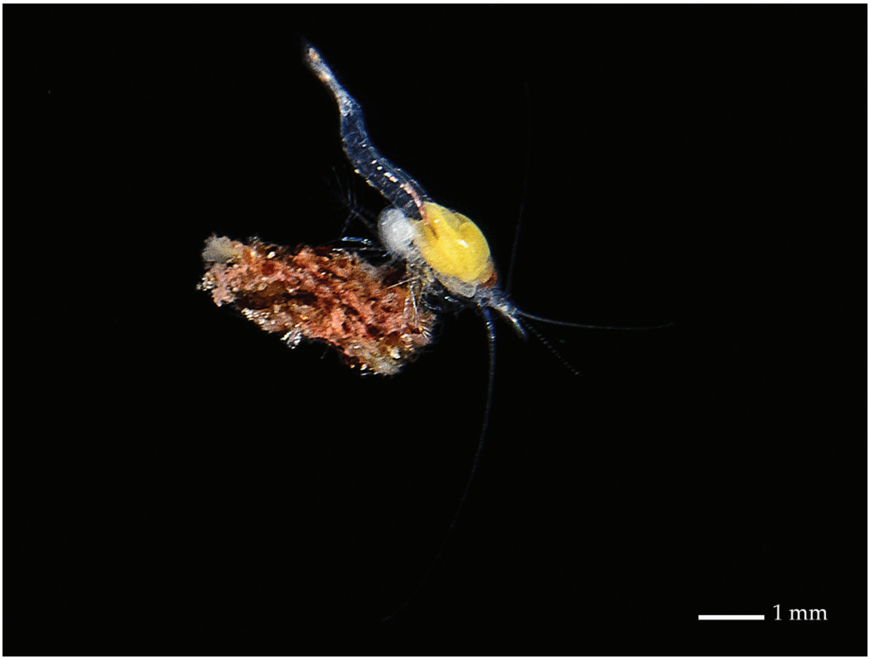

Figure 1. *A. cenotensis* holding a piece of organic detritus, typically observed during raptorial feeding. Cenote Kanún, Homún at 17 m in depth in September 2017. Photography courtesy of Benjamín Magaña-Rodríguez.

According to the National Water Commission (Comisión Nacional del Agua, CONAGUA [22]), groundwater in the Yucatan Peninsula is divided into four aquifers which comprise the states of Yucatan, Campeche, and Quintana Roo [22]. They are underlain by saltwater originated from marine intrusion [23]. The interaction of groundwater with primary and secondary geological features within the peninsula allowed for the formation of five main hydrogeological zones: the coastal plain, the cenote semicircle, the interior plain, hills and valleys and stepped basins [22]. They are highly vulnerable to pollution due to their karstic, hydraulic, and ground properties, while the northern portion is highly susceptible to impact by human activities due to the velocity at which external agents can enter [22].

The coastal plain includes barrier beaches, flood lagoons and a series of shallow bays associated with fracture systems and highly permeable semi-consolidated coastal environment coquiniferous limestone systems. Its delimitation is based on an imaginary 20 km fringe running parallel to the coastline from Campeche to Quintana Roo and is characterized by a thin freshwater layer underlain by marine water [22–24]. The cenote semicircle is delimited by a fracture system related to the Chicxulub Crater, forming an almost perfect semicircular cenote boundary surrounding a fracture system of approximately 170 km in diameter. It has lateral groundwater migration and large flow due to dissolution and subsidence along the fractures at the edges and receives a small vertical recharge [22–24]. The interior plain extends towards the northern and northeast portions of the peninsula. It is underlain by permeable limestone and contains karst forms that range from dissolution cavities to sinkholes with mature and juvenile development. It borders to the north with the semicircle of cenotes and the coastal region, and to the south with the zones of hills and valleys and stepped basins [22,24]. The hills and valleys zone represents the most complex zone due to the Puuc Cordon, its elevation and topographical relief. It is formed by highly permeable carbonates and is characterized by a fault line that divides it from the interior plain. In contrast with the other zones, cenotes are almost inexistent, although there are dry caves of great dimensions [22,24]. Finally, the stepped basins extend from the northeast portion of the peninsula near cape Catoche in Quintana Roo towards the southern border with Belize. This area contains the largest fracture concentration. The interaction of gypsum, loam, anhydrite, and limestone make it highly permeable, with poor water quality [22].

In the state of Yucatan, groundwater exhibits variations in water quality in a stratified manner, where the topmost layer is polluted through poorly designed wells used for clandestine residual discharges that reach a depth of 20 m [24]. Freshwater depth varies according to its distance from the coast, ranging from 1–5 m in depth in the coastal plain, 10–30 m in the cenote semi-circle, and 60–100 m further south. Below these depths, saltwater intrusion occurs [23,24]. The interior plain covers most of the state (52% of the surface), followed by the cenote semicircle (18%), the coastal plain (17%) and the hills and valleys zone (13%) [24].

At present, *A. cenotensis* is a threatened species listed in the Mexican Red List of Species at Risk from the Secretariat of the Environment and Natural Resources (NOM-059 SEMARNAT 2010, Secretaría de Medio Ambiente y Recursos Naturales). This category includes species in danger of disappearing in the short or medium-term by factors causing habitat deterioration or directly affecting their population sizes. It partially coincides with the vulnerable category of the IUCN Red List, which *A. cenotensis* is not part of.

Given the current urbanization and degradation processes within the Yucatan Peninsula and their associated environmental impacts on groundwater habitats [25], the monitoring of this species, and ecological information related to it, will gain importance. Thus, we present an update on the distribution of the species in several locations within the Yucatan Peninsula, and a historical summary of distribution records available for the species.

2. Materials and Methods

2.1. Bibliographic Review

To obtain the current knowledge about the distribution of *A. cenotensis*, a comprehensive bibliographic review was carried out following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines [26] on 5 February 2021. Publications and records were obtained through a comprehensive search for information in biological collections, which included the Smithsonian Invertebrate Zoology Collection (NMNH [27]) and the National Crustacean Collection from the Biology Institute, National Autonomous University of Mexico (Colección Nacional de Crustáceos del Instituto de Biología de la Universidad Nacional Autónoma de México, CNCR [28]), literary databases [29,30], scientific search engines [31–33], and online biodiversity and citizen science databases [34–36]. Inclusion criteria were (1) publications or data sets that had to explicitly mention the collection or presence of specimens in a specific location and (2) biological records that had to comprise discrete events validated through specimens stored in scientific collections with relevant geographic information. More information is available in PRISMA statements (Figures S1 and S2).

2.2. Specimen Collection

Between November 2017 and November 2020, 75 dives were carried out in cenotes from the Yucatan Peninsula lacking biological information related to *A. cenotensis*. Fifteen of these locations are found within the coastal zone of the state of Quintana Roo, while the remaining 60 are distributed within the hydrogeological units of the coastal plain, the cenote semicircle and the interior plain in the state of Yucatan as shown in Figure 2.

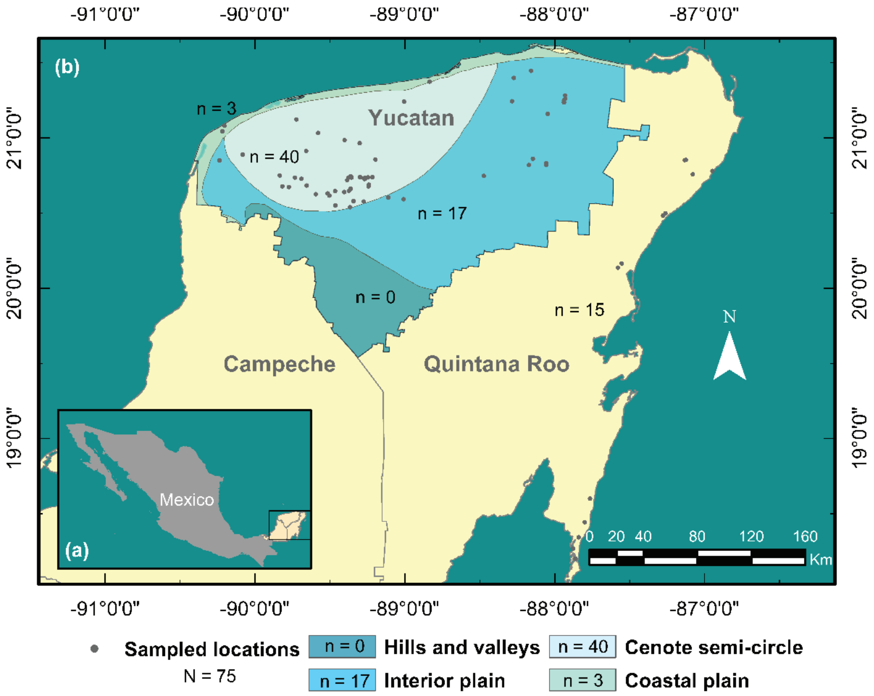

Figure 2. (**a**) Map of Mexico with Yucatan Peninsula highlighted in yellow. (**b**) Sampled locations (dots) along the coastal region of Quintana Roo, and the hydrogeological zones within the state of Yucatan [24]. Total number of locations sampled (N) is shown in the legend. Number of sampled locations per geohydrological zone (n) are shown on the map close to each region and the legend as well.

The collection of organisms was carried out manually by an average of two cave-divers or free-divers using aquarium nets and 50 mL plastic tubes in the pool (photic region), cavern (twilight region) and cave (aphotic region) of each location (Figure 3). Specimen collections were made in 52 locations at a maximum depth of 36 m, with a maximum penetration of 900 m by cave divers, and seven locations at a maximum depth of 9 m by free-divers. The specimens were immediately preserved and stored in 70% ethanol after each dive and were collected under permits: SEMARNAT/SPGA/DGVS/05263/14; and SEMARNAT/SPGA/DGVS/02068/17 issued by the Ministry of the Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales, SEMARNAT).

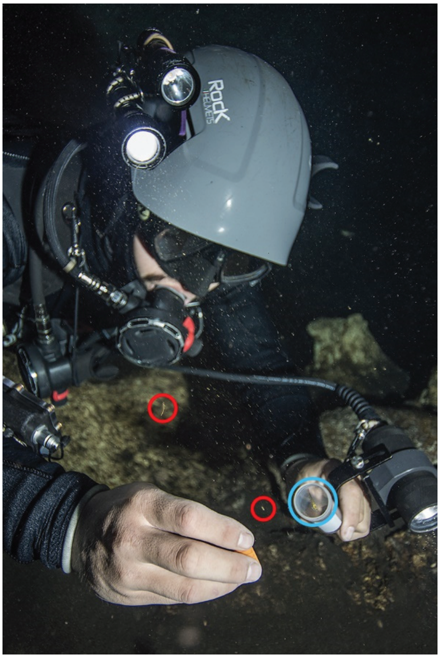

Figure 3. Luis Arturo Liévano-Beltrán collecting *A. cenotensis* (red circles) and *T. mitchelli* (blue circle) specimens from a cave in cenote Xelactún at 24 m depth on 25 February 2020. Courtesy of Kayú Vilchis-Zapata.

All organisms were identified at the species level according to Bowman [37], using stereoscopic and optical microscopes. Identified specimens were stored in the Colección de Crustáceos de Yucatán, Unidad Multidisciplinaria de Docencia e Investigación, Universidad Nacional Autónoma de México (UMDI-Sisal, UNAM) under catalog codes YUC-CC-255-11-006806-YUC-CC-255-11-006857. The obtained data comprised dates, GPS coordinates, geographical information, cenote type, depth, number of specimens collected and collection codes. Data were transformed into the Darwin Core format and made available for public access through Zenodo (https://doi.org/10.5281/zenodo.4391039, accessed on 26 February 2021) following the Global Biodiversity Information Facility (GBIF) current best practices for generalizing sensitive species occurrence data [38].

2.3. Map Ellaboration

Geospatial data/information/coordinates were extracted from obtained publications during the bibliography review. Maps were designed using QGIS v. 3.16 (QGIS Development Team, 2020). Topographic, hydrogeological, agricultural and livestock maps at

1:1,000,000 were used to illustrate and relate socioeconomic aspects with the current distribution of *A. cenotensis* in the Yucatan Peninsula (CONABIO [39]; POETY [40]).

3. Results

3.1. An Account of *A. cenotensis* Historical Distribution Records

A total of 86 publications mentioning *A. cenotensis* were reviewed (Figure S1). Thirty publications contained valuable data for the current study, 14 lacked information on the species and 39 were secondary references to previously published material/data/information missing extractable geospatial information. Furthermore, 121 records were extracted from these publications and 52 were gathered through database and collection searching (Figure S2), adding 173 records. However, 15 were accounted for in literature, and 32 did not contain extractable location information, leaving 126 validated historical records for *A. cenotensis* corresponding to 84 locations.

The fastest rate of record contributions for the species occurred from its description until the end of the seventies, with a more gradual increase until the year 2020 (Figure 4a), with a contribution of 20 records by Angyal et al. [41]. Ninety-eight records correspond to 65 locations within the state of Yucatan. The remaining 28 refer to 19 locations in Quintana Roo (Figure 4b). The main location record contributor was Bowman [37] as shown in Figure 4c. Record contributions per author for each year can be found in Table 1, and a map displaying recorded locations with their record count is shown in Figure 5.

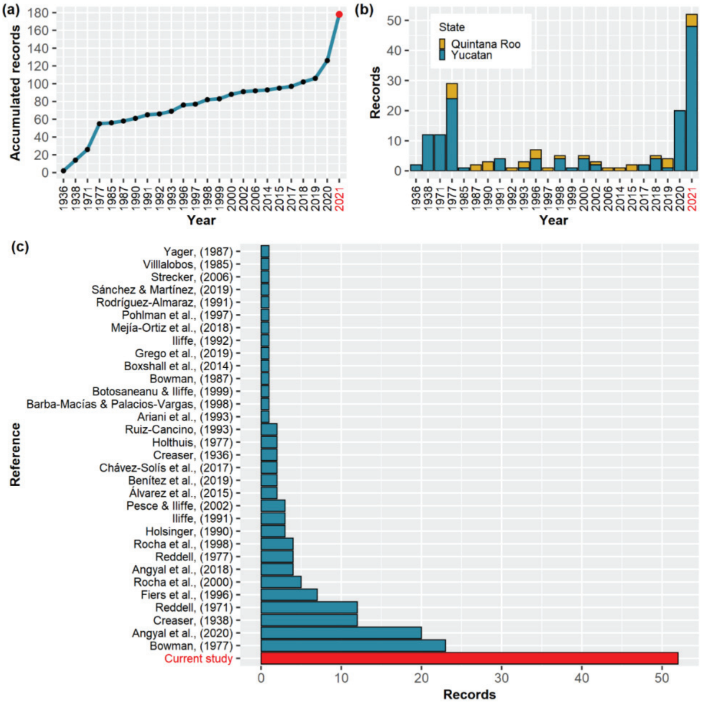

Figure 4. (**a**) Number of accumulated *A. cenotensis* records per year since species description filtered from duplicates and cross-referencing (see PRISMA statements from Figures S1 and S2). (**b**) Number of *A. cenotensis* records per year in the Yucatan Peninsula. (**c**) Author record contribution ordered according to contribution frequency. Red color indicates present study results. Main record contributors [37,41] are found in the Reference column from Table 1 along with others. Locations for each record are summarized in Table 1. A map with the recorded locations can be visualized in Figure 5.

Table 1. Locations where *A. cenotensis* records have been published per year for the states of Quintana Roo and Yucatan. Location geographical distribution and names can be found in Figure 5.

State	Year	Author	N	Locations	Reference
Q. Roo	1977	Bowman	5	69, 70, 73, 77, 81	[37]
	1987	Bowman	1	79	[42]
		Yager	1	77	[43]
	1990	Holsinger	3	74, 77, 79	[44]
	1992	Iliffe	1	79	[20]
	1993	Ruiz-Cancino	2	83, 84	[45]
	1996	Fiers et al.	3	66, 68, 71	[46]
	1997	Pohlman et al.	1	75	[21]
	1998	Rocha et al.	1	75	[47]
	2000	Rocha et al.	1	72	[48]
	2002	Pesce & Iliffe	1	75	[49]
	2006	Strecker	1	67	[50]
	2014	Boxshall et al.	1	77	[51]
	2015	Álvarez et al.	2	76, 78	[52]
	2018	Mejía-Ortiz et al.	1	82	[53]
	2019	Benítez et al.	2	76, 78	[54]
		Sánchez & Martínez	1	80	[55]
Yucatan	1936	Creaser	2	17,54	[18]
	1938	Creaser	12	4, 12, 17, 31, 39, 46, 50, 53, 54, 55, 56, 60	[56]
	1971	Reddell	11	4, 12, 17, 31, 39, 46, 50, 53, 54, 55, 56, 60	[57]
	1977	Bowman	18	3, 5, 13, 14, 17, 19, 20, 25, 27, 29, 36, 42, 46, 61, 62, 63, 64, 65	[37]
		Holthuis	2	57, 59	[58]
		Reddell	4	6, 9, 15, 30	[19]
	1985	Villalobos	1	57	[28]
	1991	Iliffe	3	38, 55, 57	[27]
		Rodríguez-Almaraz	1	49	[27]
	1993	Ariani et al.	1	27	[59]
	1996	Fiers et al.	4	2, 21, 23, 58	[46]
	1998	Barba-Macías & Palacios-Vargas	1	27	[60]
		Rocha et al.	3	21, 27, 37	[47]
	1999	Botosaneanu & Iliffe	1	24	[61]
	2000	Rocha et al.	4	21, 27, 37, 41	[48]
	2002	Pesce & Iliffe	2	23, 52	[49]
	2017	Chávez-Solís et al.	2	18, 38	[62]
	2018	Angyal et al.	4	10, 18, 43, 44	[63]
	2019	Grego et al.	1	51	[64]
	2020	Angyal et al.	20	1, 7, 8, 10, 11, 16, 18, 22, 26, 28, 32, 33, 34, 35, 40, 43, 44, 45, 47, 48	[41]

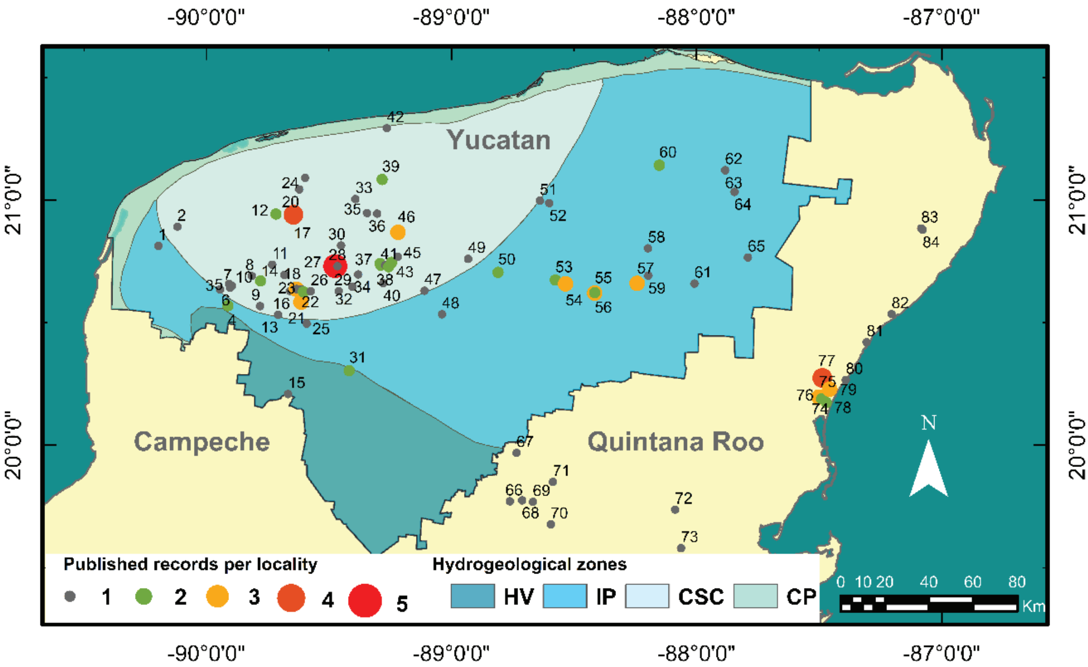

Figure 5. Known locations (dots) where at least one individual of *A. cenotensis* was previously reported in literature and/or scientific collections. Number of records/occurrences over time per location (dot color and size). Hills and valleys (HV), interior plain (IP), cenote semi-circle (CSC), and coastal plain (CP) hydrogeological zones in the state of Yucatan. Location name is presented along with the municipality where it is found (between parentheses) for each state (italics). *Yucatán:* (1) Noh Chucunchey (Celestún); (2) well (Kinchil); (3) Calchum (Kopomá); (4) well (Calcehtok); (5) Cenote de las Abejas (Kopomá); (6) San Bulhá (Kopomá); (7) San Tito (Kopomá); (8) Pool Box (Chocholá); (9) Cenote del Pochote (Opichén); (10) Dzonbakal (Chocholá); (11) Bebelchén (Sanahcat); (12) Luchil (Mérida); (13) Cenote de la Culebra (Muna); (14) Sihunchén (Abalá); (15) Aktún Chac (Sayil); (16) Dzonotilá (Abalá); (17) Cueva de San Isidro (Mérida); (18) Kankirixché (Abalá); (19) Kankirixché (Muna); (20) Sodzil (Mérida); (21) Yuncú (Mucuyché); (22) Chihuo-hol (Abalá); (23) Mucuyché (Mucuyché); (24) X'lakah (Mérida); (25) Noh Chén (Sacalum); (26) X'kakuel (Abalá); (27) Grutas de Tzab Nah (Tecoh); (28) Tza Itzá (Tecoh); (29) Chen Mul (Tecoh); (30) Chun Kapoc (Acanceh); (31) Well (Oxkutzcab); (32) Nah Yah (Tecoh); (33) Flor de Liz (Tixkokob); (34) Kampepén (Tecoh); (35) X'kokob (Tixkokob); (36) Cenote G (Tixkokob); (37) Grutas de Santa María (Homún); (38) San Juan (Homún); (39) Sambulá (Motul); (40) Kankal (Homún); (41) Chan-hoch (Homún); (42) Pozo de Santa Elena (Sinanché); (43) Xa'an (Homún); (44) Kanún (Homún); (45) Pixtón (Sanahcat); (46) Hoctún (Hoctún); (47) Ixim Ha (Huhí); (48) El Virgen (Sotuta); (49) Chihuán (Kantunil); (50) Yuhunchén (Libre Unión); (51) Xoch (Cenotillo); (52) Ucil (Cenotillo); (53) Chaac Mol (Tinum); (54) Balamcanché (Tinum); (55) Aktún Kaua (Kaua); (56) Oxolodt (Kaua); (57) X'kekén (Dzitnup); (58) well (Temozón); (59) Zací (Valladolid); (60) X'consacab (Tizimín); (61) Xtacabihá (also known as Xalaú, Chemax); (62) Cueva de Orizaba (Tizimín); (63) Cenote Orizaba (Tizimín); (64) Aka Chén (Tizimín); (65) San Diego (Chemax). *Quintana Roo:* (66) well 1 (Jose María Morelos); (67) water spring (José María Morelos); (68) well 2 (Jose María Morelos); (69) Santo Domingo (José María Morelos); (70) Cenote de Las Ruinas (José María Morelos); (71) Aguada (José María Morelos); (72) Tos Virlol (Felipe Carrillo Puerto); (73) Cenote de Juan Coh (Felipe Carrillo Puerto); (74) Naharón (Tulum); (75) Mayan Blue (Tulum); (76) Muknal (also known as Jailhouse, Tulum); (77) Aktún Ha (also known as Carwash, Tulum); (78) Odissey (Tulum); (79) Calavera (also known as Temple of Doom, Tulum); (80) Cenote Manatí (also known as Casa Cenote, Tulum); (81) San Martín (Solidaridad); (82) Muévelo Rico (Solidaridad); (83) Aktún Jaleb (Benito Juárez); (84) Tres Bocas (Benito Juárez).

3.2. New Records

We present 52 new *A. cenotensis* distribution records from cenotes in the Yucatan Peninsula (Figure 6), four in the Quintana Roo state and 48 in Yucatan. The provided records account for a 41% increase in existing records and a 62% increase in locations since the species description. Nineteen locations were previously reported in the state of Quintana Roo, and 65 in Yucatan. So far *A. cenotensis* has not been reported in the state of Campeche. The number of records obtained during this study added to those obtained through the bibliographic review adds up to a total of 178 records corresponding to 136 locations. Twenty-three in the state of Quintana Roo and 113 in Yucatán.

A. cenotensis individuals were collected mainly from cenotes that either had clear waters with no apparent smell or taste (cenotes from the cenote semicircle), a freshwater region in the cave zone above the halocline (Tajma Ha) or were at least 2 km away from the coast. However, in some locations with these characteristics the species could not be found (Figure 7). These locations include mostly cenotes from the interior plain where high touristic (Hubikú), agricultural (Saa'kal, X'bohom, cenote under papaya plantation), or livestock activities (Tzitzilá, Rancho Sac Bé) took place.

Some locations where the species was not found were cenotes with yellow or green turbid water (Crustacea, K'ax Ek), saltwater regions below the halocline in coastal cenotes (Sabtún, Dagobah, Cocom) and locations with wine-colored water in mangroves (Polac, Cauich) or the jungle. On occasions, thick white or brownish clouds with a rotten egg taste were present (Angelita, Tzitzilá, Sabtún), and slimy bacterial mats covering the walls precipitated towards the bottom when disturbed by diver bubbles (Pandora, Dagobah).

Specimens were collected in all the sampled locations within the cenote semicircle (present = 40, absent = 0) but their presence decreased towards the interior plain (present = 8, absent = 10), and were mostly absent in coastal regions (present = 4, absent = 13).

During this study, *A. cenotensis* was observed coexisting with a variety of animals that included predatory fish species like *Ophisternon infernale* (Hubbs, 1938), *Rhamdia guatemalensis* (Günther, 1864), and *Typhlias pearsei* (Hubbs, 1938), known to feed on smaller crustaceans [21,65–69]. Decapods like *Creaseria morleyi* (Creaser, 1936), (predator of *A. cenotensis*) [62], *Typhlatya dzilamensis*, Alvarez, Iliffe & Villalobos, 2005, *Typhlatya mitchelli*, Hobbs & Hobbs, 1976, and *Typhlatya pearsei,* Creaser, 1936. The isopods *Cirolana yunca*, (Botosaneanu & Iliffe, 1999), *Creaseriella anops*, (Creaser, 1936), *Metacirolana mayana* (Bowman, 1987), and *Yucatalana robustispina*, Botosaneanu & Iliffe, 1999. The amphipods *Mayaweckelia troglomorpha*, Angyal, 2018, *Mayaweckelia cenoticola*, Holsinger, 1977, and *Tuluweckelia cernua*, Holsinger, 1990. The thermosbaenacean *Tulumella unidens*, Bowman & Iliffe, 1988, and the stygiomysids *Stygiomysis cokei,* Kallmeyer & Carpenther, 1996 and *Stygiomysis holthuisi,* (Gordon, 1958). The number of coexisting species varied between locations. However, none of these species displayed populations as large as those for *A. cenotensis*, on occasions containing thousands of individuals. This makes this species an exceptionally important component within the community.

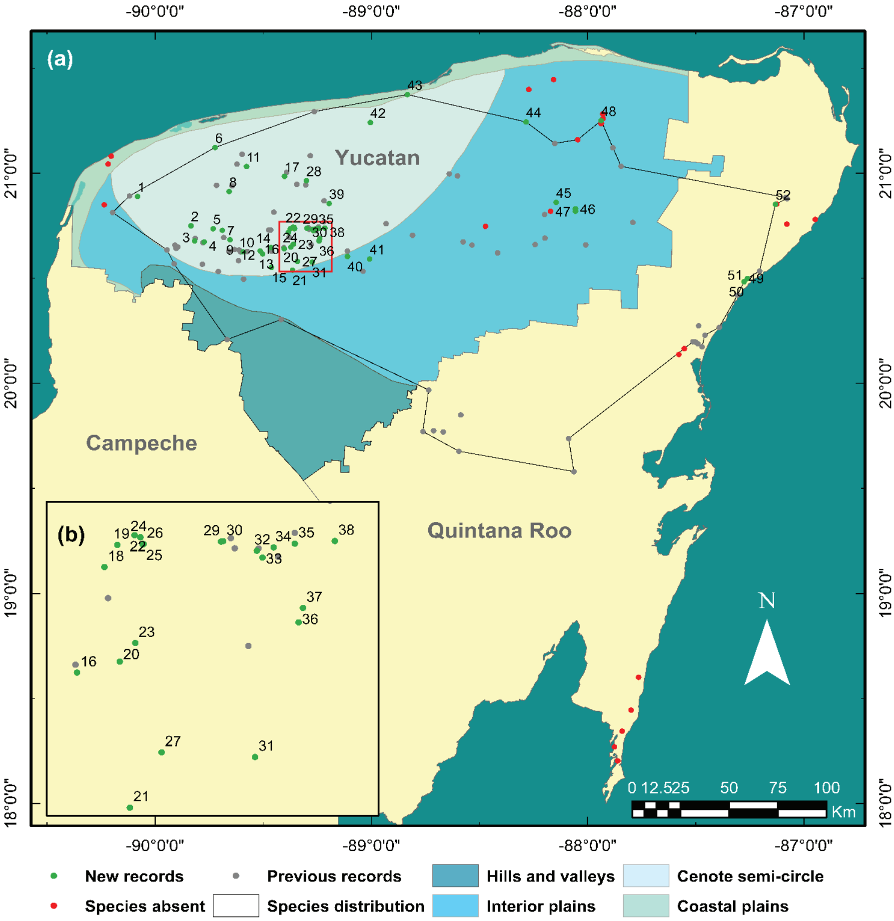

Figure 6. (**a**) New records for *A. cenotensis* (green dots), locations where the species was not found (red dots, named in Figure 7), previous records (gray dots), and current species distribution (polygon) extracted from total available data. (**b**) Close-up of stacked new records (in red square). Hydrogeological zones are also shown for the state of Yucatan. Location names are presented along with the municipality where it is found (between parentheses) for each state (italics). *Yucatán:* (1) Xelactún (Celestún); (2) San Ignacio (Chocholá); (3) X'baba (Chocholá); (4) X'batún (Chocholá); (5) Eku'he (Umán); (6) Sierra Papacal (Mérida); (7) Pebá (Abalá); (8) Huul K'in (Mérida); (9) Temozón (Abalá); (10) Yax Kis (Abalá); (11) Xoc (Mérida); (12) Itzkakal (Tecoh); (13) Itzinteh (Tecoh); (14) Cheen Cha'ac (Telchaquillo); (15) Mazucil (Tecoh); (16) Suem (Tecoh); (17) Yaax Ha (Tixkokob); (18) Boloonchojol (Cuzamá); (19) Paraíso Papacal (Cuzamá); (20) Tanimax (Tecoh); (21) X'pakay (Tekit); (22) X'tojil (Cuzamá); (23) Calcuch (Tecoh); (24) San Felipe (Cuzamá); (25) Saak Pakal (Cuzamá); (26) Ayusó (Cuzamá); (27) Uitzán (Tekit); (28) X'cohil (Cacalchén); (29) Pool Uinic (Homún); (30) Tza Ujun Kat (Homún); (31) Becal (Tekit); (32) Tres Oches (Homún); (33) Yaxbacaltún (Homún); (34) Los Huayes (Homún); (35) Chulul (Homún); (36) Oxolá (Homún); (37) San Elías (Homún); (38) Subinteh (Homún); (39) Ehbiz (Hoctún); (40) X'toho (Sotuta); (41) Tzonot Mis (Sotuta); (42) Sacahuá (Dzidzantún); (43) Cervera (Dzilam de Bravo); (44) Acancún (Panabá); (45) Santa Rita (Temozón); (46) Dzalbay (Tizimín); (47) Palomitas (Temozón); (48) Yaxcabá (Tizimín). *Quintana Roo:* (49) Tajma Ha (Tulum); (50) Buenavista (Tulum); (51) X'tabay (Tulum); (52) Hol Box (Benito Juárez).

4. Discussion

A. cenotensis is highly conspicuous in many cenotes of the Yucatan Peninsula, particularly those within the cenote semicircle. Its presence decreases towards the coastal regions and the southern edge of the Yucatan state (Figure 7). These data may be biased associated with sampling effort, considering most records come from the cenote semicircle in the Yucatan state. However, the absence/presence relationships in this study increased from the cenote semicircle towards the interior plain of the Yucatan state, with a high number of absences in the coastal regions of Yucatan and Quintana Roo.

The observed decrease in presence towards the coast may be related to environmental factors known to influence community structure, such as an uplift in the halocline due to tidal fluctuations [70]. This would convert the halocline into a mobile physicochemical barrier for the species capable of restricting its habitat. Consequently, the species would be forced to modify its feeding strategies by not being able to access bottom nutrients [71,72]. Major geological changes in the karst landscape may explain the presence decrease towards the southern edge of the state [23], where the Sierrita de Ticul fracture zone acts as an allopatric barrier to the distribution of the species.

The species usually exhibited conspicuously large population sizes, coexisting with a variety of stygobiont representatives. Groundwater ecosystems are known to be regulated through bottom-up trophic cascades [21,25,73,74]. In such habitats, *A. cenotensis* may act as an energy facilitator capable of feeding-off a combination of sources, such as soil particulate organic matter that percolates through the porous bedrock into the cave, and algal and vegetative detritus found near the cenote pool [21]. The species also serves as a readily available food source for other predatory stygobiont species [21,62,65–69]. Therefore, *A. cenotensis* may play an important role in the food-web dynamics as a mid-level prey in groundwater communities.

Over 3000 cenotes and caves have been registered within the state of Yucatan by the Secretariat of Sustainable Development of the Government of Yucatan (Secretaría de Desarrollo Sustentable del Gobierno del Estado de Yucatán, SDS). However, Steinich [75] estimated a figure of 7000 cenotes throughout the northwestern part of the Yucatan Peninsula through topographic digitalization. This would extend the current knowledge of *A. cenotensis* to approximately 4.5% of its potential habitat for the Yucatán state cenotes and 1.92% of the estimated total for the Yucatan Peninsula, without including caves or wells. Many locations remain undiscovered or lack biological information and *A. cenotensis* records. This demonstrates the species not only has a widespread distribution in Yucatan Peninsula but may also have a higher presence than current knowledge suggests. Therefore, further exploration and sampling efforts are required to describe the species distribution accurately.

Most of the locations where the species was collected can be classified as lotic cenotes according to Schmitter-Soto, et al. [25]. These contain clear, well-oxygenated water columns with sandy or rocky bottoms due to their connection with groundwater. On the contrary, we did not find the species in 24 of the studied locations, corresponding to 32.4% of the sampled total (Figures 6 and 7). Some of these locations had previous records for the species. Thus, our findings do not necessarily ensure the species' absence, but may imply less abundant populations that may require a higher sampling effort, or other phenomena which could influence the species distribution on a small scale.

The absence of the species in saltwater portions of locations found within the coastal plain may not be a matter of major environmental concern, given that salinity conditions may exceed the physiological requirements that allow for the species survival. Furthermore, saltwater stygofauna representatives such as *Metacirolana mayana*, *Tulumella unidens, Typhlatya dzilamensis* and *Xibalbanus tulumensis*, were observed in the water column at several of these locations. In contrast, the absence of the species and other stygobionts was clear in mangrove or lentic cenotes [25] with a high input of tannic acid [76] and thick white H_2S smelling clouds, characteristic of anoxic waters where bacterial sulfate reduction and organic matter decomposition take place [25,76–80]. This serves as evidence that *A. cenotensis* populations may present naturally selected physiological limitations that make them sensitive to modifications in water quality and environmental conditions under certain scenarios.

It is not clear why stygofauna was absent in some inland cenotes lacking coastal cenote characteristics within the interior plain [25]. This suggests that other non-natural factors may influence its presence. Furthermore, tourism, agriculture, and livestock activities can induce considerable impacts on karstic landscapes and groundwater biodiversity [81–88].

The municipality of Tizimin (Figure 7) is known for its agricultural and livestock activities [89–92]. Both promote land-use modification, pesticide or fertilizer use, groundwater extraction and vegetation removal. These favor soil compaction, nitrate and metabolically persistent chemical input, water table decrease, sedimentation rate and leaching or runoff of surface materials [71,86,93,94]. Climatological phenomena (e.g., precipitation, climate change, storms) can also alter abiotic conditions through surface pollutant transport, inducing potentially rapid responses in biological communities and processes [94–100]. Three records for the species came from this area from over 40 years ago [18,37,56]. Thus, the observed absence of stygofauna could be potentially linked to environmental pressures exerted by these activities. However, further research on the effect of water contaminants on stygobiont species from this region is required.

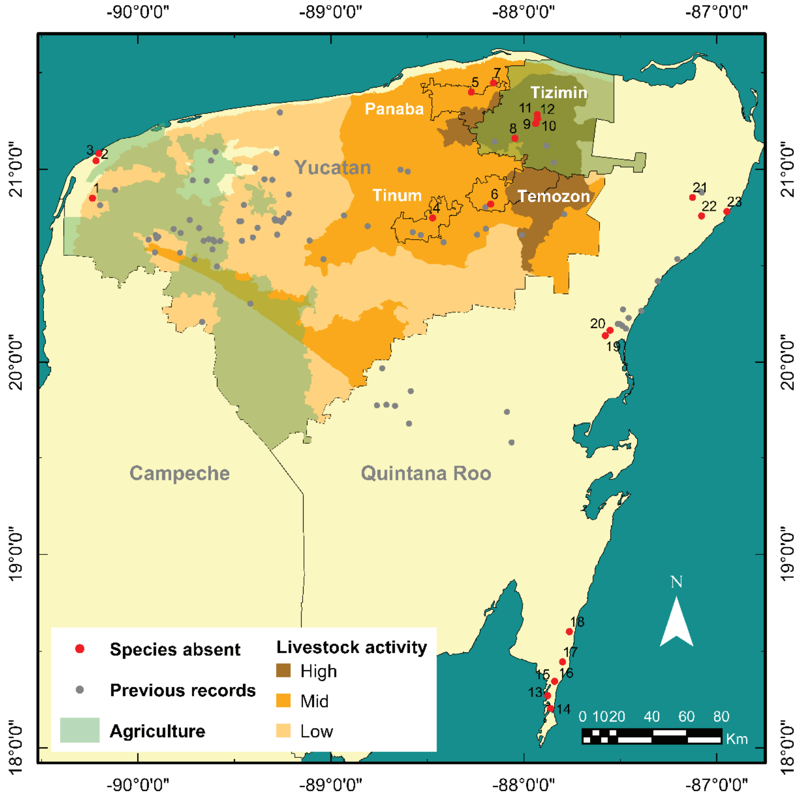

Figure 7. Regions with agricultural activity (green zones) and livestock activity intensity (brown zones) within the state of Yucatán [40,89,90]. Four municipalities in the state of Yucatan (outlined in black with white labels). Locations where *A. cenotensis* was not sighted (red dots) are numbered as follows according to state (bold italics) and municipality (in parenthesis): **Yucatan**: (1) Sabtún (Celestún); (2) Cauich (El Palmar); (3) Polac (El Palmar); (4) K'ax Ek (Tinum); (5) Rancho Sacbé (Panabá); (6) Hubikú (Temozón); (7) San Juan del Río (Panabá); (8) Saa'kal (Tizimín); (9) X'bohom (Tizimín); (10) Dzonot Aké (Tizimín); (11) unnamed cenote under papaya plantation (Tizimín); (12) Tzitzilá (Tizimín). **Quintana Roo**: (13) Pandora (Othón P. Blanco); (14) Bajo de Judas (Othón P. Blanco); (15) water spring (Othón P. Blanco); (16) water spring (Othón P. Blanco); (17) mangrove cenote (Othón P. Blanco); (18) Dagobah (Othón P. Blanco); (19) Angelita (Tulum); (20) Ka'an Lu'um (Tulum); (21) Zapote (also known as Hell's Bells, Benito Juárez); (22) Cocom (Solidaridad); (23) Crustacea (Solidaridad).

A. cenotensis was also absent in locations from the municipalities of Temozon and Tinum (Figure 7). These cenotes have suffered drastic geomorphological modifications to a great extent and currently function as ecoparks that receive a high number of visitors (>200) regularly. There is evidence that common chemical compounds found in insect repellents and sunscreen used by tourists can exert negative effects on aquatic invertebrates. These include neurotoxicity, behavioral modifications, and decreased survival [101–108]. Therefore, tourism may negatively affect the species presence. Nevertheless, previous records exist from nearby locations, one of them corresponding to the species type locality (Gruta de Balamcanché) [18,46,56]. However, tourism in this location is restricted to the dry regions of the cave, which may have softened the negative environmental pressures that touristic swimming activities have over the environment.

5. Conclusions

A. cenotensis is a widespread species in the northern portion of the Yucatan Peninsula within the Quintana Roo and Yucatan states. The species exhibits conspicuously large populations and coexists with several endemic stygobiont representatives, some of which feed on it. Therefore, its protection would exert an umbrella effect over other stygobiont representatives inhabiting groundwater ecosystems, particularly those distributed within the cenote semicircle. Nevertheless, there is a lack of information regarding the species distribution in the hills and valleys, coastal and interior plains. Thus, more data on the species from these hydrogeological zones is required.

Its large populations and an apparent sensitivity to anthropogenic activities make *A. cenotensis* a potential key species that could function as a bioindicator. Thus, its incorporation into groundwater management, monitoring and protection programs, along with water quality data, would allow local authorities to assess the environmental health of groundwater ecosystems within the Yucatan Peninsula in a broader sense. Further bioassays and ecotoxicological studies on the effects of common contaminants used in anthropogenic activities over the species would be desirable in the future for further confirmation.

Finally, most of the current information on the species comes from taxonomic and distribution records. Hence, a better understanding of the biology and ecology of this widespread species would provide valuable information related to the processes and conditions that favor colonization and survival of stygofauna in groundwater habitats.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13040154/s1, Figure S1: PRISMA flow diagram 1, Figure S2: PRISMA flow diagram 2.

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diversity

Article

Diversity of Brazilian Troglobitic Fishes: Models of Colonization and Differentiation in Subterranean Habitats

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Abstract: The Brazilian subterranean ichthyofauna is distinguished worldwide by high taxonomic and phylogenetic diversity, involving at least 30 exclusively subterranean (troglobitic) lineages. This may be explained by high native epigean diversty, allied to opportunities for colonization and genetic isolation in the subterranean biotope, thence originating troglobites. An updated list of Brazilian troglobitic lineages and a review of previous hypotheses on modes of colonization and differentiation in subterranean habitats are presented with fresh data and models. Colonization of and isolation in the subterranean biotope are independent processes, usually separate over time. Access to subterranean habitats varies from the (sub)horizontal through sinkholes and resurgences of base-level streams and vadose tributaries to the vertical by shallow and deep phreatic waters through the hyporheic zone. Phenotypic differentiation of subterranean populations originating troglobites may be achieved by various, non-mutually exclusive modes of genetic isolation, e.g., directly in base-level streams and upper tributaries through the extinction of epigean populations, due to drainage discontinuation in dry paleoclimatic phases (or by other causes), by topographic isolation due to a lowering of the regional base level and karst catchments or by parapatric differentiation. Differentiation may also be a consequence of transition from lotic to lentic waters in flooded caves, and from shallow to deep phreatic habitats.

Keywords: subterranean fishes; evolution; origin of troglobites; Brazil; subterranean biology

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1. Introduction

Subterranean (subsurface) fishes are excellent models for studies of comparative evolution. Due to the high taxonomic, ecological and morphological diversity, the Brazilian ichthyofauna is particularly suitable for this. So far, more than 30 troglobitic fish lineages (exclusively subterranean source populations—[1]) have been recognized, including 21 valid species described as troglobitic. Mattox et al. [2] presented a list of Brazilian troglobitic and troglophilic fishes (source populations in both subsurface and surface habitats, genetically connected by mutually commuting individuals [1]). This was subsequently updated by Gallão and Bichuette [3] for the troglobites. Nonetheless, the discovery and description of novel subterranean fishes continue. Troglobites usually, but not necessarily, present troglomorphisms, i.e., autapomorphies related to isolation and specialization to the subterranean life. The most frequent are the regression of visual structures and dark cutaneous pigmentation ([1,4], among others).

Brazilian species of subterranean fishes are allocated into 12 genera, seven families and three orders, thus configuring relatively high phylogenetic diversity. In comparison, the 78 species (in 20 genera) reported as troglomorphic for China [5], which has a larger total karst area, are distributed in four families and two orders, and two genera concentrate ca. 70% if this species richness [5]. The troglobitic ichthyofauna from Mexico is less phylogenetically diverse at lower taxonomic levels, but more so at the higher levels: the 11 species recorded by Proudlove [6] are distributed in six genera and families belonging to five orders. The elevated lower and higher phylogenetic levels of Brazilian subterranean ichthyofauna, both troglobites and troglophiles, could originate from a highly diversified and rich epigean (surface) fauna providing a large array of potential colonizers of hypogean habitats, thereby giving rise to troglophilic populations. Furthermore, karst fragmentation and paleoclimatic fluctuations would favor the isolation of these populations, thence originating troglobites. The extensive coastal karst of Mexico, propitious for marine colonizer adaptation and isolation in freshwaters ideal for originating troglobites [7], among others, explains the higher level of phylogenetic diversity. Only small Brazilian karst areas to the north of Bahia State and close enough to the current coastal line are appropriate for this. Epicontinental seas dating to the Early Cretaceous are too ancient [8].

Regular studies of Brazilian subterranean ichthyofauna started in the early 1980s, initially focusing on the blind catfish *Pimelodella kronei* from the Iporanga, Alto Ribeira karst area ([9–12], among others), with focus on taxonomy, morphology, ecology and behavior. Representative lineages of several genera have also been studied in detail see [13–18]. Hypotheses based on paleoclimatic models (isolation by extinction of epigean relatives during unfavorable phases—[19]) have been proposed to explain the origin of *P. kronei* [20] and *Rhamdiopsis krugi* from northeastern Brazil [21]. Topographic models were proposed for *P. spelaea* [22] and *Ituglanis* spp. [23] from the São Domingos karst area, Central Brazil.

I hereby present a review of the data from these studies, prior to discussing previous hypotheses on forms of colonization and speciation in the various subterranean habitats generating troglobitic fish species in Brazil. With the aim of explaining the high diversity of subterranean ichthyofauna, former and new data were analyzed, as a means of generating novel proposals. Robust models with high predictive potential are scientifically relevant as possible indicators of areas for further research, as well as being the means of testing the generality of hypotheses. Moreover, species presenting intra-population variability, the case of most Brazilian troglobitic fishes [15], are highly propitious for testing hypotheses on the evolution (regression) of characters/features.

Models emerge from the detection of congruent attributed patterns. I compared data on geographic distribution, degree of troglomorphism (primarily eye and pigmentation regression), habitat type and body shape, including hypotheses on apomorphic states related to specialized habitats and distribution. Data are mostly from both our studies and those of collaborators (sometimes unpublished).

2. Taxonomic Considerations

Herein, I embraced the idea of species as separately evolving metapopulation lineages, which is the common element shared by the contemporary alternative species concepts [24]. As regards species delimitation (a separate issue) among the properties acquired by lineages during the course of divergence, viz., intrinsic reproductive isolation, monophyly and diagnosability, I followed de Queiroz [24], for whom diagnosability, i.e., the presence of an exclusive set of character states (not necessarily autapomorphic), widely used by taxonomists, is the most practical operational criterion when assessing lineage separation. Based on the comparative method, it facilitates recognition of subterranean-restricted species [1].

Due to variations in taxonomic approaches and the application of Schiner–Racovitza classification, comparisons of subterranean taxonomic richness between different countries must proceed with caution. Furthermore, when differences do occur in the degree of regional richness (rich vs. poor) [25], discrimination in total diversity (trogloxenes + troglophiles + troglobites), and specifically among troglobites themselves, should become apparent.

There are several instances of cryptic lineages within species described by traditional morphological taxonomy. Morphological stasis in highly specialized ancient lineages may be a consequence of life in a stable phreatic environment over a long period, viz., the Brazilian troglobitic fishes *Rhamdiopsis krugi* and *Trichomycterus dali*. On the other hand, in the case of isolation over relatively shorter periods ("recent troglobites"), with contemporary specialization to subterranean habitats, morphological differentiation may progress faster than molecular marker evolution, due to the accumulation of mutations in structural gene systems, viz., *Ancistrus cryptophthalmus* [26,27].

Due to the emphasis on Red Lists as a basis for defining protection policies, and the tendency to include therein only nominal species from traditional taxonomy, as diagnosed by morphological characters, the case of Brazil, thence leading to legal failure in protecting genetically distinct lineages without discernible morphological differences (cryptic diversity), the aforementioned issues pose important consequences for conservation.

3. Diversity among Brazilian Subterranean Fishes

3.1. Taxonomic Diversity (Species Richness) and Distribution

Taxonomic and geographic correlations between Brazilian putative troglobitic fishes (troglomorphic and those evidently restricted to the subterranean realm) and their respective types of habitats (according to the classification by Trajano [14]) in karst areas (Figure 1), as well as their degree of troglomorphism, are listed in Table 1. The following categories of troglomorphism ("Troglom. degree" in the Table), based mainly on the eyes and melanic pigmentation, were distinguished: (1) no detected troglomorphisms (–); (2) low degree—eyes and pigmentation only slightly, but significantly, reduced compared to epigean congeners (+); (3) moderate degree—high intra-population variation, from the epigean state to its complete absence (++); (4) high degree—eyes and pigmentation conspicuously reduced, but still noticeable in at least part of the population (+++); and (5) very high—population homogeneously anophthalmic and depigmented, frequently with other morphological, physiological and/or behavioral troglomorphisms (++++). In addition to these lineages, two troglomorphic *Trichomycterus* catfishes have been reported by Gallão and Bichuette [28] for Carinhanha Co., Bahia.

Table 1. Brazilian troglobitic fishes (valid species with authors; undescribed species and cryptic lineages with references), indicating the kast areas where they occur (Figure 1) (SP = State of São Paulo; GO = Goiás, BA = Bahia; MS = Mato Grosso do Sul; MG = Minas Gerais), habitat type and degree of troglomorphism (See text for "Troglom. Degree"). Undesc. sp. = undescribed species. Gray lines: endemic to a single cave or cave system. * with adaptations to the deep phreatic zone. Grey lines highlight lneages that are highly endemic.

Species	Distribution—Karst Area	Habitat	Troglom. Degree
Order Siluriformes			
HEPTAPTERIDAE			
Pimelodella kronei Ribeiro 1907 [at least two divergent lineages—Pavan 1945, among others]	Alto Ribeira/SP	Lotic—Base-level streams	++
P. spelaea Trajano, Reis and Bichuette 2004	São Domingos/GO	Lotic—Vadose tributary	+
Rhamdia enfurnada Bichuette and Trajano 2005	Serra do Ramalho/BA	Lotic—Base-level stream	++
Rhamdia undesc. sp. (Gruta das Fadas) [Borghezan 2013]	Serra da Bodoquena/MS	Lotic—Base-level stream	++
Rhamdiopsis krugi Bockmann and Castro 2010 [two lineages—Bichuette et al. 2015)]	Chapada Diamantina/BA	Lentic—Upper phreatic zone	+++
Rhamdiopsis undesc. sp. (Toca do Gonçalo) [Trajano and Bichuette 2010]	Campo Formoso/BA	Lentic—Upper phreatic zone *	++++
Rhamdiopsis undesc. sp. (Gruta do Salitre) [Trajano and Bichuette 2010]	Cordisburgo/MG	Base-level stream	+
TRICHOMYCTERIDAE			
Trichomycterus dali Rizzato, Costa-Jr., Trajano and Bichuette 2011 [three lineages—Cordeiro 2014]	S. Bodoquena/MS	Upper and deep phreatic zones; vadose tributary	+++/++++
T. rubbioli Bichuette and Rizzato 2012	Serra do Ramalho/BA	Vadose tributary; phreatic	+++

Table 1. *Cont.*

Species	Distribution—Karst Area	Habitat	Troglom. Degree
T. itacarambiensis Trajano and Pinna 1996	Peruaçu/MG	Lotic—Base-level streams	++
Ituglanis bambui Bichuette and Trajano 2004	São Domingos/GO	Vadose tributary	++
I. epikarsticus Bichuette and Trajano 2004		Epikarst	++
I. ramiroi Bichuette and Trajano 2004		Vadose tributary/epikarst	++
I. passensis Fernández and Bichuette 2002		Base-level stream	++
I. mambai Bichuette and Trajano 2008	Mambaí/GO	Base-level streams	+
I. boticario Bichuette and Rizzato 2012			+
Glaphyropoma spinosum Bichuette, Pinna and Trajano 2008	Chapada Diamantina/BA—sandstones	Base-level streams	++
Copionodon sp. Gruna dos Torras [Bichuette et al. 2008]			+
LORICARIIDAE			
Ancistrus formoso Sabino and Trajano 1997	Serra da Bodoquena/MS	Flooded cave system	++++
Ancistrus sp. G. Fadas [Borghezan 2013]		Base-level stream	++
A. cryptophthalmus Reis 1987 [2+ differentiated lineages—Reis et al 2006]	São Domingos/GO	Lotic—Base-level streams	++
Isbrueckerichthys alipionis from Santana Cave	Alto Ribeira/SP	Upper vadose tributary	-
CALLYCHTHYIDAE			
Aspidoras albater cave form [Secutti et al. 2011]	Mambaí/GO	Base-level stream	+
PHREATOBIIDAE (sensu Cunha 2008)			
Phreatobius cisternarum Goeldi 1904	Amazonas River delta and Marajó Is.	Non-karst phreatic waters (accessible through artificial wells)	++
P. dracunculus Shibata, Muriel-Cunha and Pinna 2007	Rio Branco drainage/RO		+++
P. sanguijuela Fernandéz, Saucedo, Carvajal-Vallejos and Schaeffer 2007	Rio Guaporé drainage/RO (also in Bolivia)		
Order Gymnotiformes			
STERNOPYGIDAE			
Eigenmannia vicentespelaea Triques 1996	São Domingos/GO	Base-level stream	+
Order Characiformes			
CHARACIDAE			
Stygichthys typhlops Brittan and Böhlke 1965	Jaíba/MG	Phreatic	++++

Among the fish genera listed in Table 1, the (so far) monotypic *Glaphyropoma* and *Stygichthys* are exclusively subterranean.

The São Domingos karst area is distinguished by high fish diversity, both at the species level (more than seven troglobitic species) and above (three genera in three families—two orders). However, when also considering the neighboring Mambaí karst area, there is a jump to 10+ species located in four genera and four families. This represents one third of the total troglobitic fish diversity known so far.

Due to their many peculiarities, the familial allocation of the Amazonian *Phreatobius* has become a matter of debate. During the course of its taxonomic history, it has been progressively allocated into six familes of siluriforms. Consequently, a new separate family was erected for the genus [29]. *P. sanguijuela* is the only Brazilian subterranean fish whose distribution encompasses another country: it was originally described based on specimens from wells in the Rio Paraguá drainage system in Bolivia. The known Brazilian and Bolivian localities are ca. 290 km apart [30].

Except for *Eigenmannia vicentespelaea*, an electric fish (Gymnotiformes), and *Stygichthys typhlops*, a characin (Characiformes) like the amply studied Mexican blind tetra characins of the genus *Astyanax*, all Brazilian subterranean fishes are siluriforms (catfishes). When considering described and undescribed species, as well as diverging lineages of fishes putatively isolated in subterranean habitats, taxonomic diversity, as currently known for Brazil, involves more than 30 lineages.

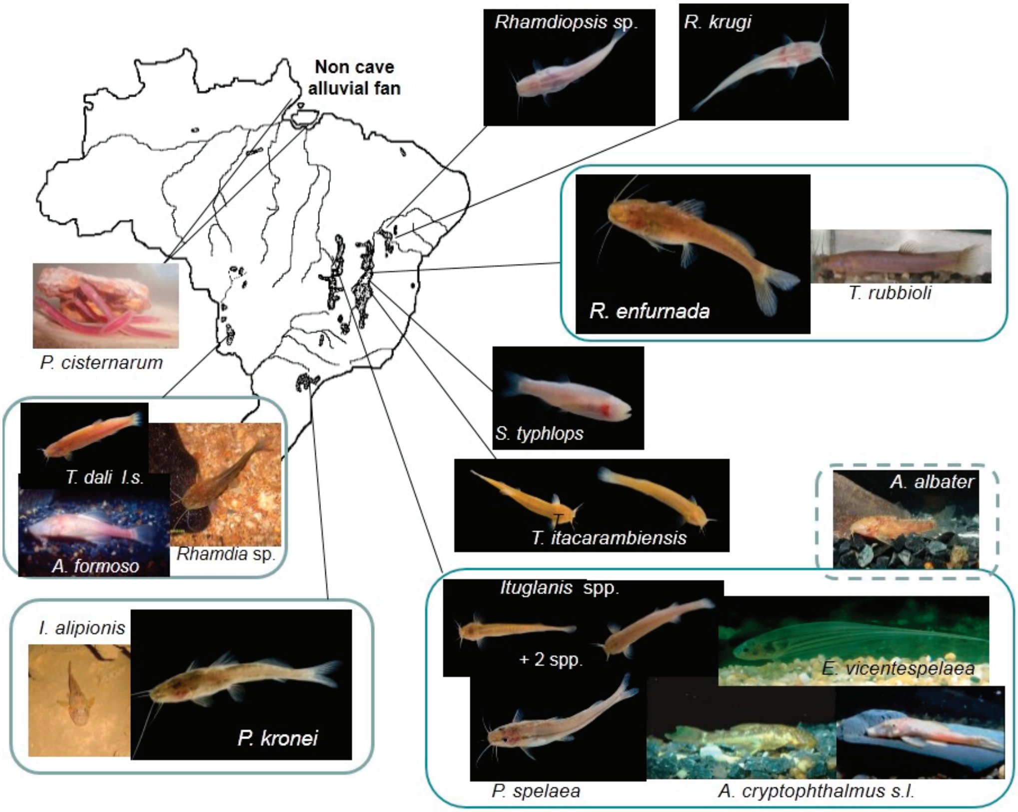

Figure 1. Distribution of selected troglobitic fishes in Brazilian karst areas. Photos: D. Fenolio (*Rhamdiopsis* sp., *Rhamdiopsis krugi*, *Rhamdia enfurnada*, *Stygichthys typhlops*, *Ituglanis* spp., *Trichomycterus itacarambiensis*, *T. dali sensu lato*, *Pimelodella kronei*), J. Muriel Cunha (*Phreatobius cisternarum*), Alexandre Camargo (*Aspidoras albater* cave form), I. Sazima (*Ancistrus cryptophthalmus*, left), E. Trajano (*Ancistrus cryptophthalmus*, right) J. Sabino (*Ancistrus formoso*, *Eigenmannia vicentespelaea*), L.M. Cordeiro (*Rhamdia* sp. Serra da Bodoquena), S. Secutti (*Isbrueckerichthys alipionis*).

These lineages have been accessed through caves (mostly in carbonate rocks) and artificial openings, such as wells. Caves are generally defined as natural underground openings in rocks that are large enough for human entry [31]. This is the operational definition adopted by the Union International of Speleology (UIS), and since it is based on the size of our own species, it is clearly anthropocentric. Caves are large spaces in networks of interconnected heterogeneous spaces of the subsurface and filled with water and/or air that constitute the subterranean realm ([32], among others). Subterranean habitats include karst and non-karst caves (e.g., lava tubes), as well as smaller spaces and interstitial habitats (such as the Mesovoid Shallow Substratum- MSS and the hyporheic). Therefore, the distribution of subterranean populations may, and usually does, surpass the boundaries of "human caves" [4].

3.2. Habitat, Morphology and Adaptations to a Subterranean Life

Brazilian subterranean fishes live in habitats as diverse as the epikarst (uppermost weathered zone of carbonate rocks, capable of delaying water infiltration, as well as storage), vadose tributaries, seasonal or permanent base-level water bodies in the oscillation zone and the upper and deep phreatic zones. While some lineages show adaptation to life in still, low-oxygen waters, and others to life in fast moving waters in typical lotic environments, there are scarce correlations between taxonomic groups and the environment they dwell in [14,15]. On the other hand, there seems to be geographic correlations with the degree of troglomorphism.

About one half of these species, mostly from base-level streams, are endemic to a single cave or cave system (a karst system is a functional unit, involving organized flow pathways forming a drainage unit with input–output zones and finite configurations—[33]). Fishes occupying the phreatic zone present the highest degree of troglomorphy and the widest distribution. A few inhabit areas hundreds of kilometers in length (e.g., *Trichomycterus dali* sensu lato, maximum distance between localities ca. 120 km; *P. sanguijuela* from localities ca. 290 km apart).

For several species, there are no reported epigean relatives living in the same karst area (e.g., *Pimelodella spelaea* endemic to a vadose tributary inside São Bernardo Cave, *Ituglanis* spp. from the São Domingos karst area and *Trichomycterus dali* sensu lato from the Serra da Bodoquena), or in the river basin encompassing the cave system occupied by the troglobitic species (*Rhamdia enfurnado* endemic to Lapa do Enfurnado, *Rhamdiopsis* undesc. sp. from Salitre Cave and *Eigenmannia vicentespelaea* from São Vicente II Cave). Nevertheless, in other cases, epigean congeners are sympatric, or even syntopic, with troglomorphic populations, viz., *Pimelodella kronei*, *Rhamdia* sp. from Gruta das Fadas and *Ancistrus cryptophthalmus*. Epigean *Eigenmannia* electric fishes have been observed in the São Domingos karst area, but not in the microbasin encompassing the cave inhabited by *E. vicentespelaea*.

The trichomycterids *Ituglanis epikarsticus*, *I. bambui* and *I. ramiroi* show adaptations to life in confined spaces, viz., their small size when compared with epigean congeners, a reduced number of vertebrae (36–37 in *I. bambui* vs. 38 in epigean species) and a reduced laterosensory canal system. A reduction in laterosensory canals (which may be fragmented or even absent in some), a common occurrence among troglobitic fishes, is also observed in some cryptobiotic epigean fishes living in confined spaces, such as clefts and crevices, or in interstitial environments, or buried in leaf litter, rocks and sand, the case of *Typhlogobius californienesis* and glanapterygines [15]. The regression of laterosensory canals (but not free neuromasts, which may be hyperdeveloped) is also observed in North American troglobitic amblyopsids and ictalurids, such as *Trogloglanis*, *Satan* and *Prietella*, as well as in *Phreatichthys* from Namibia, the Chinese *Sinocyclocheilus* and the Brazilian *Glaphyropoma*, *Rhamdiopsis* spp. (from Bahia State), *T. itacarambienses* and *T. dali* [34]. *Ituglanis passensis* is medium-sized for the genus, reaching a maximum 65 mm standard length in caves (vs. 42–122 mm recorded for six described congeneric species). However, in spite of its large size, it presents a low number of vertebrae (36) [35] and a reduced lateral line in the body. This also occurs in the other subterranean *Ituglanis* species from São Domingos/GO.

The Phreatobiidae are remarkable for their non-karst habitats in the Amazon basin. *Phreatobius cisternarum*, the first subterranean troglomorphic fish described for South America, lives in alluvial fans distributed around the Amazon delta and Marajó Island, only accessible through hand-dug wells. Epigean specimens have already been encountered in wet-leaf litter in Amapá State [36]. Reichel [37] undertook an anatomical study of this species, which was by far the most detailed for any Brazilian subterranean fish. *P. dracunculus*, even more troglomorphic than *P. cisternarum*, inhabits groundwaters also only accessible through artificial wells. It occurs in Rondônia State, ca. 1900 km in a straight line from Marajó Island. Other still undescribed phreatobiids are to be found in wet-leaf litter on the margins of the Amazonas and Negro rivers [36].

Rhamdiopsis (an undescribed species from Campo Formoso, from now on referred to as *Rhamdiopsis* sp. CF), *Ancistrus formoso*, *Trichomycterus* sp. 2 and *T. dali* sensu stricto (see below—Section 3.3) present the highest degree of troglomorphism (Table 1), with adaptation to deep phreatic zones. The first three species are homogeneously anophthalmic and devoid of any trace of dark pigmentation in vivo. Furthermore, *A. formoso* has even lost the ability to metabolize carotenoids, such as those present in commercial food for aquarium fishes (see [38]). Specialization to life in deep phreatic zones includes the following: (1) adaptation to respiration under hypoxic conditions: in its normal habitat, the skin is dark pink, becoming gradually paler under normoxic conditions in the laboratory, hence indicating facultative cutaneous oxygen uptake; (2) density-dependent mechanisms for population control (cannibalism in *S. typhlops* and *Rhamdiopsis* sp. CF, not observed in the closely related *R. krugi*); and (3) large amounts of subcutaneous fatty tissue, either scattered throughout the body or located at the base of fins, such as the pre-dorsal adipose fold retained in adult *Trichomycterus dali* stricto sensu and *Trichomycterus* sp. 2 sensu Cordeiro [16].

As already mentioned, the degree of troglomorphism correlates with geographic distribution and type of habitat, but not with taxonomy. For instance, troglobitic fishes from the São Domingos karst area, Central Brazil, are moderately troglomorphic, whereas those from the semiarid central-north Bahia State, Northeast Brazil, are highly troglomorphic (Table 1). Likewise, the most troglomorphic species are phreatobic, and the less troglomorphic are stream dwellers. This indicates the influence of common geographic and ecological factors, such as paleoclimate, geomorphology and hydrogeology, on evolution in isolation in subterranean habitats.

3.3. Hidden Diversity

Data on Brazilian subterranean fishes point to landmark morphometrics, a set of techniques with the highest discrimination potential based on shape [26], as a useful tool for uncovering hidden diversity. As an example, studies of *Trichomycterus dali, Rhamdiopsis krugi* and *Ancistrus cryptophthalmus* resulted in splitting these nominal species into two or three lineages [16,21,26].

When geometric morphometric techniques were applied to widely distributed Brazilian troglobitic fishes (*T. dali*, *R. krugi*), the differences found among sets of populations pointed to separate clades, usually associated with alternative microbasins or aquifer vicariant events. Except for *A. cryptophthalmus*, molecular data were consistent with the differences revealed by landmark morphometrics. The importance of multiple and complementary approaches to improve the robustness of species discovery hypotheses and associated descriptions was highlighted by Zimmermamm et al. [39], among others.

Geometric morphometric comparison between two populations of *Rhamdiopsis krugi* ("*Una*" and "*Irecê*"), from (currently) separate sedimentary microbasins, revealed differences in body shape corresponding to the respective site/location, hence indicating divergence in the isolation (or semi-isolation) of the two morphotypes. These results are congruent with molecular data [21].

Extending from north to south, congruent data from geometric morphometric analysis and molecular studies revealed three divergent lineages within the nominal species *Trichomycterus dali*, viz., *Trichomycterus* sp. 1, *T. dali* stricto sensu and *Trichomycterus* sp. 2 [16]. *Trichomycterus dali* stricto sensu, known from five sites, including its type locality, the Saracura Cave on the south-left margin of the Rio Salobra, is distributed throughout an area of ca. 400 km^2. It differs morphologically from the other two lineages by the unique cranial fontanel extending from the posterior half of the supraoccipital to the posterior region of the two frontal bones, with a conspicuous constriction at the meeting point of the supraoccipital and the two frontal bones. *Trichomycterus* sp. 2 (sensu Cordeiro [16]), located at a single site on the Rio Miranda basin, South Bodoquena Plateau, is characterized by an elongated fontanel, a larger number of opercular odontoids and the total absence of melanic pigmentation. *Trichomycterus* sp. 1 (sensu Cordeiro [16]), encountered at five sites across

500 km^2 on the north-left margin of the Rio Salobra basin, North Bodoquena Plateau, is the most divergent and differs from both *T. dali* sensu Cordeiro and *Trichomycterus* sp. 2, by the reduction in the cranial fontanel to the parieto-supraoccipital bone, the more posterior position of the dorsal fin and the formation of a cilinder-shaped body in the adults. The latter is due to regression of the pre-dorsal adipose fold in adults, as observed in epigean *Trichomycteus* spp. This fold is retained in the original form in the other two subterranean lineages, probably as a neotenic adaptation to life in the deep phreatic zone. *Trichomycteus* sp. 1 inhabits a vadose tributary in the Urubu Rei Cave that opens through a resurgence ca. 150 m above the regional base level (probably a nursery), with source populations in the shallow phreatic zone connected to the surface through caves, resurgences and sinkholes. The highly troglomorphic *Trichomycterus dali* sensu stricto and *Trichomycterus* sp. 2 occupy shallow to deep phreatic zones accessible through springs and caves [16].

The nominal species *Ancistrus cryptophthalmus* comprises four partially differentiated populations, viz., one at Passa Três, the type locality, another distributed between two caves of the São Vicente system and one each in the Angélica and Bezerra Caves of the Angélica–Bezerra system, Goiás State, whence the mosaic of morphological and behavioral characteristics originates [26,40,41]. The Passa Três catfish is the most distinct, without any overlap with the other populations as regards body shape [26]. *Ancistrus* armored catfish represent the only case among troglobitic fishes from Goiás State for which epigean congeners are currently present in the same microbasin, with parapatric distribution in Angélica Cave.

According to molecular studies of the hypervariable Region I of MtDNA, there was no apparent divergence between the cave populations of *Ancistrus cryptophthalmus* [27]. Mendes and colleagues [42] used Illumina sequencing technology to produce partial genome sequences of *A. cryptophthalmus* from the Passa Três and Angélica Caves and epigean *Ancistrus* specimens taken near the Angélica entrance. Disregarding variability in eye development, dark pigmentation and divergence in body shape in progress among the cave populations [27], as well as the clear separation of the Passa Três population, not only as to body shape and eye development (these fishes are born with well-developed eyes, which degenerate with growth and become externally invisible in adults [43]), but also the presence of exclusive haplotypes (H7 and H8—[42]), they proposed conspecific status for *A. cryptophalmus* and epigean armored catfish from the Angélica microbasin. Similarities between the epigean and Angélica catfish, including shared haplotypes (H1, H4 and H5), may be due to recent divergence in the cave population and/or some hybridization due to secondary contact with surface specimens. It is relevant that the studied epigean specimens were collected near the cave entrance, which is a sinkhole. Strong floods during the rainy season may carry fish into the cave, whence introgression of epigean genes into the resident cave population occurs. Since lack of proof is not evidence of absence, so far, molecular data have provided no relevant contribution to elucidate the question.

Pimelodella kronei has been found at six sites in four distinct systems in the Betari microbasin, São Paulo State, viz., the Areias de Cima and Areias de Baixo Caves (separated by a karst window, where blind catfish have occasionally been observed at night) and Ressurgência da Areias da Água Quente (=Águas Quentes) (Areias system), Ressurgência das Bombas (Bombas system), Córrego Seco Cave (Córrego Fundo system) and the Alambari de Cima and Gurutuva Caves (Alambari system). These systems were defined according to hydrogeological studies using tracing tests [44]. The Alambari de Cima population probably became extinct in the 1970s due to a pollution event caused by mining activities [34]. The Bombas population is only accessible through individuals foraging at the resurgence during the night.

On comparing specimens of the sympatric epigean *Pimelodella transitoria* with those of *P. kronei* from Areias de Cima and Bombas, Pavan [45] detected differences in morphology as to head shape, number of serrae on the pectoral spine, length of barbels and dark pigmentation. Except for pigmentation, the highest variation occurred in Areias, and the lowest occurred in Bombas (the most divergent). This was confirmed in studies of gross brain anatomy [11] and behavior [46]. Molecular studies focusing on COI and CytB haplotypes are consistent with hypotheses on at least two divergent lineages within *P. kronei*, one from Bombas and the other from the remaining populations (V. Slobodian, G.P.S. Figueiredo and J. Muriel-Cunha, pers. comm. 2016).

4. Origin and Evolution of Subterranean Fishes in Brazil

4.1. The Challenge of Colonizing Subterranean Habitats: The Establishment of Troglophilic Populations

In general, Brazilian troglobitic fishes belong to taxa comprised of typical stream dwellers in the epigean realm. Hence, it is reasonable to suppose that the colonizers of subterranean habitats were already adapted to the lotic environment, and that this state was retained by several troglobitic species as plesiomorphic traits (see heptapterid catfishes [47]).

Subterranean colonization occurs in two distinct surroundings: (1) in large spaces that impose no limitations on movement for large-sized individuals; and (2) in small limited spaces involving adaptation through body and locomotor changes, such as miniaturization, elongation and enhanced maneuverability. The first consists of almost horizontal epigean–hypogean colonization from large to large spaces through sinkholes and resurgences, as well as colonization through wide shafts, as proposed for the Mexican blind fish *Astyanax* [48]. The second would be karst habitat colonization through overlying hyporheic zones, such as interstitial spaces between sediments of the streambed, which could be considered transition zones between the surface and groundwater [33]. Vertical colonization of the subterranean realm, eventually followed by lateral dispersion throughout the aquifer, was proposed by Bichuette and colleagues [21] for *Rhamdiopsis krugi*.

With few exceptions, colonization leads to the establishment of troglophilic populations (species with source populations from both epigean and subterranean habitats, genetically connected by intercommuting individuals—[1]). If submitted to speciation processes, usually due to vicariant events, genetically isolated troglophilic populations may differentiate into troglobitic species. These processes are widely separated in time. Exceptions include the simultaneous colonization and isolation that occur in stream captures [48].

It is noteworthy that the climatic model is an allopatric model for the origin of troglobites due to isolation from epigean populations, and not for colonization [49].

4.2. Modes and Models of Isolation and Differentiation in Subterranean Habitats: Origin of Troglobites

The main allopatric models proposed to explain the origin of troglobites are: (1) the paleoclimatic model—isolation of epigean populations during unfavorable climatic phases/events, with survival in the hypogean realm, whereby species are protected from extreme climatic fluctuations [19]; and (2) the appearance of geomorphological (topographic) or hydrogeological barriers, due to karst evolution.

The paleoclimatic model was indicated for identifying the origin of *Pimelodella kronei*, by differentiation among subterranean populations isolated during dry glacial phases [20]. Secondary contact with epigean descendants of a common ancestor resulted in the present-day situation: six *P. kronei* populations sympatric with the eyed and pigmented *P. transitoria* comprising troglophilic populations in various caves. Based on differences observed in the external morphology between specimens of *P. kronei* from populations in Areias de Cima and Bombas, Pavan [45] debated a possible independent origin through the same *P. transitoria*-like ancestor. Further studies revealed differences in brain anatomy and behavior between the two, consistent with a possibly independent origin [11,46], viz., more attenuated agonistic behavior among Bombas individuals, while those of Areias presented enlarged brains and variable responses to light (vs. consistent photophobic reactions shown in epigean *P. transitoria*), hence configuring a mosaic distribution of traits. Correlations between the Areias and Bombas populations, either as sister taxa or the result of parallel evolution, are still an open question.

In the Bombas population, the high homogeneity and enhanced divergence are indications of founder effects and a small population size. Past hydrological connections facilitating dispersion of cave-adapted *Pimelodella* (a proto-*P. kronei*) may have been disrupted by landscape changes brought about by geological and depositional events. The larger size of the Areias population mirrors the variability in and proximity to *P. transitoria*. The small number of specimens collected in the Córrego Seco and Gurutuva Caves impedes interpopulation comparison.

Counterwise, the São Domingos karst area, located in the Cerrado core area (Brazilian biome with savannah-like vegetation), is divest of accentuated paleoclimatic fluctuations such as those observed in other karst areas, such as in Northeast Brazil and Alto Ribeira [50]. This could explain the relative paucity of local invertebrate troglobites. On the other hand, the diversity of troglobitic fishes could arise from other geomorphological and hydrological factors, such as rapid alluvial down-cutting and the presence of a well-developed epikarst [23].

As stated, the nominal species *Ancistrus cryptophthalmus* from the São Domingos karst area comprises four differentiated cave populations from two systems. Streams of the Angélica, Bezerra and São Vicente 2 system are base level and contiguous with epigean reaches through open sinkholes, where epigean *Ancistrus* catfish form large populations, without any physical barriers separating them from cave dwellers. On the other hand, in the Passa Três system, the stream, a vadose upper tributary of São Vicente 2 Cave, is isolated by topographic barriers, whereby morphological differentiation is consistent with the allopatric model [26]. For base-level stream populations, although parapatric differentiation is a possibility, allopatric differentiation of cave populations followed by secondary dispersion of epigean catfish cannot be ruled out.

Pimelodella spelaea dwells in a vadose stream comprising an upper tributary of the large São Bernardo Cave stream and constantly fed by infiltrated water from the epikarst. This conduit opens onto the surface through the sinkhole of a temporary epigean stream (S.Secutti pers. comm.). Eyed *Pimelodella* probably colonized the São Bernardo system when the base level was higher and the epigean tributary was permanent. With a lowering of the base level, due to either alluvial down-cutting or karst catchment, the stream became temporary, thereby leading to the extinction of aquatic communities. The only fish that successfully colonized and survived in isolation in the cave, probably due to preadaptation (in the sense of exaptation—[51]) to the subterranean life (nocturnal activity, cryptobiotic habits, predominant chemorientation, as in most siluriforms), were *P. spelaea* ancestors.

A similar case is that of the cave population of the armored catfish *Isbrueckerichthys alipionis* from Santana Cave, in the Alto Ribeira karst area. These fishes have been regularly observed for more than three decades (C.F. Lino pers. comm. 1980s; S.Secutti pers. comm. 2015) in an upper vadose stream in the Takeupa gallery, the Ronco stream, which flows into the base-level Roncador stream below. Nowadays, the Takeupa gallery is ca. 70 m below the Furnas streambed. There are several sinks from the centripetal intermittent drainage of polygonal karst basins just over Takeupa [52]. Thus, it is probable that the Rio Ronco is now fed by water infiltrated from the Furnas basin and intermittently by autogenic water from polygonal karst basins. As in the case of *P. spelaea*, these catfish may have colonized the subterranean environment from a past permanent epigean drainage that was part of what is now the Furnas microbasin. With a lowering of the regional base level and karst catchments, there was a disruption: drainage is now represented by polygonal basins fed intermittently by rainwater. Santana Cave has been intensively surveyed, and no *I. alipionis* catfish were ever seen in Rio Roncador. The strong current generated by gradient differences between the upper Rio Ronco and the base-level Rio Roncador hampers fish colonization, hence the topographic isolation. Since isolation in a subterranean realm per se, not necessarily together with troglomorphisms, defines the troglobite status [1], the armored catfish population living in Rio Ronco could be considered troglobitic.

4.3. Stream Dwellers: (Sub)Horizontal Colonization through Sinkholes and Resurgences, Eventually Followed by Isolation

Most Brazilian stream-dwelling troglobitic fishes show moderate to low degrees of troglomorphisms, regarding the development of eyes and melanic pigmentation. The plesiomorphic body shape and size indicate primary evolution in lotic habitats, where no specialization to phreatic groundwater would be required. Hence, stream-dwelling troglobites would retain the primitive body shape and so-called "normal" size (average size of the putative ancestor, inferred by examination of the probably closest epigean relative). This is the case with the two *Pimelodella* and the two *Rhamdia* species, *Rhamdiopsis* sp. from Salitre Cave, *Ancistrus cryptophthalmus* and *Ancistrus* sp. from the Gruta das Fadas, *Trichomycterus itacarambiensis*, *Ituglanis mambai* and *I. boticario*, *Glaphyropoma spinosum* and the syntopic *Copionodon* sp., the *Aspidoras albater* cave form and *Eigenmannia vicentespelaea*. My hypothesis is that (sub)horizontal colonization events occurred through cave "entrances", such as sinkholes and resurgences (spaces much larger than the fish body, thus facilitating maneuverability), as against vertical colonization through the hyporheic zone (see below). These fishes include species with the lowest degree of troglomorphism (eyes and dark pigmentation visible, although reduced when compared to epigean relatives), viz., *Pimelodella spelaea, Rhamdiopsis* from Salitre Cave, *I. mambai* and *I. boticario*, *Copionodon* sp., *A. albater* and *E. vicentespelaea*.

Caves, as defined above, are transient features within karst cycles. According to the commonest speleogenetic process, which originates most karst caves, the initiation phasis starts at the appearance of small fissures in the rock (protoconduits) at the top of the phreatic zone. These progressively become enlarged through dissolution until reaching a critical size (5–15 mm diameter). In the following enlargement phasis, a network of spaces becomes progressively available for colonization by larger and even larger organisms, until reaching the cave size in the human-centered definition (around 1 m). During this phasis, when the water flow changes from laminar to turbulent, with erosion also contributing to conduit enlargement, fast-flowing waters become available for colonization by preadapted populations living in lotic epigean habitats. Eventually, in the cessation phasis, large subterranean spaces disappear due to breakdowns or filling by chemical or clastic sediments [31,53]. This transient characteristic in karst cycles explains why stream-dwelling troglobitic fishes in general are less troglomorphic than the phreatobic ones. Moreover, in the cessation phasis, stream dwellers that have managed to adapt to the lentic conditions of the phreatic zone below may survive over a long geological period until the next cycle.

4.4. Transition from Lotic to Lentic Waters: Adaptation to Phreatic Waters

4.4.1. Stygichthys Typhlops

White piabas, *Stygichthys typhlops*, are typical troglobites of the phreatic zone in the Jaíba aquifer, with adaptations to deep oxygen- and food-poor waters [54]. Although they are currently accessible only through man-made wells, according to local inhabitants, these fishes were also found at the bottom of a certain small natural cave, which was subsequently filled with sediment to avoid vampire bats. Doubts as to their correct position within the large characine subfamily circumscribe applying the comparative method to further studies.

Unlike the slender, flexible siluriforms, allocation and accommodation to small confined spaces in interstitial habitats would require extreme changes in the characiform body plan. The maximum body size and shape of *S. typhlops* are within the range normally observed in the group, with no clear evidence of miniaturization. Hence, there is no clear evidence of karst vertical colonization through hyporheic zones. Ancestors of this fish were probably stream dwellers that colonized caves and non-cave karst spaces through connections with the surface, such as resurgences, insurgences, sinkholes and shafts. It is possible that this population had been isolated by climatic changes that disrupted epigean drainage, leading to alterations from lotic to lentic conditions. Adaptation to deep waters includes physiological and behavioral specialization, such as density-dependent cannibalism, highly vascularized skin and the loss of circadian locomotor rhythms [54].

4.4.2. Flooded Caves: Troglobitic Fishes in Serra da Bodoquena Karst Area

The Bodoquena Carbonatic Plateau stands above the plains of the Pantanal wetlands. The Bodoquena karst area is undergoing subsidence, related to the development of the Pantanal. Apart from climatic variations recorded for the Quaternary (variations in the water table level indicate a drier regional period of ca. 5000 years BP, followed by a wetter climate), tectonic subsidence would be a major contributor to the relative uprising of the water table due to a lowering of the regional base level [55]. There is a difference of almost 1000 m between the higher northern Plateau and the Rio Miranda Depression in the south. Consequently, due to an increase in the hydraulic gradient, Bodoquena subsidence progresses from south to north; hence, the relative uprising of the water table flooding caves and other subterranean habitats in the southern plains predates the observed flooding in the northern plateau.

Hydrological and topographic barriers may account for variations in subterranean populations of *Trichomycterus*. *Trichomycterus* sp. 1 sensu Cordeiro [16], while occupying the upper phreatic zone, with some individuals reproducing in a vadose cave, has retained the body shape typical of epigean *Trichomycterus* that live in lotic habitats, as well as the interopercular odontodes [family sinapomorphy], an adaptation for anchoring in fast-running waters, and vestiges of melanic pigmentation. *Trichomycterus dali* sensu stricto and *Trichomycterus* sp. 2 sensu Cordeiro, although showing adaptations to the deep phreatic zone, preferably reproduce in the less food-limited upper zone [16]. There is no evidence of miniaturization. Studied specimens of *T. dali* and *Trichomycterus* sp. 1 and sp. 2 reached lengths of 83.3, 100.8 and 98.4 mm, respectively (SL) [16,34], which are within the normal range for medium-sized epigean *Trichomyterus* catfishes.

In spite of intense efforts, no *Trichomycterus* catfishes have been encountered in the epigean streams of Serra da Bodoquena. The extinct local populations could have preceded isolation in subterranean habitats. With the lack of evidence of adaptation to small spaces, sub-horizontal colonization of cave streams through large connections with the surface (caves, sinkholes, resurgences, shafts) is supposed. Adaptation to lentic environments as subterranean spaces became flooded due to progressive subsidence of the Bodoquena Plateau from south to north explains the higher degree of troglomorphy in the southern lineage (i.e., *Trichomycterus* sp. 2). Moreover, low genetic and morphological divergence between the latter and *T. dali* may be an outcome of the simultaneous adaptation of both to deep phreatic conditions.

The same model—direct (sub)horizontal colonization of karst spaces through connections with the surface by stream-dwelling ancestors, and secondary adaptation to phreatic groundwaters due to cave flooding—applies to the subterranean armored catfishes *Ancistrus* spp. from Bodoquena. As could be expected, and based on the above-described scenario, the highly troglomorphic *Ancistrus formoso* lives in a flooded cave system in the Formoso microbasin (south plateau) [38], whereas the less troglomorphic and undescribed *Ancistrus* sp. is from Fadas Cave on the northern plateau [16].

4.4.3. Trichomycterus Rubbioli: The Problem of Sink Populations

Trichomycterus rubbioli was studied in two separate caves of the Água Clara system, Bahia State, in an upper vadose stream ca. 180 m long, fed by water infiltration inside Lapa dos Peixes, and from a side pool in Gruna da Água Clara. It shares several troglomorphisms with *T. dali* sensu lato, but at a less advanced stage of specialization (comparisons are based on *Trichomycterus* sp. 2 and *T. dali* sensu Cordeiro), viz., very reduced eyes, externally visible only as spots (vs. eyes that are absent); depigmented when alive, but still capable of light-induced melanogenesis (vs. color-unresponsive to light); barbels longer than in epigean congeners, especially the nasal one; pre-dorsal adipose fold fully developed in juveniles, but poorly so in adults (vs. developed in adults); circadian locomotor activity rhythms reduced (but not so much as in *Trichomycterus* sp. 2); frequents mid-waters and the surface. This indicates life in slow-moving waters in relatively large subterranean spaces. On the other hand, the occurrence in discontinuous caves points to dispersion throughout non-cave spaces.

After the first collections in Lapa dos Peixes (12 specimens caught), the number of observed fishes declined sharply for several months, thus indicating this to be a sink population, i.e., that which, if cut off from migrants, will eventually become extinct, contrary to source poulations that have excess production and so continue to grow if isolated (D. Culver—[56]). So far, the *Trichomycterus rubbioli* source population has not been identified, a factor compromising the formation of a hypothesis on colonization and isolation in the subterranean realm.

Sink populations pose problems for subterranean biology, due to the difficulties in distinguishing the selective regime under which the species population evolved within the chosen habitat. This can lead to ambiguous conclusions on ecology and evolution. The same applies to individuals found in peripheral habitats, such as illuminated cave sectors and artificial wells, the case with *Stygichthys typhlops* [57]. Furthermore, epigean sink populations obscure recognition of the troglobitic status (exclusively subterranean source populations) of troglomophic organisms.

4.5. Multiple-Step Model of Colonization and Differentiation in Subterranean Habitats: The Way through the Hyporheic Zone and Epikarst

4.5.1. *Rhamdiopsis* spp. from Semiarid Bahia

Besides presenting adaptations to date, morphology also supplies information about certain past evolutionary steps (phylogenetic signals), which presume evolutionary constraints. Mosaics of incongruent character states provide evidence of miscellaneous adaptive regimes over time [21]. In order to explain the coexistence of adaptations to both small, confined spaces and large ones of the troglobitic heptapterid *Rhamdiopsis krugi* from northeastern Brazil, Bichuette and colleagues [21] proposed for this species a two-step model of vertical colonization of subterranean habitats through the epikarst, followed by adaptation to the larger spaces underneath (Table 2).

Table 2. Vertical colonization of subterranean habitats by stream dwellers: schematic sequence of evolutionary steps originating present-day Brazilian troglobitic fishes.

Species	Step 1	Step 2		Step 3
Rhamdiopsis krugi	Colonization of the hyporheic zone	Colonization of the subterranean habitat beneath the hyporheic zone	Genetic isolation	——-
Rhamdiopsis sp. CF				Transition from upper to deep phreatic zone
Ituglanis bambui, I. epikarsticus, I. ramaroi		Colonization of epikarsts		Sink populations in vadose streams and the main river channel
I. passensis				Secondary adaptation to a lotic habitat due to the opening of large entrances
Phreatobius spp.		Colonization of sedimentary layers beneath the hyporheic zone		——

Step 1: Colonization of the hyporheic zone by typically stream-dwelling *Rhamdiopsis*-like catfishes was achieved through miniaturization (probably by paedomorphosis), as well as by possessing slender, elongated and flexible bodies with a reduced lateral line on the trunk, and other adaptations for energy economy in a food-poor environment, when compared to the surface, for instance, the distribution of fatty cells throughout the body. Reduction in body size may also be an adaptation for the more efficient use of energy in

extreme environments [58]. Survival and establishment of self-sustained populations in the hyporheic zone were possible due to: (1) preadaptations of epigean ancestors (nocturnal activity, cryptobiotic habits, generalist carnivory disposition and elongated bodies, typical of most siluriforms); and (2) the presence of potential prey appropriate for small catfish, in the form of a diversified community of microcrustaceans, such as copepods, ostracods and amphipods, together with micro-oligochaetes, nematoids and other minute organisms (e.g., [59,60]).

Step 2: Colonization through the hyporheic zone to the underlying subterranean habitat implied acquiring the ability to navigate in large and heterogeneous spaces. This requires adaptations that contrast with those achieved for the hyporheic zone. These comprise enhancement of sound sensitivity (highly exposed pseudotympanum), as well as a broadening of the head and snout to reduce pitching and rolling during swimming in mid-water, and which present a wider surface for free neuromasts, taste buds and other sensory structures. Isolation in subterranean habitats can occur during the dry phases of paleoclimatic cycles (such as that of the present day in the region), when epigean drainage is disrupted. Based on speleothem dating, the alternation of dry and wet periods in central Bahia over the last 210,000 years has been well documented [61]. Thus, due to the local extinction of epigean relatives, the paleoclimatic model could explain *R. krugi* speciation by allopatry [21].

In order to explain differences between the populations from two distinct microbasins (Una and Irecê), Bichuette and colleagues [21] proposed geographic isolation (a least partial) by geological (insoluble rocks between the two limestone outcrops), depositional (colmated connecting ducts) and hydrological (running waters) barriers. Since the monophyly of *R. krugi* is well supported by 24 autapomorphies [62], it is reasonable to assume a past connection through a continuous aquifer in the limestone outcrop, which was subsequently disrupted by karst denudation.

Taxonomic studies based on an extensive and detailed examination of the morphology and anatomy of *Rhamdiopsis* sp. CF point to a sister group relationship between this catfish and *R. krugi*. When compared to epigean *Rhamdiopsis*, these troglobitic species share at least 14 sinapomorphies, of which at least five can be considered troglomorphisms. These consist of the absence of eyes and an atrophied optic foramen, the very short laterosensory canal in the trunk, regression of circadian rhythms and cryptobiotic habits and extended spatial activity involving the mid-water and surface. They also have in common small-sized adults (wild-caught specimens less than 46 mm SL), a short trunk with few segments, usually 38–40 vertebrae, and a widely exposed pseudotympanum, at least in juveniles, as well as several osteological characters, such as anterior and posterior branches of the transverse process of vertebra 4 with broad bases, an undivided or weakly separated anterior portion of the spatulate-shaped posterior branch of the transverse process of vertebra 4, a parhypural fused to the complex plate formed by hypurals 1 and 2 and hypural 5 co-ossified with the complex plate formed by hypurals 3 and 4 [F.A.Bockmann and E.Trajano, unpubl. data].

Adaptations of *R. krugi* to phreatic waters are also present in the even more troglomorphic *Rhamdiopsis* sp. CF. The latter probably represents an even further step in phreatic zone colonization, viz., adaptation to a deep phreatic habitat characterized by hypoxia and energy deprivation conditions, as well as high hydrostatic pressure, representing an additional step, **Step 3**, in the previous model (Table 2). At this stage, the head and trunk have become almost entirely filled by fatty subcutaneous corpuscles, cannibalism may occur probably as a density-dependent mechanism for population control and the skin becomes dark pink, when in the natural habitat, thereby presuming facultative cutaneous oxygen uptake.

The putative sister group relationship of *R. krugi* and *Rhamdiopsis* sp. CF implies a subterranean connection between areas. *Rhamdiopsis* sp. CF data are based on specimens found in the upper zone connected with the surface through Toca do Gonçalo, which developed in the Caatinga limestones, where karstification probably begun only ca. 2.5 mi years ago. On the contrary, the contiguous Una carbonates and the Salitre Formation, present not only in Campo Formoso, but also in Chapada Diamantina, were exposed at the beginning of the Paleocene, ca. 65 mi years ago [63]. The high degree of troglomorphism and relictual status indicate an ancient origin not only for *Rhamdiopsis* sp. CF, but also for syntopic troglobitic invertebrates, such as the calabozoan *Pongycarcinia xiphidiouros* and amphipod *Spelaeogammarus trajanoae* (widespread in Campo Formoso). Their ancestors probably evolved in the Una karst and subsequently dispersed to the vicinity of the Caatinga limestones, becoming available for biological colonization through karstification. Hence, Toca do Gonçalo would be a peripheral habitat.

Aquifer connectivity throughout a large area in north-central Bahia is corroborated by the presence of amphipods of the exclusively subterranean genus *Spelaeogammarus*, with six nominal species distributed over 1200 km on the N–S axis. These species are found in several karst areas (defined by surface features), including Campo Formoso and Chapada Diamantina [63].

Dispersal throughout the deep phreatic zone of the Una limestones between Chapada Diamantina and Campo Formoso by a common ancestor would explain the adaptations of *Rhamdia* sp. CF to life in deep groundwater, whereas *R. krugi* remained at least partially associated with the upper phreatic zone. Although capable of descending to as deep as 45 m, the highest population densities of the latter are observed near the surface. According to this model, the local presence of troglobitic *Rhamdiopsis* could be expected in small discontinuous limestone outcrops between Chapada Diamantina and Campo Formoso.

4.5.2. Ituglanis Catfishes from the São Domingos Karst Area

The four troglomorphic *Ituglanis* species found in the São Domingos karst area inhabit caves in distinct microbasins, viz. (from north to south), *I. bambui* in Angélica Cave, *I. passensis* in the Passa Três, *I. epikarstikus* in the São Matheus and *I. ramiroi* in the São Bernardo (see Table 1). The mosaic distribution of morphological traits and separation of the cave systems indicate the independent origin of the four species from a common epigean ancestor, nowadays extinct in the region [23].

As mentioned, these catfishes show special adaptations to confined spaces, such as miniaturization (except for *I. passensis*), a reduced number of vertebrae and a shorter lateral line on the trunk. *Ituglanis ramiroi*, *I. bambui* and *I. epikarsticus* occur in habitats associated with the epikarst: the first two in vadose tributaries fed by epikarst waters; the latter in a permanent rimstone pool. The first specimens of *I. ramiroi* were collected in small side pools by the main river channel. The small number of specimens and the high population turnover, with very low recapture rates [64], indicate that these are sink populations, with source populations living in the epikarst. Additional evidence is provided by the occurrence of *I. bambui* at the resurgence end of Angélica Cave, ca. 6 km in a straight line from the vadose stream where they were first studied. Dispersion throughout the epikarst is the most parsimonious explanation for such a disjunct distribution inside the cave.

Colonization of the epikarst probably occurred throughout the hyporheic zone of the epigean drainage system formerly crossing the area, when the regional base level was above the present-day caves. With the opening of the caves and rapid alluvial downcutting, the regional water table dropped to its present-day position, while suspended aquifers became isolated in the epikarst of parallel microbasins [26] (Figure 1). Surviving populations would then differentiate, thereby giving rise to troglobitic *Ituglanis* species. Hence, these species can be placed in **Step 1** of our model of vertical colonization and differentiation in subterranean karst habitats (Table 2).

In contrast, *Ituglanis passensis*, medium-sized for the genus, lives in the open channel of an upper tributary, a typical lotic habitat. This is an allochthonous stream, carrying large amounts of organic matter through the sinkhole during the rainy season. Nonetheless, these catfish also present traits associated with **Step 1** in the other species from São Domingos (36 vertebrae, reduced lateral line on the trunk). Hence, I propose that *I. passensis* originated from a miniaturized ancestor living in a vadose tributary, as occurred with *I. bambui* and

I. ramiroi. When the ceiling collapsed following erosion, the large opening to the surface became the sinkhole of a small epigean stream, thereby facilitating the input of larger amounts of organic matter. Intraspecific competition in the absence of food limitation could account for the selection of larger sizes, thus leading to a reversal of miniaturization. This would be a third step in a different model (Table 2).

4.5.3. The Amazon Basin: *Phreatobius* spp.

The uniqueness of Phreatobiidae (sensu Cunha [29]) indicates ancient adaptation to a very specialized habitat (wet-leaf litter at stream margins, interstitial subterranean spaces). The subterranean *Phreatobius* populations known so far, those of *P. cisternarum*, on the one hand, and *P. dracunculus* and *P. sanguijuela*, on the other, occur at nearly opposite sides of the extensive Brazilian Amazon, at least 1900 km apart [36], thus indicating a widely distributed ancestor. The possible isolation of *Phreatobius* populations at the periphery of the huge Amazon basin was due to a lowering of the water table, as a result of drainage cut-down, associated with the Andean uplift and cycles of global sea level drops, as recorded in the Middle Miocene (~12 Ma) [65]. The vertical colonization of subterranean habitats through streambeds, followed by speciation in non-karst interstitial habitats, is a reasonable scenario (Table 2).

It is noteworthy that the *Phreatobius dracunculus* and *P. sanguijuela* sites in Rondônia State are separated by Serra dos Pacaás Novos and Chapada dos Parecis, important water dividers in the region [30]. Dispersion through a hyporheic zone with continuous drainage is a possible explanation for the wide distribution of the highly troglomorphic *P. sanguijuela* in Brazil and Bolivia at distances of up to 290 km.

5. Conclusions

The high taxonomc and phylogenetic diversity of Brazilian troglobitic (exclusively subterranean) fishes (30+ lineages in seven families and three orders, mostly Siluriformes) could be attributed to high epigean diversty, allied to opportunities for colonization and genetic isolation in the subterranean biotope.

Colonization of and isolation in the subterranean biotope are two independent processes, usually separated in time. Colonization with the establishment of self-sustained hypogean populations originates troglophiles (source populations in both epigean and subterranean habitats, genetically connected by mutually commuting individuals).

Pathways of fish access to subterranean habitats vary from (sub)horizontal colonization through sinkholes and resurgences of base-level streams (typical lotic habitats) and through vadose tributaries to vertical colonization of shallow and deep phreatic waters (lentic habitats) through the hyporheic zone.

Phenotypic (morphological, physiological, behavioral) differentiation in subterranean populations originating troglobites is achieved by various, unmutually exclusive modes of genetic isolation:

Directly in base-level streams and upper tributaries through the extinction of epigean populations brought about by drainage discontinuation during dry paleoclimatic phases (e.g., *Pimelodella kronei*, *Rhamdia enfurnada, Trichomycterus itacarambiensis*) or by other causes (*Pimelodella* undesc sp. from Fadas Cave).

By topographic isolation due to a lowering of the regional base level and karst catchments (*Ancistrus cryptophthlmus* from Passa Três Cave, *Isbrueckerichthys alipionis*).

By parapatric differentiation (possibly the case of *Ancistrus cryptophthalmus* from the Angélica system).

Adaptation to the deep pheatic zone may be a consequence of active dispersion (*Rhamdiopsis* sp. CF) or cave flooding due to the relative uprising of the water table (*Trichomycterus dali* sensu stricto and *Trichomycterus* sp. 2 sensu Cordeiro).

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Monitoring Brazilian Cavefish: Ecology and Conservation of Four Threatened Catfish of Genus *Ituglanis* (Siluriformes: Trichomycteridae) from Central Brazil

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Abstract: We investigated population parameters using the capture-mark-recapture method (*Ituglanis passensis* and *I. bambui*) and visual censuses (*I. epikasrticus* and *I. ramiroi*). The four species are regionally threatened (Brazilian Red List) and occur in caves of Terra Ronca State Park, São Domingos region, Central Brazil. We conducted seven fieldtrips across three years of dry seasons. For the capture-mark-recapture method, a 400 m long stream inside Passa Três cave, where *I. passensis* occurs and a 300 m long reach of an upper tributary where *I. bambui* occurs inside Angélica cave were divided into 20 and 17 study sections, respectively. Catfishes were hand-netted, measured (standard length), weighed, marked by subcutaneous injection of biocompatible pigments, and released. The four species show medium-to-low mean population densities (0.03 ind/m^2 0.7 ind/m^2). Capture-mark-recapture data pointed to a relatively small population size for *I. passensis*, restricted to a 1600 m long stream inside Passa Três cave (N = 719) and for *I. bambui* (N = 246; 300 m of epikarstic drainage). *Ituglanis passensis* and *I. bambui* move along relatively small areas with a restricted home range. We observed recruitment patterns and a trend of decrease in condition factor during the dry seasons for *I. bambui* but not for *I. passensis*, suggesting that epikarstic waters are probably more limiting concerning food input. *Ituglanis passensis* and *I. bambui* showed low growth and consequently high longevity, corroborated by captivity data (20 years). We suggest urgent actions and long-term monitoring projects to effectively protect this unique and endemic ichthyofauna.

Keywords: population ecology; growth; movements; São Domingos karst area; conservation; epikarst

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1. Introduction

Troglobites, characterized by exclusively subterranean species [1], show, in general, small population size [2]. In many cases, this statement is based on inference, taken from the notion of restricted geographic distribution (which applies to many but not all species) associated with low population densities rather than on data obtained through population study methods, such as capture-mark-recapture.

Available data for subterranean fish show that population densities may vary considerably, from very low to very high, and are sometimes higher than their relatives from epigean (surface) environments [3]. This variation can be partly related to the methods of study since the lowest densities are usually recorded in studies that used the visual census method [3].

There is great difficulty in estimating the absolute size of the troglobitic populations, important not only from a scientific point of view but also for conservation purposes. Most of the time, we do not know the full extent of habitats occupied by the species, and the characteristics of the subterranean realm, which is largely inaccessible, prevent accurate studies [3].

Based on literature data, Trajano (2001) proposed a classification for possible population density patterns for troglobitic fish: species with low population densities (<0.1 ind/m^2), for example, *Nemacheilus troglocataractus, Typhlichthys subterraneus, Pimelodella kronei*, *Rhamdiopsis krugi* (Poço Encantado cave population), and *Rhamdiopis* sp.; species with medium population densities (0.1–1.0 ind/m^2) [4] such as *Schistura oedipus*, *Amblyopsis rosae*, *Trichomycterus itacarambiensis,* and *Ancistrus cryptophthalmus*; and species with high population densities (1.0 ind/m^2), such as *Astyanax* spp. [5], *Poecilia mexicana,* and *Caecobarbus geertsii*. Variability in the densities is observed for *Eigenamannia vicentespelaea* population (densities varying from 0.04 ind/m^2 to 0.17 ind/m^2), tending toward low [6], and *Rhamdiopsis krugi* (densities varying from 0.07 ind/m^2 to 9 ind/m^2 indicated by personal observation (pers. obs.) from M. E. Bichuette.

Ecological characters usually associated with energy saving comprise the life cycles of many troglobites, which feature strategies tending to K in r-K continuum or early life cycle *sensu* [7]. Such strategies include production of low fecundity, infrequent reproduction, delayed reproductive maturity, slow individual growth, and high longevity [8], interpreted as adaptations to conditions of low, infrequent, and often unpredictable nutrient supply. One consequence of scarce and infrequent food is the generally small home range, with some cavefish species presenting low individual movements and therefore a sedentary behavior [9–12], saving energy. However, some studies have shown the contrary, a large home range [13,14].

Because of these attributes, low values of condition factor have been recorded for many cavefish and are attributed to scarce food availability [3], commonly observed (with exceptions) in subterranean habitats. Few studies have produced robust reliable data on growth rates and longevity in troglobitic fish [5,15] for *Caecobarbus geertsii*, [16,17] for Amblyopsidae cavefish [9,10] and [12] for cave catfishes *Pimelodella kronei*, *Trichomycterus itacarambiensis,* and *Ancistrus cryptophthalmus*, respectively. Such studies have indicated an early life cycle for these species.

Trichomycteridae (Gill, 1872) catfishes represent one of the most diverse groups of Siluriformes (Ostariophysi), with 304 species [18]. Currently, the family Trichomycteridae is considered the third richest in exclusively subterranean species, with 19 troglobites distributed in four genera: *Glaphyropoma* [19], *Silvinichthys* [20], *Trichomycterus* [21–27], and *Ituglanis* (six species) [28–31].

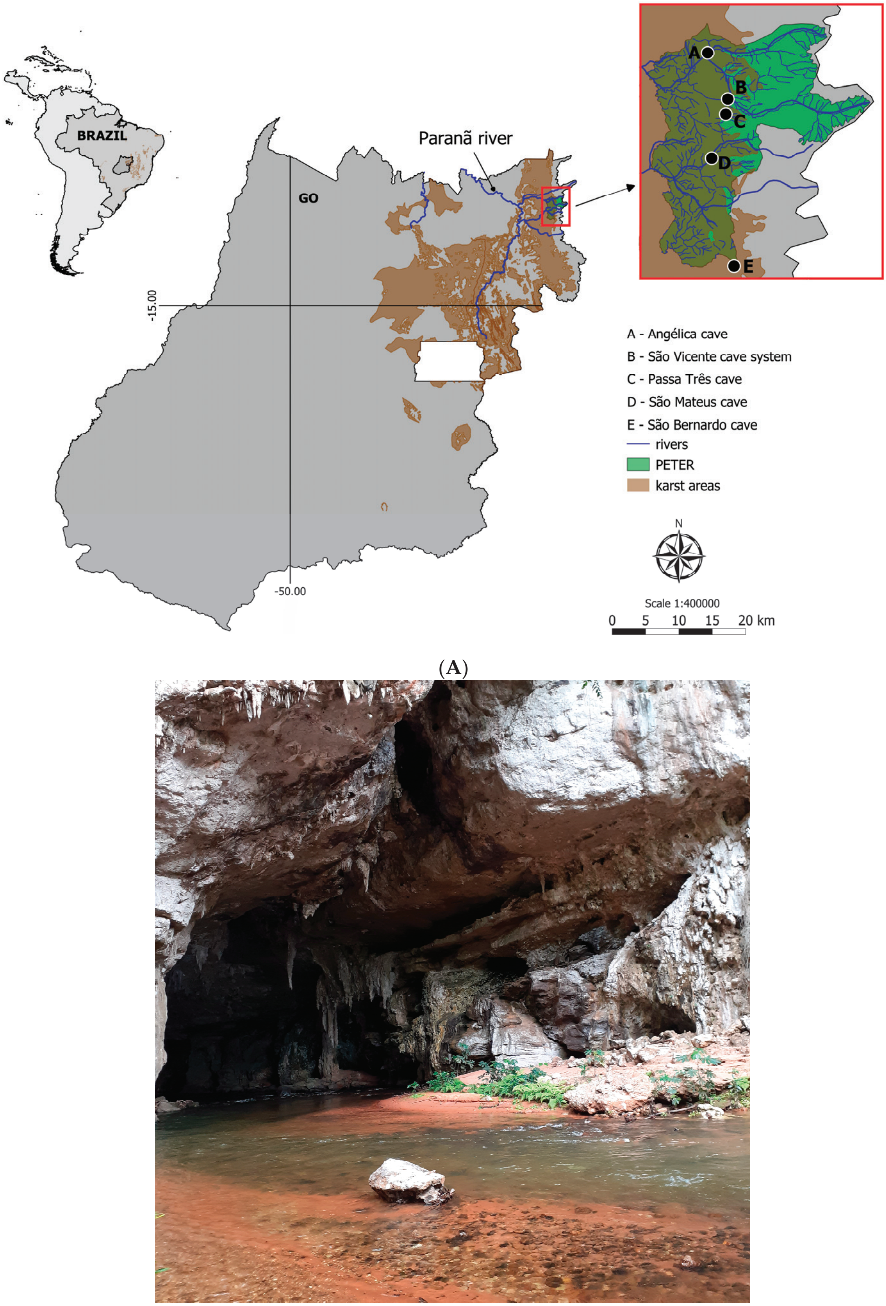
Ituglanis (Costa and Bockmann, 1993) includes six troglobitic species, all from limestone caves in Central Brazil: *I. passensis* (Fernández and Bichuette, 2002)*, I. bambui* (Bichuette and Trajano, 2004), *I. epikarsticus* (Bichuette and Trajano, 2004), *I. ramiroi* (Bichuette and Trajano, 2004), *Ituglanis mambai* (Bichuette and Trajano, 2008), and *Ituglanis boticario* (Rizzato and Bichuette, 2014), plus three undescribed species (M.E. Bichuette, pers. obs.). Most species are characterized by reduction until absence of eye and melanic pigmentation [28,29,31].

One of the places with high endemism of subterranean *Ituglanis* species in Brazil is the São Domingos karst area (upper Tocantins River basin) [32,33]. Four exclusively subterranean *Ituglanis* occur in caves from Terra Ronca State Park, and all are regionally threatened: *I. passensis*, *I. epikarsticus,* and *I. ramiroi* (vulnerable category, VU) and *I. bambui* (critically endangered category, CR) [34]. We monitored these populations with the aim to determine some population parameters: population sizes and densities, recruitment, and condition factor (body conditions). These data are useful in conservation proposals to protect and maintain these populations.

2. Materials and Methods

2.1. Study Area and Fish

The study sites were located in Terra Ronca State Park (46°10′–46°30′ S, 13°30′–13°50′ W) in the upper Tocantins River basin, State of Goiás, Central Brazil. The park is part of large limestone and sandstone landscape commonly called São Domingos karst area. More than 200 caves occur in the region, including some of the largest cave systems in Brazil (some with more than 20 km of map passageways) (Figure 1A).



(**A**)

(**B**)

Figure 1. (**A**) Map of study area, São Domingos karst area, Central Brazil, upper Tocantins River basin, source: D. M. Von Schimonsky; (**B**) sinkhole of São Vicente cave system.

The troglobitic populations of all four *Ituglanis* catfishes from the São Domingos karst area live in the upper levels of subterranean systems isolated from each other by non-karst rocks (Figure 1B). The subterranean streams run parallel westwards and dozens of kilometers downstream until their resurgence; hence, there are no subterranean connections between those populations, which seemingly evolved independently.

The *Ituglanis passensis* (Figure 2A) population is endemic to Passa Três cave, an upper vadose tributary of the São Vicente cave system, São Domingos karst area, Central Brazil. There is morphological evidence for preterit adaptation to the epikarst, indicating vertical colonization of the subterranean biotope [32].

(**A**) (**B**)

(**C**) (**D**)

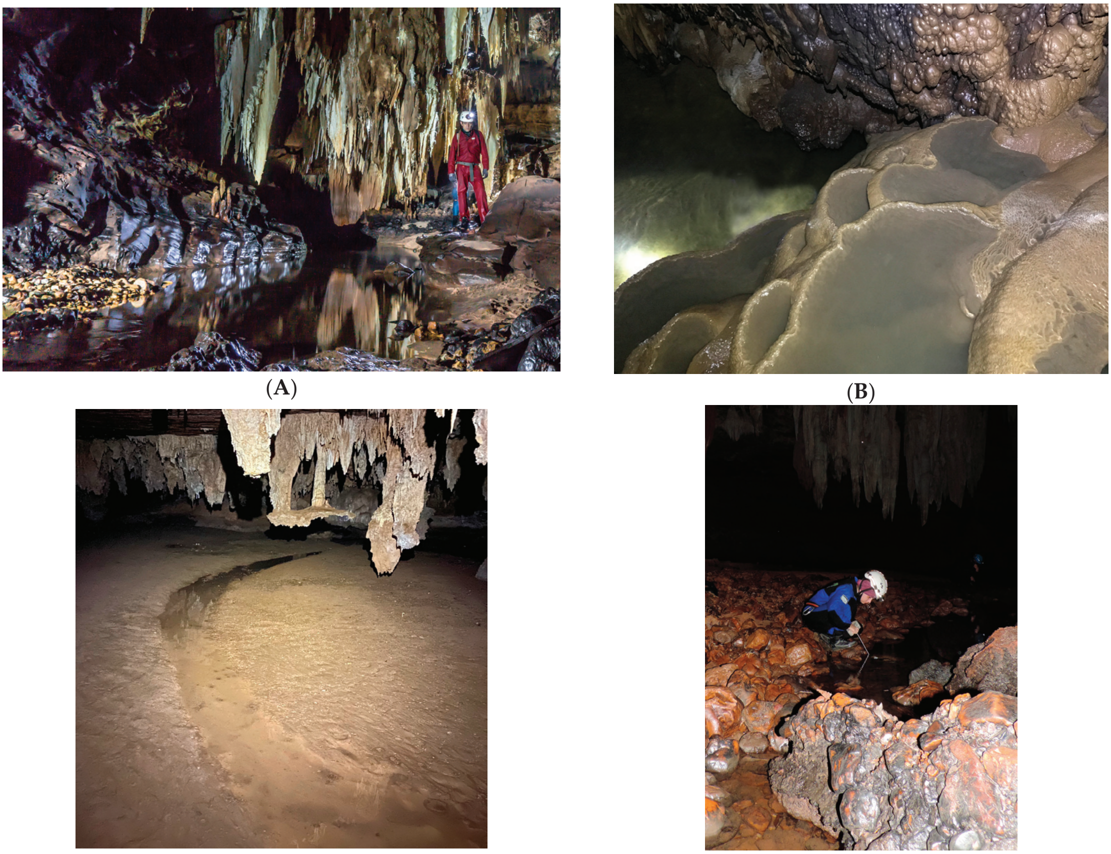
Figure 2. *Ituglanis* species from São Domingos karst area, central Brazil. (**A**) *Ituglanis passensis*; (**B**) *Ituglanis epikarsticus*; (**C**) *Ituglanis bambui*; (**D**) *Ituglanis ramiroi*. Photos: D. Fenolio.

Among the species that live and disperse through non-cave habitats, *I. epikarsticus* (Figure 2B) was the first described troglobitic fish adapted to life in the epikarst [29]. The species is known from few specimens found in a set of rimstone pools located ca. 1000 m deep inside São Mateus cave, São Domingos karst area, Central Brazil; this is the only place where such catfish are found. There is no contact between the matrix rock and the rimstone pools; therefore, there is no possibility of horizontal movements to and from non-cave habitats. One possibility for these catfish to reach the rimstone pools is through washing events from the epikarst population. *Ituglanis epikarsticus* has not been observed anywhere else in the cave, which has been explored by speleologists since the 1970s [29].

Ituglanis bambui (Figure 2C) was described based on specimens found in an upper vadose tributary inside Angélica cave, São Domingos karst area, Central Brazil, located near (ca. 200 m) the cave sinkhole [29]. Later on, several specimens matching *I. bambui* description were recovered in rimstone pools near the opposite (the resurgence) end of the cave system, approximately 4 km from the first site in a straight line. This indicates that the *I. bambui* population distributes through the epikarst above Angélica cave. Fish observed in the vadose tributary near the sinkhole may be a small cave peripherical population of the main population [32].

Ituglanis ramiroi (Figure 2D) was first known from specimens collected at side pools (Figure 3D) at the base level stream conduit in São Bernardo cave [29]. Later on, individuals were found in an upper tributary located near the surface level and fed by the epikarst aquifer, above the tributary where the troglobitic *Pimelodella spelaea* catfish lives [35].

Figure 3A–D below show the details of habitats of the four *Ituglanis* species.



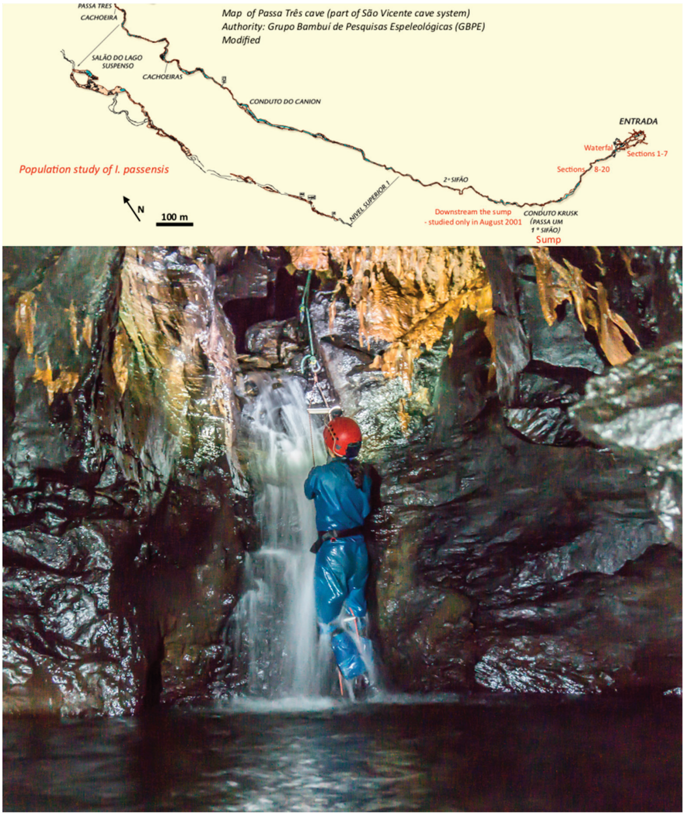
(**A**) (**B**) (**C**) (**D**)

Figure 3. Habitats of *Ituglanis* species from São Domingos karst area, Central Brazil. (**A**) Subterranean level-base stream at Passa Três cave, locality of *I. passensis* (photo: D. Menin); (**B**) rimstone pools at São Mateus cave, locality of *I. epikarsticus* (photo. A. Lobo); (**C**) upper vadose tributary from Angélica cave, locality of *I. bambui*; (**D**) pool formed by epikarstic water at São Bernardo cave, locality of *I. ramiroi* (photo: D. F. Torres).

2.2. Fieldwork

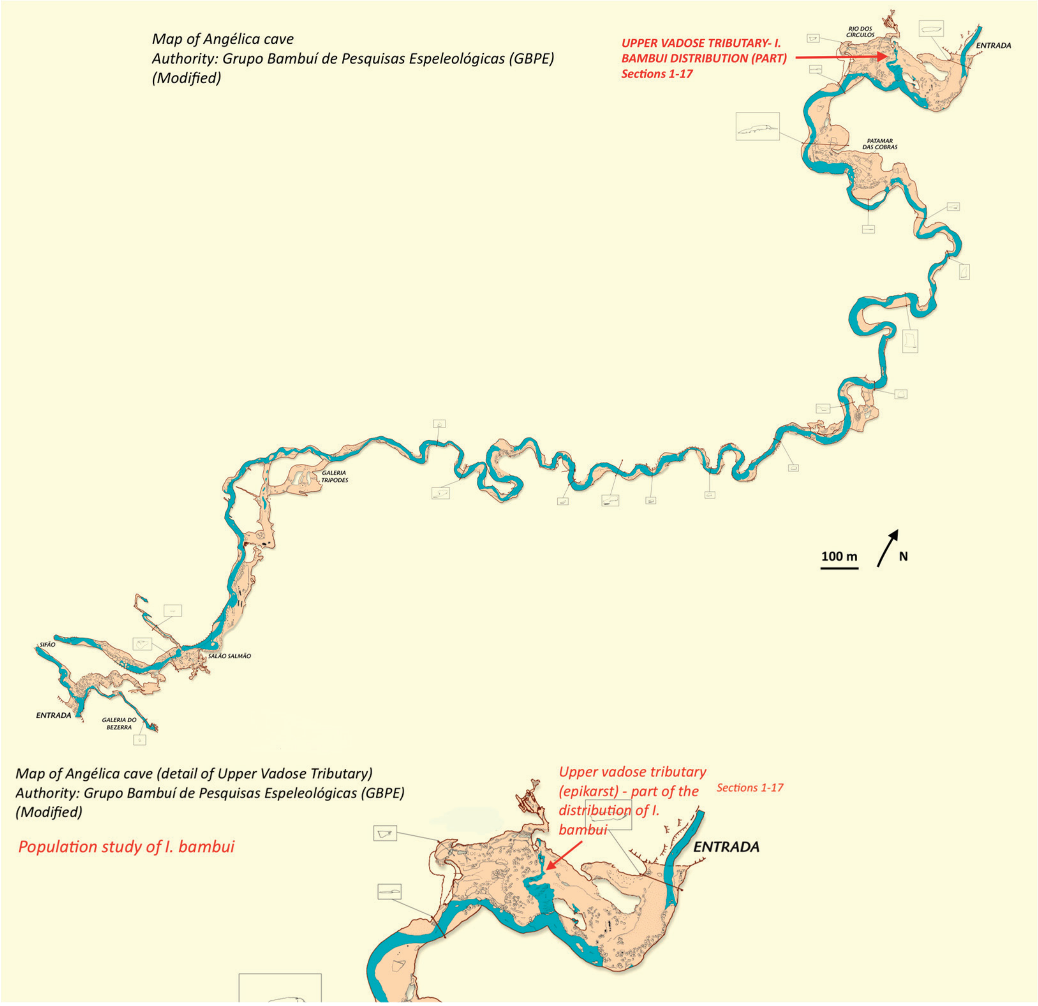
Ituglanis passensis (Passa Três cave) and *I. bambui* (Angélica cave) were studied in seven fieldtrips conducted across three years (between July 1999 and August 2001), always in the dry season, with variable intervals between each visit: 8–25 July 1999, 3–19 September 1999, 23 April–7 May 2000, 18 July–6 August 2000, 9–19 September 2000, 27 April–14 May 2001, and 5–26 August 2001. After we realized that the marks were lost after a few months due to reabsorption, we visited Passa Três cave twice (with an interval of 11 days) and Angélica cave also twice (with an interval of seven days) in August 2001. Direct observations and capture data were used in the calculations of population parameters. Fieldwork was restricted to the dry season due to the occurrence of flash floods in the rainy season.

To study the *I. passensis* population, we divided the stream conduit in 20 sections 20 m long, marked with colored plastic tape. Section 1 to Section 4 were located before a waterfall of 6 m (Figure 4A), about 100 m from the entrance to the cave (sinkhole). The remaining sections were located downstream from this waterfall towards the deeper area of the cave, ending at a short sump (a section of flooded passage), transposed only in July 2001 (Figure 4A). Using the mark-recapture method, the study covered about 400 m^2 (calculated from map), that is, ca. 1/5 of the accessible habitat occupied by *I. passensis*. In addition, visual censuses were conducted in July and August 2001 downstream from the sump. To study *I. bambui*, we divided the upper vadose tributary of Angélica cave into 17 sections (ca. 900 m^2 calculated from a map); each section was 15 m long, and widths varied between 0.80 and 10 m (Figure 4B).



(**A**)

Figure 4. *Cont.*



(**B**)

Figure 4. (**A**) Passa Três cave map and waterfall located between 7 and 8 sections. Map modified from Grupo Bambuí de Pesquisas Espeleológicas (GBPE), photo of waterfall: D. Menin. (**B**) Angélica cave map with detailed sections on upper vadose tributary. Map modified from Grupo Bambuí de Pesquisas Espeleológicas (GBPE).

On three occasions (July 1999, July or August 2000, and August 2001), we conducted visual censuses for *I. epikarsticus* at São Mateus cave (two hours of observations on each occasion) and *I. ramiroi* at São Bernardo cave (three hours of observations). We measured the areas of rimstone and side pools to estimate the densities of individuals in these populations.

We captured the specimens with hand nets after visual inspection of the habitats, including potential hiding places such as rock crevices, under stone chips and pebbles, and by revolving the bottom to dislodge buried fish. The time of inspection was 30 min per section in Passa Três cave and 30–40 min per section in Angélica cave.

After capture, the catfish were anesthetized in a solution of benzocaine, weighed (W) with a dynamometer (0.20 g accuracy), and measured (standard length, SL, in mm) with a caliper with 0.1 mm accuracy. They were marked using subcutaneous injection of biocompatible ink (nankeen). For *I. bambui*, we marked individuals larger than 23.0 mm SL; we did not capture *I. passensis* specimens smaller than 35.0 mm SL. Individual marking took place by a combination of the position of marks, in the flanks (left or right side), below the dorsal fin. For each capture, we recorded date, section, position of the marks, weight, and length. The fish were then released in the same section in which they were collected.

We measured the water physico-chemical variables at Passa Três and Angélica caves (pH, conductivity.ms.cm^{-1}, dissolved oxygen.mg.L^{-1}, temperature/°C, and salinity/%) during the population study. We established two fixed points located in the aphotic zone of Passa Três cave (section 3, 50 m downstream the sinkhole/cave entrance; section 17, 340 m downstream from the sinkhole/cave sinkhole) and in one point at the aphotic zone of Angélica cave (section 9, about 200 m from the cave entrance). For São Bernardo (*I. ramiroi* locality) and São Mateus (*I. epikarsticus* locality) caves, punctual measures were recorded. For comparison purposes, we recorded water variables in epigean drainages of Passa Três, Angélica, São Bernardo, and São Mateus.

We obtained precipitation data from 1999 to 2001 from a station located 80 km from the study area in the municipality of Posse (INMET–National Institute of Meteorology).

2.3. Data Analysis

We estimated the monthly population sizes and densities of *I. passensis* and *I. bambui* based on observed and captured individuals per each year of study (1999, 2000, and 2001). We also verified the preference of these species by upstream or downstream subterranean stream stretches. We used the Lincoln–Petersen model [36]—based on a single episode of capture, marking, and recapture—to estimate the population sizes per year (three year blocks). Standard errors of means (SEM) and confidence intervals were calculated. We used the Petersen method to develop the analysis [37].

The individual growth rates were calculated for *I. passensis* and *I. bambui* using the capture-recapture data. For this, we used the increment in body length divided by the time between the captures (days). Standard length (SL) measures presented ± 0.1 mm errors, thus we calculated the error propagation formula in subtraction, $sv = \pm(s_a{}^2 + s_b{}^2)^{1/2}$, where sv is the propagated final error, and $s_a{}^2$ and $s_b{}^2$ are the errors of measures associated with each of the measures [38], calculated in ±0.14 mm. For cases where the differences between initial and final standard lengths fell within this interval, growth was considered null.

To detect possible differences in the distribution of standard lengths and weights of *I. passensis* and *I. bambui*, which would reveal recruitment and starvation/unhealthy periods, we compared their frequency classes for each collecting occasion. The condition factor—which measures its degree of health, reflecting its feeding conditions [39]—of each individual was also calculated. For comparison purposes, 20 specimens of *I. passensis* captured downstream from the sump of Passa Três cave stream had their standard lengths and weights recorded.

The relationship between the weight and the standard length of the individuals captured in all occasions was represented by regressions $\log P = b \times (\log SL)^{-a}$, where b is the regression coefficient, and a is the point of intersection with the y-axis when (SL) is zero. The values of b were compared to 3, indicating isometric growth, and were used for the calculation of the condition factor of Fulton (K): $K = 100P.SL^{-b}$ [39], considering the data sets for each occasion of collecting.

In order to visualize trends in population/fish densities, standard lengths, weights, and condition factor across the two-year study, we created Dice-Leeras diagrams that displayed the means and the standard errors for these variables [40].

The variables—population/fish densities, standard length, weight, and condition factor—were compared through parametric and non-parametric tests (before tests, the data were tested for homogeneity of variances and normality). For comparisons of two samples, e.g., data from 1999 versus 2000, we used the two-sample t-test (for parametric data) or the Mann–Whitney test (U), and for three or more groups (treatments), we used the Kruskal–Wallis test (H, for non-parametric group of data). Then, Dunnett's post-hoc test was carried out to detect significantly different groups [41]. For these statistical analyses, we used SigmaStat software (version 2.0, 1992–1995, Jandel Corporation, San Jose, CA, USA), with α = 5%.

For *I. epikarsticus* and *I. ramiroi*, estimates of abundance and density were based on the counts using the visual census technique (VC).

3. Results

3.1. Population Size and Densities

The numbers of captured, marked, released, and recaptured individuals of *I. passensis* were grouped every two consecutive samples (Table 1). In total, 270 individuals were observed, 261 captured, and 179 released. The capture rate was 93.5%, i.e., most of the observed fish were captured. Probably due to loss of marks, there were no multiple recaptures. As expected, confidence intervals of population estimates were lower in occasions with higher recapture rates (September 2000 and August 2001). Discrepant population size estimates (between 83.8 and 719.0) were expected due to the low recapture rates (Table 2). It is important to stress that this is a minimum value since, through visual census; we observed 258 individuals in July 2001 and 168 in August 2001. The subterranean drainage downstream from the sump was formed mainly by rocky substrate, and the fish are exposed in the river bottom and not burrowed or in shelters as observed upstream.

Table 1. Population data for *I. passensis* per capture-recapture events. R, number of recaptures; RR, rates of recapture; N, population size; SE, standard error; CI, confidence interval. Lincoln–Petersen method.

	Total Observed	Captures	Released	R	RR (%)	N (SE) (CI)
July 1999	27	27	27			-
September 1999	33	33	33		0	-
April 2000	11	10	10			-
July 2000	39	34	34		0	-
July 2000	39	34	34			152.0 (43.5)
September 2000	37	34		7	20.6	(94.7–342.1)
May 2001	36	31	31			719.0 (393.7)
August 2001	48	44	44	1	3.2	(226.7–1369.1)
August 2001	48	44	44			83.8 (10.9)
August 2001	48	48		25	56.8	(70.9–104.8)

Table 2. Population data for *I. bambui* per capture-recapture events. R, number of recaptures; RR, rates of recapture; N, population size; SE, standard error; CI, confidence interval. Lincoln–Petersen method.

	Total Observed	Captures	Released	R	RR (%)	N (SE) (CI)
July 1999	27	15	11			77.0 (37.9)
September1999	42	12	12	1	9.1	(23.7–147.4)
May 2000	45	18	18			246.0 (129.8)
August 2000	64	25	22	1	5.6	(77.1–469.0)
August 2000	64	25	22			-
September2000	27	17			0	-
April 2001	89	13	11			-
August 2001	59	17	16		0	-
August 2001	59	17	16			61.3 (25.0)
August 2001	31	10		2	12.5	(30.3–446.5)

For *I. bambui*, we observed 384 individuals, captured 127, and released 90 (Table 2). The capture rate was also low, around 33.0%. The upper vadose tributary of Angélica cave is silty and clay bottomed, hampering captures because many catfish were very fast when disturbed by the collector presence. As observed for *I. passensis*, there were no multiple recaptures, and we observed high amplitude on the estimated population sizes (between 61.3 and 246) due to the very low recapture rates.

Tables 3 and 4 and Figure 5A–D respectively show population densities based on visual censuses (means and standard errors of the mean) for *I. passensis* and *I. bambui*, considering samples across the years and grouped sections for Passa Três and Angélica caves.

Table 3. Population densities (ind/m^2) for *I. passensis* per sections of Passa Três cave between July 1999 and August 2001. No catfish records from 1 to 7 sections. SEM, standard error of the mean. 0, fish-free samples.

	Sections							
Date	**8–9**	**10–11**	**12–13**	**14–15**	**16–17**	**18–19–20**	**Mean**	**SEM**
July 1999	0	0.02	0.07	0.05	0.1	0.23	0.078	0.033
September 1999	0	0.07	0.05	0.07	0.17	0.27	0.105	0.039
April 2000	0	0	0	0.02	0.02	0.15	0.032	0.024
July 2000	0	0.13	0.05	0.05	0	0.48	0.118	0.074
September 2000	0.1	0.07	0.05	0.05	0.02	0.45	0.123	0.066
May 2001	0	0.1	0.05	0	0.02	0.48	0.108	0.075
August 2001	0	0.2	0.1	0.3	0.2	0.7	0.250	0.099
Mean	0.014	0.084	0.053	0.077	0.076	0.394		
SEM	0.014	0.025	0.011	0.038	0.031	0.071		

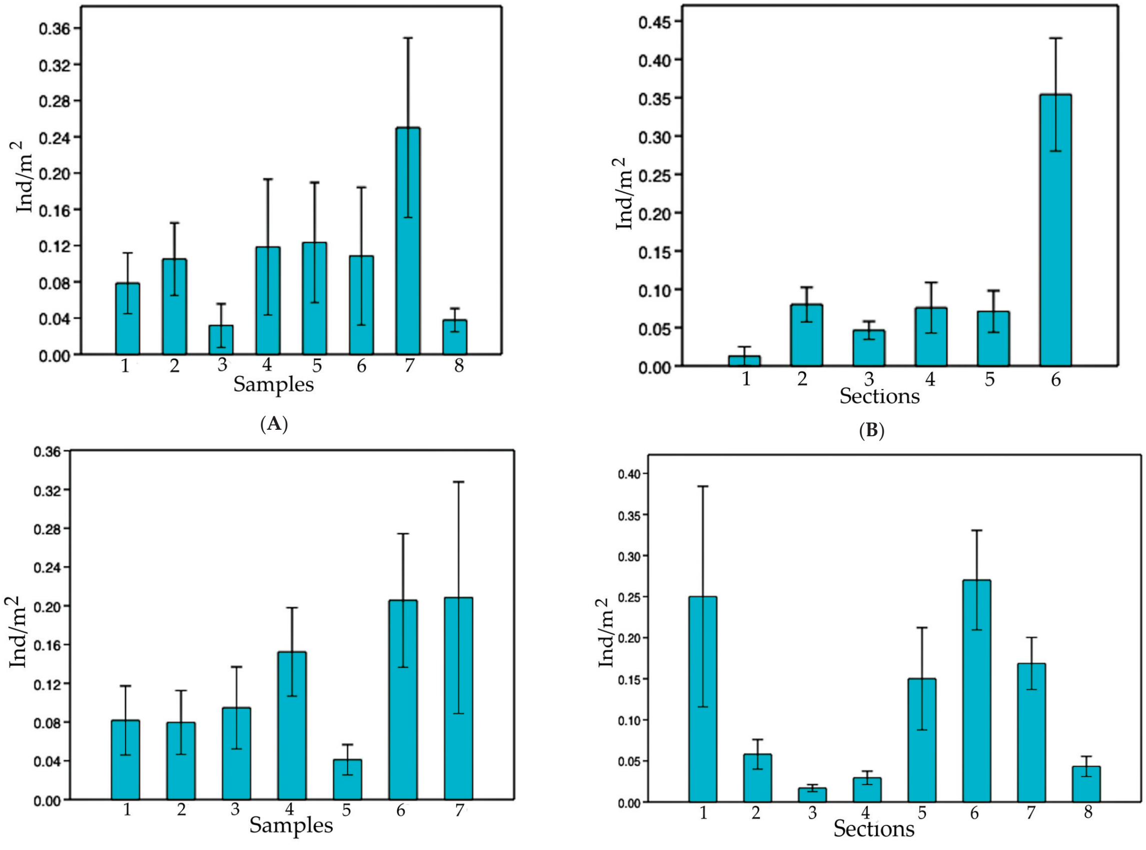
Table 4. Population densities (ind/m^2) for *I. bambui* per sections of Angélica cave between July 1999 and August 2001. SEM, standard error of the mean. 0, fish-free samples.

	Sections									
Date	**1–2**	**3–4**	**5–6**	**7–8**	**9–10**	**11–12**	**13–14**	**15–16–17**	**Mean**	**SEM**
July 1999	0.23	0	0.008	0.025	0.13	0.23	0.03	0	0.082	0.035
September 1999	0.03	0.025	0.02	0.021	0.03	0.23	0.23	0.05	0.080	0.033
April 2000	0.1	0.016	0.008	0.013	0.06	0.3	0.26	0	0.095	0.042
August 2000	0.13	0.116	0.02	0.0166	0.37	0.3	0.2	0.066	0.152	0.045
September 2000	0	0.083	0.004	0.013	0.03	0.03	0.13	0.038	0.041	0.015
April 2001	0.23	0.05	0.021	0.071	0.4	0.57	0.23	0.072	0.206	0.068
August 2001	1.03	0.116	0.037	0.046	0.03	0.23	0.1	0.077	0.208	0.119
Mean	0.250	0.058	0.017	0.029	0.150	0.270	0.169	0.043		
SEM	0.134	0.018	0.004	0.008	0.062	0.060	0.031	0.012		

We did not detect differences among the months in the three years for the population densities of *I. passensis* (Figure 5A) (Kruskal–Wallis test, H = 7.536, p = 0.274) or *I. bambui* (Figure 5C) (Kruskal–Wallis test, H = 9.533, p = 0.146), nor between the annual mean densities (Mann–Whitney test, U = 9001.0, p = 0.170).

Population densities of *I. passensis* were higher in downstream stretches (Table 3 and Figure 5B) and significantly distinct; between sections 10 and 11, and 18,19,20, the stream bottoms were formed by rocky substrate, pebbles, with some gravel and depths up to 0.40 m, offering many shelters for *I. passensis*. On the other hand, in sections 2, 8, 9, 13, 14, 15, 16, and 17, pools with sandy bottoms and depths to 1.0 m were intercalated with lotic and shallow waters. The volume of the available habitat is higher in the sections with pools, influencing the population densities of *I. passensis*. Another regulating factor was the presence of several large predator fish (110 mm standard length on average)—the erythrinid *Hoplerythrinus unitaeniatus* (Characiformes)—observed exclusively in pools downstream from section 15.

Population densities of *I. passensis* downstream from the sump (area of 400 m^2) were 0.65 ind/m^2 (July 2001) and 0.42 ind/m^2 (August 2001) (Figure 5A,B). Compared to the same extension/area upstream from the sump, the downstream densities were higher (see Table 3). However, compared to the last two grouped sections upstream from the sump (18, 19, and 20), the densities were similar or lower (August 2001, Table 3 and Figure 5B). The stream gallery downstream from the sump was relatively similar to sections 10, 11, 18, 19, and 20, with few pools and mainly rocky substrate, where the fishes were easily observed and captured. Only three or four individual *Hoplerythrinus unitaeniatus* were observed downstream from the sump (July and August 2001, respectively).



(**A**) (**B**)

(**C**) (**D**)

Figure 5. Population density means and SEM for *I. passensis* ((**A**), per samples, all sections: 1 July 1999; 2 September 1999; 3 April 2000; 4 July 2000; 5 September 2000; 6 May 2001; 7 August 2001; 8 August 2001 downstream from the sump; (**B**), per grouped sections: 1, 8–9; 2, 10–11; 3, 12–13; 4, 14–15; 5, 16–17; 6, 18–20) and *I. bambui* ((**C**), per samples, all sections: 1 July 1999; 2 September 1999; 3 May 2000; 4 August 2000; 5 September 2000; 6 April 2001; 7 August 2001; (**D**), per grouped sections: 1, 1–2; 2, 3–4; 3, 5–6; 4, 7–8; 5, 9–10; 6, 11–12; 7, 13–14; 8, 15–17).

Ituglanis bambui exhibited significantly higher densities in sections 1,2,9,10,11,12,13, and 14 (Table 4 and Figure 5D), varying from 0.04–0.27 ind/m^2. The upper tributary in Angélica cave is quite homogeneous, with a silty and rocky bottom and slow circulation of the water (lentic). Between sections 1 and 2, the number of rocky blocks was distinctly larger than in the other sections, which are probably good shelters for the fish. There are no regular predators of *I. bambui* in this tributary.

Densities of *I. epikarsticus* varied between 0.03 and 0.17 ind/m^2, whereas densities for *I. ramiroi* were larger and varied between 0.13 and 0.3 ind/m^2 (Figure 6). Additionally, there was a marked decrease in sightings of *I. epikarsticus* and, consequently, in densities throughout the study.

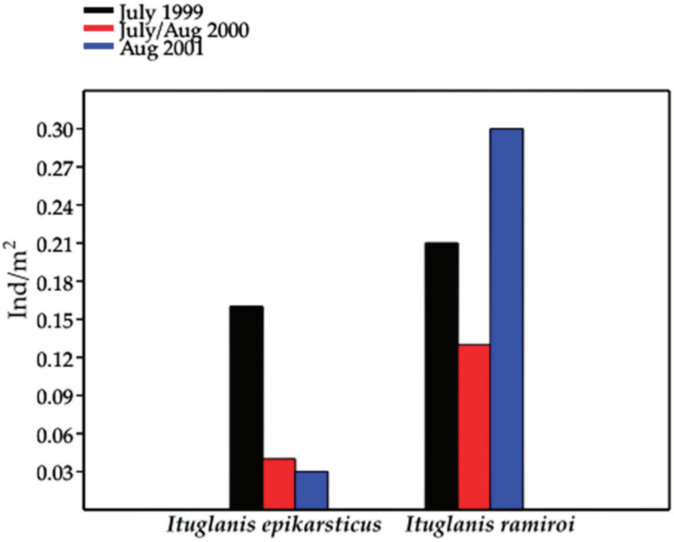


Figure 6. Population densities of *I. epikarsticus* and *I. ramiroi* based on visual census method.

3.2. Growth and Movements

Initial (ISL) and final (FSL) lengths recorded for the marked and recaptured specimens of *I. passensis* and *I. bambui* are shown in Table 5. We did not compute the recaptures recorded in August 2001 due to the short time interval between the collections in that month (seven days for *I. passensis* and 11 days for *I. bambui*). For *I. passensis*, growth rates varied between negative values, from −0.35 to 1.62 mm/month with a mean of 0.89 mm/month (sd = 0.728). Negative growth as well as the smallest increases in length was recorded for those individuals with larger body lengths. For *I. bambui*, the growth rate calculated for two specimens was 0.39 and 1.58 mm/month and, as observed for *I. passensis*, the lowest rate was recorded for the individual with larger body length.

Table 5. Individual growth for *I. passensis* and *I. bambui*. %, growth in relation to the initial standard length (ISL, in mm); *i*, time interval in days; FSL, final standard length (in mm); null, differences between initial and final standard lengths fell within the interval of the propagated final error (±0.14 mm).

***I. passensis*, Passa Três cave**	**ISL**	**FSL**	**Increment**	**%**	***i***	**Growth Rate (mm/month)**
Ind 1	54.9	57.7	2.8	5.1	52	1.62
Ind 2	68.5	69.3	0.8	1.2	52	0.46
Ind 3	40.4	43.2	2.8	6.9	52	1.62
Ind 4	59.0	61.1	2.1	3.6	52	1.21
Ind 5	60.0	62.1	2.1	3.5	52	1.21
Ind 6	66.0	65.4	−0.6	−0.91	52	−0.35
Ind 7	64.8	65.6	0.8	1.2	52	0.46
Ind 8	67.3	67.2	−0.1	−0.14	98	null
I. bambui, Angélica cave	**ISL**	**FSL**	**increment**	**%**	***i***	**Growth rate (mm/month)**
Ind 1	32.7	35.7	3.0	9.2	57	1.58
Ind 2	65.3	66.5	1.2	1.8	92	0.39

In relation to individual movements, considering *I. passensis*, out of 33 individuals recaptured throughout the study, 29 (89.9%) were recorded in the same section as the previous catch and four (10.1%) in adjacent sections. These results show individual movements of −1 (if the fish were caught at the end of one section and recaptured at the beginning of the next) to 80 m at time intervals ranging from seven to 98 days. For the four individuals of *I. bambui* recaptured, three (75%) were recorded in the same section as the previous catch and only one (25%) in the adjacent section. These results are similar to those observed for *I. passensis*, with individual movements of −1 to 60 m in time intervals ranging from 11 to 92 days.

3.3. Standard Lengths, Weights, and Condition Factor

Means and standard deviations for standard lengths and weights for *I. passensis* are shown on Dice-Leeras diagrams presented in Figures 7 and 8. For the standard lengths, we observed more than one mode on most occasions (Table 6): July 1999 (2), September 1999 (4), April 2000 (3), September 2000 (2), May 2001 (3), and August 2001, downstream from the sump (3). In July 2000 and August 2001 (upstream from the sump), we observed single modes. This demonstrates the absence of a clear pattern of recruitment throughout each year of study and may indicate multiple occasions of recruitment in the year. Multiple monthly modes were also observed for weight: July 1999 (2), September 1999 (3), April 2000 (4), and September 2000 (7). For July 2000, May 2001, and August 2001, we observed single modes.

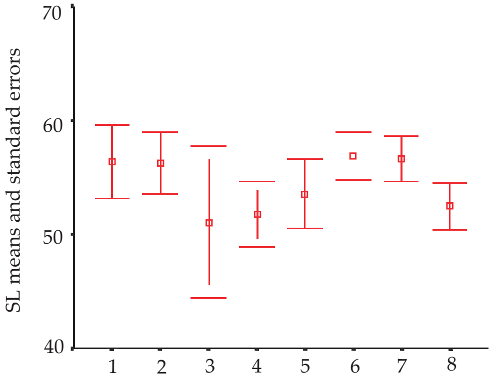


Figure 7. Dice-Leeras diagram showing the means and standard errors for *I. passensis* standard lengths (SL). 1 July 1999; 2 September 1999; 3 April 2000; 4 July 2000; 5 September 2000; 6 May 2001; 7 August 2001; 8 August 2001 downstream from the sump.

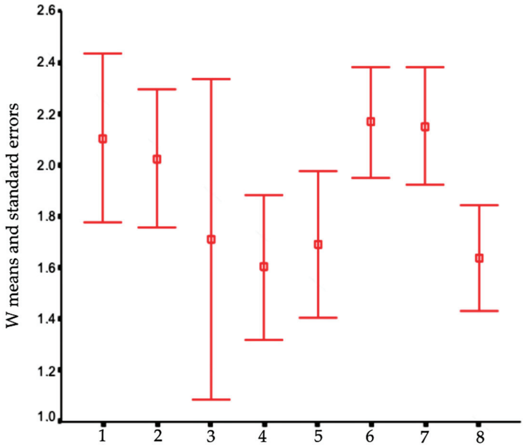


Figure 8. Dice-Leeras diagram showing means and standard errors for *I. passensis* weights (W). 1 July 1999; 2 September 1999; 3 April 2000; 4 July 2000; 5 September 2000; 6 May 2001; 7 August 2001; 8 August 2001 downstream from the sump.

Table 6. Distribution data (modes) for *I. passensis* considering standard length (SL, in mm), weight (W, in g), and condition factor (K).

Date	SL	W	K
July 1999	45.0–47.5; 57.5–62.5	1.5–2.0; 2.0–2.5	2.25–2.5; 3.0–3.25
September 1999	47.5–50.0; 52.5–55.0; 55.0–57.5; 60.0–62.5	1.5–2.0; 2.0–2.5; 3.0–3.5	1.75–2.0; 2.0–2.25; 3.5–3.75
April 2000	45.0–50.0; 60.0–65.0	0.5–1.0; 1.0–1.5; 2.0–2.5; 3.0–3.5	2.0–2.5; 2.5–3.0; 3.5–4.0
July 2000	45.0–47.5	1.0–1.5	1.25–1.5; 1.5–1.75; 3.25–3.5
September 2000	42.5–45.0; 62.5–65.0	0.5–0.75; 0.75–1.0; 1.0–1.25; 1.25–1.5; 1.5–2.0; 2.25–2.5; 2.5–2.75	1.75–2.0; 2.0–2.25; 2.75–3.0; 3.0–3.25
May 2001	52.5–55.0; 55.0–57.5; 57.5–60.0	2.0–2.5	2.5–2.75; 2.75–3.0; 3.25–3.5
August 2001 upstream the sump	57.5–60.0	2.0–2.5	2.5–3.0
August 2001 downstream the sump	48.0–50.0; 52.0–54.0; 58.0–60.0	1.5–1.75	2.0–2.25; 2.25–2.5; 2.75–3.0

The Dice-Leeras diagram shows an overlap of standard errors for the standard length means on most occasions (Figure 7), however, there was a slight trend for annual data grouping. The exception is for 2001 data, probably due to the influence of samplings downstream from the sump. In April 2000, we observed the greatest standard error interval, overlapping with all others, a reflection of the small size of the sample ($n = 10$). The small and heterogeneous samples in the occasions influenced the post-hoc tests, which did not detect any significant differences between the frequency classes. However, using t and U tests, we detected a significant difference for the standard lengths in August 2001, significantly greater downstream from the sump ($t = -2.458$, $p = 0.017$). There were no decreasing trends on lengths across the dry seasons, corroborated by the absence of significance on the statistical tests. In 2000, we recorded the lowest standard lengths, comparable to those recorded in August 2001 downstream from the sump (Figure 7). A visual analysis of the Dice-Leeras diagrams shows a partial overlap in weight for most occasions (Figure 8). Significantly greater values were recorded in July 1999 compared to those observed in July 2000 (U = 1046.5, $p = 0.022$). In contrast, significantly smaller weights were observed downstream from the sump in August 2001 in relation to upstream sump weights in May and August 2001 (U = 383.0, $p = 0.002$ and U = 469.0, $p = 0.004$, respectively).

We observed a decreasing trend in average weights during the dry season in 1999 (July to September). The smallest weights were recorded in 2000, as observed for standard lengths data; these weights were comparable with those recorded in August 2001 downstream from the sump (Figure 8).

The relationship between the logarithm of the standard length (SL) and the logarithm of the weight (W) for all captures of *I. passensis* revealed a value of b of 2.991 ($y = 2.991x - 4.948$), approaching 3, and indicating a growth near to the isometric. Considering the K distribution data, we observed more than one mode on most occasions (see Table 6): July 1999 (2), September 1999 (3), April 2000 (3), July 2000 (3), September 2000 (4), May 2001 (3), and August 2001 downstream from the sump (3). Single modes were observed only in August 2001, upstream from the sump.

Trends in condition factor (K) data for *I. passensis* are shown in Figure 9, where an overlap was found for most months, with the distinction of data blocks for the three years. We recorded a non-significant decrease in K values between July and September 1999, which could demonstrate a tendency of food restriction during the dry season. In July 1999, the body conditions were significantly greater than those of July 2000 (T = 1058.5, $p = 0.014$). In September 2000, the values were significantly lower compared to those of August 2001 ($t = -2.953$, $p = 0.004$), which shows food restriction in 2000, at least in July and September, compared to 1999 and 2001. In 2001, condition factor value for August (upstream from the sump) was significantly greater than those recorded downstream ($t = 2.625$, $p = 0.011$), probably reflecting a food restriction condition. Apparently, the sump represents a barrier for organic matter carried by floods, and the food restriction downstream must be worse than upstream.

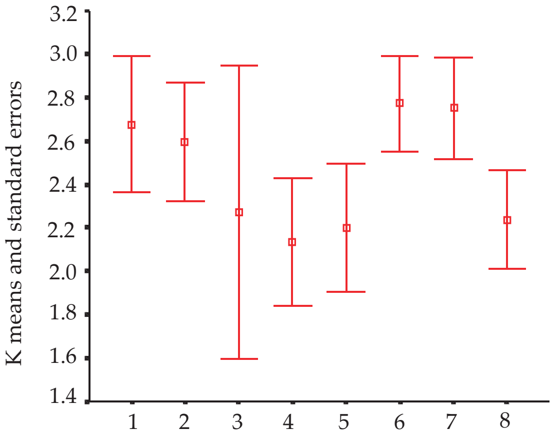


Figure 9. Dice-Leeras diagram showing means and standard errors for *I. passensis* condition factor (K). 1 July 1999; 2 September 1999; 3 April 2000; 4 July 2000; 5 September 2000; 6 May 2001; 7 August 2001; 8 August 2001 downstream from the sump.

For the standard length frequencies of *I. bambui,* we observed more than one mode on four occasions (Table 7): July 1999 (3), August 2000 (2), September 2000 (2), and April 2001 (3). In September 1999, May 2000, and August 2001 we observed single modes. These data evidenced recruitment events between April and August 2001. In contrast, multiple recruitment events occurred in 1999 and 2000.

Table 7. Distribution data (modes) for *I. bambui* considering standard length (SL, in mm), weight (W, in g), and condition factor (K).

Date	SL	W	K
July 1999	17.5–20.0; 20.0–22.5; 32.5-35.0	0–0.25; 0.5–0.75	0.50–1.0
September 1999	22.5–25.0	0–0.25; 0.25–0.5	0.38–0.50; 0.50–0.63; 0.63–0.75
May 2000	25.0–30.0	0–0.5; 0.5–1.0	0.25–0.50; 0.50–0.75; 1.0–1.25
August 2000	25.0–30.0; 45.0–50.0	0–0.5; 0.5–1.0	0.25–0.50
September 2000	22.5–25.0; 25.0–27.5	0–0.25; 0.5–0.75	0.25–0.50; 1.0–1.25
April 2001	22.0–24.0; 28.0–30.0; 30.0–32.0	0.25–0.38	0.56–0.63
August 2001	20.0–22.5	0.13–0.25; 0.25–0.38	0.38–0.50

We observed more than one mode in six of the seven occasions for weight data: July 1999 (2), September 1999 (2), May 2000 (2), August 2000 (2), September 2000 (2), and August 2001 (2), with one single mode only in April 2001.

The Dice-Leeras diagram showed an overlay of standard errors for the standard length means during each year of the study (Figure 10). The greatest standard error was registered in September 2000, reflecting the small size of the sample ($n = 17$). Significantly lower standard lengths were recorded in April 2000 compared with those observed in May 2001 ($U = 1212.5$, $p = 0.028$) and significantly greater in August 2000 compared with those observed in August 2001 ($U = 2112.0$, $p < 0.001$). Although differences among months were not significant, we noted a decrease in standard length values of *I. bambui* during the dry season.

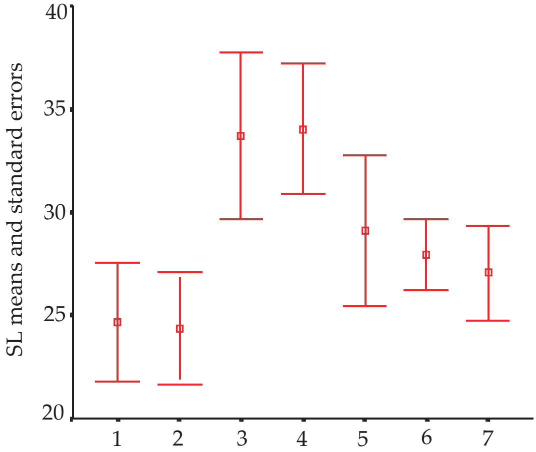


Figure 10. Dice-Leeras diagram showing means and standard errors for *I. bambui* standard lengths (SL). 1 July 1999; 2 September 1999; 3 May 2000; 4 August 2000; 5 September 2000; 6 April 2001; 7 August 2001.

Several overlays for *I. bambui* weight values were recorded, with large standard errors (e.g., August 2000, $n = 40$) (Figure 11). We did not observe any significant differences in weight across the years, even during the dry seasons of 1999 and 2000, where sharp decreases were observed (Figure 11). Similarly, no significant differences were observed between similar months (and seasons) in different years.

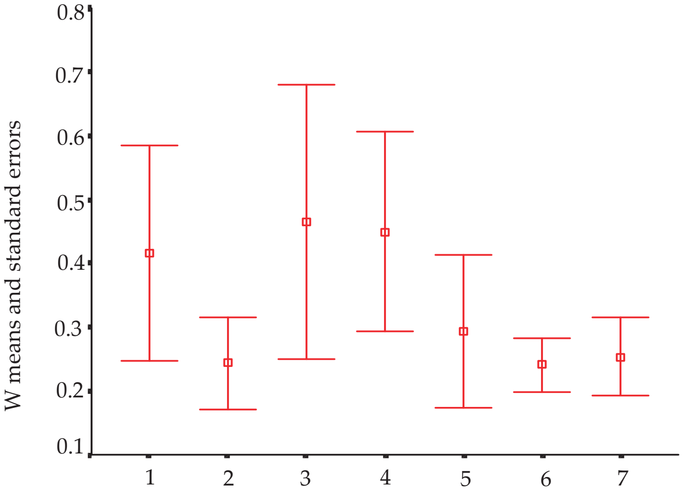


Figure 11. Dice-Leeras diagram showing means and standard errors for *I. bambui* weights (W). 1 July 1999; 2 September 1999; 3 May 2000; 4 August 2000; 5 September 2000; 6 April 2001; 7 August 2001.

The relationship between the logarithm of the standard length (SL) and the logarithm of the weight (W) for all captures of *I. bambui* revealed a value of b of 2.129 ($y = 2.129x - 3.699$), distancing 3, and indicating a growth near to the allometric. Considering the distribution data, we observed more than one mode for *K* data on three occasions (see Table 7): September 1999 (3), May 2000 (3), and September 2000 (2). Single modes were observed in July 1999, August 2000, April 2001, and August 2001.

We observed several overlays for *K* data (condition factor), with a clear declining trend in 1999 and 2000 (Figure 12), reflecting food restriction conditions during the dry season in these years (Figure 12). A significant difference recorded between July and September 1999 (T = 1085.0, p = 0.019) reinforces this hypothesis.

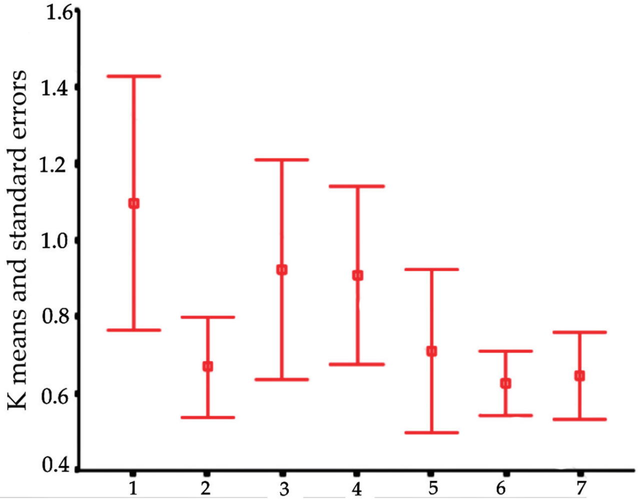


Figure 12. Dice-Leeras diagram showing means and standard errors of *I. bambui* condition factor (K). 1 July 1999; 2 September 1999; 3 May 2000; 4 August 2000; 5 September 2000; 6 April 2001; 7 August 2001.

3.4. Environmental Variables

We observed marked dry and rainy seasons during the study in the São Domingos karst area, with absence of rain in the colder months (Figure 13). Total annual precipitation values in the region varied from 1376.8 mm in 1999, 1254.9 mm in 2000, and 1210.8 mm in 2001, indicating a sharp decrease of rainfall during this period. Compared to historical rainfall data for the municipality of Posse, which experienced average annual values of 1635 mm between 1974–1989 (source: National Department of Waters and Electrical Energy and INMET), the decrease seems historic.

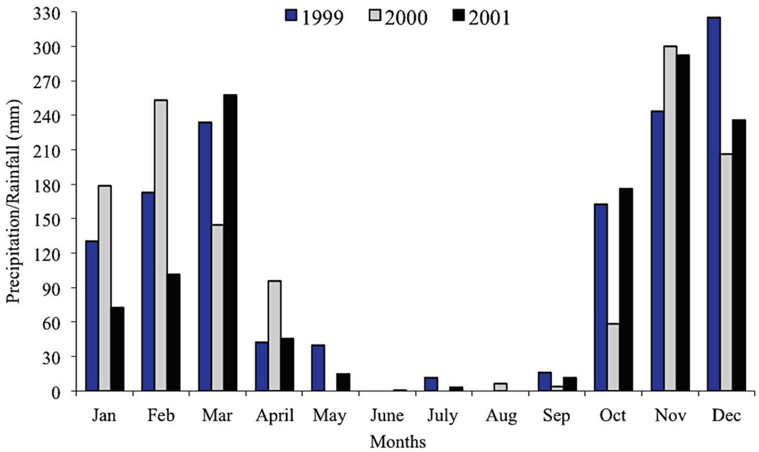


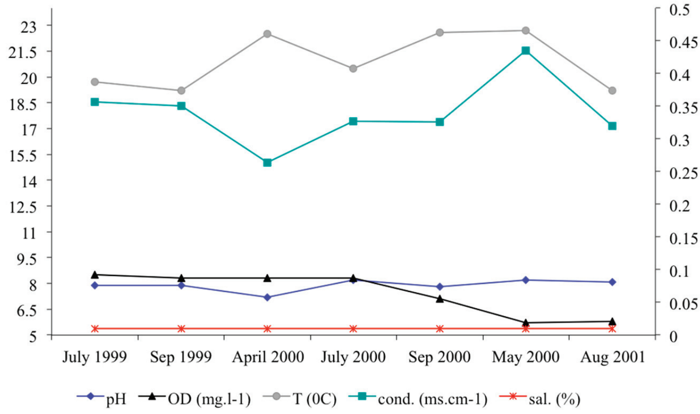
Figure 13. Monthly rainfall recorded in 1999, 2000, and 2001 at the Meteorological Station of Posse, Goiás, Central Brazil (Source: National Institute of Meteorology (INMET)).

For the four localities, we recorded high values of pH and conductivity and salinity values different from zero in most months (Table 8). The average pH values observed for Passa Três cave were lower compared to those of Angélica, São Bernardo, and São Mateus caves. The latter three caves are formed by epikarstic waters (the upper boundary of a karst system filled by percolating water), whereas, in the case of Passa Três cave, there is contact between the subterranean and the epigean drainages, which may slightly acidify the water. The same trend was observed for temperature; values recorded for Passa Três cave river were generally lower than those of Angélica, São Bernardo, and São Mateus cave drainages (with exception of September 2000). A small fluctuation was recorded in the subterranean drainages of Passa Três and Angélica caves throughout the study; conductivity, temperature, and dissolved oxygen exhibited greater fluctuation (Figure 14A,B). The minimum annual amplitude for temperature was similar for all caves (ca. 3.5 °C). Lower values of dissolved oxygen were recorded for Passa Três cave in the dry seasons of 2000 and 2001 when precipitation was lower (Figure 13).

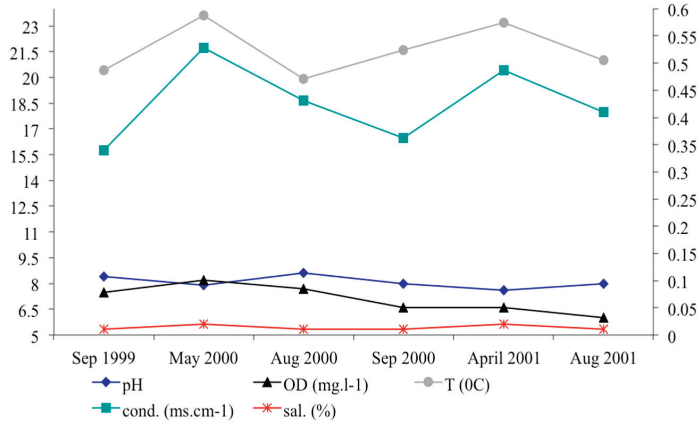
Table 8. Physico-chemical variables of Passa Três cave base level stream, Angélica cave upper vadose tributary, and epigean stretches of Passa Três, Angélica, São Mateus, and São Bernardo streams. cond., conductivity (ms.cm^{-1}); DO, dissolved oxygen (mg.L^{-1}); T, temperature (°C); sal., salinity (%); 1, 50 m downstream the sinkhole of Passa Três cave; 2, 340 m downstream the sinkhole of Passa Três cave; –, no records.

	Passa Três Cave		**Angélica Cave**
	1	**2**	
Date	(pH/cond./DO/T/sal.)	(pH/cond./DO/T/sal.)	(pH/cond./DO/T/sal.)
July 1999	7.9/0.357/8.5/19.7/0.01	8.1/0.353/8.9/19.6/0.01	–
September 1999	7.9/0.350/8.3/19.2/0.01	7.5/0.367/8.0/20.0/0	8.4/0.339/7.5/20.4/0.01
April–May 2000	7.2/0.264/8.3/22.5/0.01	7.7/0.293/8.4/21.4/0.01	7.9/0.528/8.2/23.6/0.02
July–August 2000	8.2/0.327/8.3/20.5/0.01	8.3/0.336/6.9/19.3/0.01	8.6/0.431/7.7/19.9/0.01
September 2000	7.8/0.326/7.1/22.6/0.01	7.8/0.337/7.8/21.9/0.01	8.0/0.362/6.6/21.6/0,01
April–May 2001	8.2/0.435/5.7/22.7/0.01	7.7/0.316/7.9/21.3/0	7.6/0.487/6.6/23.2/0.02
August 2001	8.1/0.320/5.8/19.2/0.01	8.4/0.323/4.6/19.2/0.01	8.0/0.410/6.0/21.0/0.01
	São Mateus cave (rimstone pools)		**São Bernardo cave (epikarstic pool)**
Date	(pH/cond./DO/T/sal.)		(pH/cond./DO/T/sal.)
May 1999	7.7/0.706/7.74/24.8/0.03		6.8/0.349/5.05/23.1/0.01
	Epigean streams		
	Passa Três stream		**Angélica stream**
Date	(pH/cond./DO/T/sal.)		(pH/cond./DO/T/sal.)
April–May 2000	7.9/0.262/6.9/22.6/0.01		7.7/0.041/8.4/24.4/0
July–August 2000	8.2/0.320/7.5/20.8/0.01		7.8/0.010/8.5/23.3/0
September 2000	7.7/0.326/6.2/22.8/0.01		7.2/0.010/8.0/25.6/0
April–May 2001	7.8/0.320/6.4/21.8/0		6.9/0.080/6.6/25.3/0
August 2001	7.7/0.307/5.8/19.2/0.01		–
	São Mateus stream		**São Bernardo stream**
Date	(pH/cond./DO/T/sal.)		(pH/cond./DO/T/sal.)
May 1999	6.6/0.025/9.25/21.9/0		5.6/0.010/8.03/24.8/0

We observed low amplitudes comparing the epigean and the subterranean stretches of Passa Três stream, even considering sites in the deeper zone (aphotic) of the cave. In contrast, we observed marked differences comparing the epigean stretches of the Angélica, São Bernardo, and São Mateus streams and their respective subterranean drainages, mainly for conductivity, temperature, and salinity values (Table 8).



(**A**)



(**B**)

Figure 14. Water physico-chemical variables. (**A**) Passa Três cave base level stream (point 1), between July 1999 and August 2001. (**B**) Angélica upper vadose tributary, between September 1999 and August 2001. The left axis represents the values of pH, dissolved oxygen (DO), and temperature; the right axis represents the values for conductivity (cond.) and salinity (sal.) variables.

4. Discussion

High pH, conductivity, and salinity values recorded for all caves from the São Domingos karst area are expected and typical of karstic drainages. Despite fluctuations of cave stream temperature during the study, their amplitudes were lower than those observed in the epigean drainages, according to the environmental stability of a hypogean environment [42]. The decline in dissolved oxygen values across the three years is probably related to a decrease in annual rainfall. We observed an accentuated decrease in the volume of watercourses of Angélica cave throughout the study. In the case of Angélica, São Mateus, and São Bernardo caves, the drainages where *Ituglanis* spp. occurs are isolated from the base level drainages (and principal), reducing the influence of surface drainages.

The low rates of recapture observed for *I. passensis* and *I. bambui* are probably related to the loss of marks. For *I. bambui*, which occurs in an upper vadose tributary of Angélica cave, recapture rates were markedly lower than rates recorded for *I. passensis*, which occurs in a base level stream. This difference is related to the fact that the population of *I. bambui* probably represents a small sample of a larger population distributed throughout the epikarst, since specimens morphologically similar were recorded in pools about 4 km away from the studied tributary. A population study of troglobitic amphipods in the U.S., which occurs in pools formed by infiltration water and base level rivers, also showed a similar pattern [43].

The estimated population size for *I. passensis* shows that this species has a relatively small population. Population sizes, based on mark-recapture studies as herein, also varied from very small to comparable to relatively small populations of epigean species. The population size of the troglobitic trichomycterid, *Trichomycterus itacarambiensis*, from eastern Brazil (5000 m of habitat extension) varied from 1500 to 2000 individuals. The cave catfish *Pimelodella kronei* from southeastern Brazil (5000 m of habitat extension) reached 900–1200 adults; the armored catfish *Ancistrus cryptophthalmus* from the same region (São Domingos karst area) reached 20,000 individuals in 8000 m of habitat extension at Angélica cave and 1000 individuals at Passa Três cave (2000 m of habitat extension) [4].

There is very little information about the population size of epigean trichomycterids, and most of that information was based on indirect observations. However, it is possible to see the amplitude of differences in relation to troglobitic populations. Shoals of 40,000 individuals were observed for *T. migrans* from Colombia, and more than 7000 individuals of *Trichomycterus areolatus* were captured across three years in two streams from Chile [44]. While small abundances were recorded for *Trichomycterus* sp. from southeastern Brazil (with 341 individuals in three sites of a small stream six-time sampled [45]), and for the benthonic catfish *Pygidianops amphioxus* from igarapé of northern Brazil (with 136 individuals in a 1150 m long sampled in 11 occasions [46]), its distribution is the broadest for the genus, reaching 900 km in a straight line [47]. One study used the capture-mark-recapture method for *T. brasiliensis* from southeastern Brazil, however, the population size estimated by Lincoln–Petersen was 38.5, and the absolute abundance was 148 individuals [48]. This study was conducted for a short period (two occasions in the same month), thus these discrepant values would be expected.

Densities recorded for the four *Ituglanis* species (Passa Três, Angélica, São Mateus, and São Bernardo caves) were always smaller than 0.7 ind/m^2 and, in general, smaller than 0.11 ind/m^2. According to [3], these values should be considered as medium-to-low. Low densities were recorded for other troglobitic trichomycterids, such as *T. itacarambiensis* from Brazil (between 0.15 and 0.20 ind/m^2, mark-recapture method) [10] and *T. conradi* from Venezuela (0.01 ind/m^2, visual census method) [49]. Medium to high densities were recorded for the armored catfish *Ancistrus cryptophthalmus* (Loricariidae) from the São Domingos karst area: 0.63 and 1.0 ind/m^2 for Passa Três and Angélica caves [12] through the capture-mark-recapture method and for *Aspidoras mephisto* (Callichthyidae), 5–6 ind/m^2 [50] through the visual census method. As expected, higher densities were recorded for epigean trichomycterids: 0.7 ind/m^2 for *Trichomycterus brasiliensis* from streams of southeastern Brazil [48]; 3–12 ind/m^2 and 18–25 ind/m^2 for *Trichogenes longipinnis* in two coastal rainforest streams from southeastern Brazil [51].

The increase in population densities of *I. passensis* according to the distance to the epigean environment (sinkhole of Passa Três cave) may be related to the greater availability of organic matter (and substrate for aquatic invertebrates, food items of *I. passensis*) close to the sump or even due to the drastic floods that can carry the fish downstream. The higher abundance of *I. passensis* downstream from the sump corroborated this idea. Trajano (1997) also observed higher densities for *T. itacarambiensis* downstream in Olhos D'água cave, where the availability of food is apparently higher; in this case, the downstream stretches are closer to the epigean environment. Another factor that limits the distribution of *I. passensis* upstream (in sections 1 to 7) is the waterfall between sections 7 and 8, reaching 6 m high in the dry season when the water level is lower (Figure 4B).

For *I. bambui* from Angélica cave, we noted an increase in densities toward the deepest part of the cave; however, this increase is upstream from the drainage and does not seem to be related to the amount of food. There was a very low concentration of organic matter upstream. The higher number of rocky blocks observed in these places can offer shelters for catfish, although we did not observe any regular predator of *I. bambui* in the vadose tributary.

Small fluctuations observed in the population size and densities for both *I. passensis* and *I. bambui* throughout the dry season were also observed for the armored catfish from Passa Três and Angélica caves (*Ancistrus cryptophthalmus*) [12] but only in the year 2000. This was probably related to higher precipitation rates and consequently higher food input to caves.

Due to the quite different environmental conditions in which hypogean and epigean populations live, differences in the use of space, including individual movements, are expected. The low rates of recaptures recorded for *I. passensis* and *I. bambui* did not allow robust analysis of the movements, however, we can make some inferences. *Ituglanis passensis* and *I. bambui* showed movements in an extension of 80 and 60 m, respectively. Smaller fish tend to have limited movements [52], which would explain the limited movements observed for *I. bambui*. Similar results were observed for *Trichomycterus itacarambiensis*, who remained in an area of about 100 m along the river [10]; this species is under a marked food restriction in dry seasons. The absence of seasons with marked food restriction in Passa Três cave explains the existence of more limited movements of *I. passensis* compared to *T. itacarambiensis*. The heptapterid *Pimelodella kronei* is a mid-sized catfish, with SL reaching 150 mm (significantly greater than trichomycterids studied herein) and fewer and shorter movements than *T. itacarambiensis*, *I. passensis,* and *I. bambui* [9], suggesting that seasonality can be one of the main factors influencing home ranges. There are few studies about movements of epigean trichomycterids. A massive migration of juvenile *Trichomycterus barbouri* catfishes on floodplains from Bolívia was recorded and the authors considered that each individual could migrate upstream more than 300 km [53]. The reason for this migration seems unclear, and the authors suggest that sites more upstream would be more appropriate for survivorship and development to reach sexual maturity.

The slow growth rates observed for *I. passensis*, 0.89 mm/month on average, and *I. bambui*, 0.39 and 1.58 mm/month (for two recaptures), are higher than those observed for the troglobitic catfish *Trichomycetrus itacarambiensis*, 0.06 mm/month [10], and for the armored troglobitic catfish *Ancistrus cryptophthalmus* (0.35 to 0.7 mm/month on average) [12], and are close to those observed for the troglobitic catfish *Pimelodella kronei*, 1.0 mm/month on average [9]. Compared to the epigean trichomycterid catfish *Trichomycterus chiltoni* and *Bullockia maldonadoi*, which have high rates of growth [44], the rates registered for *Ituglanis* spp. feature low growth, which would imply high longevity. Laboratory data for *I. bambui* and *I. passensis* reinforces this hypothesis since two individuals of these species survived for 20 years in captivity (from May 1999 to December 2019) (M.E. Bichuette, pers. obs.). Although we did not quantify the food availability for *Ituglanis* cave catfish, it is quite clear that it is much lower than in epigean habitats. This explains the low growth rates and the high longevity expected for *I. passensis and I. bambui*, a precocial lifestyle.

Monthly distributions of frequencies of weight and condition factor suggest that, for *I. passensis*, there was no direct relationship with seasonality. However, those individuals that were located downstream from the sump were thinner than those living upstream. This fact reinforces the idea that the sump blocks organic matter. For *I. bambui*, we observed a decline in condition factor values during the two-year dry seasons. In contrast to the situation observed in Passa Três cave, food was scarcer in Angélica cave, with apparently decreased availability by the end of the dry season. This low condition factor across the study period indicates a regime of food restriction intensified during the dry season. Similar results were reported for the troglobitic catfishes *Trichomycterus itacarambiensis* [10] and *Pimelodella spelaea* [35] and for the armored troglobitic catfish *Ancistrus cryptophthalmus* [12].

Conservation Remarks

Considering these particularities of *Ituglanis* from the São Domingos karst area, we can discuss their conservation status. The caves where *Ituglanis* spp. are found are within the Terra Ronca State Park limits, which already offers some kind of protection. However, much of the area was not yet acquired, and the management plans for touristic use of the caves are under study. The four species studied herein are inserted in the Brazilian List of Threatened Fauna: vulnerable category (VU) includes *I. passensis*, *I. epikarsticus,* and *I. ramiroi*, and the critically endangered category (CR) includes *I. bambui* [33]. *Ituglanis passensis* is the least threatened of all, having once presented greater populations and with no touristic visitation in Passa Três cave. The threat to *Ituglanis bambui* is mainly from tourism, since Angélica cave is intensively visited and the upper vadose river gallery where the fish live is close to the cave entrance with easy access. Although *I. bambui* is probably distributed throughout the epikarst, it seems that the main population is distributed in the studied stretch; urgent actions are necessary to control and plan tourism, effectively applying a management plan for cave use. *Ituglanis epikarsticus* and *I. ramiroi* are also endangered species, and urgency is needed on population monitoring studies since they occur in specific microhabitats of epikarstic waters of São Mateus and São Bernardo caves. Tourists also intensively visit the locality of *I. epikarsticus* (São Mateus cave), and the place where this species lives (rimstone pools) represents the only passage to access other cave galleries. São Mateus is one of the most touristic caves of Terra Ronca State Park, which probably impacts these fish by carrying organic matter from the surface, muddying the water and trampling the dams. The pool where *I. ramiroi* occurs in the São Bernardo cave is out of the way of tourists, and, apparently, they are protected despite the very easy access. In addition to long term monitoring, we suggest urgency in the execution of the management plans for Terra Ronca State Park at São Domingos karst area, Central Brazil, mainly for the Angélica and the São Mateus caves, where visitation is relatively intense. More expressive supervision of governmental agencies is urgent at Terra Ronca State Park for effective protection of its cave fauna. Monitoring is fundamental for protective measures for cave organisms. We suggest that funds from environmental studies that affect caves and karst areas in Brazil be invested in long-term monitoring and conservation projects for cave fauna.

5. Conclusions

In conclusion, *I. passensis* and *I. bambui* populations are relatively small; population densities of the four studied *Ituglanis* species may be considered medium-to-low for troglobitic fish standards. *I. passensis* and *I. bambui* are sedentary species, with small home ranges; the low rates of individual growths recorded for *I. passensis* and *I. bambui* may indicate high longevity for these species. The pattern of recruitment and the trends in weight and condition factor decreasing for *I. bambui* during the dry season are probably related to progressive food depletion, which was not marked for *I. passensis*. *I. bambui* is under a more restrictive regime than *I. passensis*, probably due to isolation in an upper vadose tributary.

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Data Availability Statement: Data from this study is available publicly.

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Subterranean Waters of Yucatán Peninsula, Mexico Reveal Epigean Species Dominance and Intraspecific Variability in Freshwater Ostracodes (Crustacea: Ostracoda)

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Abstract: The Yucatán Peninsula is a karstic region, rich in subterranean environments with a diverse crustacean stygobiont fauna. In order to gain insights into the biological evolution of the subterranean environments of this region, we evaluated the ostracode species composition of caves and cenotes in five independent sampling campaigns (2008, 2013, 2017–2019). Using morphometric analyses, we evaluated inter-population morphological variability; using molecular analysis based on mitochondrial COI and nuclear 18S rDNA, we evaluated genetic differentiation in selected species. The observed fauna is composed of 20 (epigean) species, presenting a lack of strict stygobionts. Morphometric analyses discriminated up to three morphotypes in each of the three most abundant species: *Cytheridella ilosvayi*, *Alicenula* sp. and *Cypridopsis vidua*. High intraspecific morphological variability was found either in shape or size. Phylogenetic analysis based on COI demonstrated the existence of three lineages on *C. ilosvayi*, with high support (>0.9). The 18S rDNA sequences were identical among individuals of different populations. A lack of congruence between the genetic markers precluded us from postulating speciation in subterranean environments. It is likely that Late Pleistocene—Early Holocene climate variation related to sea level and precipitation was forcing agent for epigean ostracode dominance in subterranean environments of the Peninsula.

Keywords: Ostracoda; Yucatán Peninsula; cenotes; mitochondrial marker; geometric morphometrics

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1. Introduction

Subterranean waters are critical environments for biodiversity. The combination of unique abiotic features such as stable and relatively low temperatures, geologically driven water chemistry, space restriction and low energy and food availability, challenge the success of most taxa [1–4]. Aquatic species have been able to colonize subterranean environments through morphological, behavioral and physiological adaptations [4–6], but given the highly selective environment, the number of species per site (i.e. α diversity) and their ecological interactions are usually limited [1,7–9]. Species inhabiting subterranean waters are usually isolated, with a limited capacity of dispersal. These conditions may drive large differences in species composition between regional sites (high β diversity) [10,11]. Speciation and endemicity are therefore common in these environments and are promoted by events such as vicariance and spatial ecological partition of the environment [11–13].

Global patterns of biodiversity and endemicity in subterranean waters are largely varied among regions and are influenced by factors such as subterranean water availability, the geological and climatic history of the region and the capacity of the organisms to succeed in these environments [2,13–15]. In tropical regions for example, the biodiversity of obligate aquatic cave dwellers is overall lower than that in temperate regions [16,17], but large tropical regions remain unexplored.

The Yucatán Peninsula is a calcareous platform located in southern Mexico; it is partially surrounded by the Gulf of Mexico and the Caribbean Sea. The Peninsula has one of the largest underwater cave systems in the world. This system is characterized by extensive interconnected caves throughout the Peninsula [18]. Physical and chemical properties of the subterranean system, such as water level and ionic water composition are strongly influenced by the proximity and connectivity with marine environments, particularly the Caribbean Sea [19–21].

In the Yucatán Peninsula groundwaters become frequently exposed by the continuous dissolution of limestone [22–24] and underground waterways become exposed. The resulting karstic phenomenons are locally named “cenotes” (“dzonot” in mayan language, meaning “hollow with water”). Currently, more than 8000 cenotes are estimated to be sparsely distributed in this region (Secretaría de Desarrollo Sustentable, Yucatán census). In the northern portion of the Peninsula, cenotes are almost the unique source of freshwater and represent an important interaction between groundwater (hypogean) and surface environments (epigean) [25,26]. Given their geomorphological features, subterranean habitats of the region can be caves (partial or completely flooded) or cenotes (deep wells), sometimes with extensive networks of cave passages [27]; in some of them, marine waters can underlie the freshwater table [28].

Biotic diversity in subterranean waters of the Peninsula is dominated by crustaceans and the region is recognized as one of the foremost sites for this group with more than 40 obligate subterranean species from fresh and anchialine waters, also known as “stygobiont” species [27].

Freshwater ostracodes are micro crustaceans with a maximum length of 7.5 mm [29] (but mostly <5 mm), characterized by having their body enclosed between two calcareous valves [30]. Ostracodes are typical of almost all aquatic systems, including ephemeral water deposits, subterranean and interstitial waters, semiterrestrial environments and a wide variety of epigean water systems [31]. In subterranean environments, ostracodes have been documented worldwide [32–37] and are characterized by high levels of endemism and a number of morphological adaptations including reduction of size, pigmentation loss, elongation of sensorial structures and reduction of setae [33]. Globally, the study of subterranean ostracodes is still under development, but evidence from karstic systems in Europe [32,33,38] and the arid regions of Australia [39] have demonstrated a high level of diversity. In Western Australia (Pilbara region), subterranean ostracode fauna can be more diverse than that in surficial environments [39–41].

Studies of ostracode and crustacean species from cenotes of the Yucatán Peninsula have revealed high species diversity, with a variety of ecological interactions between epigean, hypogean, and anchialine fauna [26,27,42–44]. For ostracodes, a sampling campaign undertaken in cenotes in 1936, revealed the presence of 23 freshwater species in Yucatán, out of which 13 of them were described as new to science [45]. More recently, sampling campaigns undertaken in 1989 [46] and 1998 [47] in cenotes located along the Caribbean Sea coast (<5 km separated from the coast) revealed the presence of two anchialine species in the water column, below the halocline. On the Yucatán Peninsula, therefore, integrated studies aimed to understand species compositions are required to further test ecological and evolutionary hypotheses, such as epigean and hypogean species interactions, colonization, speciation events and endemicity. In this study, (i) we evaluate ostracode faunal composition from subterranean habitats (cavern type and cenotes) of the Yucatán Peninsula; (ii) we evaluate morphological variability in valves and using molecular analyses, we assess the genetic differentiation occurring among subterranean ostracodes, and

(iii) we discuss the role of past climate variations (since the Late Pleistocene) in structuring the composition of subterranean ostracode fauna of the Peninsula.

2. Materials and Methods

We sampled a total of 32 cenotes in 25 settlements of the Yucatán Peninsula (Yucatán, Campeche and Quintana Roo federal states), Mexico, during independent sampling campaigns performed in 2008 (eight sites) and 2013 (ten sites) and between 2017 and 2019 (five sites in 2017, three sites in 2018 and six sites in 2019) (Figure 1, Table S1). Each site was visited only once, as each campaign took place in different regions of the Peninsula.

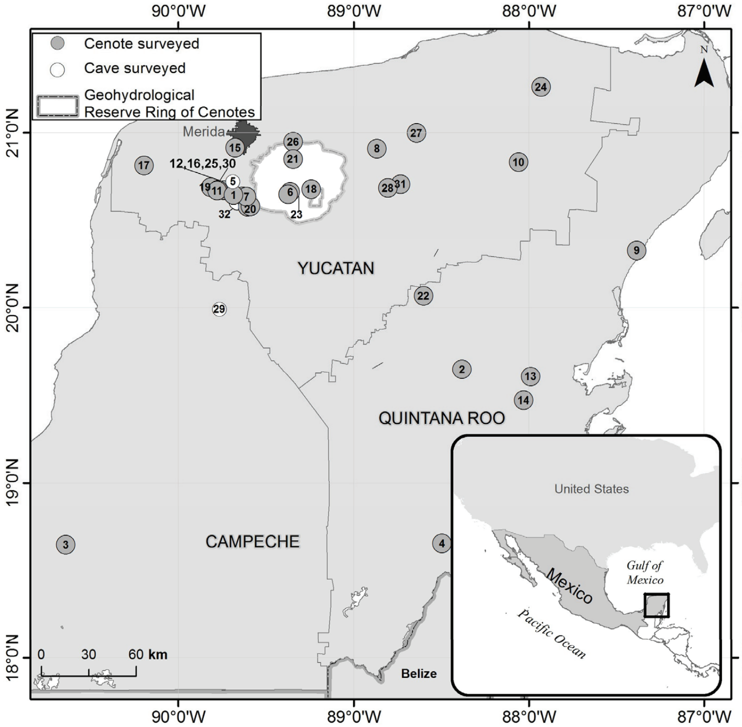


Figure 1. Map of the Mexican federal states of the Yucatán Peninsula and location of the 32 studied caves and cenotes. Details of the numbered cenotes can be found in Table S1. The light area within the Yucatán state represents the Geohydrological Reserve in Yucatán, while the dark area depicts the urban extension of the city of Merida.

2.1. *Sampling, Valve Extraction, Counting and Identification*

Biological samplings were carried out using two different methods. During the years 2008 and 2013, sampling was undertaken by the Instituto Tecnológico de Chetumal (ITCH), Chetumal, Quintana Roo, Mexico and El Colegio de la Frontera Sur (ECOSUR), Unidad Chetumal, Chetumal, Quintana Roo, Mexico. During these campaigns, biological samples were collected from the littoral zone, water column and deepest bottom of cenotes. At littoral areas, samples were taken from submerged vegetation using a hand net of 250 µm mesh size. In the water column, samples were collected with vertical tows and horizontal trawls with a 150 µm mesh and a 20 cm wide net. Sediment samples were collected with an Ekman grabfrom the deepest bottom; only the uppermost 2 cm of sediment of each grab were collected to avoid fossil or subfossil shells. Samples fixation was done *in situ* with 96% ethanol and then preserved on ice. Thereafter, samples were kept in refrigeration at −4 °C for seven days to preserve DNA from degradation. Samples were deposited at ECOSUR, Unidad Chetumal, and ITCH. Sites geolocations (latitude and longitude), including elevation, were determined with a Garmin GPSmap 60c navigator. For sites sampled in the year 2013, maximum water depth was measured with a Fishfinder GPSMAP 178C echosounder (Garmin Corporation, Taipei, Taiwan); for systems sampled in 2008, we lack measures of depth lack.

Ostracode specimens sorting and counting were carried out in 50 cm^3 of wet sediment, using a stereomicroscope. Only organisms with complete soft parts or well-preserved valves were extracted. For species identification, organisms with complete soft parts were dissected and mounted in a mixture of glycerol and formaldehyde (1:1). Preparations were sealed with Entellan mounting media. Shells were stored in micropaleontological slides. Valves and appendages were measured with a stage micrometer adapted to an Olympus light microscope. Non-dissected material was preserved in plastic tubes in 70% ethanol. All studied material was deposited at the zooplankton reference collection from ECOSUR, Unidad Chetumal.

Samplings between 2017 and 2019 were conducted by Cenoteando, a research group team from the Unidad Multidisciplinaria de Docencia e Investigación Sisal, Mérida, Yucatán, México (UMDI-Sisal), Facultad de Ciencias, Universidad Nacional Autónoma de México, Mérida, Yucatán, México (UNAM). Sediment samples from different depths of the cenotes and associated submerged cave passages were collected during scientific SCUBA cave dives in 50 mL sample tubes and in 1000 mL plastic bags. The sediments collected corresponded to the uppermost layers of the cave floors. Sample fixation was done *in situ*, by replacing all water with 96% ethanol; then, the sediment samples were preserved in ice. Large samples were filtered in situ using a 150 µm mesh size net. Thereafter, samples were kept in refrigeration at −4 °C. Species extraction was carried out at the UNAM UMDI-Sisal facilities. Small portions of sediment, diluted with ethanol, was examined using a Nikon SM Z800 stereomicroscope.

Ostracodes and other minute invertebrates such as thermosbaenaceans, stygiomysids, mysids, and amphipods were selected. Only ostracodes with well-preserved valves were extracted. The individuals selected were preserved in labelled sample tubes in 96% ethanol and were sent for further analyses to the Instituto de Geología of the UNAM in Mexico City and to the ITCH. The remaining sediment samples were deposited at the Laboratorio de Ecología y Manejo de Costas y Mares of UMDI-Sisal, UNAM.

In the case of the non-submerged caves X'tacumbilxuna'an, Yumku, and Peba, sediment samples were collected in dry cave passages from freshwater ponds. Samples were processed and deposited, as described previously.

Scanning electron microscope (SEM) analyses were conducted for selected and representative species with a Jeol Jsm-6010 plus/LA (Tokyo, Japan) scanning electron microscope from ECOSUR, Unidad Chetumal.

An Olympus BX51 microscope (Tokyo, Japan) was used for light photographs of specimens using the stacking method.

Species identification was conducted with species keys provided by Karanovic [31] and original species descriptions. Taxonomic classification follows Cohuo et al. [48].

2.2. Morphometric Analysis of Ostracode Valves

Morphological variability between populations and subterranean habitats was evaluated with geometric morphometrics. Valve shape analysis was conducted in the three species most frequently found in our sampling sites: *Cytheridella ilosvayi* Daday, 1905, *Alicenula* sp., and *Cypridopsis vidua* (O.F. Müller, 1776). For *C. ilosvayi*, we evaluated a total of 13 individuals from seven populations (caves Peba and Yumku and cenotes Huul K'in, Yokdzonot, Azul (in Campeche), Azul (in Quintana Roo) and Xoch). We decided to use female right valves for this analysis as they were the most abundant and in better preservation state. Although the left valve is more stable in ostracodes, for *C. ilosvayi*, right valve has demonstrated morphological stability and accurate results on inter- and intra population comparisons [49,50]. For *Alicenula* sp., specimens were mostly identified by using empty disarticulated valves on the populations evaluated. We used 12 female left valves from seven populations (cenotes Dzonbacal (San Antonio Mulix), Dzonotila (Mucuyché), Huul K'in (Mérida), Calcuch (Sabacché), Kankirixché (Mucuyché), Oxolá (Chichi), and X'batun (San Antonio Mulix) including populations of other *Alicenula* species (e.g., *Alicenula yucatanensis* Macario-González et al., 2018) and for *C. vidua* 17 female left vales from seven populations (cave X'tacumbilxuna'an (Bolonchen) and cenotes Colac (Balantún), Noh'Chunck (Chunchumil), Oxolá (Chichi), Sabakha (San Antonio Sodzil), Xoch (Cenotillo), and X'batun (San Antonio Mulix) were used. Only adult well-preserved valves were used in the analysis. Given that *Alicenula* sp. and *C. vidua* lack enough landmarks (type I and II) in their valve surface, we used for all three species an outline approach based on the elliptic Fourier method (EFA). The outlines of the specimens were created as two curves, starting at the maximum anterior and posterior curvature of the valves. Considering the differences in the valve shapes of the target species, 104 semilandmarks were drawn, equidistantly spaced for *C. ilosvayi*, 106 for *Alicenula* sp., and 98 for *C. vidua*. Semilandmarks were created manually in the tpsutil32 and tpsDig2 software packages ([51]; https://life.bio.sunysb.edu/morph/).

A set of 20 harmonics that best describe the shape of the valves were calculated with the EFA3D software (3D elliptic Fourier; https://life.bio.sunysb.edu/morph/soft-outlines.html). The harmonics were invariant to the conditions of the valves, such as size, rotation, and position. A principal component analysis (PCA) with the harmonic scores was conducted to visualize the patterns of shape variations, and EFA coefficients were used for valve shape reconstruction. Significant differences in shape morphologies between morphotypes were assessed with MANOVA and canonical variates analysis (CVA). For MANOVA, morphotypes represented by two or more specimens were included in the analysis, and Bonferroni correction was applied to the resulting *p*-values. CVA was performed using the PCA scores, and, as groups of interest, we used the associations resulting from the harmonics scores in the PCA morphospace. As CVA interpretations can be strongly affected by the number of variables (when variables exceed the number of specimens per group) [52], we used only those components that describe 90% of data variability. PCA, MANOVA and CVA were performed with the PAST version 3 software (Oslo, Norway) [53].

The resulting geometric morphometrics analysis did not uncover differences in individual size. A length—height analysis was then performed to provide a graphical representation of size differences among populations. Valve size analysis was conducted using 17 specimens of *C. ilosvayi* from eight populations, 19 specimens of *Alicenula* sp. from eight populations, and 30 specimens of *C. vidua* from seven populations. For this analysis, we used adult well-preserved and partially broken valves, as far as the length and height were clearly observable. We measured the total length (L) and height at the middle of the valve (H) of each specimen in each of the three groups. The L/H ratio was then calculated to identify changes in the lateral shape of the valves. The normality of the measurements was assessed by Shapiro-Wilk tests. As the data sets did not fit a normal distribution, differences between populations in length, height, and L/H ratio were assessed with non-parametric Kruskal—Wallis tests. In order to provide a graphic representation of the relationship between length and height for all species, scatter plots were generated. Descriptive statistics such as mean, median, standard deviation, and maximum and minimum measures of the length and height of each group were calculated.

2.3. Molecular Analysis: DNA Extraction, Amplification and Sequencing

To test genetic differentiation in subterranean waters (cenotes and caves), we performed phylogenetic analyses, using two independent genetic markers—the mitochondrial COI and nuclear 18S rDNA gene. Molecular analysis was conducted exclusively on *C. ilosvayi*, as it is the unique species that we were able to collect complete specimens (valves + soft parts) of. Given the practical absence of genetic sequences for this species, and to provide a reference framework for species delimitation, we included individuals of six epigean populations. Subterranean habitats corresponded to Cave Yumku, Cave Peba, Cenote Azul (in Campeche), Cenote Azul (in Quintana Roo) and Cenote El Padre. Epigean environments are represented by lakes characterized by different trophic conditions. The following lakes are oligotrophic: Bacalar and Señor, while the remaining ones—Tres Garantías, Campeche, Caobas and Perdida—are eutrophic lakes.

From each of these systems, three individuals were used for molecular analysis. The valves of each organism were removed and stored in micropaleontological slides, and the soft parts were further used for DNA extraction.

DNA extraction was carried out according to the methodology proposed by Macario-González et al. [54]. For the COI gene, PCR amplification was conducted with universal primers (LCO1490: 5′ -GGTCAACAAATCATAAAGATATTGG- 3′ HCO2198: 5′ -TAAACTTCAGGGTGACCAAAAAATCA- 3′) [55], and the settings recommended by Prosser et al. [56] were followed. For the 18S rDNA gene, we used the primers 18S-F1: 5′ -TACCTGGTTGATCCTGCCAG-3′ and 18 S-R9: 5′-GATCCTTCCGCAGGTTCACCTAC-3′ to amplify a gene region of about 1100–1200bp (base pairs). We followed the procedure proposed by Yamaguchi and Endo [57].

A volume of 4 µL of DNA was used as a template for amplifications. PCR products were visualized in 1.5% agarose gel, and sequencing was done with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 automated DNA sequencer. Chromatographs were manually checked, and sequences were edited using CodonCode Aligner (v. 9.01, Codon Code Corporation, Centerville, MA, USA) where necessary. All new sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank) with the following accession numbers: MW018845-MW018862 for COI and MW018806-MW018816 for 18S rDNA (Table S2).

2.4. Molecular Analysis: Sequence Alignment and Phylogenetic Analysis

COI and 18S sequences were aligned in MEGA software version 10.1.8 [58], the Clustal W algorithm was used for COI [59], while non-coding sequences from 18S were aligned with the MUSCLE algorithm. Sequences were trimmed manually when necessary. To avoid stop codons, COI sequences were translated into amino acids using the ORF finder from the NCBI page. All COI and 18S sequences are newly obtained in this study. With the purpose of comparison and in order to provide robustness to the analysis, we also used five sequences from closely related species of *C. ilosvayi*: two COI sequences are from *Paracythereis opesta* (Brehm, 1939), which are newly obtained in this study, two sequences are from a putative *Cytheridella* species from GenBank (accession numbers: *Podocopida* sp.1 and 2—MG449867 and MG449978) and an 18S sequence from *Metacypris digitiformis* Smith amd Hiruta, 2004 (accession numbers: AB674964). Two ostracode species where used as the outgroups for the COI and 18S data sets, respectively (accession numbers: *Limnocythere inopinata* (Baird, 1843)—AJ534412 and *Limnocythere* sp.—AB076635).

For phylogenetic analysis, the best-fitting model of molecular evolution and the partition scheme, under the corrected Akaike information criterion (AICc) [60] was estimated for the COI and COI-18S concatenated database using Partition Finder 2 on XSEDE [61].

A Bayesian approach was implemented using MrBayes on XSEDE [62]. According to the best-fitting model, the following partitions were included: SYM + I for the first COI codon position, GTR for the second COI position, K80+G for the third COI position, and GTR for the 18S gene segment. Two runs of one million generations and four chains sampled for every 1000 generations were used. The first 25% of the resulting trees corresponding to the initial phase of the Markov chain were discarded as burn-in. All analyses were implemented in in CIPRES Science Gateway [63].

3. Results

3.1. Ostracode Species Composition in Subterranean Waters of the Yucatán Peninsula

A total of 20 ostracode species, belonging to four families, were recovered from subterranean habitats (caves and cenotes) of the Yucatán Peninsula (Figure 2, Table 1). We found ostracodes in all 32 systems sampled. Table 1 summarizes the taxonomic classification and distribution of the species in the study areas. The species *C. vidua* and *C. ilosvayi* were the most widely distributed with 15 and 13 records, respectively. For all other species we collected mostly empty shells, mainly from bottom sediments. As a result, we were unable to confirm the taxonomic classification for at least eight species, which remain in open nomenclature.

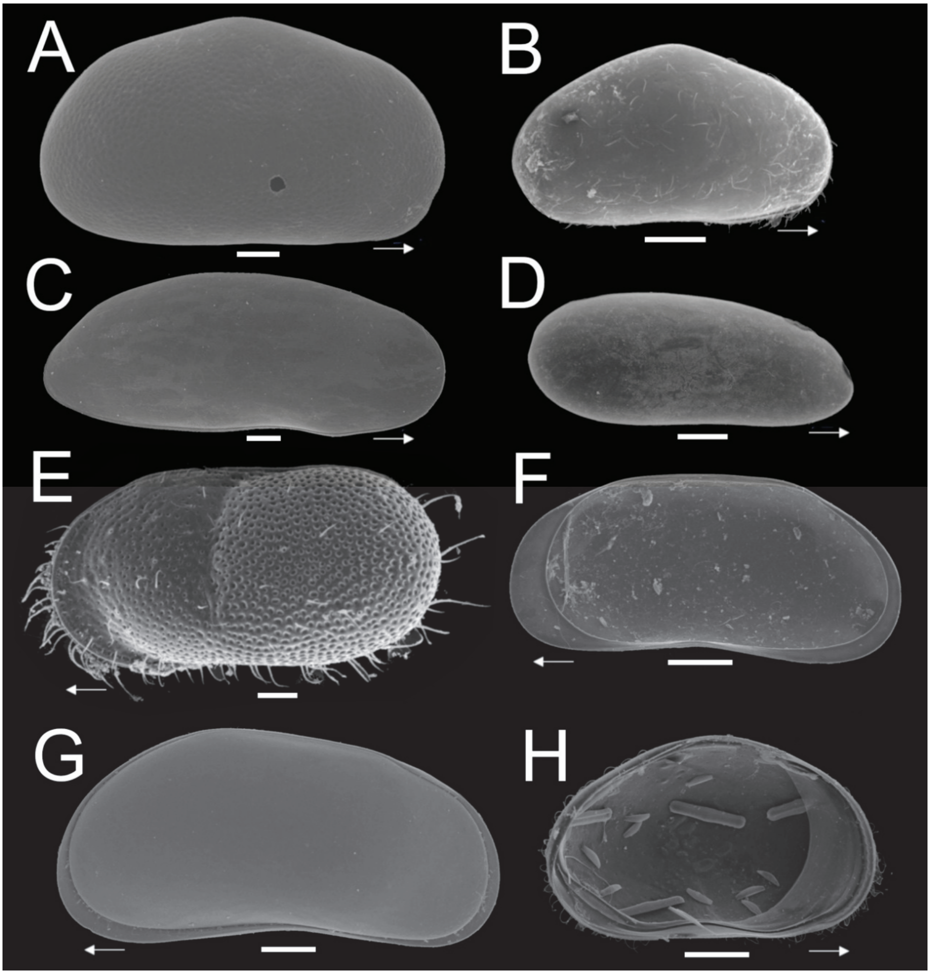


Figure 2. SEM plate analysis of ostracode species of subterranean environments of the Yucatán Peninsula. (**A**) *Heterocypris* sp; (**B**) *Cypridopsis vidua* (O.F. Müller, 1776); (**C**) *Diaphanocypris meridana* (Furtos, 1936); (**D**) *Alicenula* sp.; (**E**) *Cytheridella ilosvayi* Daday, 1905; (**F**) *Pseudocandona* sp.; (**G**) *Pseudocandona antilliana* Broodbakker, 1983; (**H**) *Cypridopsis vidua* in ventral position, with remains of diatoms. White arrows show the anterior position of valves. Scalebar = 100 µm.

Table 1. Ostracode species composition of 32 cave and cenotes of the Yucatán Peninsula.

Taxon	Family	Cenote/Cave
Alicenula sp.	Darwinulidae Brady and Robertson, 1885	Calcuch, Dos Palmas, Dzonotila, Kankirixché, X'Batún
Alicenula yucatanensis Macario-González et al., 2018		Chihuo-Hol, Dzonbacal, Galeana, Kankirixché, Tanimax, Xtojil, Huul K'in, Oxolá.
Darwinula stevensoni (Brady and Robertson, 1870)		Abala, Dzonotila, Huul K'in, Oxolá, Pol Box, Sabtún-1, X'kokob, Xoch, Yumku
Vestalenula pagliolii (Pinto and Kotzian, 1961)		Azul (in Campeche), Azul (in Quintana Roo), X'Batún
Chlamydotheca unispinosa (Baird, 1862)	Cyprididae Baird, 1845	Antum
Cypridopsis sp.		Cave X'tacumbilxuna'an, Tanimax
Cypridopsis niagranensis Furtos, 1936		Abala
Cypridopsis vidua (O. F. Müller, 1776)		Abala, Antum, Azul (in Quintana Roo), Colac, Dzonotila, Galeana, Huul K'in, Noh'Chunck, Sabakha, Sabtún-1, Tzitzila, X'Batún, Xoch, Yokdzonot, Yumku
Diaphanocypris meridana (Furtos, 1936)		Colac, Oxolá
Heterocypris sp.		Dzalbay
Strandesia intrepida Furtos, 1936		Azul (in Campeche), Azul (in Quintana Roo)
Cypria sp.	Candonidae Kaufmann, 1900	Cave X'tacumbilxuna'an
Cypria petenensis Ferguson et al., 1964		Azul (in Campeche)
Cypria cf. *pseudocrenulata* Furtos, 1936		El Padre
Keysercypria sp.		Dzonotila
Keysercypria xanabanica (Furtos, 1936)		Xtojil
Pseudocandona antilliana Broodbakker, 1983		Xoch
Pseudocandona sp.		Dzonotila, Sacalaca
Cytheridella ilosvayi Daday, 1905	Limnocytheridae Sars, 1925	Antum, Azul (in Campeche), Azul (in Quintana Roo), Cave Peba, El Padre, Galeana, Kankirixché, Sabtún-1, Xtojil, Xoch, Yokdzonot, Yax Há, Yumku
Cyprideis sp.	Cytherideidae Sars, 1925	Xoch

The cenote Xoch was the site with the highest species richness (number of species per site) in the region, with five species. The occurrence of *Cyprideis* sp. in this cenote revealed the influence of the marine environment on species composition. Species richness in other cenotes varied from one to three species. In cave environments such as Yumku, Peba and, X'tacumbilxuna'an, between one and two species were observed.

All taxa recorded in the sampling sites are epigean fauna. The species composition is integrated with species widely distributed in tropical America or cosmopolites such as *Diaphanocypris meridana* (Furtos, 1936), *Chlamydotheca unispinosa* (Baird, 1862), *Pseudocandona antilliana* Broodbakker, 1983, *Darwinula stevensoni* (Brady and Robertson, 1870) *C. vidua*, and *C. ilosvayi* and regional endemics such as *Alicenula yucatanensis* Macario-González et al., 2018, *Cypridopsis niagranensis* Furtos, 1936, *Cypria petenensis* Ferguson et al., 1964, *Strandesia intrepida* Furtos, 1936, and *Keysercypria xanabanica* (Furtos, 1936). The latter species (*K. xanabanica*) is the only species currently known to be distributed exclusively in cenotes of the northern Yucatán Peninsula.

3.2. Shape and Size Morphometric Analysis of Selected Ostracode Species from Caves and Cenotes

The principal component analysis based on the first and second components of elliptic Fourier coefficients, explain more than 59% of total data variation for *C. ilosvayi* (Figure 3A), *Alicenula* sp. (Figure 3B), and *C. vidua* (Figure 3C). For *C. ilosvayi*, the first and second principal components of the PCA explained 85% of total data variation. Patterns in morphospace suggested the presence of three morphological forms that seem not to be randomly distributed but, rather, associated with habitat types (Figure 3A). Cave systems Yumku and Peba were associated with the first morphotype (type CIL I), in which the outline describes elongated organisms with a well-developed brood pouch, exceeding the dorsal margin, and with the anterior margin slightly pointed. The second morphotype (type CIL II; Figure 3A) is related to cenotes, with the water surface directly in contact with the exterior. These organisms are narrow, elongated, and square—shaped, with an almost straight dorsal margin (without overlapping of the brood pouch) and a broadly rounded anterior margin. The third morphotype (type CIL III; Figure 3A) is considered a valve deformation, as it was observed in a single specimen. It was characterized by a narrowly developed and totally compressed brood pouch area. This specimen belongs to cenote Yokdzonot and contrasted markedly with the other forms. Due to the low number of specimens recovered, we cannot provide a reliable conclusion on the nature of this form. A MANOVA test showed significant differences (Wilks' lambda test: $\Lambda_{\text{Wilks}} = 0.0014$, $F = 151.7$, $p < 0.006$) between CIL I and CIL II morphotypes. Jackknifed rate classification was 83% correctly assigned for this species. Positions of CIL I and CIL II morphotypes on the CVA plot were clearly differentiated and no overlap was observed (Figure 4A).

The first and second components of the PCA explained 94% of the total shape variation of the ostracode *Alicenula* sp. Two forms were recovered from the morphospace, mainly associated with PC1 (Figure 3B). Similarly, to *C. ilosvayi*, the forms were not randomly associated but clearly corresponded with well-defined water systems. The cenotes Dzonbacal and Dzonotila were related to the morphotype AI, whereas all other cenotes were associated with the second morphotype AII (Figure 3B). An observed difference in the forms was the anterior margin, which is rounded in the AI form and slightly pointed in the AII form. The MANOVA test revealed significant differences (Wilks' lambda test: $\Lambda_{\text{Wilks}} = 0.0001$, $F = 2164$, $p < 0.0004$) between the AI and AII morphotypes. Jackknifed classification rate was 100% correctly assigned. On the CVA plot, morphotypes did not overlap (Figure 4B).

In *C. vidua*, the first and second components explained only 59% of shape data variability, but a notable trend was depicted in the PCA morphospace, with three morphotypes (Figure 3C). Two of them (type CIV I and CIV II) were distributed in cenotes with waters directly in contact with the exterior, whereas the third morphotype CIV III, inhabits in the cave environment of X'tacumbilxuna'an. A MANOVA test showed significant differences between *C. vidua* morphotypes (Wilks' lambda test: $\Lambda\text{Wilks} = 0.0003$, $F = 33.92$, $p < 0.003$). Classification rates were 100% correctly assigned in the confusion matrix. The CVA plot clearly discriminates three morphotypes, as no overlaps were observed (Figure 4C).

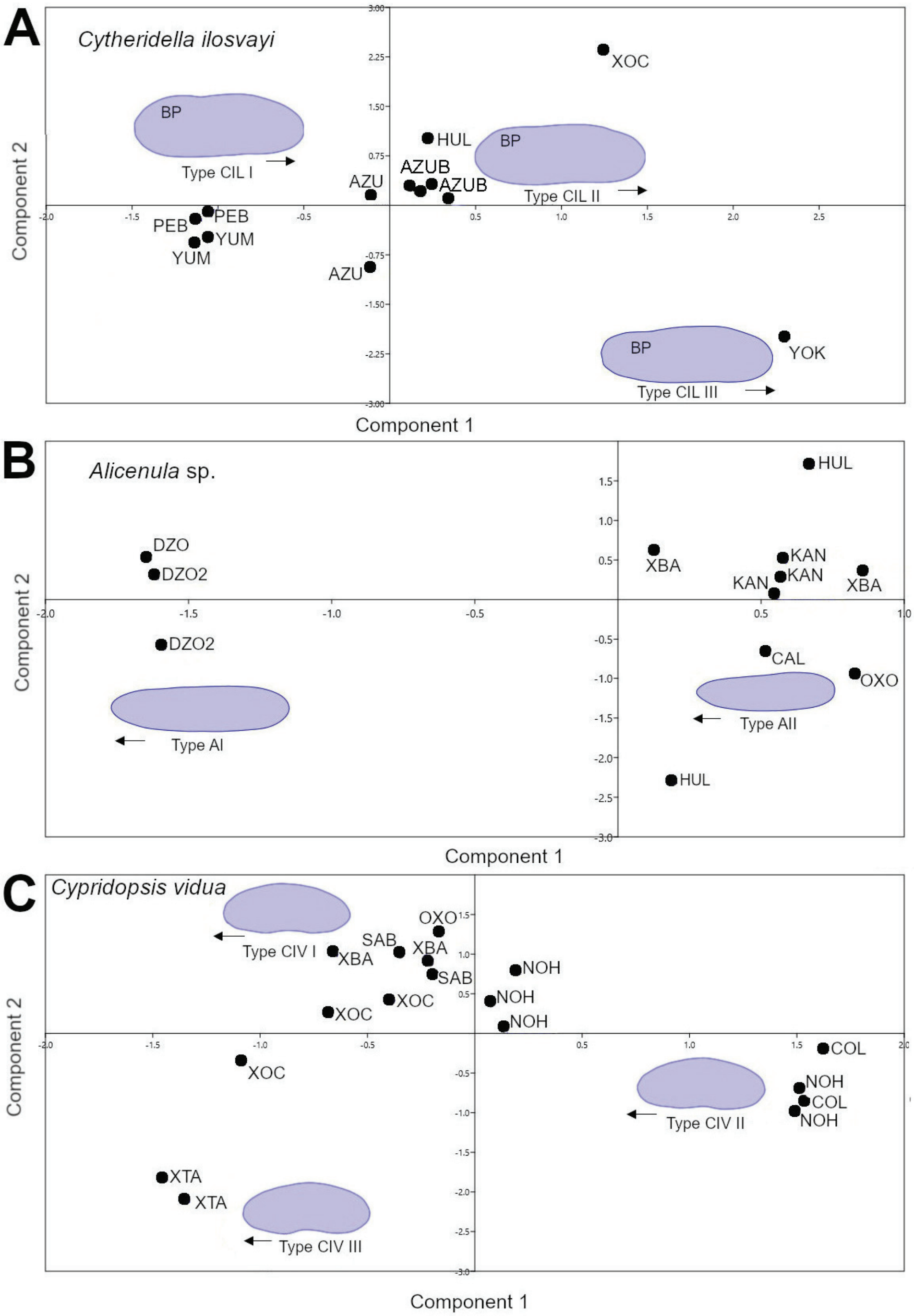


Figure 3. Principal component analysis based on the first and second components of elliptic Fourier coefficients. (**A**) *Cytheridella ilosvayi*; (**B**) *Alicenula* sp.; (**C**) *Cypridopsis vidua*. For site codes see Table S1. Abbreviation BP = brood pouch. Black arrows represent anterior the position of valves.

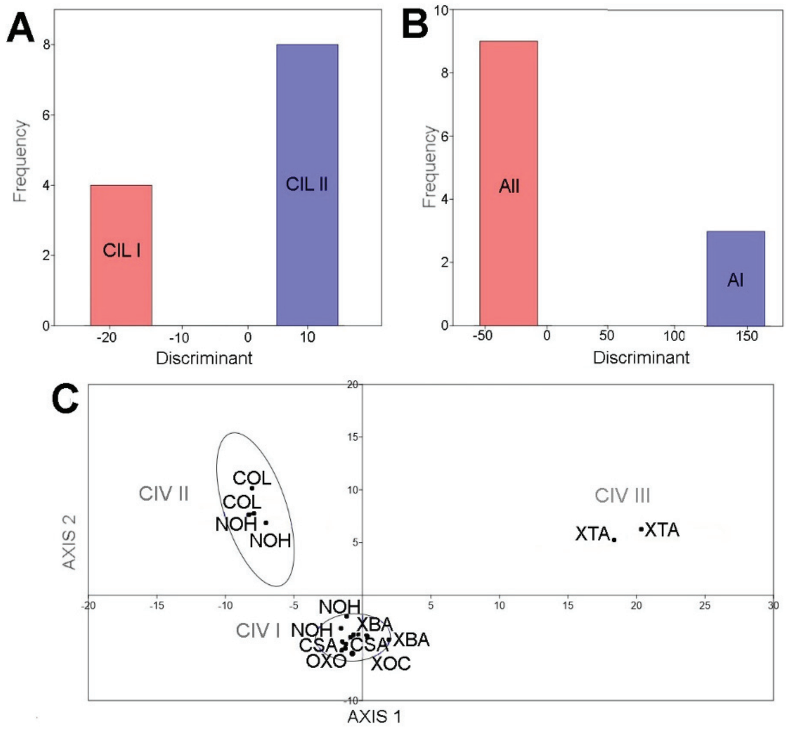
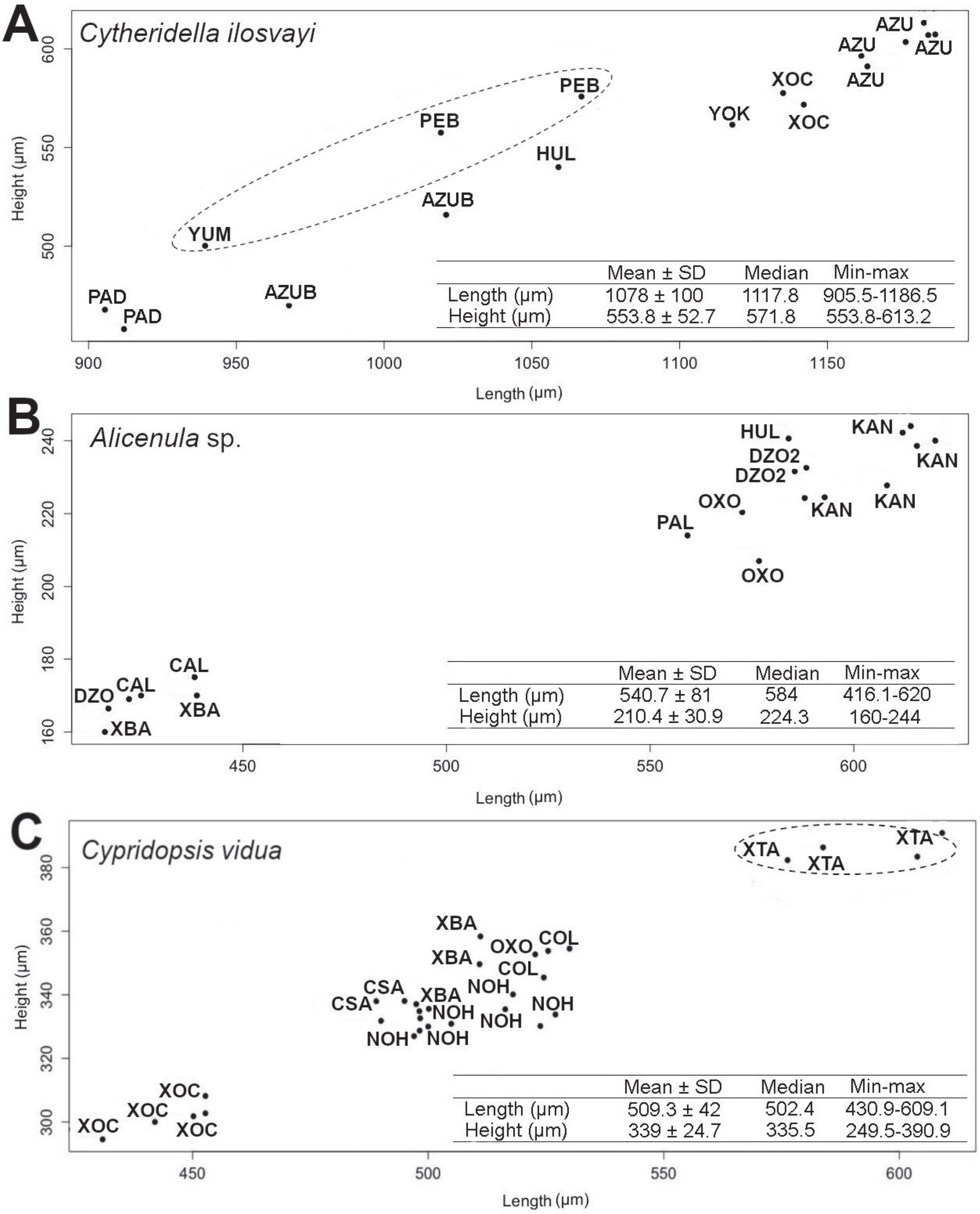


Figure 4. Canonical variate analysis plots. (**A**) *Cytheridella ilosvayi*; (**B**) *Alicenula* sp.; (**C**) *Cypridopsis vidua*. Rate of correct classification was > 83% for all species. Abbreviations: CIL I and II, AI and AII and CIV I, II, and III correspond to morphotypes identified in the PCA based in elliptic Fourier coefficients. For site codes see Table S1.

As elliptic Fourier analysis did not recover variability in valve size, a morphometric analysis based on length and height was conducted. Data dispersion in the scatterplot for *C. ilosvayi* showed overlapping positions between the populations of different habitat types (cenotes and caves; Figure 5A). Comparisons of length, height and L/H ratio, showed no significant differences between the size of the eight populations evaluated (Kruskal–Wallis test, length: $H_2 = 15.19$, height: $H_2 = 15.13$, L/H: $H_2 = 11.99$; all $n = 17$, p-value > 0.05).

The scatterplots for *Alicenula* sp. and *C. vidua* showed well-differentiated positions between populations. For *Alicenula* sp. two groups were identified, differing in size. Populations of Calcuch, X'batun and Dzonbacal were short specimens (<450 μm length and 180 μm height), whereas all other populations were consistently larger with sizes > 550 μm length and 200 μm height. Length, height and L/H ratio of *Alicenula* sp. differed significantly between populations (Kruskal–Wallis test, length: H2 = 16.36, height: H2 = 15.25, L/H: H2 = 13.27; all $n = 19$, p-value < 0.05; Figure 5B).

Data dispersion of *C. vidua* shows three main groups in the scatterplot. The Population of cenote Xoch was the smallest, with individuals < 470 μm in length and 180 μm in height. All other populations of cenotes conformed the second group. The population of cave X'tacumbilxuna'an was the largest of the data set with individuals > 570 μm in length and 370 μm in height. Length, height and L/H ratio differed significantly within *C. vidua* populations (Kruskal–Wallis test, length: H2 = 25.32, height: H2 = 25.24, L/H: H2 = 20.59; all $n = 30$, p-value < 0.05; Figure 5C).



	Mean ± SD	Median	Min-max
Length (µm)	1078 ± 100	1117.8	905.5-1186.5
Height (µm)	553.8 ± 52.7	571.8	553.8-613.2

	Mean ± SD	Median	Min-max
Length (µm)	540.7 ± 81	584	416.1-620
Height (µm)	210.4 ± 30.9	224.3	160-244

	Mean ± SD	Median	Min-max
Length (µm)	509.3 ± 42	502.4	430.9-609.1
Height (µm)	339 ± 24.7	335.5	249.5-390.9

Figure 5. Scatter plot of length and height of (**A**) *Cytheridella ilosvayi*, (**B**) *Alicenula* sp. and (**C**) *Cypridopsis vidua* populations of cenotes and caves of the Yucatán Peninsula. Descriptive statistics of the length and height of each ostracode species are shown in the tables. Abbreviations of each population correspond to a site code (see Table S1). Dashed circles indicate cave populations.

3.3. Molecular Analysis of Cytheridella ilosvayi Epigean and Hypogean Populations

For COI alignment after excluding the primer region and editing the sequence, the consensus length of sequences was 442 bp (base pairs) and no insertions, deletions, or stop codons were observed. For *C. ilosvayi*, the final COI data set was integrated by 16 new sequences of three populations from subterranean habitats and six populations from epigean environments and also 2 sequences from GenBank (Podocopida).

Phylogenetic analysis based on COI and Bayesian Inference (BI) resulted in a tree topology that discriminates three lineages with high support (>0.9; Figure 6). Populations of freshwater and oligotrophic lakes and cenotes located relatively close to the Caribbean Sea coast, constituted the first lineage. Populations of lakes from southern Yucatán Peninsula, characterized by extensive littoral areas and eutrophic conditions, are the second lineage. The third lineage is composed of populations from cave environments Yumku and Peba, both in the northern part of the Peninsula.

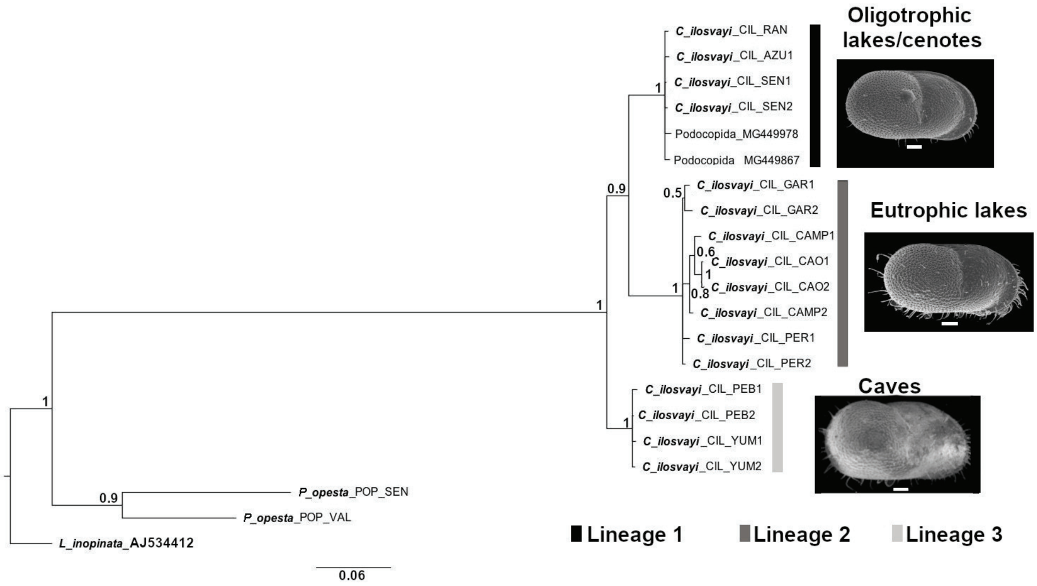


Figure 6. Phylogenetic relationship in *Cytheridella ilosvayi* populations of oligotrophic and eutrophic lakes, cenotes and caves in the Yucatán Peninsula. COI tree topology is based on Bayesian Inference (BI). Numbers above branches represent Bayesian posterior probabilities. Abbreviations on tree topology correspond to GenBank species identification code (see Table S2).

The 18S data set comprised 11 sequences of 1078 to 1170 pb length, and they show extremely low variation between populations; most sequences were identical among species. This made it impossible to differentiate intraspecific groups, based on this gene. The concatenated COI-18S data set consisted of 16 sequences of 2238 pb length, and the resulting tree topology was highly concordant with COI tree topology. The lineages discriminated by independent COI analysis were also uncovered by the COI-18S tree (Figure S1). This is expected as the COI sequences were responsible for the variability of COI-18S data set.

4. Discussion

4.1. Low Diversity and Epigean Species Dominance on Ostracode Assemblages of Subterranean Environments of the Yucatán Peninsula

The Yucatán Peninsula is a region where the biological diversity of freshwater ostracodes in epigean environments is high (>30 spp.), compared to other tropical regions of the American continent such as Central America (~25 spp.), the Caribbean Islands

(~40 spp.) and the transition zone between the Neotropics and the Nearctic (Central Mexico; ~20 spp.) [45,48,54,64,65]. This region is indeed considered a site of (micro) endemism, with more than 70% of the species restricted to single lakes (e.g. *Cypretta spinosa* Cohuo et al. 2013) [61] or limnological regions (e.g. *S. intrepida, C. petenensis*) [66]. In subterranean waters, however, the observed α- diversity is relatively low, with an average of two ostracode species per site and with a total of 13 genera and 20 species in the 32 systems evaluated. The low biodiversity in ostracodes of subterranean waters of the Yucatán Peninsula, at this stage, is coincident with the global trend observed for subterranean assemblages, in which species richness is higher in temperate regions of higher latitude, compared to the tropics [16,67]. In groundwaters of the karstic regions of Europe such as Romania, a total of 19 genera and 42 ostracode species have been recorded [68] in an area of a similar extent to Yucatán. The number of ostracode species found in our study, may, however, represent a gross underestimation of the biological diversity of subterranean waters in the Yucatán Peninsula, as our sampling sites represent less than 1% of the total subterranean systems available in the region. Particularly, the presence of ostracodes in all systems evaluated (caves and cenotes and its subterranean passages) may be an indicative of a higher diversity in the region, as far as more systems are evaluated.

Surprisingly, the ostracode assemblage in the cenotes and caves of the Peninsula was constituted exclusively by epigean species, while stygobionts are lacking. All of the fauna collected has also been observed in lake environments and about 40% of these species display wide distributions in the tropical America or are cosmopolitan (generalists) [48,69].

At the regional scale, in the northern Neotropics (southern Mexico, Central America, and the Caribbean), subterranean ostracodes have been studied mainly in the Yucatán Peninsula [45–47] and the Antilles (including the Caribbean coast of Venezuela) [70–73]. These studies have demonstrated that the region harbors stygobiont fauna in subterranean waters in both fresh and anchialine environments. In freshwaters, three endemic genera have been described so far in the Caribbean: *Caribecandona* Broodbakker, 1983, *Cubacandona* Broodbakker, 1983 and *Danielocandona* Broodbakker, 1983 [73]. The first two genera (with six species) have been found exclusively in Cuba and Hispaniola [73], reflecting endemicity in the older islands of the Caribbean. The genus *Danielocandona* was found only on the northern coast of Venezuela [73]. In the Yucatán Peninsula, stygobiont ostracode species in freshwaters have not been found yet, but in anchialine environments, two stygobiont species have been recorded, so far. These species belong to the marine order Halocypria and are likely endemic to the Peninsula. *Humphreysella mexicana* (Kornicker and Iliffe, 1989) was first found in cenote Mayan Blue, near Tulum in Quintana Roo [46], and was originally described as *Danielopolina mexicana* Kornicker & Iliffe, 1989 [46]. Similarly, *Spelaeoecia mayan* Kornicker and Iliffe, 1998 was first found in cenote Mayan Blue [47]. These species have been recently collected in other localities of the submerged cave system "Ponderosa" and cenotes Muknal, Bang, 27 steps, and Cristal, located along the Caribbean coast of the Yucatán Peninsula, always in marine water habitats, under the halocline [74].

The low numbers of obligated cave dwellers found in freshwaters in previous sampling campaigns and the present study on the Yucatán Peninsula, may reflect the scarcity of or difficulty in collecting these organisms with standard sampling methods, or with the current sampling effort. For instance, the two known stygobiont species of the region (*H. mexicana* and *S. mayan*), were collected during scientific cave diving expeditions using plankton nets [46,47], suggesting that this alternative collection method could increase the opportunities of finding new stygobiont species. However, freshwater ostracodes, with the difference of anchialine species, which can be planktonic, are mostly benthic or nektobenthic. Therefore, the intensification of bottom sediments and ecological niches explorations, particularly in cenotes and caves walls, may increase the opportunity of finding stygobionts. Furthermore, a detailed analysis of the soft parts of the currently collected organisms may reveal stygobiont species.

4.2. Morphological Variability of Freshwater Ostracodes in Subterranean Waters of Yucatán Peninsula

Geometric morphometrics is a technique widely used in freshwater ostracodes, in both shell and appendages, to evaluate morphological variability within species populations as an environmental effect [75,76] or to postulate new species on the basis of the integrative taxonomy paradigm [77,78]. Understanding the source of variability in ostracodes, is fundamental for their use as bioindicators of present and past environments and to gain insights into the biological evolution of a region.

In the Neotropical region, the use of geometric morphometrics in ostracodes is rare; and most studies have been used to clarify the taxonomic status of the species (e.g., *Cypridopsis silvestrii* (Daday, 1902)) [79] or to evaluate the influence of the environment in the phenotype (e.g., *Argentocypris fontana* (Graf, 1931) and *Limnocythere rionegroensis* Cusminsky and Whatley, 1996 [80,81].

Cytheridella ilosvayi is the neotropical species in which the use of geometric morphometrics has been more intensively applied. For this species, large-scale biogeographical patterns [49,50], taxonomic issues [50], and the influence of the environment, i.e., climate and hydrogeochemistry on the shell and appendages [82], have been tested. These studies have illustrated that *C. ilosvayi* displays morphological variability in both shell and appendages between populations of its distribution range (southern Mexico, Central America, the Caribbean, and South America) [48,49]. Outline is the most important valve shape attribute that varies most significantly between geographical regions [50]. Given the large and disjunct distribution areas of *C. ilosvayi*, valve variability has been attributed to biotic (type of reproduction, dispersal) and abiotic (water physics and chemistry) features of the host environments [49,82].

In the Yucatán Peninsula, the geometric morphometric approach effectively discriminates morphotypes. For instance, Wrozyna et al. [82], identified two morphotypes in epigean environments: one of them is attributed to *C. ilosvayi*, and the other to an undescribed species. In the subterranean waters of the Peninsula, similarly, our study reveals two well-defined morphotypes of *C. ilosvayi*, significantly differing in shape, but not in size. The CIL I morphotype, corresponding to cave environments, is distinctive as it displays poor ornamentation in the valve surface (the anterior first third of the valve is almost smooth) and a prominent brood chamber that exceeds the dorsal margin of the shell. These characteristics (attributed to their life in caves) differentiated them from the other forms of the Peninsula, and therefore, represents the third morphotype of *C. ilosvayi* in the region. This is relevant as at the continental scale, excluding Yucatán species, only two morphotypes have been recognized in adult specimens (Florida and Brazil—Colombia) [49,50,82]. Therefore, high morphological variability in Yucatán can be assumed. The presence of *C. ilosvayi* in the caves of Yucatán represents the first record of the species in subterranean environments, as previously it was documented in lakes, rivers, cenotes, springs, flood plains, and coastal areas [49,83]. For the CIL II morphotype which is widely distributed in the region, we could not establish a morphological relationship with the morphotypes identified by Wrozyna et al., [82], particularly because a full description of those morphotypes is not presented.

The low number of specimens evaluated (13 individuals) in *C. ilosvayi* may, however, represent a source of bias for geometric morphometrics, as outline analysis requires a number of specimens (independent data) to consider assumptions to be statistically robust. For ostracodes, however, geometric morphometric analysis has shown acceptable results with a relatively low number of specimens, such as 27 [84] and 29 [74], particularly in studies of paleoenvironments, where shell recovery can be a limiting factor. For cave and cenotes (mostly small-sized and oligotrophic), valve recovery of a single species can be complex and not always possible with standard methods. For instance, contrary to what was expected (as subterranean waters are low energy), the valves recovered in our samplings were not always in a good preservation state, and this may be related to ecological interaction as predation, which may affect valve integrity.

For the *Alicenula* sp. and *C. vidua* populations, valve shape variation between the morphotypes identified in the PCA plot, was significant. Furthermore, for both species, valve size analysis (length/height ratio), resulted in significant differences between the populations. This reveals that morphological variation is relevant in these species and its related to both valve size and shape. In comparison to their epigean relatives, the most prominent pattern is valve reduction. For *Alicenula* sp., epigean forms have a length range of 560 to 580 μm [54], and in our study forms of cenotes Xbatun, Dzonotila and Calcuch, the valve size of the organisms ranges from 420 to 450 μm. Specimens from cenote Kankirixché, however, were even larger than those in epigean environments, with a lengthof more than 600 μm. For darwinulid ostracods (such as *Alicenula* sp.), valve shape and size have been demonstrated to be highly conservative in epigean environments, even across large geographical areas [85]. In the Yucatán Peninsula, molecular evidence has highlighted that minimal changes in valve structure and size may indicate different species [54]. Currently, there are two *Alicenula* species described so far in Yucatán: *A. yucatanensis* and *A. serricaudata* [48]. Differentiation between these species is difficult based on morphological or morphometric attributes of valves only. Nevertheless, two well-defined groups were recovered with both the elliptic Fourier method and length—height analysis. The two groups identified were closely match, with the overall shorth and low *A. yucatanensis* (514 μm length, 198 μm height) and *A. serricaudata* (572 μm length, 210 μm height), but size discrepancies were observed in particular in *A. yucatanensis.* A definitive taxonomic position of *Alicenula* species inhabiting subterranean environments of Yucatán cannot be provided at this stage and the ecological significance of the presence/absence of a particular species, the valve size reduction or variability and the possible co-occurrence in a single system, must be carefully tested and should be accompanied by soft part analysis and studies of genetic divergences.

Similarly, for *C. vidua*, the variability of cenotes and cave populations with respect to epigean fauna is evident. The size of epigean individuals ranges from 550 to 650 μm [54], whereas, in subterranean waters, we recorded individuals less than 450 μm in cenote Xoch; most specimens have a length ranging between 480 and 530 μm. As *C. vidua* is a cosmopolitan species, the morphological features of shell and genetic diversity are variable, and the morphotypes may represent a complex interaction between genotype and environment. In the Yucatán Peninsula, three *Cypridopsis* species have been reported so far (*Cypridopsis rhomboidea* Furtos, 1936; *C. niagranensis*; *C. vidua*), with distinct characters mostly in appendages and reproductive structures [45,54]. Furthermore, for *C. vidua* cryptic speciation has been documented in the Peninsula with at least three genetic lineages, that cannot be fully discriminated by morphometric analysis [54]. For this species uncertainty in species identification remains, as we were unable to collect well-preserved and complete organisms and corroborate their taxonomic identity.

For *Alicenula* sp. and *C. vidua*, an integrative approach using morphological, morphometric, ecological, and molecular analysis is highly recommended to clarify the influence of the environment on shell structure and to evaluate diversity [54], particularly for their use as bioindicators in paleoenvironments.

4.3. Genetic Differentiation of Cytheridella ilosvayi in Subterranean Waters of the Yucatán Peninsula

In the Yucatán Peninsula, there are currently known two species belonging to the genus *Cytheridella*: *C. ilosvayi* and *Cytheridella americana* (Furtos, 1936) [45,48]. Based on morphometric differentiation, Wrozyna et al. [50] postulated the existence of a statistically differentiated morphotype of *C. ilosvayi* in Yucatán, but it remains uncertain whether the species is *C. americana* or an undescribed species. In ostracodes, valve morphology can be strongly influenced by the physical and chemical water properties of the systems. Most importantly, valve variations attributed to the environment are size, ornamentation, and shape [78,86–88]. Appendages are also subject to phenotypic plasticity as a response to local or regional environmental factors [78,89]. For ostracode species, therefore, minimal changes in both hard and soft parts must be evaluated on the basis of the integrative taxonomy approach [90]. Molecular analysis has been demonstrated to be a fundamental technique to complement the morphological determinations, and the combination of them have proved to be reliable for species delimitation in the Yucatán Peninsula [54].

For the *C. ilosvayi* individuals of our study, it remains uncertain whether the morphological interpopulation variability is related to local environmental factors of caves and cenotes or derived from speciation processes.

Phylogenetic analyses undertaken in *C. ilosvayi* specimens of subterranean environments and epigean populations shows three main groupings on tree topology based on COI, and with high Bayesian support (0.9). This suggests the existence of three genetic lineages in the region. Furthermore, these lineages corresponded to well-defined habitat types, i.e., cave populations, cenotes/oligotrophic lakes, and lakes with eutrophic conditions. This specificity for habitat types may also indicate an ecological separation between lineages. COI has been widely used for aquatic species identification and has a high resolution for (cryptic) species delimitation [91,92]. This gene, therefore, suggests the existence of three independently evolved lineages in the Peninsula, associated to habitat types, that may represent different species. Differentiation between habitat types for *C. ilosvayi* must, however, be taken with caution as this species is considered broadly tolerant to environmental conditions, as it is widely distributed in the tropical America. A well-defined ecological differentiation of morphotypes, must be accompanied by controlled conditions and experiments such as transplantation or translocation.

The 18S gene is highly intraspecifically conservative and is also effective in species-level identification, even with single nucleotide polymorphisms [93,94]. Intraspecifically, 18S sequences similarities close to 100% have demonstrated to be an appropriate criterion for species delimitations in aquatic groups such as copepods [95]. For *C. ilosvayi,* the 18S gene sequences were identical between populations (100% similarities). For this gene, the lack of differentiation may further suggest that reproductive intermixing occurs between populations. Therefore, lineage discrimination is not supported by the 18S gene in *C. ilosvayi*.

The lack of concordance between the mitochondrial and nuclear markers in *C. ilosvayi* populations, represents a challenge to determine speciation in subterranean populations.

Concatenated COI-18S analysis revealed, however, Bayesian posterior probabilities of 0.7 for *C. ilosvayi* lineages; the evidence for genetic differentiation within the group is, therefore, weak. Given the lack of additional morphological evidence, particularly of soft parts, we consider that there is not enough evidence to support diversification in subterranean environments for *C. ilosvayi* in the Yucatán Peninsula.

Regarding the taxonomic identity of *Cytheridella* specimens of Yucatán subterranean waters, based purely on valve size, we assume that *C. ilosvayi* is the most appropriate name, as they average 1078 µm in length and 553 µm in height ($n = 17$), which is coincident with most records of this species in tropical America [83]. Conversely, *C. americana* valve sizes range 550 µm in length and 310 µm in height [45]. At this stage, we are unable to test the hypothesis of Wrozyna et al., [50], on the existence of an undescribed *Cytheridella* species in lakes (or cenotes) of Yucatán and to resolve this taxonomic issue. Genetic sequences of *Cytheridella* of other different regions of the American continent will be necessary.

4.4. Late Pleistocene and Early Holocene Climate as the Driver of Subterranean Biodiversity in the Yucatán Peninsula

Aquatic environments of the Yucatán Peninsula during the Late Pleistocene and Early Holocene have been influenced by regional and local events, mainly related to climate fluctuation. From a regional perspective, environments of the Peninsula and, particularly, subterranean systems have been strongly influenced by the relative position of the sea level. Evidences from multiple biological and non-biological indicators, such as corals and speleothems, suggest that during the Late Pleistocene and, particularly, the Last Glacial Maximum (LGM), the marine water level in the Caribbean decreased by ~120 m, relative to today's levels [96,97]. This suggests that the water level in cenotes, was much lower than today. Evidence from sedimentary sequences of Lake Petén Itzá, the largest and oldest system in the Yucatán Peninsula, supports that events such as Heinrich Stadials and deglacial were characterized by precipitation decreases and periods of severe droughts [98,99].

In terms of freshwater availability, Lake Petén Itzá decreased during the Heinrich stadial 1 by about 50 m with respect to their current water level, with a reduction of about 80% of the total area [100–103]. This estimation allows us to postulate that water systems in the entire Yucatán Peninsula were severely affected by similar magnitudes as Lake Petén Itzá [98,99,101]. Few systems must have remained filled during the dry spells of Late Pleistocene—Early Holocene, with water chemistry that was likely saturated with minerals as a consequence of evaporation rates.

For subterranean waters, the lowering of the sea level and the prevailing inland dry conditions, might reduce subterranean water availability and caused modifications of the chemical composition of the systems by rock weathering, i.e., increased conductivity by calcium and magnesium incorporation. These conditions may have triggered the reduction of groundwater sources and the millennial-lasting complete isolation of subterranean environments. Long-lasting systems isolation, together with environmental variability, is recognized as a driver for biological diversification worldwide [104,105]. Currently, in the Yucatán Peninsula, a high number of freshwater endemic and stygobiont species have been recorded so far, in groups such as Copepoda, Thermosbaenacea, Stygiomysida, Mysida, Isopoda, Amphipoda, and Decapoda [26,74,106,107], all with well-developed capabilities to inhabit temporarily or permanently the water column.

The low number of freshwater endemic and stygobiont species in Ostracoda of subterranean waters of Yucatán, can be related to the ecological behavior of the group and the environmental fluctuations of the late Pleistocene and Holocene [108]. For instance, ostracodes can be considered benthic species adapted to exploit sediments, and with limited capacities of swimming in the water column.

Sedimentary sequences of the Yucatán Peninsula have revealed that the sea-level rise was progressive after the drastic decrease of the Late Pleistocene. During the Holocene, bottom sediments were subject to strong environmental alterations given sea level fluctuations. Data from the currently submerged speleothem of the Yucatán Peninsula have revealed periods of either increased/decreased water level during 8.9, 8.6, 8.4, and 6.0 ka BP (before present) [109]. The frequency of bottom sediments instability in subterranean environments might have represented an important limiting factor for benthic species success, i.e., adaptation and diversification.

In cenote Aktun Ha, located ~8 km away from the Caribbean sea coast, the fossil record of *C. ilosvayi* and *D. stevensoni* during the last ~6.8 ka, has revealed phases of relatively high ostracode abundance during low water stands (relative to lower sea levels). The increase of water levels following a sea-level rise of about 4 m at 6.5 ka BP, drove the extinction of *C. ilosvayi* in Aktun Ha, likely due to the effect of marine water intrusion in the subterranean aquifer [110]. Conversely, *D. stevensoni*, remained present in the system despite hydrological alterations [110]. The ecological response of *C. ilosvayi* and *D. stevensoni* in cenote Aktun Ha may illustrate the regional effect of sea level variability in benthic assemblages on a regional scale, with some species more susceptible to subterranean environmental change and others with better capacity to tolerate them.

In isolated sites such as Yumku and Peba, which are systems not directly connected to the subterranean aquifer, *C. ilosvayi* demonstrated morphological variability in carapace structure, as well as a clear genetic differentiation compared to their relatives in cenotes and lakes. This may be indicating different evolutionary pathways for species of subterranean environments in which marine intrusion has shown none or little relevance in the geological history. These sites are, therefore, highly important for a comprehensive evaluation of the biological evolution of subterranean species associated with sediments in the region. The exclusive occurrence of epigean species in freshwaters in cenotes and caves of Yucatán may, therefore, be reflecting the ecological alteration of the Late Pleistocene and Early Holocene climate variability in the region [111].

Colonization of epigean species is a common trend in subterranean environments worldwide, and in most systems, a combination of epigean and hypogean fauna can be observed [8,68,112]. Evidence from different aquatic groups reveals that colonization of subterranean environments is explained by active and passive models [31,32,113]. Active models describe that: (i) the species found refugium from climate variations (the climate-relict model), or strong ecological competitive pressure; (ii) generalist species colonized subterranean environments even in the absence of climate or ecological stressors (the active colonization model), and (iii) marine species invaded land through subterranean connections [31,113]. Passive models suggest that colonization occurred by transportation, either by biotic or abiotic factors [31]. The evidence observed from the freshwater species assemblages of subterranean environments in the Yucatán Peninsula is, by far, not definitive; given the combination of a high percentage of generalist species and epigean regional endemics and the practical absence of microendemics and obligate cave-dwellers, we can infer that active and relatively recent colonizations (Holocene) may have taken place in the Yucatán Peninsula. Such colonization may be occurring at different time scales (with different lineages) and may be occurring polytopically, as it has occurred in subterranean zooplankton assemblages worldwide [32].

5. Conclusions

The Yucatán Peninsula is a karst region with high availability of subterranean waters and hypogean biodiversity. The dominance of epigean species and low freshwater ostracode species richness characterize the subterranean waters of this region. About 40% of the species are generalists widely distributed in the American continent, and the remaining 25% are regional endemics. All other species remain in open nomenclature, given the limitations in the taxonomic analysis based on soft part analysis. The absence of stygobiont species in freshwaters, can be related to the applied sampling methods or effort in the field. For instance, in this study, we used scuba diving, sediment analysis, and water filtration, but the increased use of sampling and analysis of bottom sediments and cenotes walls, may represent an appropriate method for hypogean species recovery, given the benthic habits of the ostracodes. Morphometric analyses of valve shape based on the elliptic Fourier analysis and valve size using length—height determination, allowed us to capture the morphological variation between populations. The three species evaluated demonstrated morphological differences between populations. Cave (epigean) populations display the highest morphological variation (shape and size) with respect to species of cenotes. Molecular analysis is a powerful tool for understanding interpopulation variability in *C. ilosvayi*. This species displayed three molecular lineages within subterranean and epigean habitats, but we lack conclusive evidence of species diversification in caves and cenotes. The climate history of the region is assumed to be the main driver for species composition. Subterranean marine intrusions, as well as severe droughts that occurred during the Late Pleistocene and Early Holocene, likely modified water and sediment chemistry, which may have had a direct effect on benthic species composition.

Supplementary Materials: The following are available online at https://www.mdpi.com/1424-2818/13/2/44/s1, Table S1: Location data of the studied cenotes and caves, Table S2: GenBank identification codes and accession numbers for COI and 18S rDNA sequences of *Cytheridella ilosvayi* populations, Figure S1: Phylogenetic tree of concatenated COI-18S dataset on *Cytheridella ilosvayi* populations of cenotes, caves and lakes in the Yucatán Peninsula. COI tree topology based on Bayesian Inference (BI); numbers below branches represent Bayesian posterior probabilities; abbreviations on tree topology correspond to GenBank species identification codes (see Table S2).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to their usage in the ongoing study. Sequences of this study are available from GenBank (accession numbers MW018845-MW018862 for COI and MW018806-MW018816 for 18S rDNA).

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diversity

Article

Hypogean Communities as Cybernetic Systems: Implications for the Evolution of Cave Biotas

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Abstract: Ramón Margalef proposed in 1968 that ecosystems could be better understood if they were viewed as cybernetic systems. I tested this hypothesis in the case of hypogean ecosystems using available pieces of evidence. I looked on how information on feedbacks, stability, succession, organization, diversity, and energy flows in the hypogean environment fit the cybernetics hypothesis. The results were that there are convincing arguments that the application of the concept of cybernetics in biospeleology can be beneficial to broadening our understanding of cave biota in terms of their structure. I also make the case that this approach can provide more clarity about how cave biota has evolved through time and the implications for their conservation.

Keywords: cybernetics; ecology; cave biology; biospeleology

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1. Introduction to the Concept of Cybernetics in Ecology

Cybernetics can be defined as the interdisciplinary approach of exploring regulatory systems, structures, constraints, and possibilities. The term cybernetics was first introduced in the modern scientific literature by Norbert Wiener in his book “Cybernetics or Control and Communication in the Animal and Machine” [1]. He used it as a form of transfer of information. The origin of the term “cybernetics” [2] is as found in Plato’s “The study of self-governance”. There he used the word *κυβερνητικός* or *kybernetes* for helmsman, steersman, rudder, rudder-man, or skipper. The Latin form was *gubernetes* or, later, *gubernator*. Although Plato’s application of the term was to mean effective government as an art [3], Wiener’s definition concerned the scientific study of control and communication among animals and machines [4,5].

The concept of cybernetics in ecology was first formally introduced by Margalef [6], although the idea had been floating around for some time [7]. Margalef contended that cybernetics was a more holistic and elucidating way to look at ecological communities. Although this terminology might be considered by some as just a buzzword, this approach continues to be used by theoretical ecologists and system engineers [3,8–11]. For Margalef [6], the ecological approach was to look at cybernetics as a model for understanding representations of life (from cells to individuals to ecosystems) where feedbacks, stability, organization, diversity, and energy flows are interacting elements that are both the factors and the results of highly dynamic systems. How these elements interact is via information transfer. In information theory, that means quantification, storage, and communication of information. That information could be in the form of trophic energy, reproductive behavior, and evolutionary processes via molecular data, etc. [12].

I will test Margalef’s hypothesis that ecosystems can be interpreted as cybernetic mechanisms by scrutinizing his proposal using material provided by the scientific literature. Some of these data were previously published in Romero [13] but never examined under these parameters, to which I added new information generated in the last ten years. I will use the term epigean for any environment exposed sunlight and hypogean to any environment under perpetual darkness, with one being the opposite of the other. One exception will be dealing with some caves with a portion exposed to sunlight due to their geological conditions.

2. Cybernetics in Biospeleology

2.1. Metaphysical Barriers

To fully understand how a cybernetic model can be applied to biospeleology, we need to recognize two metaphysical barriers. One is the stereotype that the hypogean ecosystems are mostly closed ecosystems or ecological islands, *sensu* [14]. The other is the artificial and rigid segregation of hypogean forms of life based on archetypes or *Baupläne*.

There are numerous arguments against the concept of caves as isolated environments. The first thing we must recognize is that unlike oceanic islands, hypogean ecosystems are not separated by long distances from other ecosystems. Therefore, they are just part of the same general ecosystem above them with the caveat that the hypogean one is under perpetual darkness. This is, therefore, the only commonality to all caves. In any case, generalizations on the concept of caves as an island would lead to stereotypes that will not fit with the diversity of caves worldwide.

Hydrogeologists have described the interactions between river waters and hypogean ones as essential to understanding the flow and biochemical nature of groundwaters [15]. Gers [16] provided evidence that there are exchanges of organic matter and living organisms between caves and the soil above them, documenting an active migration of arthropods not only from the epigean environment to the cave but also from the cave to the epigean environment with the food webs of both ecosystems interlinked. Similar connections have been found for the cave beetle *Speonomus hydrophilus*, which utilizes the primary producers' energy from the epigean environment [17]. The continuous gradation of species in their distribution has also been documented. That is the case of marine caves that penetrate the ocean directly into karstic areas of 100 m or more in length. Different species of mysid crustaceans distribute themselves based on light intensity and salinity [18].

Thus, it should not be surprising that many taxa thought to be found exclusively in epigean ecosystems are also represented in hypogean ones [13], (p. 159). The very different nature of caves further substantiates the hypogean ecosystems' nature as open ones in tropical vs. temperate ecosystems [19–21].

The other issue that creates some metaphysical barriers regarding understanding the hypogean ecosystem as cybernetics is its jargon. One of the major problems in speleology is the proliferation of terms to describe either organisms that inhabit the hypogean ecosystem based on their spatial distribution and/or different portions of the ecosystem itself. Although terms are useful to identify ideas, objects, or mechanisms, an overabundance of them leads toward confusion and, above all, the misleading interpretation of nature as a series of well-defined compartments. The reality is a different one: In nature, all is in flux. Although basic terminology such as herbivore and plankton is well understood, others can be confusing and highly artificial. Romero [13], (pp. 130–132) already mentioned how artificial the hyperclassification of cave organisms is based on their phenotype. More recently, Martínez and Mammola [22] further emphasized this point by showing statistically that these hyperclassifications were a barrier for communication among biospeleologists themselves. The hyperclassification of cave organisms induce the idea that they are instead fixed elements as continually evolving (or having the ability to evolve rather rapidly) within the system. After all, species are not members of a periodic table [13], (p. 166).

In the early twentieth century, ecologists confronted these problems when they stopped looking at natural associations as static components of nature and viewed them as rather dynamic systems in both time and space [6]. That is when the concept of succession [23] was fully adopted. Hence, the term ecosystem became universally accepted as one in four dimensions, i.e., the three spatial ones plus time.

Having clarified these two confounding variables in biospeleology, we can move forward in testing Margalef's hypothesis for understanding the hypogean ecosystem as a cybernetic system.

2.2. Feedbacks

Margalef [6], (pp. 4–5) wrote that "A simple example of an elementary cybernetic mechanism, in the form of a negative feedback loop, is the classical one of a predator and its prey. Organisms are the bearers of huge amounts of information. Since they can be destroyed but cannot be produced from nothing, any regulatory mechanism implies an initial overshoot". He illustrated this point by the fact that the predators reduce excess prey and the number of predators themselves are also regulated by prey availability. The Lotka-Volterra equations can quantify this correlation [13].

This may be the most difficult of Margalef's assertions that can be tested for the hypogean ecosystem. The reasons are two-fold. First, there are not that many prey-predator relationships in the hypogean ecosystem reported in the scientific literature. Second, it is a well-established ecological principle that energy decreases as it moves up trophic levels because energy is lost as metabolic heat when organisms consume the organism from lower trophic levels. That is why a food chain can usually sustain no more than six energy transfers before all the energy is used up. Consequently, endotherms (birds and mammals) use more energy for heat and respiration than ectotherms and have to seek food outside caves. This is because of the insufficient amount of food resources present in many subterranean environments.

That is why we need to take a broader view of the transfer of energy into the hypogean ecosystem to visualize better how the transfer of information does occur in that ecosystem. The connectivity issue among the different cave ecosystem elements and how energy and information flow from one to another has been little studied. This is surprising since these ecosystems are supposed to be simplified because of the lack of primary producers' abundance. Thus, let us explore these issues in detail.

Contrary to perceptions based on caves studies in temperate regions [24], many caves are very rich in nutrients. That is particularly true in tropical regions [21], and some are even chemoautotrophic [25–29]. They are considered primary producers thanks to the bacteria that produce organic matter by oxidizing sulfur. Moreover, one of the most striking examples of the opportunistic nature of life in the hypogean ecosystem is the presence of phototrophs in caves, including algae, lichens, and plants (liverworts, mosses, ferns, and seed plants) [13] (pp. 67–69, 73–76) and [30].

Examples of animals in caves that demonstrate interesting cases of transfer of energy (i.e., information) are many. For species that enter or use caves as temporary habitats, their relationship with those habitats is more complex than it may seem. For example, there are several species of harvestmen (Opiliones) that spend the daytime in caves to leave at night to prey on insects. One species of harvestmen of the genus *Goniosoma* from Brazil is found on different portions of the caves depending upon the vegetation outside, showing that climatic factors may influence cave organisms distribution [31]. Interestingly enough, this species of opilion is preyed upon inside the cave by insects and spiders [32]. Another example is that of cave salamanders that utilize resources from both the cave and the epigean environment [33]. According to numerous reports, snakes regularly predate on bats in tropical and subtropical caves [34–36].

Oilbirds (*Steatornis caripensis*) in South America and several species of swiftlets of the tribe Collocaliini in southern Asia, South Pacific, and northeastern Australia, are found in caves. They are permanent residents of caves during the day for resting and nesting but are the only bird species that have developed echolocation abilities to navigate the caves [37,38]. Similar to many nocturnal birds, they have very sensitive eyes, which they use mostly outside the caves to forage [39]. Yet, the development of echolocating and olfactory abilities is a major adaptation to life in caves which requires major neurological rewiring [40]. More importantly, from an ecological perspective, the droppings of these bird species and those of cave bats greatly influence the cave's ecology. Studies at Cumaca Cave in Trinidad, W.I., indicate that oilbirds were a major component of the ecology of that cave occupying the cliffs of most of the largest halls and displacing the bats to the smaller galleries and towards the end of the cave [41–43]. The droppings of the oilbirds in that cave are prominent. Although no quantitative studies have been conducted in that regard, it is difficult to imagine that such abundant organic material does not influence that particular cave's ecology.

The best example of an organism playing a major role in cave ecology is the case of bats that roost in caves. Bat guano generates rich and complex invertebrate communities, particularly in tropical caves [44,45]. Bat guano has also been accounted for as a source of food for cavefish [46] and salamanders. Fenolio et al. [47] reported coprophagy in salamanders from an Oklahoma cave and found that their nutritional value was comparable to their invertebrate preys. They further suggested that bat guano may play an essential role as a food source among other cave vertebrates.

The effect of bat guano on microbial fauna must also be significant, but that is largely unstudied. Explorers of tropical caves know very well that caves with high levels of guano deposits have higher temperatures. This anecdotal observation has been confirmed empirically. Baudinette et al. [48] found high and relatively constant temperature levels in caves inhabited by large bat colonies. Such heat was part of the microclimate created by the bats themselves, which, in turn, generates better conditions for maternity.

The complexity of trophic structure in caves is another factor that is little studied. For example, Graening [49], while looking at six subterranean stream habitats in the Ozarks, found that there were three trophic levels in those underground streams. The first one was formed by a detrital food base of clastic sediment, bat guano, and surface inputs, a second trophic level created by detritivores, primarily crustaceans and amphibians, and a third, top-level one, of predators, mostly fishes.

Sometimes the input of energy can come from unexpected sources. That is the case of plant roots. In many karstic areas, tree roots penetrate the substrate to the phreatic levels to obtain water. Their root mats form diverse and abundant biomass. Jasinska et al. [50] reported 41 species of aquatic hypogean organisms, including annelids, arthropods, and fish from a cave in Australia that had root mats in their waters. Their study concluded that the root mats were the primary source of energy for all these organisms.

All of these examples indicate two things. One is that cave ecosystems cannot be considered as closed ecosystems. They take considerable input from external sources. Although their output is not comparable even when many organisms that exit the caves reproduce in those niches as it is the case of bats or cave birds, we can conclude that it is asymmetric. The other one is that there is much more that we can learn by conducting more ecological studies in tropical and subtropical regions where the presence of large populations of cave animals such as bats, play a significant role in those ecosystems. Since the vast majority of biospeleologists who have historically conducted fieldwork do so in temperate regions, the scientific literature is biased towards colder parts of the planet. That is what accounts for the apparent—but false—generalization that caves lack both primary producers and prey-predator relationships.

2.3. Stability and Succession

It has long been assumed that the hypogean ecosystem is very stable, a perception based mainly on a historical circumstance. That circumstance is that most ecological observations in caves have been drawn from studies conducted in temperate areas when diversity and biomass tend to be low. Therefore, significant variations in caves' ecological conditions located in those latitudes are difficult to ascertain, mainly when conducting short-term studies. The standard concept of stability in ecology is derived from MacArthur [51], which, in the interpretation by Margalef [6], (p. 11), presupposes the existence of alternative pathways for energy flow, chosen according to the imposition of external circumstances.

When it comes to the cybernetic model, Margalef suggested that there were two types of stability. The first one is when the system achieves a steady state under stable conditions; in the second, the system is much more robust because of its ability to display a more considerable resistance to external changes to the system in their origin. The latter is characterized by higher energy flow and a smaller number of interacting elements. In cybernetic terms, the first system, its next state is predictable from within the system because that system contains much information, and new events add small amounts of information. Margalef argued that the future is less predictable for the second type because the system has less information, and each event represents a relatively important source of information [6], (pp. 11–12).

As Margalef himself proposed, instead of stability, we should be talking about the "frequency of fluctuations" [52]. This makes sense in the case of biospeleological studies since the long-term ecological research in caves is rare and, if so, is based on historical data. Take, for example, changes in the morphology of the cave catfish *Rhamdia quelen* of Trinidad's island. Romero et al. [43] were able to document extraordinary morphological changes in this fish's cave population in less than 50 years. They used museum specimens and meteorological data to map those changes through time. Those changes were most likely the result of changes in the rainfall regime in the area over time.

There is an essential distinction between caves in temperate vs. tropical and subtropical areas. In the tropics, although there are no significant changes in temperature throughout the year, periodic (yearly) floods are not only common but also predictable as part of the external factors affecting the hypogean biota, particularly the aquatic one. Although water regimes' changes do occur in temperate areas, they are more spaced in time and smaller in intensity [13], (p. 161). The ability to withstand those fluctuations have a particular impact on whether or not they can respond to those variations rapidly and why the concept of phenotypic plasticity is so crucial for survival and evolution.

Due to the apparent lack of primary producers, it has long been believed that caves lack any expression of meaningful ecological succession (a phenomenon first and mostly studied for vegetation transformations). This has led to the idea of the so-called "stability" of the cave ecosystem. Much has been written alleging that the cave ecosystem is stable. For example, Langecker [53], (p. 135) characterized caves as "an environment that is relatively stable in its climatic characteristics", and Boutin and Coineau [54], (p. 434) affirmed that "the relative temporal stability of subterranean habitats, postulated for a long time by many authors, has been demonstrated in many particular cases and constitutes one of the generally accepted paradigms of biospeleology".

Still, those blanket generalizations are not supported by data. Perhaps because of the apparent lack of primary producers, cave biologists think there is no succession, and therefore, the system is a 'stable' one. Moreover, succession tends to be slower in temperate ecosystems than in tropical, humid ones. That is why life spans of animals tend to be longer in ecosystems with slow succession instead of those with rapid ones. Subsequently, we find very long-life spans among some troglomorphic organisms in temperate caves as is the case for amblyopsid fishes [55,56]. Moreover, tropical ecosystems, because they have higher levels of energy, allow for more fluctuations and more rapid succession, which in turn accelerate the pace of evolution, both at the individual (species/population) level and at the ecosystem one [57].

Yet, you do not need primary producers for succession to happen. Milanovich et al. [58] documented succession in an abandoned mine in Arkansas that had been recently invaded by the slimy salamander (*Plethodon albagula*) for nesting. Ashmole et al. [59] described faunal succession in the lava caves of the Canary Islands. This phenomenon has also been reported for relatively short periods in marine caves [60]. Mammola and Isaia [61] provided an elegant description of how this phenomenon occurs among cave arachnids.

It is too bad that caves are terrible places for fossilization; otherwise, they could provide us with interesting ecological succession clues. Since bats are such an important source of energy for many caves, one can only wonder how their explosive radiation in the Eocene [62] may have changed the caves' ecological landscapes.

2.4. Organization

From an information theory viewpoint, which is essential to understand ecosystems from a cybernetic standpoint, the hypogean ecosystem defies an intuitive understanding that we may have from other ecosystems about their structures. For example, in aquatic ecosystems, it is relatively easy to separate plankton from the benthos, or in terrestrial ones, plant from animal communities despite their interconnectivity. Several factors complicate blanket generalizations for the hypogean environment: One is the limited space available in caves in comparison, for example, with oceans. That is true even for large caves. The other is that except for the hypogean ecosystems with chemoautotrophic bacteria as primary producers or tropical caves with some plants at the entrances, it is always more challenging to differentiate boundaries. Things get even more complicated when we consider that most of the energetic input in tropical and subtropical caves come from outside sources such as bat droppings. All this further emphasizes the concept discussed earlier that the hypogean ecosystem is not a closed one. Thus, we need to examine what is the evidence of any ecosystem structure in the hypogean ecosystem.

Little work has been done on the structure and information and energy transfer in the hypogean ecosystem. Most cave studies have seen caves as authentic islands of much reduced dimensions, in comparative terms, and one-dimensional representations [63]. However, caves have spatial and temporal dimensions that attest to their complexity (despite their apparent simplicity). Bussotti et al. [64] used a multifactorial sampling to examine the distribution of species assemblages within three different caves in Southern Italy over 11 months. They found a pattern of change in the structure of the assemblages along the exterior-interior axis and areas that suggested a highly complex structure of the biotic community.

From a spatial viewpoint, the typical cave (if there is such a thing) has five spatial-conceptual axes. First, the terrestrial-horizontal one on which we find many terrestrial invertebrates being the most evident to the casual observer. The length of the cave defines the second one. It is well known that community structure and biodiversity distribution changes throughout the cave's length, and the lengthier the cave, the more complex that structure can be [13], (p. 166). Even for relatively small caves, we see a spatial segregation between competing species [12]. That is the case of the Cumaca cave in Trinidad or the Guácharo cave in Venezuela that was discussed earlier. The oilbirds occupy areas closer to the entrance in detriment of bat colonies that occupy deeper areas [41]. The third is vertical and is mostly defined by the differences between the biodiversity found on the ground and roosting on walls or the ceiling of a cave. This is an important dimension since roosting animals, whether they are bats or birds, usually provide large amounts of nutrients to the cave generating ample grounds for increased biodiversity [65]. Moreover, these animals typically move daily from inside the cave to the epigean ecosystem. They represent one of the most important facilitators of the interactions between the hypogean and epigean ecosystems. The fourth is water: Whether a cave is permanently or periodically flooded with water makes a significant difference not only in its biotic composition but also in its dynamic and community structure. The fifth dimension that we need to consider is that of the outside ecosystem that influences the cave. Whether it is the terrestrial community outside the cave determining the species composition and abundance of animals that are frequent on both sides of the equation or water flowing in and out of the cave (including marine caves), they have a tremendous impact in cave ecology [5], (p. 166).

More recently, Hutchins et al. [66] have also questioned the old paradigm that food webs in hypogean ecosystems are simple, limited to one or two trophic levels, and composed of generalist species spatiotemporally patchy food resources and pervasive energy limitation. Their fieldwork generated data that chemolithoautotrophy "has been fundamental for the long-term maintenance of species diversity, trophic complexity, and community stability in this subterranean ecosystem, especially during periods of decreased photosynthetic production and groundwater recharge that have occurred over geologic time scales".

The way bats are distributed in a cave influences patchiness in that cave because of the heterogeneous way their droppings are deposited. This phenomenon has also been observed among mysid crustaceans, which deposit organic material in a patchy manner [67]. Another remarkable example of the complexity of ecosystem structure in caves can be conveyed by looking at the ecological role played by the mite *Coprozercon scopaeus*. This species was found in the feces of the wood rat

(*Neotoma floridana magister*) in Mammoth Cave, Kentucky. This is a remarkable example of a species whose life cycle seems to be restricted to the hypogean ecosystem. Individuals of this subspecies defecate in the same sites (usually about 1 m away from each other), providing a patchy source of nutrients [68].

In addition to these spatial dimensions, we also need to see caves from a temporal perspective. From a geological viewpoint, caves have evolved in many ways depending upon their geology, location, and climate. Logically we can expect that organisms living in them have co-evolved. Unfortunately, and unlike the epigean environment, we lack a meaningful fossil record that can give us an idea of how those changes occur beyond the most recent ice ages. The closest we have come to it is the case of *Paleozercon cavernicolous*, a species of mite known only from specimens embedded in calcium deposits of a stalagmite near a cave entrance [69]; yet that does not mean that these organisms lived exclusively in caves.

However, there is more than the eye of a casual observer can see when it comes to structures associated with caves. Campbell et al. [70] proposed to view caves as an example of an ecological dendritic network. They defined dendritic networks as those spatial environments in which both the branches and the nodes serve as habitat and where the specific spatial arrangement and hierarchical organization of these elements interact with a species way of moving and distributing, which, in turn, will affect their abundance and community interactions. Since most caves do show a certain geometric similarity with this type of structure, their approach seems reasonable. Furthermore, they proposed that one of the reasons for the high rate of endemism in cave biota is precisely these habitats' spatial organization.

This further supports the argument that there is structure in the hypogean ecosystem.

2.5. Diversity and Energy Flows

As defined by the number of species per locality/area, the concept of diversity is related to the notion of ecosystem structure. Yet, it is not the same thing. In avoiding typological thinking, we need to look closer at the idea of ecotypes since several studies have shown that when using the biological species concept, some cave populations show a high level of genetic similarities to their epigean ancestor despite drastic phenotypic differences [13], (pp. 132–139).

The other important consideration is that since a portion of the energy is lost every time one organism passes its energy (information in cybernetic terms) to another, the more complex the ecosystem, the less efficient it is in preserving it. That is particularly important in the hypogean ecosystem, usually described in temperate areas as energetically poor [24].

This brings us to the Ashby's Law of Requisite Variety. This law was initially devised by the English psychiatrist and cybernetic pioneer W. Ross Ashby [71]. He postulated that the more variable the operating environment, the more variable a system must be. In other words, a system will fail unless its variability matches or surpasses that of its environment. Imagine that you are trying to keep in balance a broom from its top on the tip of one of your fingers. You know that you have to move your hand regularly to keep up with the highly unpredictable movement of the broom to keep it in equilibrium. Otherwise, the broom would fall.

An ecosystem has to have the ability to respond to fluctuations if it is to maintain its essential characteristics in the long term. For ecosystems such as hypogean ones, this is a complicated issue because, in general, particularly in temperate climates, they tend to be less complex and, therefore, less plastic when it comes to the system's adaptive nature. That also means that the ecosystem elements must show enough plasticity to make the system itself more plastic if it were to survive fluctuations. That brings us to the evolutionary consequences of adapting to the conditions of the hypogean ecosystem.

Romero [13], (pp. 150–156) provided empirical data and reasoning to support the idea that the phenotypic characters among many hypogean organisms are achieved via phenotypic plasticity. Moreover, that genetic rearrangement that carries such changes is triggered by parameters in the hypogean ecosystem's physical environment, such as lack of light and flood events. This, in turn, supports the idea that the characters (or lack thereof) that we usually associate with the hypogean environment result from natural selection. Thus, phenotypic plasticity often provides a reproductive advantage over a genetically fixed phenotype because environmentally induced phenotypes have a higher probability of conforming to prevailing environmental conditions than genetically fixed ones [72].

The convergent nature of troglomorphic characters further supports this explanation. Convergent evolutionary patterns are strong evidence of adaptation via natural selection [73].

Plasticity can (and should) be maintained in fluctuating environments, especially when fluctuations in the environment are predictable to some extent as exemplified in the case of the Cumaca cave mentioned above. In another longstanding generalization about the cave environment, there is the belief that caves are so constant that no ecological fluctuations occur. However, this view has been challenged for some time. Hawes [74], for example, was the first to provide specific examples of flooding being a periodic event in caves leading toward fluctuations in their ecological conditions. He showed how, despite other factors such as temperature and lack of light being constant, periodic floods provide conditions of a fluctuating environment and play a role in colonization events.

Thus, it is not surprising that the cave organisms for which phenotypic plasticity has been demonstrated are all aquatic: Crayfish, fishes, and salamanders. Fluctuating environmental conditions are the case in tropical caves where there are constant (but predictable) fluctuations in water level due to drastic seasonal changes in rainfall regimes [13], (p. 156).

Finally, when it comes to energy flows, we must look at the concept of biomass. When dealing with the biomass concept for hypogean ecosystems, we also need to take a broader view. Biomass is usually defined in ecology textbooks as the total mass of organisms in a given area or volume. However, as we saw throughout this article, one major component of the hypogean ecosystem can be the droppings from organisms such as bats and birds that reside in caves. If we were to apply the above definition of biomass, we would be excluding the energetic contribution of those droppings to the entire ecosystem. This will be shortsighted. Therefore, for the definition of biomass, we need to include these droppings as organic material consumed and the process by living organisms that end up being utilized by other living organisms. Only then we can include them as information in cybernetic terms.

3. Conclusions

All the data and concepts presented here show a remarkable similarity between hypogean ecosystems and cybernetic systems. The question is, what do we have to gain from applying these cybernetic structures to a hypogean ecosystem?

First, a cybernetic approach can help us systematize hypogean ecosystems, which, in turn, will make us understand better how they work, especially when it comes to the interplay between different biotic and abiotic factors.

Second, this cybernetic characterization will allow us to recognize better—and even quantify—the diversity in ecosystem structures worldwide, as well as when it comes to different latitudes and the biome with which hypogean environment interacts.

Third, a cybernetic approach will help us open up more to understand the interaction between hypogean ecosystems and their surrounding environment to see them as open instead of a closed ecosystem.

Although Margalef [6], (p. 24), was ambivalent about the immediate descriptive application of cybernetic approaches to the understanding of ecosystems, and that we still have some holes to fill in our biospeleology knowledge, he reasoned that because (a) they tend to be too complex and (b) we are far from understanding all of its variables, I would argue that the hypogean ones are an excellent laboratory for such endeavor. First, they tend to be less complex than, for example, a tropical rain forest or a coral reef, and, second, the smaller the cave, the easier it would be to understand how they operate from a cybernetic viewpoint. Moreover, with the use of artificial intelligence that can generate models of fluctuations to visualize how they operate under different circumstances, that could be an excellent tool for conservation purposes. The reason for that is that we can better represent the role that different organisms play in the cave community as an instrument to convince the proper authorities of the importance of preserving each element of that community [13], (p. 182). Tesler's Theorem, as Douglas Hofstadter [75] puts it, goes as follows "Artificial Intelligence is whatever hasn't been done yet".

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diversity

Article

Diversity of Olfactory Responses and Skills in *Astyanax Mexicanus* Cavefish Populations Inhabiting different Caves

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Abstract: Animals in many phyla are adapted to and thrive in the constant darkness of subterranean environments. To do so, cave animals have presumably evolved mechano- and chemosensory compensations to the loss of vision, as is the case for the blind characiform cavefish, *Astyanax mexicanus*. Here, we systematically assessed the olfactory capacities of cavefish and surface fish of this species in the lab as well as in the wild, in five different caves in northeastern Mexico, using an olfactory setup specially developed to test and record olfactory responses during fieldwork. Overall cavefish showed lower (i.e., better) olfactory detection thresholds than surface fish. However, wild adult cavefish from the Pachón, Sabinos, Tinaja, Chica and Subterráneo caves showed highly variable responses to the three different odorant molecules they were exposed to. Pachón and Subterráneo cavefish showed the highest olfactory capacities, and Chica cavefish showed no response to the odors presented. We discuss these data with regard to the environmental conditions in which these different cavefish populations live. Our experiments in natural settings document the diversity of cave environments inhabited by a single species of cavefish, *A. mexicanus*, and highlight the complexity of the plastic and genetic mechanisms that underlie cave adaptation.

Keywords: fieldwork; wild fish; comparative biology; behavior; troglomorphism; olfactory test; infrared movies; amino acids; chondroitin; plasticity

1. Introduction

A very broad diversity of fauna (micro-organisms, insects, vertebrates) lives in underground environments in a more or less permanent manner. Among various niches in the subterranean milieu, caves are emblematic and attractive to human exploration. Species living there permanently display striking phenotypic convergences in their morphology, physiology, or behaviors, with the hallmarks of troglomorphism being the loss of eyes and pigmentation [1]. Caves are often considered as an extreme environment. In the absence of photoautotrophic production, the quantity of food available is limited or irregular, the space available is finite, and reproduction seems difficult. Finding food and mates in the absence of vision are the two main challenges faced by cave animals and must limit cave colonization and survival. The evolutionary forces at play during cave adaptation and the respective contributions of natural selection and genetic drift, along with the evolutionary mechanisms, are still a matter of debate [2]. Biologists currently aim at disentangling the roles of genetic mutations and phenotypic plasticity, or epigenetics, in the process. Finally, the observation that some species or lineages have repeatedly adapted to the cave environment while some others never did may support the questioned idea of "pre-adaptive traits" that might favor adaptation to permanent darkness (e.g., [3,4]).

During evolution, most epigean representatives of species that became cave-adapted have become extinct, leaving the underground lineages the only representatives of their taxon, which hampers comparative or genetic studies. The teleost fish *Astyanax mexicanus* is one of the few exceptions to this rule [5,6]. Therefore, the surface-dwelling and cave-dwelling morphs of this species are increasingly used in evolutionary studies to address the developmental, genetic, or genomic mechanisms of morphological evolution and behavioral adaptation [2,7–9]. The surface form (SF) lives in the rivers of the southern United States and Central America, while the blind and depigmented cave form (CF) is endemic to caves in a karst region located in the states of San Luis Potosi and Tamaulipas in Mexico. There, 30 identified caves host *Astyanax mexicanus* cavefish populations. They are distributed into three geographically distant groups located, respectively, in the Sierra de El Abra, the Sierra de Guatemala, and the Sierra Colmena [5,10,11]. All populations of cavefish and surface fish are interfertile, indicating that they are conspecific [12,13]. In nature, the hybridization phenomenon has also been observed and documented [14]. In addition, crosses between geographically distant populations of cavefish can lead to eyed F1 offspring [13], indicating that different mutations are involved in ocular regression in different cave populations and suggesting that some of these populations have evolved independently. However, the evolutionary history of *A. mexicanus* cave populations is still poorly understood because of the geographic dispersion of caves, the lack of knowledge on the underground aquifer network, and the possibility of surface fish introgressions into caves as well as cavefish migrations between caves using underground flows. Recent studies have indicated that initial cave colonization by *A. mexicanus* surface-like ancestors occurred very recently, less than 20,000 years ago [15,16], prompting evolutionary biologists to revise some views about the (rapid) mechanisms of cave adaptation. Like most cave-adapted animals, *A. mexicanus* cavefish present sensory specializations to life without vision. The brains and sensory systems of surface fish and cavefish differ (reviewed by the authors of [7]), along with their sensory systems: Cavefish have more taste buds [17], more neuromasts [18], and larger olfactory epithelia [14,19,20]. From a behavioral point of view, these mechano- and chemosensory specializations are associated with vibratory attraction behavior to locate moving objects [18] and to an excellent sense of smell to detect low concentrations of food-related odors [20], respectively.

Most of the results described above were obtained in the laboratory, often on one or two lab-raised cave population(s). It is therefore important to extend the studies to other populations, and to validate the results on wild animals in order to avoid possible misinterpretations. Going to the field to observe the natural environment of fish, taking samples, and filming behaviors can help answer questions or revise preconceived ideas [21,22]. For instance, it is often stated that caves are a food-poor environment. However, analysis of stomach contents of wild individuals from the Pachón cave has shown that juveniles feed on small arthropods, and adults on decaying materials and bat guano. Overall and contrary to common belief, Pachón cavefish seemed relatively well fed [23]. Another study revealed that growth curves and age/size relationship are comparable in wild surface fish and wild cavefish, again indicating that cave environment is probably not as food-poor as it may seem [24]. In fact, depending on the location of the cave, its topography, and the hydraulic regime, the amounts of carbon flux can sometimes be of the same order as those reported for surface rivers [1] and, most importantly, the carbon content in the mud sampled from different *Astyanax* caves can show up to three-fold variation [24]. Energy sources can come from percolating water, animals entering caves and depositing their excrement or dead bodies, or rivers that overflow during the rainy season and carry organic matter.

These energy sources are both spatially and temporally variable [1]. During several cave expeditions, our team noticed the diversity of local environments between caves—and also between pools within a single cave—reinforcing the idea that field comparisons on fish biology between different caves can be as interesting as the comparison between cavefish and surface fish.

During a field trip in 2013, we carried out, for the first time, experiments of olfactory behavior in situ, in the Subterráneo cave [14]. We had the idea to use a small, light, compact, inflatable children's plastic pool, which was easy to bring on the field. With a rudimentary perfusion system and an infrared camera, we showed that only fish with eyeless phenotype and large olfactory epithelia swam toward an odor source consisting of a food extract. Thanks to this experience and after visits to many other caves, we set up a more complex behavioral experiment, with the aim of systematically assessing olfactory skills and responses of *A. mexicanus* cavefishes inhabiting different Mexican caves. During expeditions in 2016, 2017, and 2019, we performed olfactory tests in the Pachón, Sabinos, Tinaja, Chica, and Subterraneo caves. We found that wild adult cavefish from these five caves showed very variable responses to the three different odorant molecules they were exposed to, with Pachón and Subterraneo cavefish showing the highest olfactory capacities, and Chica cavefish showing no olfactory responses to the odors presented. We discuss these data with regard to the environmental conditions in which these different cavefish populations live.

2. Materials and Methods

2.1. Field Experimentation in Five Different Caves: Constraints and Criteria for Choice

Caves chosen to carry out olfaction experiments had to fulfill several criteria: (1) Reasonably easy access and climbing challenges, as the total weight of experimental equipment was approximately 25 kg carried in backpacks and each location had to be visited twice on two consecutive days. (2) Sufficient space inside the cave to install three experimental plastic pools near the water. Thanks to our field experience, we excluded some caves. For example, Curva's ceiling is too low, Chiquitita's entrance is too narrow, as it is located in a big tree's root [10], Toro is a fault in the rock, and Molino and other Guatemala caves are too challenging in terms of climbing ([11] and team observations). (3) Caves already known and visited by the team in the past were preferred to plan the precise place where to install plastic pools and to anticipate troubleshooting. (4) Good representative sampling of diverse local environments (e.g., rocky and muddy caves). (5) Good representative sampling of diverse cavefish population histories (e.g., with or without surface gene flow, or mountain range in which the cave is located). (6) Possibility to compare with our lab studies performed on Pachón cavefish.

Consequently, we decided to perform experiments in four caves: Pachón and Sabinos (fully troglomorphic fish morphotypes in muddy caves), Chica (introgressed fish population in "dirty" cave), and Subterráneo (introgressed population in rocky cave). We also performed some preliminary experiments in Tinaja (fully troglomorphic fish morphotypes in rocky or muddy cave ponds).

2.2. Cavefishes in the Wild

Olfactory behavior tests were carried out during three field trips to San Luis Potosi and Tamaulipas States, Mexico, in March 2016, March 2017, and March 2019, in five cave localities. Fieldwork Mexican permits 02438/16, 05389/17 and 1893/19 (to SR and Patricia Ornelas-Garcia) were delivered by the Secretaria de Medio Ambiente y Recursos Naturales. The history of the discovery and precise descriptions of *Astyanax* caves are given by the authors of [5,11].

The Pachón cave is located in altitude near the village of Praxedis Guerrero (22°37′ N latitude and 99°01′ W longitude, about 16 km SW of Ciudad Mante), in the north Sierra de El Abra, and is easy to access [11]. The cave is small, and the water is stagnant on a muddy bottom. Fish from the Pachón cave (named here CF-Pachón) present a fully troglomorphic type.

The Sabinos cave is located near the village of El Sabino, in the central Sierra de El Abra (22°06′ N latitude and 89°56′ W longitude, about 13 km NNE of Ciudad Valles) [11]. Villagers installed a padlock grid to exploit this cave and the access is chargeable. The entrance is majestic and the succession of two pits involves bringing harnesses and ropes to abseil down. Fish from the Sabinos cave (CF-Sabinos) are also fully troglomorphic.

The Chica cave is located at the south Sierra de El Abra, on the property of a farmer, at about 21°52′ N latitude and 89°56′ W longitude, near the village of El Pujal. It is easy to access, and the entrance and the first cavity are wide. Fish from the Chica cave (CF-Chica) are phenotypically diverse because of surface fish introgression and hybridization. We worked on cavefish from the Chica superficial pool, which are the most troglomorphic/least introgressed among the three natural pools of this cave.

The Subterráneo cave is located in the Micos region, in the Sierra de Colmena (22°03′ N latitude and 99°14′ W longitude, about 10 km SSW of Micos). Access to this cave is not difficult and the entrance is easy, at the level of the polje or sugar cane field. Fish from the Subterráneo cave (CF-SubT) also occasionally hybridize with surface fish. Hence, they show variable levels of eye regression and pigmentation.

The Tinaja cave is very close to the Sabinos cave, also located in the territory of El Sabino (entrance is free). The cave is located at 22°05′ N latitude and 89°57′ W longitude, about 10.5 km NE of Ciudad Valles on the Rancho de La Tinaja. Access is via a sugar cane field and permission must be obtained from the owner before crossing it. The cave entrance is accessible after a 2-h hike through a thorny tree forest and a dry canyon covered with jungle. According to Eliott, 2016 [6], the underground hydraulic systems of Tinaja, Sabinos and Sótano de Soyate caves are connected. It has been suggested indeed that the cavefish populations of Tinaja and Sabinos are genetically close [25,26]. The cave entrance is majestic. Climbing equipment is not needed but hiking is difficult due to a very slippery mud covering a stony soil. The air is charged with CO_2 and renders physical effort somewhat difficult. Fish from the Tinaja cave (CF-Tinaja) show a full cave morphotype.

2.3. Fishes from the Lab Facility

Laboratory *Astyanax mexicanus* surface fish (origin: San Salomon spring, Reeves County, TX, USA) and cavefish (Pachón population) were obtained in 2004 from the Jeffery laboratory at the University of Maryland, College Park, Prince George's County, MD, USA. Here, we also used F1 hybrids, which were the progeny of a cross between a Pachón female and a surface fish male. Colonies were maintained at 22 °C (cavefish and F1 hybrids) or 26 °C (surface fish) on a 12:12 h light:dark cycle. In the present paper, lab-raised fish are named Lab-Pachón, Lab-SF, and Lab-Hyb, respectively. SR's authorization for use of *Astyanax mexicanus* in research is 91–116 and the Paris Centre-Sud Ethic Committee protocol authorization number related to this work is 2017-04#~8545. The animal facility of the Institute received authorization 91272105 from the Veterinary Services of Essonne, France, in 2015.

2.4. Sampling and Photography

Wild and lab fish were caught with a net (hand net or seine). In order to record their phenotypes after the behavioral tests, wild cavefish were photographed individually in a small aquarium or a plastic support with a graduated ruler and immediately returned to their pond of origin. Total body lengths were measured from these pictures using the ImageJ software. In the Pachón and Sabinos caves, we also weighed the fish using a portable balance. Taring of the balance was carried out with a glass of water, and each fish was weighed inside the glass of water.

2.5. Odor Choice

The general principle of the experiment was to place eight fish in an experimental square plastic pool, and to successively perfuse three odorant compounds, each in a different corner of the pool (Figure 1). Each odor perfusion was preceded and followed by a perfusion of water, and a water counterflow flowed permanently from the corner opposite to the perfusion of odor. The odors used were L-alanine and L-serine amino acids, and chondroitin (all from Sigma). They correspond to degradation products of organic compounds assimilated to food odors and serve as attractants to fish.

The rationale for choosing alanine and serine as odorant molecules were: (i) To compare them with the results already obtained in the laboratory [19,20]); (ii) due to the fact that attraction and food-searching behavior are easier to identify than repulsion behavior; (iii) because finding food is a matter of survival for these fish and the sense of smell probably plays a major role in this quest. Indeed, previous experiments performed in the lab had shown that responses to alanine and serine are olfactory-mediated in *Astyanax* larvae [20].

We chose working concentrations of 10^{-7} M for alanine and serine because it is intermediate between the detection threshold for larvae of surface fish (10^{-5} M) and Pachón cavefish (10^{-10} M) in the lab. We also reasoned that it would be "risky" to use a very low concentration close to the cavefish detection threshold in noncontrolled cave/field experimental conditions (in particular, water cleanliness, more or less charged with natural odors).

As chondroitin induces freezing in zebrafish [27], we tested this molecule in the lab assuming that *A. mexicanus* would also adopt a freezing behavior [28,29]. Surprisingly, both surface fish and Pachón cavefish instead showed a pronounced and persisting foraging behavior (see Video S1). Preliminary tests in the lab showed that Pachón cavefish have a chondroitin detection threshold at 10^{-4} M.

2.6. Setup Design

With the conditions for setting up and carrying out the experiments in situ being difficult, the setup was meant to be light, easy to assemble and dismantle, and not too bulky. Plastic pools (Intex, 85 cm × 85 cm × 23 cm; thoroughly rinsed several times in the lab before use to eliminate inorganic volatile odors) were inflated and placed on the ground after adjusting for horizontality with mud or rocks if necessary. They were filled with 40 L of local cave pond water where cavefish swim. Before pouring into the plastic pools, water was filtered on a coffee filter paper to remove large suspended particles and to ensure cleanliness and good video quality.

A tripod was placed near the corner #1 of the plastic pool. Two infrared lamps (IR Torch 850 nm, (Maketheone, LA, USA) and the syringe holder were attached to the tripod at a height of 110 cm. The pool was enlightened from above to reduce shadows and filmed from the top to avoid blind spots. For the 2016–2017 campaigns, we built our own infrared camera using a Raspberry pi 3 model B and a Pi-NoIR V2.1 camera. We used a 5-V power bank to supply electricity to this camera, which was controlled from a computer connected in ethernet via a SSH connection. Using Python 2.7.9 and the PiCamera V1.12 package, we wrote two scripts (available at https://github.com/julienfumey/PiCaveRecord) to frame on the pool and to record the video. Videos were converted from h264 to mp4 with VLC software [30]. In 2019, to avoid having to use a computer in caves and to save time, we opted for an infrared hunting camera (nature camera Full HD WK-590, VisorTech, Nairobi, Kenya) which records films in AVI format.

For each plastic pool, we constructed a set of four tubing lines consisting of four 50-mL syringes connected to a 180-cm-long medical solution administration tubing (Infusion device, Intrafix® SafeSet; B. Braun; inside volume of 20 mL) and terminated by a 0.6-mm-diameter needle (protected by a plastic cap to avoid wounding the fish). The opening of the perfusion was controlled by a Luer stopper and the perfusion rate was regulated by the needle. The end of the tubing was attached to a metal guide (which did not touch the water) to hold the needle in the corner of the plastic pool. Each perfusion line was guided to its respective corner.

A set of three pools was installed in the visited cave, with eight cavefish in each ($n = 24$ total). The installation of three experimental setups was completed in ~4 h by four people. Fish were left for a 20-h habituation period (from approximately 5 p.m. day 1 to 1 p.m. day 2). The next day, behavioral tests were carried out in the dark (IR recordings) and in silence, in parallel, by three experimenters.

2.7. Procedure

Chemicals were weighed in the laboratory on the day before field trip departure. In a hermetically closed 50-mL tube, 10 mg of L-alanine (CAS 56-41-7, Sigma-Aldrich, Saint-Quentin Fallavier, France), 10 mg of l-serine (CAS 56-45-1, Sigma-Aldrich), and 5 mg or 110 mg of chondroitin (CAS 9082-07-9, Sigma-Aldrich) were each placed (a series of three tubes of powder per cave were thus prepared in advance). Solutions were prepared extemporaneously by adding 50 mL of filtered cave water to each tube. Chondroitin concentration was 10^{-4} M and 10^{-3} M, respectively. For alanine and serine working solutions, a second dilution (3 µL in 50 mL) was prepared to obtain 10^{-7} M solutions. For experiments performed in the lab with surface fish, we prepared a different dilution (300 µL in 50 mL) to obtain 10^{-5} M alanine or serine solutions.

The experimenter seated near the pool took care not to move and not to speak during the whole experiment. To start the experiment, filtered water (=control) was perfused from tubing #1 and #3 (i.e., opposite corners), and then from tubing #2 and #4 (i.e., opposite corners), for 6 min each. The aim of this step was to accustom fish to flow and possible vibrations of perfusions and to reduce subsequent nonspecific responses. Indeed, thanks to their lateral line, fish perceive and are attracted by vibrations [18].

Then, water was perfused for 6 min from tubing #1 and #3. Solution flow from the two syringes was initiated simultaneously. When syringes emptied, 50 mL of alanine solution was added to syringe #1 and water was added again for counter-flow in syringe #3 (Figure 1, left panel, orange for alanine). After completion of alanine perfusion, the test continued with a new water perfusion from corners #1 and #3. Experimenters took care to ascertain a continuous flow by filling syringes with water or odor solutions before they were completely empty to avoid the introduction of air bubbles into the system.

The same principle was then applied for the perfusion of water, serine or water, and water at corners #2 and #4 (Figure 1, middle panel, green for serine). Finally, water, chondroitin or water, and water, were perfused at corners #3 and #1 (Figure 1, right panel, blue for chondroitin).

With the exact same setup and procedure, we performed series of experiments in the lab, in a dark and soundproof room, using animals from our breeding facility.

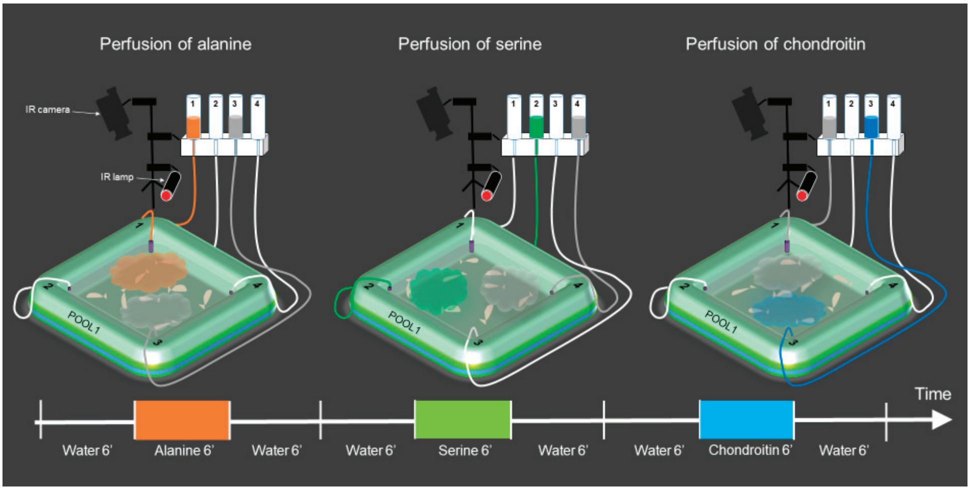


Figure 1. Establishment of a behavioral setup and protocol to test cavefish olfaction in the field. The setup allows testing olfactory responses of eight adult fish in inflatable plastic pools in the dark under infrared recordings. Three different odors were perfused sequentially by gravity flow at different corners of the plastic pool, according to the indicated timeline. See Methods and the first paragraph of Results for details.

2.8. Video Scoring

A total of ~60 h of infrared videos was recorded. We first attempted to analyze them with an automatic multiple tracking video software [31], which unfortunately did not detect the fish correctly.

The reasons were probably numerous: There were eight fish in the pool, the film was in infrared, the contrast of fish on white background was not strong enough, the lamps sometimes produced lighting reflections on the water surface, and the pool edges generated blind spots. Thus, we could not satisfactorily extract the time spent by each fish in each zone and turned to manual scoring.

We first established that, after perfusion of 50 mL of blue-colored water (with a counterflow), the blue color occupied roughly half of the arena, i.e., a triangle formed by the corner from which the perfusion arrived and the diagonal of the plastic pool (Figure S1A). We therefore considered this half of the pool as the "odorant area" and its opposite half as the "water/control area". Videos were tracked manually through frame-by-frame analysis using Windows Media Player. The number of fish present in each half of the pool was counted every 15 s during the whole experiment. The odor Preference Index (PI) was calculated for each odor using the formula: (Fish count (odor area) − Fish count (water area)/Total fish count (Figure S1B). When all fish were in the odorant area, PI = 1 (suggesting attraction effect); when all fish were in the opposite corner, PI = −1 (suggesting repulsion effect); and when fish were distributed evenly/randomly in the two parts of the pool, PI = 0 (suggesting no effect).

2.9. Data Analyses and Statistics

When the odorous solution was poured into the syringe, 20 mL (=dead volume contained inside the 180 cm long tubing) of water flew out before the actual odorant solution entered the pool. For each perfusion, we calculated the speed of the flow to determine odor (or water) perfusion duration. Although tubing were the same length and volume perfused by gravity flow was always 50 mL, the perfusion duration (theoretically 6 min) was appreciably variable both between the four perfusion lines, between the successive water/odor/water perfusions, and between the three plastic pools in a given cave. To overcome this problem, we normalized time and calculated PI means over periods corresponding to 25% of the total perfusion time for each sequence. Thus, we obtained four PI means for each perfusion duration. To determine the statistical significance of the behavioral responses, we used Friedman nonparametric test by ranks for repeated measures in order to compare PI variations along time, during water perfusion before odor, during odor perfusion, and during water perfusion after odor. We also used Wilcoxon Mann-Whitney tests to determine the statistical significance of each PI distribution against PI = 0 (no effect) for each time segment.

Fish sizes and weights were also compared using a nonparametric Wilcoxon–Mann–Whitney test.

Statistical analyses were performed using R 3.6.1 [32] in Rstudio environment [33] with rstatix package, version 0.5.0 [34]. Plots were generated using ggplot2 package [35] from tidyverse open-source R packages [36].

3. Results

3.1. Methodological Considerations: Testing Olfactory Responses in the Lab versus in the Wild

Our previous analyses of olfactory skills and behaviors in *A. mexicanus* were mostly performed on larvae, in small 9- × 13-cm U-shaped "olfaction boxes" containing 150 mL of water, and under laboratory-controlled conditions and standards [19,20]. We had also performed a preliminary experiment on adult fish in the Subterráneo cave but the insights were limited because, among other difficulties, a single "odor" consisting of crushed food pellets was tested as odorant cue [14]. Here, to reach our goal of testing several relevant odors on adult cavefishes inhabiting several caves, we developed a novel setup and a novel experimental procedure adapted to the field.

First, we carried out laboratory experiments to establish and validate the experimental setup (Figure 1 and see Methods) and to study adult fish olfactory behavioral responses. We reasoned that such results would also help us interpreting data obtained in caves. Cavefish from our animal facility originated from the Pachón cave but have been raised under markedly different conditions (food, light, water quality) for several generations [37], and they might have been affected by captivity and environment.

Moreover, laboratory experiments were the only option we had to study surface fish olfactory responses. In our experience, it has been impossible to test wild surface fish in the field. Besides the difficulty and time needed for catching wild fish in rivers, they are highly sensitive to manipulations and stress and do not behave "normally," displaying most signs of their stress repertoire [25]. In addition, due to daylight, setting up an experimental test near the river is complex, with a risk of predation on fish by wild animals during the habituation period or destruction/stealing of the equipment.

Finally, we also carried out tests on lab-generated F1 Hybrids. We previously showed that F1 Hybrids larvae have a relatively poor odor detection threshold, similar or even below surface fish skills [19]. In the Subterráneo and Chica caves, wild fish present a wide variety of intermediate hybrid-like phenotypes. It was therefore interesting to perform tests on adult F1 Hybrids in the lab to compare with result obtained on larvae and on wild cavefish populations where hybridization occurs.

3.2. Responses to Odors in Laboratory-Raised *A. mexicanus*

The results of laboratory experiments are presented in Figure 2 (n = 8 plastic pools averaged for each graph, hence 64 fish were tested for each morphotype).

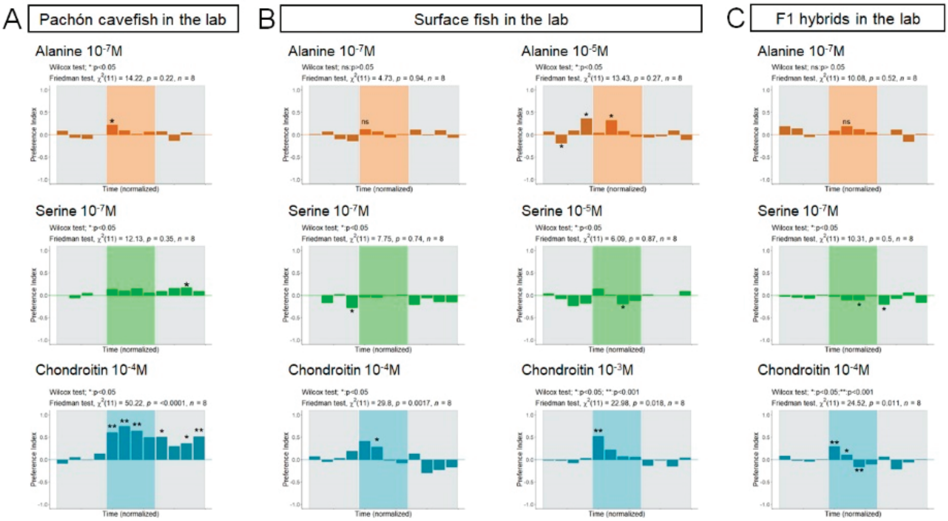


Figure 2. Testing olfactory skills in the laboratory; Olfactory responses of Pachón cavefish (**A**), Surface fish (**B**) and F1 hybrids (**C**) to the indicated odors at the indicated concentrations using the described olfactory setup in laboratory conditions (n = 8 for each); The preference index (PI; positive values suggest attraction) is shown as a function of time. Time intervals corresponding to odor perfusion are shaded. Asterisks on graphs indicate significance as compared to no response (i.e., PI = zero) for a given time interval (Wilcoxon Mann-Whitney test). The results of Wilcoxon and Friedman tests to probe the significance of the response across time with repeated measures are also indicated.

In agreement with previous experiments on larvae, only Lab-Pachón showed a positive attractive response to the low concentration of alanine 10^{-7} M (Figure 2A, orange). A modest but significant response was observed during the first quarter of alanine perfusion time (PI = 0.22; Wilcoxon test, p = 0.008; but Friedman test, NS), suggesting that Lab-Pachón detected the odor as soon as it arrived in the arena but were not attracted for more than a few minutes. On the other hand, Lab-SF did not respond to alanine 10^{-7} M but were significantly attracted by the higher concentration of 10^{-5} M alanine during the second quarter of perfusion time (Figure 2B, orange; PI = 0.32; Wilcoxon test, p = 0.008; but Friedman test, NS). These data suggest that, like larvae, adult Lab-Pachón have a better olfactory detection threshold than Lab-SF for alanine. Finally, Lab-Hyb did not respond to the low (10^{-7} M) alanine concentration, and were not further tested for a higher concentration (Figure 2C, orange).

Serine elicited very little, if any, response on surface and cave adult *Astyanax* (Figure 2A–C, green; Wilcoxon tests and Friedman tests, all NS). Of note, Lab-Pachón were present in the odor perfusion zone in a delayed manner, i.e., when water was subsequently perfused from that corner (PI = 0.16; Wilcoxon test, $p = 0.008$), suggesting that the response to serine may have different kinetics compared to the response to alanine. Surprisingly, transiently negative PIs were observed for both Lab-SF (10^{-5} M serine, PI = −0.2; Wilcoxon test, $p = 0.008$) and Lab-Hyb (10^{-7} M serine, PI = −0.07 and −0.03; Wilcoxon test, $p = 0.008$ and 0.02), which was an unexpected result (see Discussion).

Chondroitin provoked strong positive responses in all lab-raised fish, as shown by statistical significance with both Wilcoxon tests and Friedman tests (Figure 2A–C, blue). Lab-Pachón showed intense response to 10^{-4} M chondroitin and were present in the perfusion zone as soon as the odor arrived, with very high PIs (PI = 0.6/0.7/0.6/0.5; Wilcoxon test, $p = 0.0004$ at all times). The attraction was persistent, since fish remained in the odor zone during the water perfusion that followed. Lab-SF showed a comparatively more modest response to 10^{-4} M chondroitin (PI = 0.27; Wilcoxon test, $p = 0.008$), but a strong attraction for the higher chondroitin concentration of 10^{-3} M (PI = 0.52; Wilcoxon test, $p = 0.0004$). Finally, the Lab-Hyb were present in the odor zone during the first half of the chondroitin perfusion (PI = 0.29 and 0.1; Wilcoxon test, $p = 0.0004$ and 0.008) but then seemed to avoid the area (PI = −0.17; Wilcoxon test, $p = 0.0004$). Thus, all morphotypes/genotypes were attracted by chondroitin, but persistence and intensity of the response was particularly spectacular with Lab-Pachón (Video S1). Overall, these laboratory experiments showed that our setup allowed us to measure olfactory responses on adult *A. mexicanus* in a reliable manner.

3.3. Responses to Odors in Caves, in Wild A. mexicanus Cavefish Populations

3.3.1. Pachón Cave

Despite the thick mud, it was easy to install three plastic pools near the natural main pool (Figure 4B). CF-Pachón were of relatively small size (mean: 4.6 cm) compared to other caves studied (Figure 3A, purple) and they were all eyeless and depigmented (Figure 3D). Fish used for olfaction experiments came from the small lateral pool in 2017 and from the main pool in 2019. These two pools communicate with each other when the water level is high and the fish can swim and mix between these two pieces of water. There was no difference in size (or phenotype) between the fish tested during the two expeditions (Figure 3B, purple), suggesting that the local conditions have been stable across the years in the cave. The fauna encountered there and the feeding habits of the CF-Pachón have been described by Espinasa et al., 2017 [23].

Despite fair conditions for installation of the olfactory setup, CF-Pachón from two experimental pools out of the six recorded presented an obvious place preference behavior (one in 2017 and one in 2019), for unknown reasons (Figure 5). These data were thus discarded from the analyses shown in Figure 6A. Contrary to Lab-Pachón, CF-Pachón did not respond to alanine 10^{-7} M. Even more so, the preference index reached negative values during the perfusion. Conversely, CF-Pachón were strongly and significantly attracted by serine 10^{-7} M (PI = 0.41) and by chondroitin 10^{-4} M (PI = 0.55), as shown both by Friedman and Wilcoxon statistical tests (Figure 6A, green and blue). Moreover, the type of behavioral response elicited by chondroitin in the field was similar to the food-seeking behavior recorded in the lab (Video S2). Although slightly surprising because the alanine and serine responses seem divergent between the Lab-Pachón and the CF-Pachón, these data confirm, in the field, that CF-Pachón have excellent olfactory detection skills.

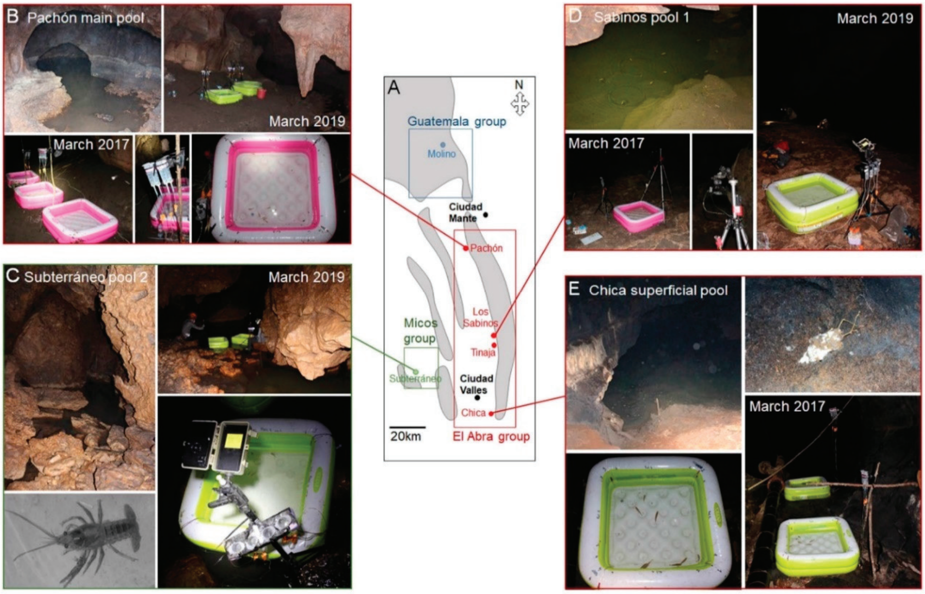


Figure 3. Cavefish sizes and phenotypes in different caves; (**A**) Sizes (left graph) and body mass indexes (BMI, right graph) of the cavefish individuals tested for olfactory responses in the Pachón (purple), Sabinos (brown), Subterráneo (red), and Chica (grey) caves. Asterisks indicate significant differences (Mann–Whitney test); (**B**) Comparison of sizes of the cavefish tested for olfactory responses in different field campaigns, in 2017 and 2019, showing that the condition of the fish tested did not vary; (**C**) Comparison of sizes of cavefish in different water ponds in a same cave locality, showing that fish condition varied, probably due to local trophic, environmental and/or genetic parameters; (**D**) Fish phenotypes are fully troglomorphic in the Pachón and Sabinos caves, whereas hybrid-type fish with small eyes (arrowheads) and some pigmentation can be seen in Subterraneo (pool 2) and Chica (superficial pool) caves. They result from hybridization with introgressed surface fish. Of note, in Subterraneo, surface fish enter by the cave entrance at the polje level, whereas, in Chica, surface fish enter the cave by the bottom. Hence, in both caves, the most troglomorphic fish are larger.

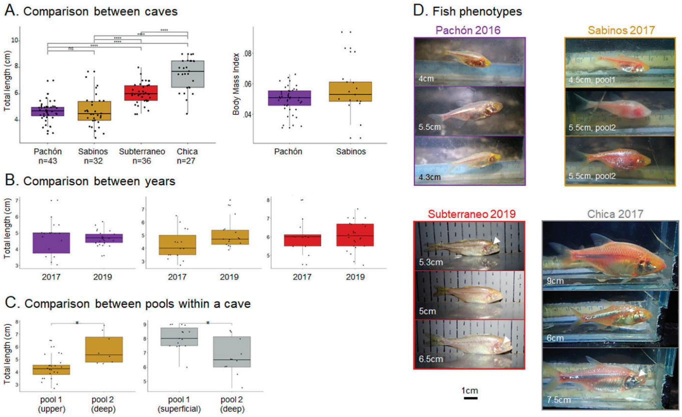


Figure 4. Sampling cavefish olfactory skills in their natural environment; (**A**) Simplified map of the region of Ciudad Valles, Mexico. Mountain ranges are in grey. The locations of visited caves are indicated by colored circles. The color code indicates the geographical group where they belong; (**B**) Fieldwork in the Pachón cave. The main pool was muddy and the water level was low. Note that in March 2017 and March 2019, the three plastic pools were installed and processed in the best possible reproductive manner, on the "beach" along the main pool. Close-ups show the Luer-lock perfusion system and the eight fish installed in one of the experimental plastic pools; (**C**) Work in pool 2 of the Subterráneo cave, located just after the 3-m pit. This pool offers a rocky substrate. There, we repeatedly observed the presence of crayfishes (predators on adult cavefish). The bottom right panel shows the infrared, hunting-type camera, used in our 2019 campaign; (**D**) Work in Los Sabinos cave. Pool 1 of this cave had crystal-clear water. Again, note that olfactory setups were installed in the same place in March 2017 and 2019, respectively, i.e., on a rocky plateau just above pool 1. A close-up shows the Raspberry Pi camera used during our 2017 campaign. (**E**) Testing olfaction on fish from the Chica superficial pool. The top photos show the guano slope leading to the superficial pool, which was very rich in organic material (decomposing bat cadaver). On the bottom left panel, note the large size of the eight fish (compare with the equivalent picture in Pachón, and see Figure 3).

Figure 5. The place preference problem during cave experimentation. Graphs showing the position of the fish along the whole ~1 h protocol for the n = 6 experiments performed in the Pachón cave. The bars represent the preference of fish toward the serine perfusion side (left column) or the chondroitin perfusion side (right column). In theory, the eight fish should swim across the arena and distribute or explore randomly except for the response to the considered odor. However, in some instances (here, plastic pools 2 and 6), it was not the case: The fish remained in the same part of the plastic pool throughout the experiment, showing place preference for unknown reasons. These data were excluded from analysis.

3.3.2. Sabinos Cave

The first fish pool encountered, i.e., the upper pool, lies under a large arch and contains shallow water that disappears under the rock (Figure 4D). Exploration there revealed a large (20–30 m), continuous body of water under a low ceiling, hosting a significant cavefish population. The water was clear with a muddy substrate. Juvenile cavefish (size 1–2 cm) were observed, indicating that reproduction occurs. Large isopods and numerous mysid shrimps were present. Just above this natural pool, a natural rocky stage allowed us to install three plastic setups in the same place in 2017 and 2019 (Figure 4D). From there, a corridor led into a large and relatively low room where a second fish pool, or bottom pool, was encountered. Bats were numerous (observed in 2013, 2017, and 2019). The air was loaded with spores and the soil was covered with patches of microorganism covered with insects. CO_2 was not measured but was probably high.

All fish in the Sabinos cave had a fully troglomorphic phenotype (Figure 3D). Fish used in the experiments were sampled from both the upper and the bottom pools. Like in the Pachón cave, their size and body mass index was modest (Figure 3A, brown; mean: 4.7 cm) and did not vary between 2017 and 2019 samplings (Figure 3B, brown), suggesting that the two experimental series were performed on fish of similar condition. We noted that CF-Sabinos found in the deeper pool were significantly larger (Figure 3C, brown) and more corpulent (Body Mass Index, Wilcoxon test, $p = 0.006$) than those fished in the upper pool, suggesting that they were older [24] and/or better fed. However, this observed difference in condition could not be correlated to their olfactory responses. As in the Pachón cave, we observed a strong place preference bias in two experiments out of six, hence these data were discarded (Figure S2). When tested for olfactory responses, CF-Sabinos were attracted neither by alanine nor by serine (Figure 6B). However, they did spend time in the odorous part of the arena after chondroitin perfusion (PI values between 0.46 and 0.28; Wilcoxon test, $p = 0.02$; but Friedman test, NS), suggesting that they were, although moderately, attracted to this molecule (Figure 6B).

3.3.3. Subterráneo Cave

A 20 min descent in a boulder tunnel filled with organic waste (including of very large size like trees) carted inside during the rainy season leads to a first small fish pool, where almost all individuals are surface-like (*A. mexicanus*, poecilids, some cichlids, presumably washed in during flooding) and where troglomorphic fish are rare (see Simon et al., 2017 [24]). Then, the tunnel continues and leads to a small 5-m pit which descends directly into the natural pool 2, with clear water on a rocky substrate, where CF-SubT swim. All fish used and measured in 2013 [14], 2016, and 2019 came from this room (Figure 4C). The ground of the cave at this level was relatively horizontal but it was covered with pebbles. As there was no mud to flatten the ground, we installed plastic pools on small flat mounds (Figure 4C).

As we previously described [14], CF-SubT presented mixed phenotypes in terms of eye size and pigmentation as a result of hybridization with introgressed surface fish, and most of them were not fully troglomorphic (Figure 3D). We found that CF-SubT were significantly larger than CF-Pachón and CF-Sabinos (Figure 3A, red; mean: 6 cm), suggesting that they may have been overall slightly older [24] and/or in better nutritional condition. In addition, the CF-SubT tested in 2017 and 2019 were of similar sizes (Figure 3B, red). During the olfaction tests, the CF-SubT were present in the 10^{-7} M alanine perfusion area approximately 2 min after the odor arrived and they remained in this zone during the entire perfusion time (PI = 0.25/0.26/0.19; Wilcoxon test, $p = 0.007/0.118/0.025$) (Figure 6D). Conversely, they did not significantly respond to serine. Finally, CF-SubT were attracted by chondroitin (but note that the concentration perfused was higher than in other caves: 10^{-3} M), since they stayed in the odorous area with high preference indexes (PI between 0.48 and 0.33; Wilcoxon test, $p = 0.025/0.007$). Thus, overall, CF-SubT showed significant olfactory skills and responses in their natural settings. Of note, in videos, it was impossible to make any correlation between olfactory responses and the degree of troglomorphism exhibited by individual CF-SubT.

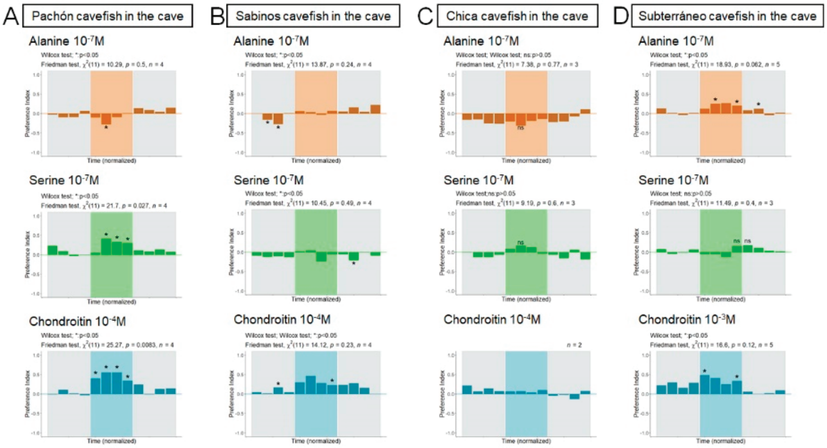


Figure 6. Olfactory responses of cavefishes in natural cave settings; (**A**–**D**) Olfactory responses recorded in the Pachón (**A**), Sabinos (**B**), Chica (**C**), and Subterráneo (**D**) caves; Odors and concentrations are indicated, as well the number of replicates. The preference index (PI; positive values suggest attraction) is shown as a function of time. Time intervals corresponding to odor perfusion are shaded. Asterisks on graphs indicate significance as compared to no response (i.e., PI = zero) for a given time interval (Mann–Whitney test). The results of Wilcoxon and Friedman tests to probe the significance of the response across time with repeated measures are also indicated.

3.3.4. Chica Cave

The Chica superficial pool (or pool 1) hosting cavefishes is a large body of water with a guano slope bank, probably very rich in organic content due to a large bat colony and influx of organic materials from the surface during the rainy season (decaying bats, vegetal debris were observed; Figure 4E). A corridor with a flat rocky floor allowed us to set up experiments just above this superficial pool (Figure 4E). Further down, Chica pool 2 could be reached by following the underground river, which cascaded after a pit [11], and also contained fish that were phenotypically less troglomorphic and more surface-like or hybrid-like than the fish in superficial pool 1 (not shown).

The superficial pool CF-Chica tested were not fully troglomorphic, i.e., some individuals showed some degree of pigmentation and had tiny to small eyes (Figure 3D), probably as a result of hybridization with surface fish. CF-Chica were, by far, the largest and most corpulent fish that we tested among the different caves visited (Figure 3A, grey and Figure 3D). The individuals fished for the olfaction experiments originated from the superficial pool, where the most extreme sizes (mean: 8.1 cm, max: 9 cm) were encountered (compare with CF-Chica from pool 2; mean: 6.8 cm, Figure 3C, grey). In this cave, olfaction experiments were performed once, in 2017. Hence, only $n = 3$ plastic pools were recorded. For the CF-Chica, Friedman tests indicated that the Preference Indexes did not vary between the perfusions of water and amino acids, and the Wilcoxon tests indicated that they were not different from 0 (Figure 6C). A video problem at the end of one recording resulted in $n = 2$ for chondroitin, for which we could not perform statistical tests. However, for chondroitin, the pattern was flat along the whole perfusion sequence, and there was no such behavioral foraging response as visually observed in other caves. These results showed that CF-Chica did not respond to the odors used in our tests, including chondroitin that elicited strong responses in all other cavefishes tested as well as in Lab-SF and Lab-Hyb.

3.3.5. Tinaja Cave

The first, very small, Tinaja "perched pool" was encountered after a 20-min walk and consisted of a small piece of crystal-clear water retained between rocks (Figure 7A). We found fully troglomorphic

CF-Tinaja there (approximately $n = 20$–30) in two consecutive years (2016 and 2017). After another 20 min of hiking down, the tunnel narrows before reaching a fault, at the bottom of which lies Traverse Lake (or Tinaja pool 1) (Figure 7B). A small natural beach allowed for experimental installation, but the space was small and inconvenient (~3 m wide). Drops of water constantly falling from the ceiling generated a very thick and sticky layer of mud. The water was cloudy. In such conditions, it was difficult to install the olfaction setup. This was partly achieved in March 2016, with only $n = 2$ plastic pools and with video recordings performed in the light (Figure 7B). The size of the fish was 6.1 cm on average ($n = 12$ measured). In 2017, we aimed to complete the Tinaja study and we performed $n = 1$ additional olfactory test directly in the small natural "perched pool 1." Although we cannot provide statistical support ($n = 2$ and $n = 1$, respectively), the video analyses suggest that that CF-Tinaja were not attracted by any of the three perfused odors (data not shown).

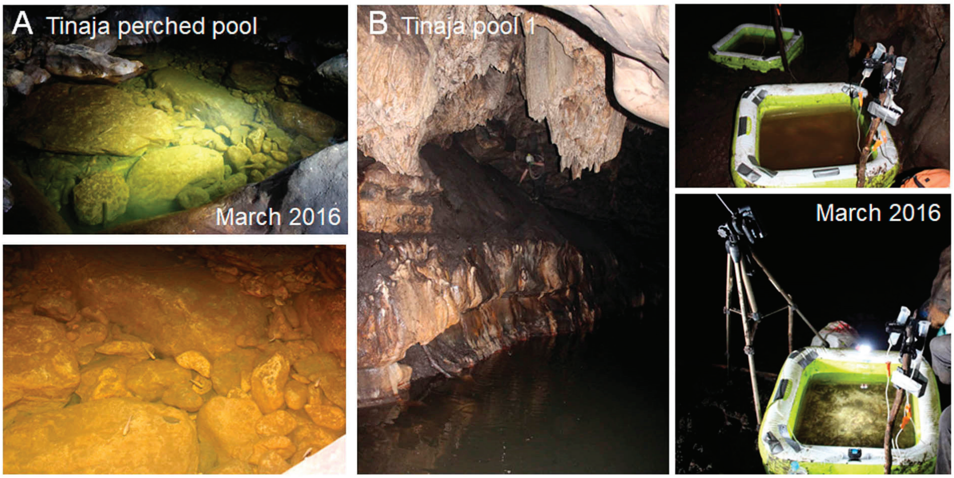


Figure 7. Experimentation in the Tinaja cave; (**A**) single test was "manually" performed in Tinaja perched pool, a very small (~3–4 m^2) and shallow natural pool where water is retained between rocks. There, over the years, we repeatedly observed 20–30 fully troglomorphic fish, presumably trapped there during the rainy season. The water is crystal clear; (**B**) Preliminary experiments were performed in March 2016 in Tinaja pool 1 (called Traverse Lake), a relatively large and muddy water reservoir. At the time, films were recorded in the light (instead of in the dark with infrared) and the perfusion system and the odorant molecules perfused were still under tuning. This type of expedition was of paramount importance to improve and establish the final experimental setup and design we used successfully in 2017 and 2019 in other caves.

4. Discussion

4.1. Testing Cavefish Olfaction in the Field: A Challenge

To our knowledge, this is the first study reporting systematic, precise, and quantified olfactory responses to specific odorant molecules in behavioral tests with subterranean animals in their natural environment (but see [14,22]). The trip organization, transport of equipment, and difficulties of access, together with the struggle of setting up experiments in "natural laboratories" which were different each time and handling the unexpected, means that few researchers risk of this kind of experimentation. The interpretation of the results must also be approached with caution because the experimental parameters are far from being fully mastered or even known. Of note, we found that establishing and pretesting of protocols in the lab are mandatory steps, and the comparison between lab and field results can help reach conclusions. Finally, this type of field behavior work needs to extend over the long term, with continuous improvements and feedbacks. For example, the difficulties we encountered in Tinaja in 2016 allowed us to improve the setup. From that expedition, we concluded that the plastic pools needed to be perfectly clean and the cave water needed to be filtered before being poured into the arena or else the videos would be difficult to interpret, that we should not use rechargeable batteries for infrared lamps because the Mexican electrical network sometimes fluctuates, that we should systematically include a water counterflow at the corner opposite to odor perfusion to remove biases of lateral line driven behaviors, and that we should not plan for 2-day-long experiments in a cave where access is difficult. Hence, our Tinaja 2016 experiments ($n = 2$ only) were not performed in the dark, did not have the same perfusion sequence nor the same odor concentrations, and were not included in the main results of the present paper, but they ended up being very instructive for improving the setup and protocols, and they allowed us to draw preliminary conclusions (see Figure 7).

4.2. Alanine, Serine and Chondroitin Elicit Variable Behavioral Responses in Wild and Lab-Raised Adult A. mexicanus

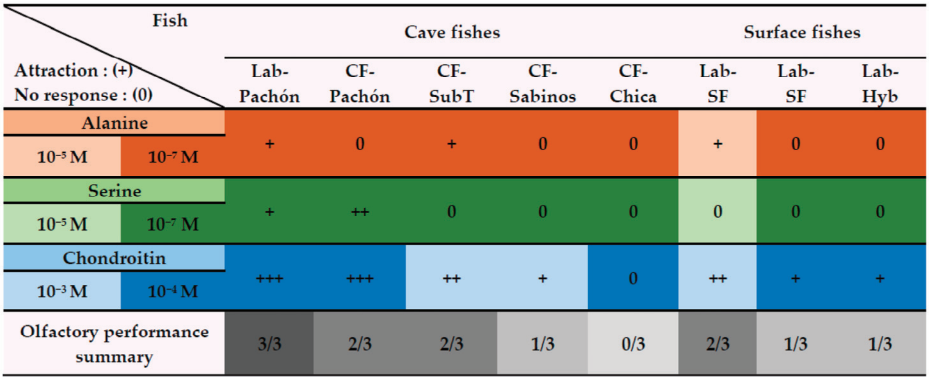
The choice of odors presented to fish was critical. Whereas alanine and serine amino acids are “classically” used in fish olfaction experiments because they correspond to food degradation products and therefore serve as food-related attractive cues (e.g., [38–40]), the use of chondroitin is more novel.

Chondroitin sulfate is a glycosaminoglycan composed of a chain of alternating sugars and a component of the cartilage matrix. It is also present in zebrafish skin mucus, where it was recently discovered to serve for the long-searched active molecule of the alarm substance [27,41]. Indeed, zebrafish strongly react to chondroitin with a typical alarm behavior, including erratic swimming and freezing, and the activated brain regions are the same as those activated after presentation of skin extracts [27]. In *A. mexicanus*, it was therefore unexpected that chondroitin would elicit (1) attraction and (2) foraging, and even more so, (3) the same response in the two morphs. Along with the idea that the alarm substance is species-specific [42], our findings suggest that its composition in zebrafish and *Astyanax* must be markedly different. Moreover, early studies have suggested that alarm reaction is indeed present in surface fish but mostly lost in cavefishes, at least those originating from the Pachón and Chica caves [28,29,43]. Here, surface fish and Pachón cavefish strongly reacted to chondroitin, but Chica cavefish did not, further suggesting that this molecule is not part of the alarm substance in the species. Rather, it seems to correspond to a foraging cue, which is in line with *Astyanax* being carnivorous, eating carcasses and responding to cartilage odors. In fact, we ended up considering chondroitin as a sort of positive control: All types of fish tested, in the lab and in the field (except CF-Chica, see below), adopted an intense food search behavior, almost leaking or sucking the extremity of the tube where chondroitin flew in (Supplementary videos). Of note, chondroitin was the third and last odor presented in the protocol because we reasoned that the more modest responses elicited by amino acids may be lost after the strong stimulation caused by chondroitin. Finally, response to chondroitin also validated, in a way, the whole test, and we were confident that absence of response to amino acids sometimes observed was true because fish reliably and repeatedly responded to chondroitin at the end of the behavioral assay.

Regarding amino acids, the results we obtained with adult fish in the lab globally corroborate what has already been observed with 1-month-old larvae [20]: The adult Lab-SF had a lower olfactory detection threshold than the Lab-Pachón (Table 1). The results confirm that older and larger adult fish do not have a better detection threshold than 1 month-old-larvae [19], reinforcing the idea that olfactory detection threshold is not directly or exclusively linked to the size of the olfactory organs, or to the intrinsic properties of the olfactory system. The results also confirm that the olfactory system of the 1-month-old larvae tested in our previous studies was functionally mature.

For reasons explained earlier, adult surface fish (and F1 hybrids) could only be tested in the lab. We considered their olfactory performance to be poor (Table 1). They did respond to chondroitin-albeit less intensely than Lab-Pachón, but barely responded to amino acids, even at high concentrations. An explanation to this mitigated result might be that the olfactory response is slightly blurred by schooling behavior, even in the dark [25,44]. Indeed, for these fish, we observed transient statistically significant PI scores (positive or negative) before or after odor perfusion (see Figure 2B,C) that cannot correspond to true and specific olfactory responses, and that probably resulted from the grouping of all eight fish in the same area of the arena at some time points of some experiments. These observations further confirm the appropriateness of using 1-month-old surface fish—which do not yet school intensely—for olfactory tests with these morphotypes. Of note, such an interference of schooling in olfactory responses is irrelevant in the case of cavefish who have lost schooling behavior, as first observed by Parzefall in the field [22].

Table 1. A summary of the results obtained in all the cave and laboratory olfaction experiments. Attractive response (+) or no response (0) is summarized for each cavefish or surface fish population, when recorded in the field or in the lab (as indicated in the different columns) for the 3 different odors studied (as indicated in the different rows; orange/alanine, green/serine, blue/chondroitin). Light and dark colors indicate response to the high concentration (light color, lower detection capacities) or the low concentration (dark color, better detection capacities) of the odor considered. The last line (grey shades) indicates to how many odors, out of the 3 tested, the fish have responded.



Fish Attraction : (+) No response : (0)	Cave fishes					Surface fishes		
	Lab-Pachón	CF-Pachón	CF-SubT	CF-Sabinos	CF-Chica	Lab-SF	Lab-SF	Lab-Hyb
Alanine 10^{-5} M / 10^{-7} M	+	0	+	0	0	+	0	0
Serine 10^{-5} M / 10^{-7} M	+	++	0	0	0	0	0	0
Chondroitin 10^{-3} M / 10^{-4} M	+++	+++	++	+	0	++	+	+
Olfactory performance summary	3/3	2/3	2/3	1/3	0/3	2/3	1/3	1/3

Finally, the comparative responses of Pachón cavefish from the lab and from the field to amino acids is also worth discussing. Lab-Pachón responded positively (=attraction, like larvae) to alanine, whereas CF-Pachón responded negatively (=repulsion). Moreover, Lab-Pachón response to serine was weak or delayed (if any), whereas CF-Pachón response was strong and immediate. Thus, both lab-raised and wild animals originating from the Pachón cave seem able to detect very low concentrations of amino acids (here, 10^{-7} M), thereby confirming their excellent olfactory skills. However, unexpectedly, their responses to a given odor can differ in nature or intensity. The first hypothesis we can draw relates to the distinct environment in which the lab and wild fish were grown and live, e.g., the water parameters or diet could be at the origin of their difference in reaction. Indeed, the water used in the lab tests (tap water bubbled for 24 h) is "cleaner" than the water used in the field, which, although filtered, is loaded with dissolved organic compounds. It has also been proposed that fish move toward or away from a given amino acid depending on the concentration as well as the age and the species of fish. For example, cysteine acts as a repellant for 1-month-old juvenile zebrafish (3×10^{-5} M; [45]) but as an attractant for rainbow trout (10^{-6} M; [39]). Moreover, olfactory conditioning or learning seems to play an important role in the expression of behavioral responses [46,47]. In the Pachón cave, juveniles feed on micro-arthropods and adults feed on decomposing organic debris and mud [23]. Conversely, in the lab, larvae are fed with micro-worms and artemia nauplii, and juveniles and adults are fed with granules that contain by-products of fish, cereals, vegetables, and crustaceans. Lab-raised fish eat very little decomposing food since they finish their meals in a few minutes. Such differences in feeding experience and odor exposure might explain the differential responses of Lab-Pachón and CF-Pachón. The repulsive behavior after alanine perfusion in the natural cave may suggest that CF-Pachón are exposed to an unpalatable organic matter that gives off alanine. We do not know the amino acids compositions of different organic materials found in this cave. It is therefore difficult to determine which one might be the source of this repulsion. Overall, the sometimes-contrasting responses that we observed in the lab and in the wild reinforce the interest and necessity to assess behavioral repertoires in the natural environment to discuss ecological or evolutionary relevance.

4.3. Not All Cavefish Respond the Same

Our field observations in five different caves lead to one main conclusion: Cavefish from different caves showed very distinctive responses when presented with exogenous odor cues at low concentrations (Table 1). For those that did respond (CF-Pachón, CF-SubT), we can propose that they have augmented olfactory skills when compared to their surface conspecifics, which confirms our previous studies [14,20]. For those which did not respond as well (CF-Sabinos) or did not respond at all (CF-Chica, CF-Tinaja), several questions arise. Is the lack of response due to local environment, previous experience and/or fish condition and nutritional status? Or is it due to experimental conditions?

(1) Let us start with the latter—perhaps less biologically interesting—hypothesis. We cannot rule out that CF-Chica did not respond because the cave water used (although filtered) was so loaded with debris and dissolved organic materials and endogenous olfactory cues that their olfactory system was saturated and could not detect the low concentrations of amino acids or even chondroitin perfused (see Figure 4E). The same holds true in the Tinaja cave, where carbon content in the mud is high (32%, to be compared to 9.2% in the main pool of Pachón) [24], and, maybe to a lesser extent, in the Sabinos cave (see description in Results). A way to test directly this hypothesis would be to transport "clean" tap water into the caves to perform the experiments. However, we feel that the interest of such experiments would be rather limited, as we anticipate that they would be flawed with other problems and quite demanding in terms of logistics. Nevertheless, future studies examining the physicochemical parameters and the exact nature of compounds present in the water will be important to answer these questions.

(2) The environment-dependent and experience-dependent hypothesis is more scientifically exciting to discuss adaptation to the environment. It implies that the expression of olfactory skills depends on local environment, such as the configuration and ecological parameters of the cave and previous olfactory experiences of the fish related to these local environmental parameters, including possible interactions with other sensory systems and fish nutritional status and motivation to find food.

Indirect support for this hypothesis comes from our finding that CF-Pachón and CF-SubT, which do not belong to the same geographical group of caves and correspond to independently evolved cavefish populations [5,11,48], both show significantly augmented olfactory skills. Thus, this trait most probably corresponds to an evolutionary convergence. This result further suggests that the evolution of food-related (and maybe pheromonal, not addressed here) odor sensing is of paramount importance for cavefish life, adaptation, and survival in the dark. On the other hand, population genetic studies have shown that El Abra cave populations (Pachón, Sabinos, Tinaja, Chica) are genetically close and share many polymorphisms [25,48,49], suggesting that CF-Sab or CF-Tinaja probably carry all or part of the mutations in "olfaction genes" that genetically determine CF-Pachón olfactory skills. However, they do not necessarily express these skills as a consequence of plasticity, with expression depending on the environmental conditions at the precise time and location where we measured olfactory responses. The most striking pieces of evidences in favor of this possibility are discussed below.

CF-Pachón were the smallest among all fish assayed in this study, in agreement with previous measurements [24]. They live in a low-carbon cave (9.2% in the mud), with limited influx of organic matter from the surface. There, foraging must be challenging and strongly olfactory-driven. Conversely, CF-Chica were the largest and biggest among the cavefish that we tested and measured. The very large bat colonies in this "dirty" cave probably render foraging easy, together with fast growth and a long lifespan. CF-Tinaja are also large and old [24] and live in a carbon-rich (31.9%) environment [23]. Strikingly, and in line with these very different trophic environmental conditions, CF-Pachón and CF-Chica (and CF-Tinaja, according to preliminary results) were at the two ends of the spectrum of olfactory responses that we observed.

Vibration attraction behavior (VAB) is thought to help cavefish locate food droppings or vibrating objects at the surface of the water [18,50]. VAB is mediated by neuromasts, and therefore corresponds to a mechanosensory modality. In the field, VAB responses are variable in different caves and even in different ponds of a single cave [51]. In cave ponds where insects are numerous or where percolating water drops are abundant, food search is probably strongly guided by VAB, and less so by the chemosensory olfactory modality. Our results fit well with this idea. Indeed, in the Tinaja cave, where it literally "rains," or in the Sabinos cave, where we personally witnessed abundant water drops over pool 1 to which the CF-Sab were systematically attracted (SR and A. Alié, pers.obs.), the olfactory sense and skills may not be solicited as much as in the Pachón or Subterráneo caves, where vibrating objects are much rarer and food must be found by the nose. Thus, we propose the possibility of a balanced use of different sensory modalities and expression of sensory skills depending on local conditions. Of note, fish may preferentially use one or the other (or both) sense across their lifespan to find a proper diet and to adapt to different locations where they can swim in a cave, or even across seasonal fluctuations of local ecological conditions.

Finally, cavefish genetics and the hybridization with introgressed surface fish is also a parameter to consider. Whereas Pachón, Sabinos, and Tinaja cavefish are fully troglomorphic (see Figure 3), CF-SubT and CF-Chica correspond to "hybrid-like" populations and carry surface fish alleles (see [14]). Interestingly, the entrance of surface individuals into these two caves does not proceed by the same end: In Subterráneo, surface fish are washed inside the cave from the surface by flooding, whereas, in Chica, surface fish enter the cave by a bottom resurgence. This probably explains why, in Chica, the pool 2 (deeper) fish, which carry more surface alleles, are smaller than the pool 1 (upper) fish, which are less hybridized and more troglomorphic and cave-adapted. In any case, we cannot exclude that the important gene flow from the surface that exists in Chica [25,48] might counteract the effects of cave alleles, favoring the evolution of augmented olfactory skills. Hence, the absence of olfactory responses in CF-Chica might correspond to truly modest olfactory capacities. Deciphering the effects of genetics (presence of surface alleles) from the effects of environmental parameters on plasticity (food-rich cave) or from experimental conditions (odor-saturated water during test) is impossible at this stage. However, it must be noted that the CF-SubT, which also carry surface alleles, responded much better than CF-Chica to the odors we presented: They strongly responded to both alanine and chondroitin. Therefore, in this later case, the effects of hybridization and introgression of surface alleles appears very limited on the behavioral phenotype.

5. Conclusions

Almost 80 years have passed since the first descriptions of ecological conditions in which Chica cavefish live by Charles Breder [52]. Since then, fieldwork in *Astyanax* caves has continuously brought novel information on caves topography, population genetics, and cavefish biology in general. Recently, important examples of insights on the evolution of cavefish behaviors or the evolution of their immune system started from field observations [53,54]. Here, by bringing the "behavior room" into the field, we highlighted the diversity and complexity of the mechanisms that underlie cave adaptation and documented the diversity of cave environments inhabited by a single species of cavefish, *A. mexicanus*. Our data confirm the classical proposal of sensory compensations to the absence of vision in the dark. Indeed, overall cavefish showed lower (i.e., better) olfactory detection thresholds than surface fish. However, the picture appears more complicated than simply "cavefish smell better." The next challenge will be to disentangle the effects of genetics, plasticity, environment, and their interactions in the evolution of cavefish olfactory system. Then, of course, more fieldwork will be needed to refine behavioral observations and to further describe the diverse natural ecological conditions in which cavefish live.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/10/395/s1, Figure S1: Rationale for olfactory scoring. Figure S2: Place preference problem in the Sabinos cave. Video S1: Behavioral response of Pachón cavefish to chondroitin in laboratory settings. Video S2: Behavioral response of Pachón cavefish to chondroitin in natural settings.

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Article

Discovery of Two New *Astyanax* Cavefish Localities Leads to Further Understanding of the Species Biogeography

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Abstract: The *Astyanax* species complex has two morphs: a blind, depigmented morph which inhabits caves in México and an eyed, pigmented surface-dwelling morph. The eyed morph can also be found in a few caves, sometimes hybridizing with the cave morph. This species complex has arguably become the most prominent model system among cave organisms for the study of evolutionary development and genomics. Before this study, 32 caves were known to be inhabited by the cave morph, 30 of them within the El Abra region. The purpose of this study was to conduct new surveys of the area and to assess some unconfirmed reports of caves presumably inhabited by troglomorphic fish. We describe two new localities, Sótano del Toro #2 and Sótano de La Calera. These two caves comprise a single hydrologic system together with the previously described cave of Sótano del Toro. The system is inhabited by a mixed population of troglomorphic, epigeomorphic, and presumably hybrid fish. Furthermore, *Astyanax* cavefish and the mysid shrimp *Spelaeomysis quinterensis* show a phylogeographic convergence that supports the notion that the central Sierra de El Abra is a biogeographical region that has influenced the evolutionary history of its aquatic community across species. The presumptive location of the boundaries of this biogeographical region are identified.

Keywords: *Astyanax*; *Spelaeomysis*; *Troglomexicanus*; *Speocirolana*; Toro cave; Sierra de El Abra; troglomorphy; troglobite; stygobite

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1. Introduction

Cave organisms have been a choice model for many evolutionary biologist due to their independent loss of vision and pigmentation. The genetic and developmental controls on troglomorphic features are best understood in the blind Mexican tetra [1]. While most recent authors consider it to be the cave morph of *A. mexicanus* De Filippi 1853, some authors gave it the status of species, *Astyanax jordani* Hubbs & Innes 1936. Most notably, *A. jordani* is still used in the IUCN Red List and listed as Vulnerable [2]. Both surface and cave morphs remain inter-fertile, making the species complex well-suited for experimental manipulations [3]. With the genomes of both morphs already sequenced, *Astyanax* is one of the main contributors in the understanding of cave evolution, and is also recognized as an influential model system in evolutionary developmental biology [4,5].

Mitchell et al. [6] described 29 cave localities for troglomorphic *Astyanax*, all of which occur within the El Abra region (which includes the adjoining Sierra de El Abra, Sierra de Guatemala, and Micos area), in the states of San Luis Potosí and Tamaulipas in Northern México. Two additional cave localities, Granadas and La Joya [7,8], have been described for the closely related species of *Astyanax aeneus* Günther, 1860 in Guerrero, Southern México. The last and most recently discovered locality was Cueva Chiquitita [8], from Sierra de El Abra (Figure 1).

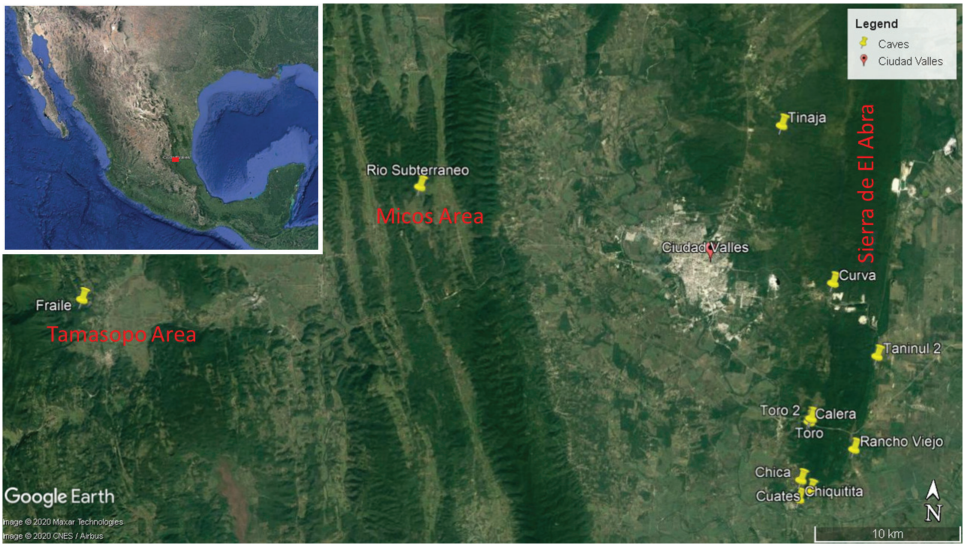

Figure 1. Cave locations. Tinaja and Curva caves are in the central Sierra de El Abra. Taninul, Calera, Toro, Toro #2, Rancho Viejo, Chica, Chiquitita and Cuates are in southern Sierra de El Abra. Río Subterráneo is in the Micos area, still within the El Abra general region. Cueva del Fraile is in the Tamasopo area. Pachón Cave is outside the map boundary, 70 km north of Curva, in the northern Sierra de El Abra. Molino and Caballo Moro are about 110 km north of Curva, in the Sierra de Guatemala. Map showing location of Ciudad Valles, San Luis Potosi. Google Earth, earth.google.com/web/.

Despite the presence of thousands of caves, there are no known troglomorphic *A. mexicanus* ssp. *jordani* sites south of the Río Tampaón. Cueva Chiquitita is a spring found at the southernmost edge of the Sierra de El Abra, approximately 200 m from the Río Tampaón. Cueva Chiquitita is inhabited by surface, troglomorphic, and hybrid fish. It is also likely that there is a continuous underwater passage between Cueva Chiquitita and one of the most cited *Astyanax* cave localities, Cueva Chica [6,8]. Subsurface hydrologic connections allow for both troglomorphic and surface fish to migrate between El Abra cave localities. These connectivity is supported by gene flow [9] and geologic [10] studies.

Previous reviews of caves inhabited by troglomorphic *Astyanax* suggested areas that could yield new discoveries, in particular the southern Sierra de El Abra [11]. This is a low-lying area that has little potential for deep caves. Most caves were explored in the 1970's, and cavers at that time may have put less effort into describing shallow caves that would nevertheless be of biological interest [6,8]. Supporting evidence for the possibility of new discoveries was the aforementioned recent discovery of Cueva Chiquitita in this area [8].

Previous mitochondrial DNA analyses on *Astyanax* cavefish have shown the presence of two broadly different clades that have been termed mitochondrial lineages A and B [12]. Caves harboring populations with haplotype B mitochondrial DNA (Sabinos, Tinaja, Piedras and Curva) are restricted to an area in the central Sierra de El Abra [13]. El Abra caves are also inhabited by the mysid cave shrimp *Spelaeomysis quinterensis* Villalobos, 1951. Histone H3 DNA sequences of them also showed that mysid shrimp from those caves in the central Sierra de El Abra were quite different from other cave populations [13]. This phylogeographical convergence supports the hypothesis that the central Sierra de El Abra is a biogeographical zone, perhaps with barriers for either cave-to-cave or surface-to-cave gene flow, that have modulated the evolutionary history across species in the aquatic community. Interestingly, Sótano del Toro is located between the central and the southern Sierra de El Abra. Determining which stygobitic community it belongs to will allow for a better understanding of where the biogeographic barriers are located.

Another area that was identified [11] that could yield interesting new discoveries was the Tamasopo area and, in particular, Cueva del Fraile. This cave is 24 km west of the nearest *Astyanax* cave population in Río Subterraneo, in the Micos area [11]. This locality is in a drainage isolated from the El Abra region hydrology by the 100 m waterfall of Tamul. If there is a cave-adapted population in the Tamasopo area, it would have to be from an independent evolutionary event, and thus of great scientific interest.

2. Materials and Methods

Sótano del Toro and Sótano del Toro #2 were visited on March, 2019 and on January 2020. Sótano de La Calera, near Sótano del Toro, was explored in June 2019 and in January 2020. Cueva del Fraile was visited in February and shortly after in March 2019. Cueva del Rancho Viejo, Taninul #2, and Tinaja were visited in January 2020. Figure 1 shows a map with the caves described in this article. Description of the caves and their precise ubications can be found in [6,11].

To explore Sótano del Toro #2, Sótano de La Calera, Taninul #2, and Tinaja, single rope techniques were employed to ascend and descend their up to 30 m pits. Some pools received special attention with the help of mask, snorkel and underwater scuba lights. Sótano de La Calera and Sótano del Toro #2 were surveyed using a DistoX2, and in the case of Toro #2 using the TopoDroid app on a tablet. Survey data were processed using Compass and Walls, and drawn using Adobe Illustrator.

To capture specimens, baited traps or hand-held nets were used. Photographs were taken in the field with the help of a small glass tank and a Canon EOS100 camera. A small fin clip was taken with scissors for DNA studies from all specimens. Voucher samples were stored at the Colección Nacional de Peces, IBUNAM, Mexico.

In accordance with the Ethical Guidelines for the Use of Animals in Research and collecting permit # SGPA/DGVS/1893/19 from the Secretaría del Medio Ambiente y Recursos Naturales, México issued to Patricia Ornelas-García, only five specimens were euthanized from Sótano del Toro #2, which were stored in 100% ethanol. Five other fish were kept alive in the laboratory of Patricia Ornelas-García for breeding in captivity, which can serve as a stock for future studies. All other specimens collected were returned unharmed to their source pool.

Astyanax mitochondrial 16S rRNA was sequenced from 5 individuals collected in Sótano del Toro #2. For comparison, DNA sequences were aligned to sequences from localities with reported [12,14] haplotypes of mitochondrial lineage A (2 Comandante surface river, 2 Sótano del Molino, 2 Sótano del Caballo Moro, 2 Cueva del Pachón, 1 Cueva Chica, and 4 Cueva Chiquitita) and from localities with haplotypes of mitochondrial lineage B (4 Rascón surface stream, 2 Tamasopo surface stream, 2 Cueva de Los Sabinos, 4 Cueva de la Tinaja, and 1 Cueva de la Curva). For mysid shrimps, the Histone H3 was sequenced from a single individual from Toro #2. It was then compared with sequences from localities with reported [13] haplotypes of clade A (2 Pachón and 1 Cueva Chiquitita) and from haplotypes of clade B (3 Tinaja and 1 Sabinos caves). The use of two different markers for comparison between *Astyanax* and mysid shrimp aligns with previous studies [13].

Genomic DNA samples were obtained following standard methods for DNA purification using Qiagen's DNeasy® Tissue Kit by digesting a fin clip of the individual in the lysis buffer. Markers were amplified and sequenced as a single fragment using the 16Sar (CGCCTGTTTATCAAAAACAT) and

16Sb (CTCCGGTTTGAACTCAGATCA) primer pair for 16S rRNA [15] and the H3aF (5′ ATGGCTCGT ACCAAGCAGACVGC 3′) and H3aR (5′ ATATCCTTRGGCATRATRGTGAC 3′) for histone H3 [16]. Amplification was carried out in a 50 µL volume reaction, with QIAGEN Multiplex PCR Kit. The PCR program consisted of an initial denaturing step at 94 °C for 60 s, 35 amplification cycles (94 °C for 15 s, 49 °C for 15 s, 72 °C for 15 s) and a final step at 72 °C for 6 min in a GeneAmp® PCR System 9700 (Perkin Elmer). PCR amplified samples were purified with the QIAquick PCR purification kit and directly sequenced by SeqWright Genomic Services. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher™ 3.0. All external primers were excluded from the analyses. BLAST was used to identify GenBank sequences that resemble the specimens. Sequences were aligned with ClustalW2.

3. Results and Discussions

3.1. Southern Sierra de El Abra

3.1.1. Background on Sótano del Toro, Cueva del Rancho Viejo and Taninul #2

Sótano del Toro is the smallest and shallowest of the known eyeless *Astyanax* caves. It is possible to stand in the entrance sink and see the 0.5 m wide by 2.5 m long pool. A gallery can be seen continuing underwater. Light reaches this small pool, as can be seen in Figure 2. The cave was visited in 1969, when three days of work resulted in only about eight fish being collected. On March 2008, three cavefish were obtained for genomic work [9]. Previous reports [11] conclude that "None of these fishes appeared to have hybridized with surface *Astyanax*, even though Cueva Chica (which has large amounts of surface and hybrids) is located only 4 km to the south". They further report that "Another cave may exist in the vicinity, but it was not found in 1974 by local guides who knew of it".

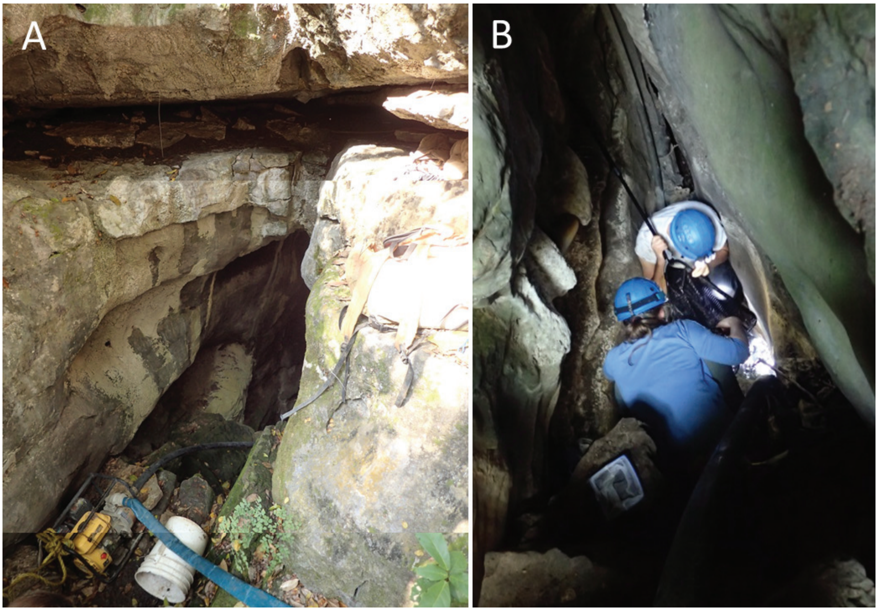

Figure 2. Sótano del Toro. (**A**); Entrance sink descends to a 0.5 m wide by 2.5 m long pool. (**B**); Light reaches this small pool. Locals use a water pump (A) to extract water for the local farm.

The Cueva del Rancho Viejo is near a small resurgence called Nacimiento del Rancho Viejo, which only discharges water after a rain. The cave was explored by Elliott et al. in 1974. A climbdown of

8 m reaches the first pool, 5-10 m deep. A low gallery 100 m long reaches a second pool. Eyed *Astyanax* and cichlids were observed and collected at that time [11]. During the dry season, no water comes out of the resurgence, and fish are isolated in pools that lie in complete darkness. This resurgence is of interest because it is only 3.6 km east of Sótano del Toro, and 4.2 km northeast of Cueva Chica, so they could be hydrologically related. Before this study it was unclear if troglomorphic fish were also present in this cave.

Cueva de Taninul #2 is 6 km northeast of Sótano del Toro. This cave is only 100 m away from the fresh water, non-sulphur resurgence pool at the hotel Taninul. This cave is about 200 m long, and has a 30 m pit. At the bottom of this pit there is a sump. This sump is at about the same level as the resurgence. There were no published records of fish inhabiting this cave before this study.

3.1.2. Results from Taninul #2, Cueva del Rancho Viejo, and Toro Area

Taninul #2 was explored to the final sump, where half a dozen fully eyed and pigmented fish were observed. They were comparable to surface fish found above ground, and were responsive to light. No troglomorphic fish were seen, despite careful observations while snorkelling with an underwater lamp.

We visited Cueva del Rancho Viejo on 6 January 2020. About 10 fully pigmented fish were observed in the pools. A baited trap was left with the aim of attracting troglomorphic fish, since they have enhanced smell [1,17]. The trap was then recovered on the 10th of January, 2020. Around 15 individuals were collected, but all had normal eyes and pigment. Fish were released unharmed after careful observation. Despite two days of effort and a baited trap, we found no evidence that troglomorphic or hybrid fish inhabit these caves. This supports the 1974 observation that only epigeomorphic fish inhabit this cave [11].

Exploration of the area around Sótano del Toro yielded two new entrances to caves with troglomorphic *Astyanax*: Sótano del Toro #2 is only 68 m west of Sótano del Toro, and Sótano de La Calera is 239 m south of Sótano del Toro #2.

Sótano del Toro #2 consists of a vertical pit of about 12 m. For the first 6 m the pit is very narrow, just 1 m wide. Then it opens into the roof of a large chamber that is 20 m long. In this chamber there is a 10 m diameter pool with fish (Figures 3 and 4). This pool is a sump heading in the general direction of Sótano del Toro. From the lake room several short dry galleries bifurcate, one of them being a 30 m crawl that reaches a low ceiling gallery with water, also inhabited by fish (Figure 5). This water passage heads in a southerly direction for another 20 m until a sump is reached, at which point it appears to continue in a direction that would suggest a possible connection with Sótano de La Calera. It is noteworthy that the walls near the sump are covered with fine mud inhabited by scores of nicoletiid insects, *Anelpistina quinterensis* (Paclt, 1979).

Sótano de La Calera has a 6 m pit entrance (Figure 6A). During the dry season when the cave was explored, galleries were interspersed with many pools varying from just 0.5 m long to over 10 m, some inhabited by *Astyanax* fish. Apart from the *Astyanax* and the nicoletiid insects, this cave system is inhabited by the mysid shrimp *Spelaeomysis quinterensis*, as well as *Speocirolana* isopods. The downstream gallery of Sótano de La Calera heads south for about 77 m via low ceiling crawlways until a sump is reached. This sump has not been explored by divers, and could lead to a significant extension of the system to the south. The upstream gallery has been mapped for 186 m and heads north, in the direction of Sótano del Toro #2 (Figure 7). Beyond the mapped limit, another 15 m have been explored until a crawlway is reached that is covered with fine mud and inhabited by scores of nicoletiids. There are also several unexplored side passages in Calera.

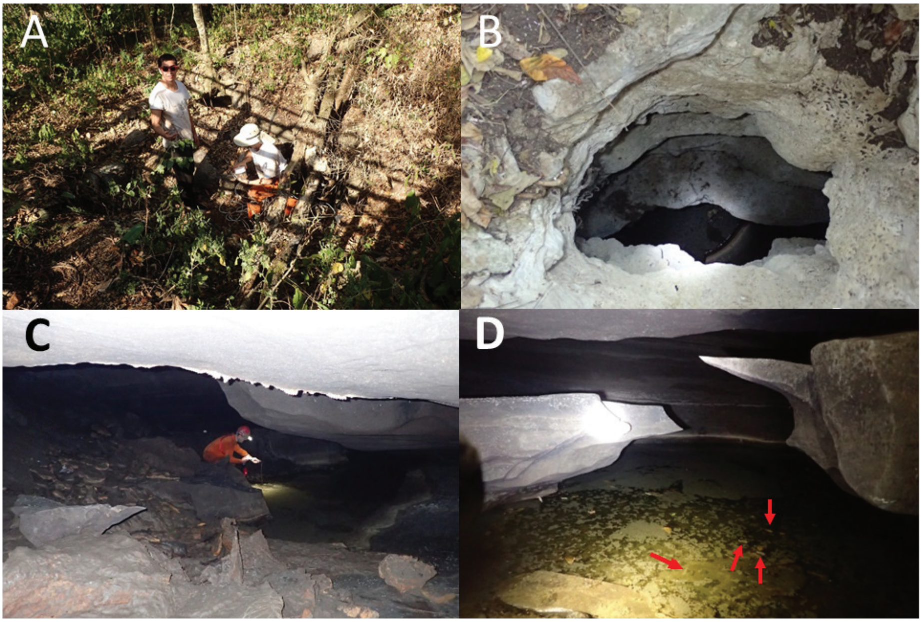

Figure 3. Sótano del Toro #2. (**A**); Entrance pit. (**B**); Narrow portion of the entrance pit. (**C**); Typical galleries within the cave. (**D**); Entrance lake. Arrows point to fish.

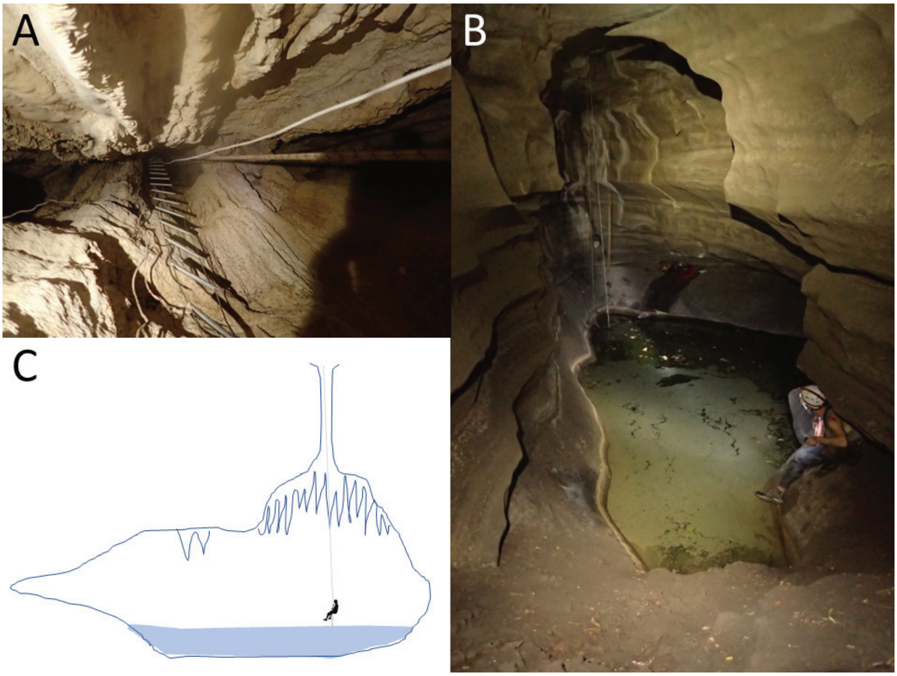

Figure 4. Sótano del Toro #2. (**A**); looking up to the narrow portion of the entrance pit. (**B**); Entrance lake. (**C**); Profile drawing of the entrance pit and lake.

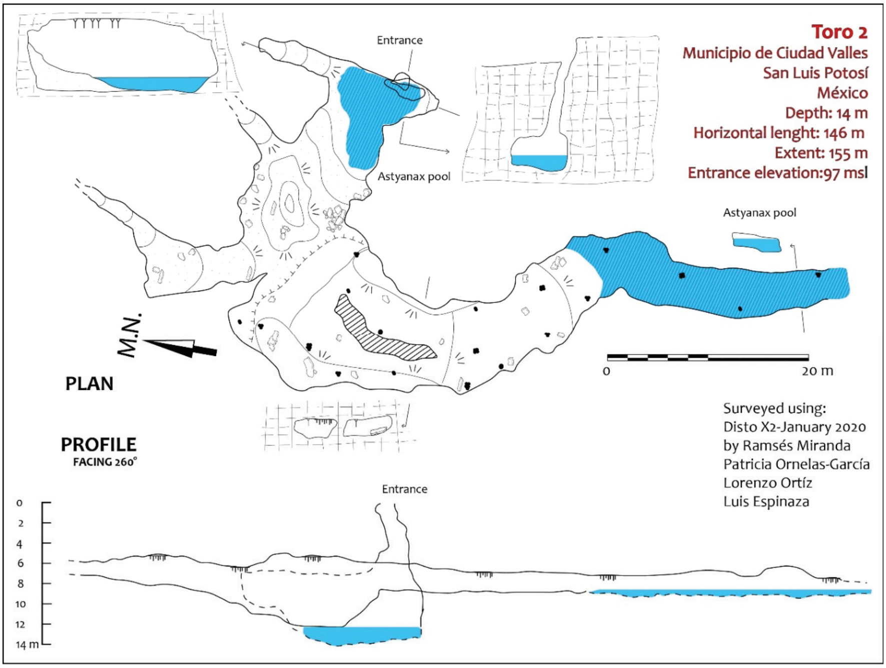

Figure 5. Map of Sótano del Toro # 2.

Figure 6. Sótano de La Calera. (**A**); Entrance pit. (**B**); One of the many pools with fish in this cave. (**C**); Fully troglomorphic fish. (**D**); Potential hybrid between troglomorphic and epigeomorphic fish. Notice reduced and embedded eyes.

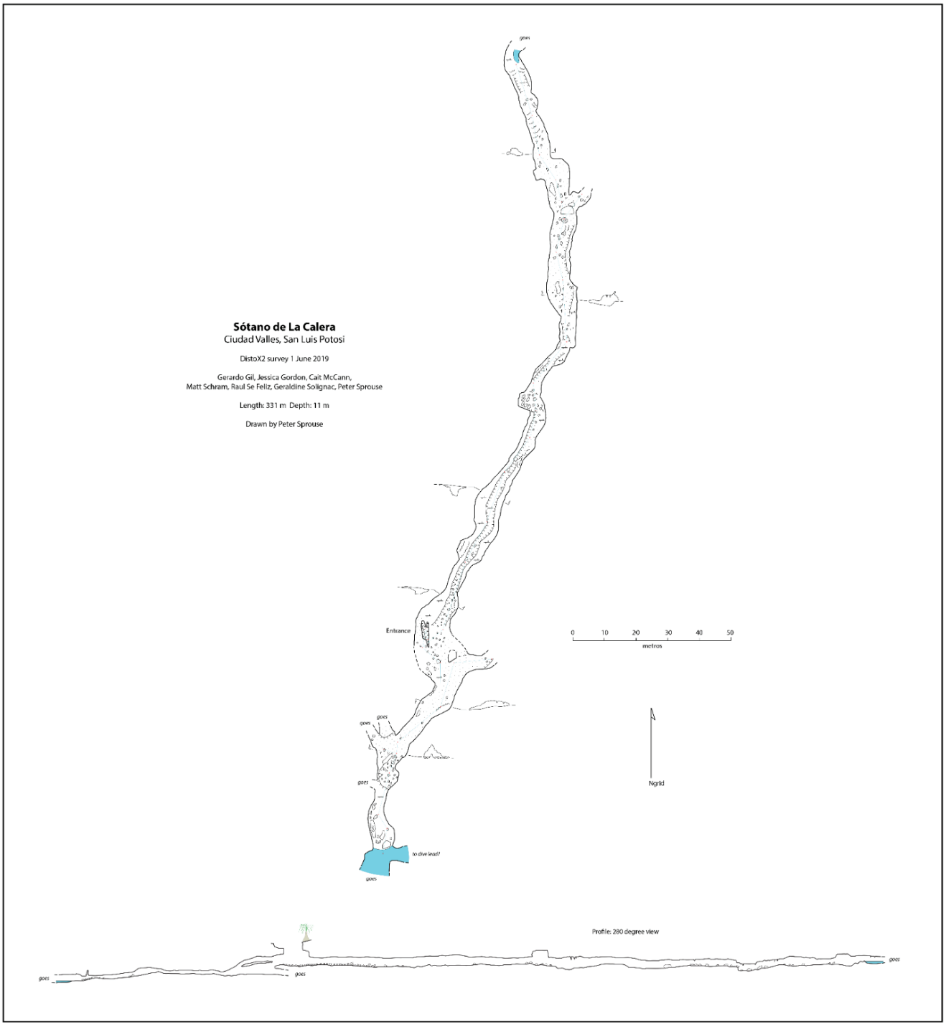

Figure 7. Map of Sótano de La Calera. During the dry season, galleries contain many pools, some inhabited by *Astyanax* fish. During the rainy season it becomes a flowing stream and fish can freely swim along its galleries and to Sótano del Toro and Sótano del Toro #2

The entrances to Sótano del Toro #2 and Sótano de La Calera are only 239 m from each other. Considering that Calera extends north for about 200 m, heading directly towards Sótano del Toro #2, and that Sótano del Toro #2 extends south for about 40 m south, it is likely that the muddy sections inhabited by nicoletiids are the opposite sides of a short sump. We suggest that both caves are a single cave system. These two caves also may be connected to Sótano del Toro, only 68 m away. It probably is a single hydrologic system with three entrances (Figure 8). During the rainy season, fish can probably travel easily from one section to the other.

Figure 8. Topographic map showing the close proximity of Sótano del Toro, Sótano del Toro #2 and Sótano de La Calera. Also shown in the map are the town of La Calera, from which the cave receives its name, and the town of El Nacimiento, which means "spring". This spring may be one of the potential sources of surface fish found in the cave system. In dark blue is the line plot of Sótano de La Calera. Notice that it is heading directly towards Sótano del Toro #2. While sumps prevent passage without diving gear, these three caves likely form a single hydrologic system, and fish can move from one to the other during the rainy season. Modified map from INEGI data, F14D11, El Pujal, 1:50,000 topographic sheet.

From the point of view of the *Astyanax* biology in the Toro system, our observations differed significantly from previous reports [11] of a cave inhabited exclusively by troglomorphic fish. Instead, we found that the population is a mixed population (Figure 9). Some were highly depigmented, with characteristic pinkish-white coloration and no external eyes. Other individuals were identical to surface fish, fully pigmented, with large eyes, and responsive to light. But many individuals had high variability in the eyes, with individuals having phenotypes such as small eye size (Figure 9), closed pupil, embedded eye (Figure 6D), and eye mostly absent. Introgression between the surface morph and the cave morph is suggested by the presence of individuals that are highly depigmented, but with eyes (Figure 9G,H,K) or individuals with pigment, but reduced eyes (Figure 9J).

None of these three caves has a large bat population. No large streams enter them. While the entrances may provide debris that can serve as food for the fish, large amounts of food are likely only in the pools directly under the entrances, and not throughout the cave system. Troglomorphic, epigeomorphic and presumed hybrids are found throughout the caves. Future studies may resolve if there are significant different ecological pressures in the different pools to promote intraspecific variability, although since during the rainy season fish from all pools are intermixed, we doubt it will have a large impact in the overall population.

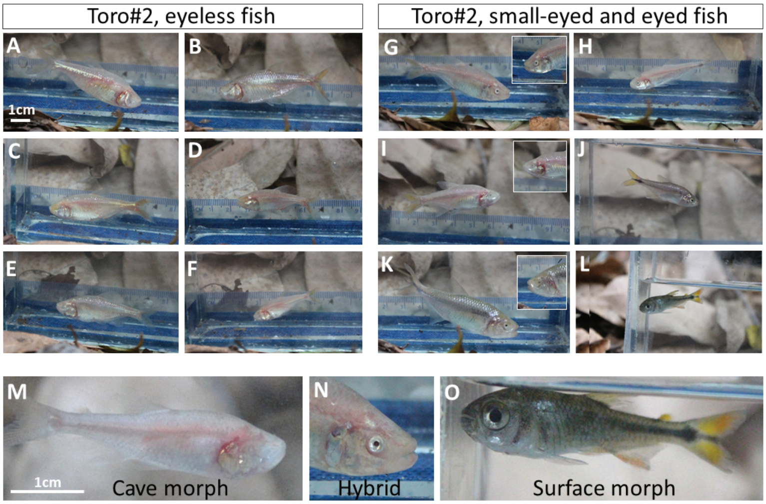

Figure 9. High variability in the eye and pigmentation level within the population inhabiting the Toro system. (**A**–**F**); Eyeless fish. (**G**–**L**); Small eyed and large eyed fish. (**M**–**O**); Higher magnification of representative morphs. Introgression between the surface morph and the cave morph is suggested by the presence of individuals that are highly depigmented, but with eyes (**G**,**H**,**K**) or individuals with pigment, but reduced eyes (**J**). Asymmetry is evident in some individuals (**K**) in which on one side they may have an eye, but on the other is eyeless (inset photo). Scale bar in A is for (**A**–**L**) and scale bar in M is for (**A**–**O**).

Five troglomorphic fish from the Toro system were genotyped. DNA amplification of mitochondrial 16S rRNA produced a 572 bp sequence. All specimens presented a haplotype A (GenBank# AP011982.1) identical in sequence to fish from Pachón, Chica, and Chiquitita caves and from the local surface *Astyanax* (Figure 10). When compared to the mitochondrial haplotypes B of fish from Rascón and Tamasopo surface streams, Toro specimens differed by 2–3 bp. When compared to the mitochondrial haplotypes B of cavefish from Sabinos, Tinaja, and Curva, they differed by 5 bp (red arrows in Figure 10). It is thus supported that *Astyanax* Toro specimens have a mitochondrial DNA most similar to haplotypes A, with specimens being identical to individuals from other southern El Abra caves like Chica and Chiquitita.

In the mysid shrimp from the Toro system, the H3 fragment was 328 bp long. Two clades or haplotypes (GenBank # MH422492, MH422494) were found, in support of Kopp et al. [13]. The two mysid shrimp haplotypes differed by 36 bp (10.9%). The first clade A included specimens from Pachón, Toro #2, and Chiquitita. The clade B included Tinaja and Sabinos. Thus, similarity of sequences among populations did not follow geographical proximity between caves. Specimens from the northernmost (Pachón) and the southernmost (Toro #2 and Chiquitita) portions of the Sierra de El Abra were identical. Likewise, the specimens from the central Sierra de El Abra (Sabinos and Tinaja) were identical. It is thus supported that the mysid shrimp from Toro #2 cave has an H3 DNA most closely related to the clade A, with their sequence being identical to individuals from other southern El Abra caves such as Cueva Chiquitita.

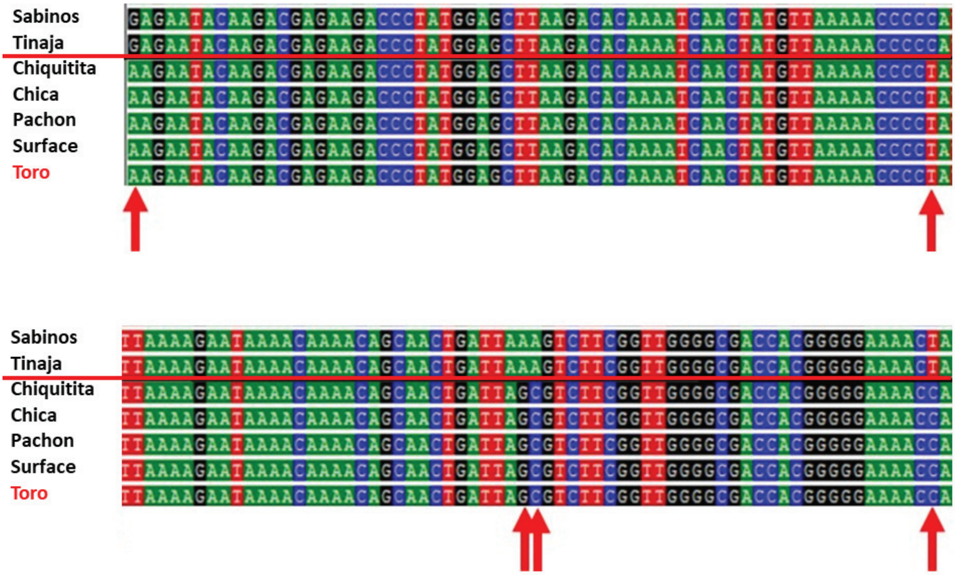

Figure 10. Fragment of the *Astyanax* mitochondrial 16S rRNA. Individuals from Toro system have identical sequence to populations harboring haplotype A (Cueva Chiquitita, Cueva Chica, Pachón caves, and Río Comandante surface river). Populations harboring haplotype B (Sabinos and Tinaja caves) have five bp disagreements in this fragment, indicated by red arrows.

3.1.3. Discussion: Southern Sierra de El Abra

Our results revealed some interesting aspects of the cave populations in the southern Sierra de El Abra. Previous reports have described Sótano del Toro as a small cave with a population composed of only troglomorphic *Astyanax*. Instead, we have found this to be a cave system with over 350 m of passages, three different entrances, and many environmentally variable pools inhabited by a mixed population of troglomorphic, epigeomorphic and presumably hybrid fish, similar to those found in Chica, Cuates and Chiquitita caves. Toro system cavefish also have the mitochondrial A haplotype found in the aforementioned southern Sierra de El Abra cave populations, and in surface fish from the streams that surround Sierra de El Abra. They are distinct from the cavefish populations of the central Sierra de El Abra, and the surface fish from Rascón.

What is the origin of the surface fish in the Toro system? The cave is heading directly south, towards Chica, Cuates, and the resurgence of Chiquitita. The Chiquitita spring has been hypothesized to be the source and entrance point of surface fish to these caves [8]. The Chiquitita resurgence is about 5.6 km to the south. Another alternative is that the cave system changes its direction and veers to the west to a spring near the town of “Ojo de Agua”, about 1.3 km away. Regardless of the surface fish’s source, the Toro system shows that they have the potential to penetrate deep into the cave systems. These surface fish are swimming upstream starting at a spring. They are not washed in. Furthermore, surface fish were found in galleries in complete darkness late in the dry season, so they must have been there for many months. Their presence in large numbers in a cave far from any surface stream input implies that, to a certain extent, they can survive and reproduce alongside the cave morph. While they may be slimmer than troglomorphic fish, they are somewhat successful in reproducing, as evidenced by the high number of apparent hybrids observed.

It has been argued that surface fish at Cueva Chica have been able to survive and successfully reproduce because of the mild selective conditions due to an ample food supply from the large bat colony in this cave [6]. The Toro system shows that even without a large input of food, surface fish can still be part of the reproductive community over the span of six months or more. This contrasts drastically with the view that surface fish are to be considered accidentals, destined to die soon after, such as we have personally witnessed in the Río Subterráneo cave in the Micos area. This result is corroborated by the Cuates cave population, also in the southern El Abra, where fish of both morphs cohabitate.

A recent study has estimated that all cavefish populations are probably recent, less than 20,000 years old [18]. It has been argued that the incongruence of the mitochondrial DNA phylogeny with phylogenies obtained with several independent nuclear loci does not support the existence of two cavefish lineages [18]. Genomic studies have further shown a much more complex evolutionary history with reticulation, with recent and historical gene flow both within and between cave and surface populations where "a simple bifurcating tree does not fully capture the history of these populations" [19]. Regardless of whether or not there are cave-adapted fish lineages, results show that caves harboring populations with haplotype B mitochondrial DNA (Sabinos, Tinaja, Piedras and Curva) are restricted to a distinct area in the central Sierra de El Abra [13]. The Toro system is now recognized as belonging to the southern Sierra de El Abra, which harbors *Astyanax* mitochondrial haplotype A. A consistent pattern was corroborated with the stygobitic mysid shrimp, also suggesting that those in the central Sierra de El Abra (Sabinos and Tinaja) harbor a separate haplotype different from the rest of the Sierra de El Abra populations (Pachón, Toro #2, and Cueva Chiquitita).

Phylogeographic results obtained from the mysid Shrimp, *Spelaeomysis quinterensis*, are consistent with the results of mitochondrial studies in *Astyanax*. This suggests that the geographic distribution of mitochondrial haplotypes in *Astyanax* is not stochastic. It has been suggested that northern populations like Pachón, Molino, and Caballo Moro and the southern Sierra de El Abra populations have haplotype A, while the central populations have haplotype B because they represent different colorizations and/or because they have been affected by recent introgression from surface populations [20]. It has also been suggested that the disparity between nuclear and mitochondrial DNA could be explained by linkage to paternally or maternally inherited adaptive characters of distinct populations [20]. The present data also support that the Sierra de El Abra has distinct biogeographic areas, with partial barriers that affect evolutionary histories and generate evolutionarily significant units across different species of the aquatic cave community. Since the Toro system is the northernmost cave of the southern Sierra de El Abra group and Curva cave is the southern-most cave of the central group, it is now recognized that the border between these two groups is probably between 21°58′ N and 21°54′ N.

We propose a model to explain the biogeography of the Sierra de El Abra, in which its low-lying southern portion is like a sponge or sieve where conduits have few barriers for surface fish entering at resurgences along the Río Tampaón (28.5 m above sea level masl) or into the Río Valles (48 masl). Chiquitita's bottom is at 27 masl., Chica at 30, Cuates at 37, and Toro at 88 [11]. All these caves have mixed populations with epigeomorphic fish and troglomorphic fish with mitochondrial A haplotypes. In the central Sierra de El Abra caves, populations are highly troglomorphic, with little incidence of hybridization, and harboring mitochondrial B haplotypes. Kopp et al. [13] have proposed a biogeographic divide that isolates both *Astyanax* cavefish and mysid cave shrimps in the central Sierra de El Abra caves from those in the southern Sierra de El Abra. The southernmost cave population of the central Sierra de El Abra group is Curva, whose bottom is at 113 masl. The central and southern portions of the Sierra de El Abra are separated by distance and altitude barriers, plus there is the remnant of a relict canyon where the Río Tampaon/Valles crossed farther north before plate tectonics elevated the southern portion [10]. This relict canyon is used by the highway that connects Ciudad Valles with Tamuin (Figure 11A). It has been estimated that the Río Tampaon/Valles changed its northern course to its current locality about 2.78–0.69 mya [10]. The authors argued that the southern portion of El Abra was elevated, and cave erosion could not start until after that time [10]. Caves north of this southern, low-lying sponge may effectively be isolated from surface fish immigrants, with the exception of Sótano de Yerbaniz and Sótano de Matapalma, where surface fish are flushed into entrance pits [11].

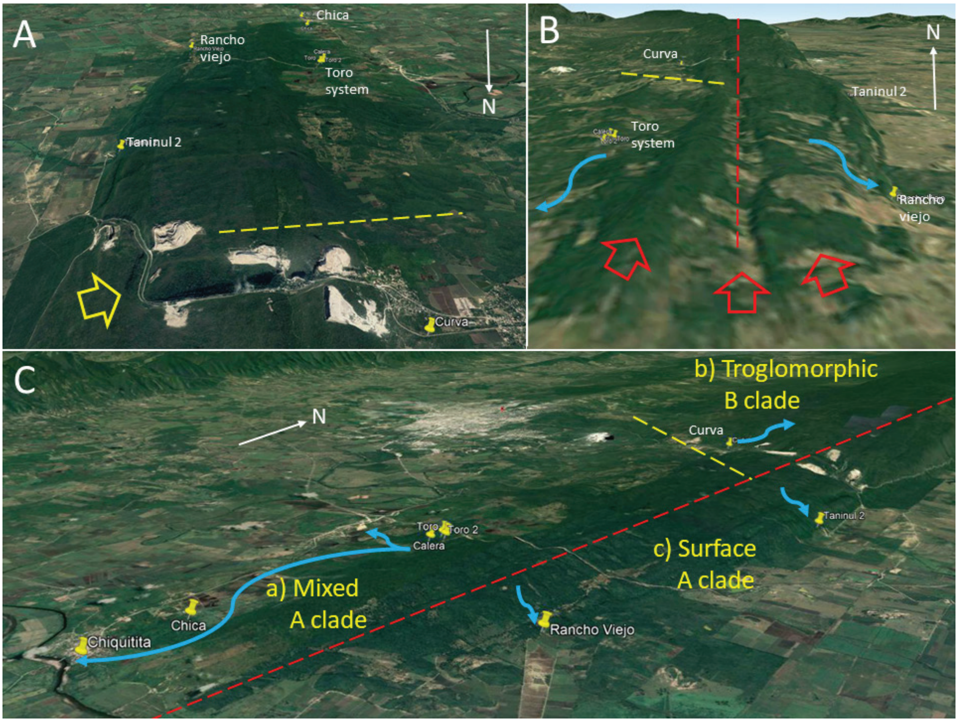

Figure 11. Model of biogeographical barriers (dashed lines). (**A**); There is a difference in altitude (Toro at 88 masl and Curva at 132 masl) and a fossil canyon (Yellow arrow) that divides the central and low-lying Southern sections of the Sierra de El Abra (**B**); The southern El Abra ridge is made of two crests and a valley (Red arrows). It is proposed that these tectonic structures may have promoted cave development resulting in two watersheds (blue arrows). (**C**); The combination of these two biogeographic barriers has prompted the development of three differently isolated communities: (a) In the low-lying southwest, *Astyanax* populations are mixed, and both cavefish and mysid shrimp harbor haplotype A markers; (b) To the north of the canyon, *Astyanax* populations are mainly troglomorphic, with little hybridization with surface fish, and both cavefish and mysids harbor haplotype B; (c) To the east, only surface fish are encountered in the caves. Map showing location of Ciudad Valles, San Luis Potosi. Google Earth, earth.google.com/web/.

A restriction to this low-lying sponge model of easy fish dispersal lies to the east. Both Cueva del Rancho Viejo and Taninul # 2 have a bottom at about 50 masl. Neither of them has evidence of cavefish. Only surface fish that derived from the nearby local surface streams inhabit them. Barriers on the east flank of the Sierra de El Abra for *Astyanax* cavefish dispersal seem to be prevalent throughout the range. None of the caves or resurgences on the eastern side have troglomorphic *Astyanax*. Along the southern Sierra de El Abra ridge there are two crests divided by a valley. This is probably linked to a synclinal and two anticlinal folds that comprise the southern El Abra limestone ridge (Figure 11B). We suggest that these ridges and valley may create a divide for cave development that effectively subdivides the hydrologic systems: A western one that drains toward the Río Valles at the town of Ojo de Agua and/or to Río Tampaon at Chiquitita resurgence, and an eastern one that drains to Cueva del Rancho Viejo, Nacimiento del Río Choy, and/or other nacimientos on the eastern escarpment.

In conclusion, we suggest that the combination of tectonics, altitude difference, ancient canyons, ridges, and valleys has affected the karst development of caves in the central and southern Sierra de El Abra. There are barriers for cave-to-cave and surface-to-cave migration and gene flow. Such barriers have affected whole stygobitic communities, as evidenced by the congruence of phyletic markers in both cavefish and mysid shrimp. For *Astyanax*, this has resulted in a group of caves in the central Sierra de El Abra with few instances of hybridization with surface fish and troglomorphic fish harboring mitochondrial haplotype B. In the southwest, there is a population of mixed troglomorphic, epigeomorphic and assumed hybrids which harbor haplotype A, and to the east there are caves with strictly epigeomorphic fish which also harbor haplotype A (Figure 11C). Finally, the central Sierra de El Abra is isolated from the northernmost Pachón cave which harbors the same A mitochondrial haplotype as the southernmost caves studied here. Mitchell et al. [6] proposed a drainage divide, in which the northern Sierra de El Abra drained to Nacimiento del Río Mante and the central and southern portions drained to Nacimiento del Río Choy. The exact location of this barrier is close to Sótano del Venadito, which as of today remains to be genotyped.

3.2. Central Sierra de El Abra

3.2.1. Background on Sótano de la Tinaja

A general rule has been invoked to understand the distribution and barriers for dispersal of cavefish [6]. Mainly, that downstream barriers may not be effective to prevent dispersal of cavefish of fish or their eggs because they can simply be flushed down waterfalls. On the contrary, upstream waterfalls may act as effective barriers. Thus, fish may be absent from pools in high passages but not from the lower passages. Based on previous descriptions [6,11], there is one conspicuous exception. Sótano de la Tinaja is described as having cavefish in many pools in the upper levels of the cave, but not below the "Downstream Canyon". This canyon descends steeply from −31 to −82 m depth in a series of four pits, the longest being 18 m. This gallery reaches a large lake room 107 m long, 9 m wide, and 30 m high. The surface of this lake marks the deepest surveyed point in the cave. This lake has yielded a stygobitic shrimp, *Troglomexicanus perezfarfantae* Villalobos, 1974. Curiously, no cavefish have been sighted here. This lake appears to be the major collecting point for flood waters. With the exception of the lake at the cave's deepest point, cavefish have been encountered in many pools and lakes in all the major passages" [11]. It is surprising that biologists visiting the caves in the 1970's did not report the presence of cavefish in the lower levels [11], since there is apparently no barrier for the fish to flow downstream. Therefore, we carried out a new expedition to explore this lake.

3.2.2. Results from Sótano de la Tinaja

Contrary to previous reports [11], *Astyanax* cavefish were abundant in this lake. While no precise method was used for estimating density of fish, a review of underwater videoclips taken in the field showed at least one cavefish for about every 5 m^2. The Tinaja lake has a surface area of about 1000 m^2.

We also observed abundant *T. perezfarfantae* shrimp and the mysid shrimp S. *quinterensis* at densities of about one shrimp of either species every 5 m^2 (Figure 12A,B,E). Less abundant were *Speocirolana pelaezi* Bolivar & Pieltain, 1950 isopods (Figure 12C). Both in the field and later in the laboratory, where cavefish and shrimp were hosted together alive, it was noticed that cavefish did not respond or initiate feeding behavior when close to the shrimp. Cavefish could even bump with a *T. perezfarfantae* shrimp in their swimming paths and not induce feeding behavior. When *Astyanax* was provided with commercial fish food, they immediately conducted feeding behavior, indicating that it was not lack of hunger that prevented them from feeding on the shrimp. Mysid shrimps have also been seen to cohabitate with *Astyanax* at other cave pools in Caballo Moro, Pachón, Sabinos, and the Toro cave system.

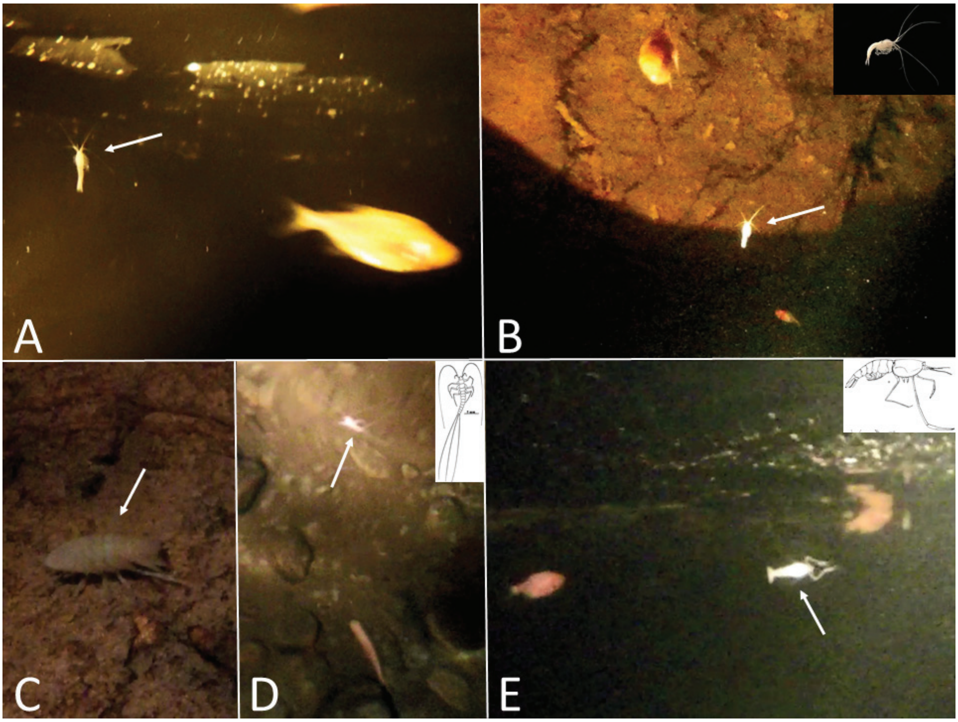

Figure 12. Stygobites and troglobites that cohabitate with *Astyanax*, but nonetheless were not seen to actively stimulate feeding behavior on the cavefish despite swimming less than 20 cm from them. (**A**,**B**); Mysid shrimp *Spelaeomysis quinterensis*. (**C**); Isopod *Speocirolana pelaezi*. (**D**); Nicoletiid insect *Anelpistina quinterensis* using surface tension to walk above water. €; Decapod shrimp *Troglomexicanus perezfarfantae*. (**A**), Cueva de Los Sabinos. (**B**–**E**), Sótano de la Tinaja. Inserts in (**B**,**D**,**E**) show higher quality images of organisms of the same genus. All photos were taken in the field. Arrows point to the arthropods.

Another troglobite that did not trigger feeding behavior was the terrestrial nicoletiid insect *A. quinterensis*. While normally it is seen in mud banks, on one occasion an individual was filmed on the water using surface tension to walk. Cavefish swimming just about 20 cm under them did not respond (Figure 12D). On the contrary, a surface cricket that jumped into a pool immediately attracted cavefish and was eaten.

3.2.3. Discussion: Central Sierra de El Abra

Previous reports [6,11] specifically said that "no cavefish have been sighted" in the deepest lake of Tinaja cave. Our results showed that, on the contrary, this lake has an abundant population of cavefish. Downstream underground waterfalls do not appear to prevent cavefish dispersal since they can simply be carried down waterfalls. Since the previous authors were able to see the shrimps that inhabit this lake and thus were giving proper attention to the organisms inhabiting this lake, it is unclear if they simply just failed to notice the fish, or abundance of fish was much lower when they explored this portion of the cave. Of ecological interest, it was also found that while *Astyanax* cavefish have evolved a host of enhanced senses to find food in the darkness [1,17], potential stygobite and troglobite prey may have also coevolved to reduce detection or being preyed upon. Thus, they live side by side with *Astyanax* in the same pools and lakes. It may be that adult shrimp are not the standard food of cavefish, and thus they can cohabitate in this lake together.

3.3. Tamasopo Area

3.3.1. Background on Cueva del Fraile

Cueva del Fraile is two km west of the town of Agua Buena, San Luis Potosí, México. It is in the Tamasopo valley, which is isolated upstream from the general El Abra region by the 100 m high waterfall of Tamul. In a review of *Astyanax* biogeography [11], it was reported that in 1991 three cavers from San Luis Potosí, México, explored Cueva del Fraile and that "about 30 to 50 m inside there was a pool up to 1 m deep, with 15 or 20 pale, eyeless fishes. They got a close look at the fishes in the 2 by 6 m pool, which seemed to be the beginning of a stream passage, but their light was flickering and they had to retreat." The report further says that on November, 2017, Juan Cancino Zapata, a speleologist from Río Verde, San Luis Potosí, and a member of Asociación Potosína de Montañismo y Espeleología (APME), searched for the cave at the request of one of the original explorers. He obtained GPS coordinates and photos. While this was the same cave recalled by the original cavers, Juan Cancino Zapata did not see any fish.

3.3.2. Results from Cueva del Fraile

This is a complex cave which, to our knowledge, has yet to be mapped. Our exploration showed that Cueva del Fraile is a resurgence, active only during the rainy season (Figure 13A). The cave is about 200 m away and 50 m above a resurgence that flows year-round (Figure 13B). It is possible that both are connected hydrologically, and that Cueva del Fraile is the high-water discharge conduit. Many galleries in Cueva del Fraile show evidence of becoming phreatic, i.e. with water up to the ceiling during the rainy season (Figure 13D). In many sections fresh mud covers walls and ceiling, suggesting that large amounts of water can circulate through its passages, facilitating movements throughout the cave of any stygobitic organisms inhabiting it. Being a resurgence, water flows from the end of the cave, which is at higher altitude, to the entrance.

Galleries are typically at least 5 m high and wide. A few meters after the penumbra zone, a left trending gallery bifurcates. This is the shortest gallery. Several permanent pools are crossed, until a sump is encountered. In the right gallery a maze-like section divides again into two main galleries. The left one crosses several pools until reaching an estimated 15 m pit with a lake at its bottom. Exploration stopped here. The other main gallery also has several pools. It ends at a large room about 30 m high that is covered with fresh mud and blocked by a large collapse from which water flows out of cracks in the rainy season.

This cave has many pools, some of them quite large (about 15 m long), which remain full of water throughout the year (Figure 13C,D). During the rainy season the pools are connected in a mostly continuous, water filled conduit. Some pools are inhabited by stygobitic isopods similar to those found in the El Abra caves inhabited by *Astyanax* (Figure 13E). Bats are also encountered over many of these pools, possibly providing food for cave-adapted fish. When compared to the El Abra caves, Cueva del Fraile would appear to provide a perfect habitat for cavefish [21,22]. However, despite careful searching in two independent trips, including snorkeling with scuba diving lights in one of the sumps, we did not find cavefish in any of the pools.

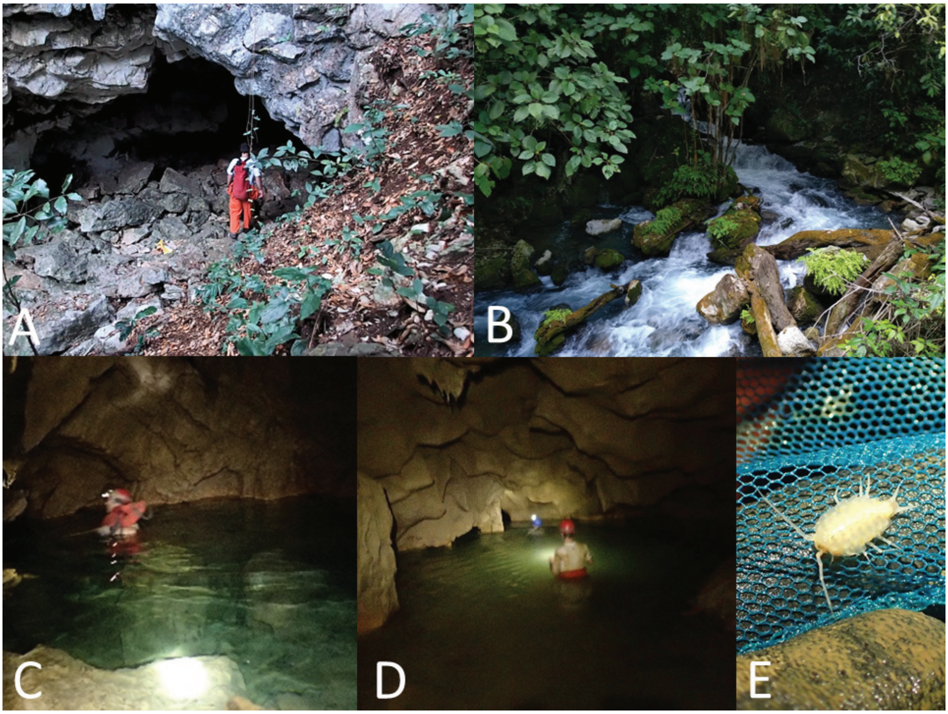

Figure 13. Cueva del Fraile in the Tamasopo area. Despite this cave apparently having habitats perfectly suited for *Astyanax* cavefish, none were found. (**A**); Entrance to Fraile cave. (**B**); year-round active resurgence near Cueva del Fraile. (**C**,**D**); Cueva del Fraile has many pools and lakes. (**E**); Aquatic cave-adapted isopod found in some of these pools. Notice that the roof on D is covered with mud, and shows erosion typical of passages where water reaches the ceiling during the rainy season.

3.3.3. Discussion: Tamasopo Area

Due to epigean *Astyanax* showing nyctophilia, i.e. a preference for the darkness [7], surface fish are adept at entering and colonizing the cave environment. In the El Abra region, fully pigmented and eyed fish of presumed surface origin have been found in Cueva de El Mante, Cueva del Rancho Viejo, Cueva Chica del Arroyo Seco, Taninul #2, and Nacimiento del Río Coy ([6,11], and results herein). Additionally, of the 33 described caves with troglomorphic cavefish, 13 have presumed surface fish. Therefore, surface fish are found in at least 18 caves in the El Abra region. Epigean *Astyanax* fish are found throughout the streams of México. Likewise, karstic areas with thousands of caves are found throughout México. This should offer scores of potential sites for the colonization to the underground by *Astyanax*. Nonetheless, if we exclude the two caves in the state of Guerrero which are inhabited by a different species, *A. aeneus* [7,23], all cave adapted *A. mexicanus* ssp. *jordani* populations are found exclusively in the El Abra region. With so many potential places available to establish cave-adapted populations, why is it that only the El Abra area has troglomorphic *A. mexicanus* ssp. *jordani*? It would appear that for some reason, this area had unique and exceptional conditions that allowed for an evolutionary event that culminated in a singular wave or waves of successful adaptation(s) to the underground environment.

The El Abra region is comprised of the Sierra de El Abra and the Sierra de Guatemala, two continuous limestone ranges, plus the adjoining karstic area of Micos, which is only one valley away from the Sierra de El Abra. This close proximity allows for gene flow [9] of adaptive genes among caves. We assume this is done either by direct underground migration between cave systems as underground drainages are eroded and sequestered, or by local surface populations potentially carrying some cave adapted alleles as "stepping stones".

In an effort to better understand if there was a "singularity" in the El Abra region for *Astyanax* cavefish adaptation, Cueva del Fraile in the Tamasapo valley was visited. Cueva del Fraile had an unconfirmed report of eyeless, depigmented fish [11]. The Tamasopo valley is in a different hydrologic system from the rivers in the El Abra, isolated by the 100 m vertical waterfall of Tamul, which prevents upstream migration from the Río Tampaón. Caves in the Tamasopo valley represent a separate karst area from the Sierra de El Abra, and that hydrologic isolation should have prevented significant flow of adaptive cavernicole genes from El Abra. Therefore, finding a cave-adapted fish population in the Tamasopo valley would indicate a truly independent evolutionary event.

After two failed exhaustive searches for cavefish in Cueva del Fraile, it is our opinion that this cave has no population of eyeless fish. The 1991 report [11] which stated that at about 50 m inside the cave there were eyeless fishes should be considered erroneous. We do not assume that the population went extinct. The report says that although they observed the fish up close, their light was flickering and they had to retreat. Thus, there is the possibility that they actually could not precisely identify the morphs of the fish due to the light limitations, which were already flickering just 50 m away from the entrance. Our assumption is that in 1991 Cueva del Fraile, being a resurgence, probably had surface fish which managed to swim upstream and enter the cave, getting stranded in this pool near the entrance. Many surface fish get pale when kept in the darkness for extended periods. We assume the fish they saw were simply accidental surface fish and not permanent or stable inhabitants of the cave.

Our observations do not support that a troglomorphic population exists deeper in the system. The reason is that barriers for the dispersal of fish tend to be upstream when vertical waterfalls are encountered, not downstream as fish get flushed down waterfalls, especially if passages become phreatic. Cueva del Fraile is a resurgence, and thus at the bottom of the underground hydrologic system. The base of a hydrologic system should be the most accessible part for fish. We propose that with so many suitable large pools available during the dry season in Cueva del Fraile, eyeless fish are not encountered simply because no population of cavefish inhabits this hydrologic system. Our conclusion is supported by the November 2017 report from a speleologist and APME member who searched for the cave at the request of one of the original explorers and also did not see any fish.

Was there a "singularity" in the El Abra region for *Astyanax* cavefish adaptation? While absence of evidence is not evidence of absence, our results further support that the El Abra region experienced exceptional conditions that allowed for a unique evolutionary event. This is supported by decades of studies by cavers and cave biologists throughout México that have failed to find any troglomorphic *A. mexicanus* ssp. *jordani* in caves outside of the El Abra region [6,7,11].

4. Conclusions

(1) Two new caves have been found to host cavefish, Sótano del Toro #2 and Sótano de La Calera. These two caves likely interconnect with Sótano del Toro, making up a single hydrologic system.

(2) Previous reports had suggested that Sótano del Toro was inhabited by only troglomorphic fish. Our results show that on the contrary, this system is inhabited by a mixed population of fully eyed and pigmented fish to fully eyeless and depigmented fish.

(3) There is a correspondence in phylogeographical patterns between *Astyanax* cavefish and the stygobitic mysid shrimp *Spelaeomysis quinterensis*. *Astyanax* mitochondrial DNA and mysid histone H3 DNA sequences show that, in both species, cave populations in the central Sierra de El Abra are broadly different from other cave populations. (see also [24]). This phylogeographical convergence supports the notion that the central Sierra de El Abra is a biogeographical zone with effective barriers for either cave-to-cave or surface-to-cave gene flow, which have modulated the evolutionary history of its aquatic community.

(4) The southern portion of the Sierra de El Abra, which includes the Chiquitita, Chica, Cuates, and Toro caves, is a low-lying karst area with apparently few barriers to fish movement, including immigrant surface fish. While undoubtedly there is a lot of underground hydrologic connectivity between many caves [10], there are also biogeographical barriers. Mitchell et al. [6] proposed that there was a hydrological divide somewhere in between Cueva del Pachón and the central Sierra de El Abra caves. Kopp et al. [13] further proposed that there was a biogeographic divide that isolated the central from the southern Sierra de El Abra, which affected both *Astyanax* cavefish and mysid cave shrimps. Our study helped to pinpoint that this divide is somewhere between the Toro and Curva caves. We further propose that there is a third biogeographical barrier to the east. While resurgences on the east side of the El Abra have surface fish and habitats apparently amenable for troglomorphic fish, it appears blind cavefish have not been able to colonize them.

(5) Previous reports had suggested that there were no cavefish in the deepest lake of Tinaja cave. This was an anomaly as downstream barriers are not effective for the dispersal of cavefish since fish (or eggs) can simply be flushed down waterfalls. Our results showed that, contrary to the report, this lake has an abundant population of cavefish.

(6) While *Astyanax* cavefish have evolved a host of adaptive features to help them find food in the darkness, they cohabitate with two stygobitic crustaceans: *T. perezfarfantae* shrimp and the mysid shrimp S. *quinterensis*. Cavefish can be seen swimming barely centimeters away from these crustaceans. Despite being perfectly sized potential prey, they were not seen to activate feeding behavior. Likewise, a troglobitic nicoletiid insect, A. *quinterensis*, was seen walking over water using surface tension without attracting the cavefish. It would appear that while cavefish have evolved to have an enhanced sense of smell [17] and fine-tuned vibration attraction behavior [25,26], among other adaptations to find food in the darkness, potential stygobite and troglobite prey have also coevolved so as to not be detected or be preyed upon.

(7) Report of a cave (Cueva del Fraile) hosting troglomorphic *Astyanax* in the Tamasopo valley, which is outside the general El Abra and Micos contiguous areas, was rejected. Despite decades of cave exploration by cavers and biologist, not a single cave outside the El Abra hydrologic area has been found to host troglomorphic *A. mexicanus* ssp. *jordani*. This supports the concept that the El Abra region experienced exceptional conditions that allowed for a unique evolutionary event resulting in the adaptation to the cave environment by these fish.

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Are the Neglected Tipuloidea Crane Flies (Diptera) an Important Component for Subterranean Environments?

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Abstract: Usually, biospeleological studies focus on cave-specialist taxa showing strong adaptation to the subterranean environment, as their unusual morphological and ecological features represent intriguing case studies. On the other hand, species occurring in subterranean environments but without marked adaptations have been generally overlooked, probably because they are thought to be accidental and not very important for the ecosystem. Particularly exemplificative is the case of Tipuloidea crane flies (Diptera), which although abundant, are rarely considered in biospeleological studies. Here, by analyzing the crane fly occupancy, we observed that individuals occur within the shallowest areas of subterranean environments throughout the year, with a peak of presence during hot season. Crane flies occupy dark and warm areas close to the connection with surface and with smoother walls. Furthermore, we observed that the presence of crane flies is positively related to the abundance and the richness of cave predators, highlighting their importance for the sustainment of the local community. With this study we aim to stimulate future researches on these important, but still neglected cave species.

Keywords: Diptera; *Limonia*; *Limonia nubeculosa*; *Neolimonia*; Trichoceridea; cave community; biospeleology; cave biology; prey; food resources; occupancy; abundance

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1. Introduction

Studies on cave adapted species have gained large interest during the last decades, a trend demonstrated by the growing available literature [1–5]. The high interest in subterranean environments and its biota is often related to the peculiar study-cases offered to taxonomists, ecologists, and evolutionary biologists [6]. Indeed, subterranean environments have represented (and still represent) a source of unknown and bizarre organisms that help understanding adaptation and evolution in extreme environments [7–9]. The appearance of troglomorphic characters (e.g., reduction in pigmentation, anophthalmia, and elongation of appendages) is related to the peculiar ecological conditions of subterranean environments, which are very different from those found in any surface ones [1]. The subterranean environments show high stability and have a natural microclimatic gradient making them an excellent natural study-system [10–12]. The most obvious difference is the lack of light, a condition which not only contributes in reducing the fluctuation of the subterranean microclimatic conditions, but also impedes the colonization of plants, therefore strongly limiting the availability of food resources [1]. The lack of light also means that the sense of sight is useless and subterranean species often rely on alternative senses to perform their natural activities [13–15]. A missing day–night cycle in subterranean environments promotes a prolonged stability of the inner microclimate, which is more evident in the deepest areas not reached by incoming light [10,16].

A large number of species with different degree of adaptations can be found within subterranean environments. The most specialised species are called troglobionts; these animals are usually found in the deepest areas laying in complete darkness and are unable to reproduce in surface environments [17]. Troglobionts often show the well-known morphological, physiological (e.g., resistance to starvation), and behavioral (e.g., loss of fear) adaptations occurring in cave animals [18–20]. Troglophiles can maintain stable populations in subterranean environments, but are still able to exit and reproduce in both surface and subterranean environment [6]. These animals can be found throughout the subterranean environment (but generally not too far from the connection with surface) and might show specific traits suitable for subterranean life [21–23]. Trogloxenes are species usually found within the first meters from the cave entrance, as these animals usually do not show evident adaptations to the cave environment, and thus are unable to persist in the deepest parts [24]; these species can successfully reproduce only outside caves or in proximity of the entrance [6,17]. The species showing higher degree of adaptation to subterranean life (troglobionts and troglophiles) have been intensively studied and used as model species in a large number of researches, from macro-ecology to evolutionary studies [25–28]. On the other hand, the interest on trogloxenes has often lagged behind, probably because these species have been thought to have little importance for the subterranean environment [29].

This misleading idea has been proven wrong by recent research, which highlighted that some species traditionally considered to be “occasional” actually show strong ecological relationships with the underground environment, and play a major role in subterranean ecosystems [30–32]. Some of such neglected species are the Tipuloidea crane flies (Diptera). Crane flies represent a large taxonomic group (>3300 described species for Palaearctic; [33]) with at least two genera, *Limonia* Meigen, 1803, and *Neolimonia* Alexander, 1964, widespread in Italy [34]. Generally, crane flies show similar size and habitus: the typical features are an elongated yellow-brownish abdomen and wings with particular venation and dark pigmentation [35] (Figure 1A). Crane flies inhabit forested areas [36,37], but are also found in subterranean environments, particularly from spring to autumn, where they shelter from external unsuitable climatic conditions (i.e., when it is too hot and dry) [38–41]. Considering the lack of specific adaptation to cave life, crane flies are usually observed in areas not too far from the connection with the surface, where they can form dense aggregations (Figure 1B) [39,42]. This condition probably makes tipuloids one of the most abundant taxonomic groups in the cave-entrance; thus, representing the most abundant prey for several cave-dwelling predators [43–46].

Given the scarce quantitative ecological information on crane flies, our aim is to study the use of subterranean environments by these species and evaluate its potential relationships with cave-dwelling predators, as crane flies may represent an important food resource in an ecosystem characterized by constant scarcity [1]. Although, in the literature, only the presence of *Limonia nubeculosa* Meigen, 1804 is generally reported within Italian subterranean environments [30,39,47], the broad distribution of multiple species with similar habitus makes challenging their identification without manipulation [34,35]. Therefore, to avoid potential misidentification, we refer here to the whole superfamily Tipuloidea. We focused in assessing the major environmental features related to the occupancy of tipuloids within subterranean environments. Despite no specific adaptation toward the cave life, crane flies regularly frequent these environments [30,39], and previous studies suggested that ecological relationships with the cave environment can be extremely strong even for some species traditionally considered to be “accidental” [31,48]. Furthermore, we evaluated relationships between the distribution of crane flies and cave predators, as crane-flies can represent a major food resource in prey-deprived environments [30,43].

Figure 1. Two photos showing crane flies in subterranean environments: (**A**) two individuals mating and (**B**) high density aggregation. Photo credit: Enrico Lunghi.

2. Methods

2.1. Study Sites and Surveys

Fifteen subterranean environments located in the north of Tuscany (Italy) have been monthly surveyed throughout a year (2013). Three of them are semi-natural environments, one is a subterranean drainage tunnel, while 11 are natural caves (Table 1). These sites show a specific microclimatic gradient, going from the entrance to the deepest sectors of caves [10]. The microclimate of subterranean environments is affected by the influence of external climatic conditions especially through the opening connecting them with the surface [1]. Consequently, the areas close to the entrance have microclimatic conditions similar to those found outside. On the other hand, the external influences become weaker with depth and the microclimate of the deepest areas do not experience the same variability, but rather high stability [11,12,16]. We surveyed the 15 subterranean environments following a standardized procedure [49]. The environments were subdivided in sections of three linear meters (hereafter, sector) from the entrance (i.e., the main connection with the surface) to the deepest point reachable without the use of speleological equipment. At the end of each sector, multiple environmental data on both cave morphology (height, width, and wall heterogeneity) and microclimate (temperature, humidity, and incident light) were recorded [10]. Sector height and width were recorded using a laser meter (Anself RZE-70, accuracy 2 mm), while the wall heterogeneity was estimated by unrolling a one-meter length string following the vertical wall shape, then with a tape meter, we measured the linear distance between the two string extremities [50]. A Lafayette TDP92 thermo-hygrometer (accuracy: 0.1 °C and 0.1%) was used to record sector temperature and humidity, while a Velleman DVM1300 light meter (minimum recordable light: 0.1 lx) was used to record the minimum incident light. For further details on data recording refer to Lunghi, Corti, Mulargia, Zhao, Manenti, Ficetola and Veith [49].

Table 1. List of the surveyed subterranean environments. For each Site we provide: Latitude (N), Longitude (E), Elevation (m a.s.l.), Origin (N = natural; S = semi-natural; and A = artificial), and the number of surveyed Sectors. Latitude and longitude are shown with reduced precision to increase species protection [51].

Site	Latitude	Longitude	Elevation	Origin	Sectors
Site1	44.03	10.25	872	N	5
Site2	43.97	10.53	91	N	11
Site3	43.93	11.16	624	N	10
Site4	44.04	10.25	889	N	5
Site5	44.04	10.25	875	N	3
Site6	43.92	11.14	286	N	4 *
Site7	43.92	11.14	319	N	17
Site8	44.00	10.82	948	S	4
Site9	44.00	10.82	853	S	5
Site10	44.00	10.82	850	S	7
Site11	44.04	10.86	744	A	6 *
Site12	43.92	11.16	699	N	2
Site13	43.92	11.16	715	N	14
Site14	43.97	11.16	492	N	20
Site15	44.06	10.31	556	N	8

* During particular periods, some sectors were inaccessible due to flooding.

We surveyed each sector to assess the presence of crane flies through Visual Encounter Survey (VES) [52]; sectors were surveyed with similar effort to limit bias due to imperfect species detection [53]. Adopting the same methodology, we also recorded the presence and the abundance of five predators (traditionally considered to be troglophiles or trogloxenes) usually occurring on cave walls: the cave salamander *Hydromantes italicus* (Dunn, 1923), and four spider species, *Meta menardi* (Latreille, 1804), *Metellina merianae* (Scopoli, 1763), spiders of the family Agelenidae C. L. Koch, 1837, and *Amaurobius ferox* (Walckenaer, 1830) [10,48,54].

2.2. Statistical Analyses

We first estimated the detection probability of crane flies; the detectability of small cave species usually is <1 [53,55]. We calculated the species detection probability on the base of 35 pairs of survey performed within 14 days; this ensures to meet the assumption of population closure [55]. We built a model including the linear distance from the entrance (hereafter, depth) as potential variable affecting the species occupancy suitability. Starting from this, we built a second model adding the wall heterogeneity as potential variable affecting species detection. Models were compared following the Akaike Information Criterion (AIC) [56] and the best one (with lowest AIC value) used to estimate the detection probability of crane flies.

We then used a binomial generalized linear mixed model to assess which environmental factors influence the occupancy of crane flies in subterranean environments [57]. Their presence/absence was used as dependent variable, while the morphological and microclimatic features of cave sectors, along with depth, as independent variables; site and sector identity were used as random variables. We included as further independent variables the interaction between month of survey and microclimatic features. Considering the imperfect detection of tipuloids (see Results), we weighted their absence with the estimated detection probability [58]. The values of AIC corrected for small samples (AICc) were used to estimate a set of positive Akaike weights w_i summing to 1, and used to rank the models; the one with the highest weight was the best [59]. The significance of variables included in the best AICc model was tested using the likelihood ratio test [60].

Moreover, to assess the relationships between the occurrence of crane flies and cave predators we also built two linear mixed models [61]. We used predators' abundance (total N of individuals) and richness (total N of species), respectively, as dependent variables, the presence/absence of crane flies as independent variable, cave, and sector identity as random factors.

3. Results

We performed a total of 1417 cave sector surveys (Table 1) and we detected crane flies in 709 of them; both the number of occupied sectors and their average depth increased during the hot season (Figure 2). The microclimatic features at which Diptera occurred were: air temperature, average = 12.18 ± 3.08 (SD), max = 25.7, min = 2.5; humidity, average = 89.16 ± 4.95, max = 96.5, min = 47.2; and minimum illuminance, average = 0.53 ± 3.22, max = 58.2, min = 0.

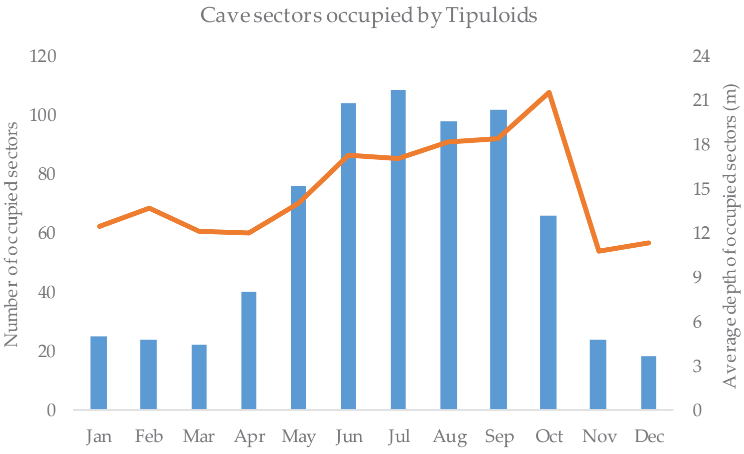

Figure 2. Number and distribution of occupied cave sectors. The plot shows the monthly number of cave sectors occupied by crane flies and their average depth (linear meters from the connection with surface).

The best model estimating detection probability of crane flies did not include the covariate wall heterogeneity (AIC = 625.49 vs. 626.63); the model suggested that crane flies have a detection probability of 0.58 (SE = 0.05).

The best-AIC model suggested that the occurrence of crane flies within caves was significantly related to temperature, illuminance, sector depth, wall heterogeneity, to the month of survey and its interaction with temperature (Tables 2 and 3). Crane flies occupied sectors close to the cave entrance, showing low illuminance, relatively high temperature and smooth walls; during the hot season, the species occupied colder sectors.

Table 2. Five best corrected Akaike Information Criterion (AICc) models relating crane flies presence within cave sectors. We considered as dependent variable the presence of the species. We used as independent variables: sector Width, Height, Depth, wall heterogeneity (Het), sector humidity (Humid), illuminance (Lux) and temperature (Temp), Month of survey and its interaction between microclimatic features (×). For each continuous variable, the regression coefficient is reported if the variable is included into a given model. For categorical variables and interactions, + indicates that the variable or the interaction is included into the model. If not shown in table, the variables were not included in the first five best AICc models. The best model is highlighted in bold.

Independent Variables Included in the Model									**df**	**AICc**	**Δ-AICc**	**Weight**
Depth	**Het**	**Humid**	**Lux**	**Temp**	**Month**	**Width**	**Height**	**Temp × M**				
−0.085	−0.07		−0.62	0.18	+			+	29	589.9	0	0.164
−0.084			−0.62	0.18	+		0.07	+	29	590.4	0.44	0.131
−0.079	−0.07	−1.98	−0.64	0.20	+			+	30	590.9	0.99	0.100
−0.084			−0.59	0.18	+	−0.06		+	29	591.3	1.39	0.082
−0.078		−2.03	−0.62	0.2	+		0.06	+	30	591.3	1.40	0.081

Table 3. Parameters related to the presence of crane flies within cave sectors. The significance of variables included in the best AICc model are shown; significant variables are in bold.

Factor	***B***	**χ^2_1**	***P***
Temperature	0.18	37.98	<0.001
Month		267.57	<0.001
Sector	−0.09	0.01	0.001
Wall heterogeneity	−0.07	13.83	<0.001
Lux	−0.6	22.04	<0.001
Month × Temperature		27.79	0.003

Finally, we found a strong, positive relationship between the presence of crane flies and both predator species' abundance ($F_{1,1295} = 4.71$, $P = 0.03$) and richness ($F_{1,1295} = 12.95$, $P < 0.001$).

4. Discussion

Crane flies occurred within the subterranean environments throughout the year, although there was a clear peak during the hot season (Figure 2). These species, as many other taxa without specific adaptations, probably seek refuge in subterranean environments when outdoor climatic conditions become too dry and hot [10,30,39]. Indeed, when species are strongly dependent on fine scale environmental conditions [62], they need to track suitable microclimate when the local conditions become too harsh [63]. Occupancy was the highest in areas not far from the connection with the surface, where microclimatic conditions are more influenced by external climate [10]. Dipterans, as many other trogloxenes, usually actively seek habitats closely connected to cave entrances, likely because they are phototactic and lack specific adaptations to cope with the peculiar environmental conditions found in the deepest areas (e.g., darkness and food scarcity) [64]. Considering the wide range of microclimatic variables at which Diptera occurred, it is likely that multiple species of Diptera occurred within the studied subterranean environments, but detailed studies are required to ascertain this hypothesis. Furthermore, the buffered microclimatic conditions found in the shallowest areas are probably suitable enough for crane flies; indeed, individuals mate and occur in large numbers (Figure 1). Tipuloids seems to be less linked to the subterranean environments if compared with Trichoceridea, as these Diptera have been usually found in areas distant from the connection with surface [65,66]. Cave walls having less irregularities seem to promote the occupancy of crane flies. A smooth wall may facilitate the attachment of individuals, so they probably choose this feature to facilitate their resting. On the other hand, a wall with pronounced irregularities may shield their presence from surveyor view, and reduce the overall species detection [53]. However, this hypothesis was not confirmed by the analysis of detection probability, as we did not find relationships between crane-fly detectability and wall heterogeneity.

Both richness and abundance of cave predators were positively related to the occurrence of crane flies. Subterranean environments in temperate areas are often food deprived [1], and cave predators have to develop specific adaptations to cope with that. Indeed, some species are able to reduce their metabolism to save energies and withstand prolonged starvation periods [67], others develop specific morphologies to store energy [68], while some others exit and forage on the surface [63]. Crane flies in subterranean environments can represent a large proportion of local biomass [69], thus being an important food resource for the local community; sure enough, dipterans represent one of the main food resources for multiple species [44,45] (Figure 3), as we often saw live and dead ones on different spiders' webs.

Figure 3. *Oxychilus draparnaudi* (Beck, 1837), a facultative cave-dwelling snail, feeding on a dead crane fly. In caves, crane flies are not only important for species directly preying on them, but also for the many scavengers. Photo credit: Simone Giachello.

Crane flies can be highly suitable prey for many cave predators. First, individuals show a particular aggregative behavior, and during the harsher months, they can form dense "clouds" on the cave walls (Figure 1B). The reason of such behavior is still unknown, but it provides to predators an easy way to catch multiple prey with minimum effort. For example the cave salamanders of the genus *Hydromantes* Gistel, 1848, catch their prey darting their protrusible tongue [70,71]; therefore, if they target a dense aggregation of dipterans they could even catch multiple individuals with one "shot". In fact, crane flies are the most abundant prey of European cave salamanders [72]. A second advantage is given by location. Compared to the surface, subterranean environments represent a safer place for many species, not only because the suitable microclimatic conditions, but also because the lower presence of potential predators [73–75]. Some predators mostly living in subterranean environments can exit caves for foraging, but this exposes them to several risks (e.g., predation or unsuitable climatic conditions), while hunting inside caves is likely safer [43,63].

This was the first study only focusing on the ecology of crane flies within subterranean environments of Italy. Even though many open questions remain, our study highlighted the importance of expanding ecological analyses toward often neglected cave-dwellers. Indeed, there are still so many gaps in the knowledge of the ecology and distribution of crane flies for both their surface and subterranean phase. For examples, we still have no information about species richness of crane flies within subterranean environments, their phenology and whether spatial segregation occurs. Indeed, in this study we attributed all Diptera observed within subterranean environments to the superfamily Tipuloidea, while similar species of the superfamily Trichoceridea can also occur in caves [66]; thus, further refinements are required to better comprehend the occurrence of the different Diptera in subterranean environments and their habitat preference. Nonetheless, we only analysed the main determinants of their occurrence, leaving unexplored all the causes affecting their abundance and aggregation. It would be also interesting to evaluate the energetic contribution provided by crane flies assessing their role in the food web, and evaluating how the flow of individuals between outdoor and subterranean environments affects the functioning of these ecosystems [76].

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Abiotic Community Constraints in Extreme Environments: Epikarst Copepods as a Model System

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Abstract: The general hypothesis that the overall presence or absence of one or more species in an extreme habitat is determined by physico-chemical factors was investigated using epikarst copepod communities as a model system, an example of an extreme environment with specialized, often rare species. The relationship between the presence or absence of epikarst copepods from drips in six Slovenian caves and 12 physico-chemical factors (temperature, conductivity, pH, Ca^{2+}, Na^+, K^+, Mg^{2+}, $NH_4{}^+$, and Cl^-, $NO_2{}^-$, $NO_3{}^-$, and $SO_4{}^{2-}$) was explored. Statistical analyses included principal components analysis, logistic mixed models, stepwise logistic multivariate regression, classification trees, and random forests. Parametric statistical analyses demonstrated the overall importance of two variables—temperature and conductivity. The more flexible statistical approaches, namely categorical trees and random forests, indicate that temperature and concentrations of Ca^{2+} and Mg^{2+} were important. This may be because they are essential nutrients or, at least in the case of Ca^{2+}, its importance in molting. The correlation of Cl^- and $NO_3{}^-$ with copepod abundance may be due to unmeasured variables that vary at the scale of individual cave, but in any case, the values have an anthropogenic component. This contrasts with factors important in individual species' niche separation, which overlap with the community parameters only for $NO_3{}^-$.

Keywords: extreme environment; niche; epikarst; classification trees; Copepoda; random forests

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1. Introduction

1.1. Background

Extreme environments, such as polar ice caps, hypersaline lakes, acidic peatlands, hyper-arid deserts, groundwater aquifers, and caves, often harbor a specialized fauna that is both numerically rare and patchily distributed. Rarity is typical in extreme environments for which resource scarcity is at least part of what makes them extreme. This resource scarcity is, with the exception of chemoautotrophic sites [1] and sites very near the surface [2], a universal characteristic of aphotic habitats, which lacks primary productivity. In addition, sites with extremes of temperature, such as polar ice caps, or aridity, such as hyper-arid deserts, are also typically very low primary productivity habitats. Sampling rare species and populations of such extreme environments presents unique statistical challenges itself [3], and analysis of ecological relationships among species and between species and their environment is especially difficult given the low numbers of individuals of any one species. Whilst classic ecological techniques like Canonical Correspondence Analysis [4] and Outlying Mean Index [5] address the relationship between species occurrences and abiotic parameters, the spatial distribution of taxa (i.e., occupancy) is still an unexplored question. This question is interesting because it addresses the question of the overall constraints to adaptation to extreme environments.

In extreme environments, there is often a suite of species that show specialized physiological, morphological, and behavioral adaptations to the environment. There are numerous examples in other extreme environments, such as stomatal modification of desert dwelling plants [6], and heat-resistant enzymes in species living in thermal vents on the ocean floor [7]. Convergent adaptation in extreme environments is perhaps best developed, or at least best studied, in the numerous species found in caves and other subterranean habitats. Species specialized for and isolated in subterranean environments typically have elaborated extra-optic sensory structures as well as reduced or absent eyes and pigment [8]. This convergent morphology is an expression of the constraints imposed by aphotic environments. Such communities of specialists, whether in thermal vents, caves, or other extreme environments, are often cited as examples of convergence and parallelism [9–13]. The study of the whole community of morphologically convergent species, rather than individual species, has yielded insights into not only adaptation but also community function (e.g., [14,15]).

1.2. Epikarst as a Model Extreme Environment

Epikarst is the shallow part of karst areas, where stress release, climate, tree roots, and geological processes (especially dissolution of rock) fracture and enlarge rock joints and cracks, creating a more porous zone than the underlying limestone [16]. It is directly beneath the soil, and retains water from infiltration, typically in a 3–10 m thick zone (Figure 1). It is subject to periodic drying and to periodic flushing of water that dislodge the plankton sized organisms that inhabit it [17]. Epikarst is aphotic, low in organic carbon [18] with habitat spaces that limit size and shape of animals occupying the space [19].

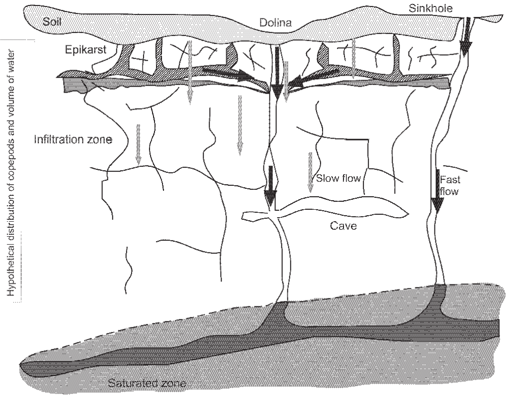

Figure 1. Conceptual model of epikarst. Gray arrows indicate the direction of slow water flow and black arrows are faster flow paths. From [18]. Used with permission of ZRC SAZU, Založba ZRC.

Copepods are the predominant group found in epikarst throughout the world, but other crustacean taxa occur regularly, including Amphipoda, Ostracoda, and Syncarida [2]. Sampling of the epikarst fauna is necessarily indirect, and the least biased samples are those that capture the invertebrates found in epikarst water dripping into caves [20,21]. Due to this indirect sampling, most environmental information is about the physical and chemical characteristics of the dripping water. A universal feature of such epikarst samples is that a significant number of samples have no animals (e.g., [20,22–24]).

1.3. Aims and Goals

The question we wish to examine is whether these rare, specialized communities are constrained in their spatial distribution by physical and chemical factors. It is common that not all extreme environments of a particular kind, e.g., caves, harbor a specialized fauna. In any region, a number of caves have no specialized fauna, although caves without species are not always reported (but see [25]). The absence of a specialized fauna from a particular site may be because of barriers to dispersal to the site, historical accident, or because the physico-chemical conditions at the site are unsuitable for the fauna in general.

Our hypothesis is that differences in chemical and physical factors among extreme habitats provide an explanation for the presence or absence of a specialized fauna from a particular site. To test this, we analyzed an extensive data set on the occurrence of copepods specialized for life in a shallow subterranean habitat—epikarst, sampled in central Slovenia [20,26]. Pipan [26] took > 250 monthly chemical and biological samples from dripping epikarst water in six caves in central Slovenia. Slightly more than half of these samples lacked copepods. The question then is how the physico-chemical parameters differed between the two kinds of samples (those with and without fauna), and whether there was a biological explanation for the difference. These epikarst copepod communities are especially appropriate for analysis because they are diverse and with variable densities among and within subterranean patches [27]. Thus, we can not only elucidate the important community-wide parameters, but we can also compare these parameters with the individual species parameters that differentiate their niches.

2. Methods and Materials

2.1. Study Area and Sampling Procedures

The study area is in the western Dinaric Mountains of Slovenia. Six caves were studied—Črna jama, Dimnice, Pivka jama, Postojnska jama, Škocjanske jame, and Županova jama (see [20]).

Direct sampling of epikarst habitats is not possible due to the small size of the cavities and their inaccessibility. Thus, the epikarst water and epikarst fauna must be sampled indirectly by taking samples of the percolation water that drips directly from the ceiling. To do this, water from trickles was directed through a funnel into a collecting container. On two sides, the plastic containers have 5 × 5 cm openings covered with a 60 μm diameter mesh to retain animals in the container. At monthly intervals, the water and the organisms in the containers were collected and preserved in 4% formalin. This sample is likely biased in favor or smaller organisms since they are more easily dislodged by water currents [1,2], but it is less biased than pool samples because there is differential survival and reproduction in pools [2]. For the purpose of this study, only the presence or absence of adult copepods was used for analysis. Juveniles were not included because adults should be a better representation of a permanent population [1,2]. In addition, a series of chemical and physical measurements were made at the time of the biological sampling.

Temperature and conductivity were measured in situ by a conductivity meter (WTW^{TM} Model LF 91); pH was measured by a pH meter (WTW^{TM} Model 323). Water samples for ionic analysis were collected and stored in plastic containers and kept at 4 °C prior to measurement. An ion chromatograph (Metrohm^{TM} Model 761 Compact IC) was used to analyze the concentrations of major ions. Chloride, nitrite, nitrate, phosphate, and sulphate were determined using an anion separation column. Sodium, ammonium, potassium, calcium, and magnesium were analyzed using a cation separation column. Concentrations of PO_4^{3-} were always below the level of detectability of 0.05 mg/L, and therefore not included in the analysis. Detection limits of ions ranged from 50 to 100 μg/L.

Sampling (Table 1) began in Postojnska jama, but when five months of sampling produced relatively few copepods, sampling was switched to other parts of the Postojna-Planina Cave System (Črna jama and Pivka jama). Sampling in three other caves in the region (Dimnice, Škocjanske jame, and Županova jama) was performed for the whole period. Five drips were sampled in each cave, except in Postojnska jama, where 10 drips were sampled. The copepod fauna in the studied drips shows no temporal correlation for each drip [21], and samples within and among drips are treated as independent.

Table 1. Summary of sampling coverage of the six study caves, by month. An X indicates that all of the drips in the cave were sampled for physico-chemical parameters, at least once. Data from [26].

Month	Postojnska Jama	Pivka Jama	Črna Jama	Škocjanske Jame	Dimnice	Županova Jama
Jan		X	X	X	X	X
Feb		X	X	X		
Mar					X	X
Apr	X					
May	X			X	X	X
Jun	X			X	X	X
Jul	X			X	X	X
Aug	X					
Sep	X			X	X	X
Oct	X	X	X	X	X	X
Nov		X	X	X	X	X
Dec		X	X	X	X	X

2.2. Statistical Methods

Summary statistics and exploratory analyses.—Stratified means and standard errors were calculated for each physico-chemical variable where strata were identified as each distinct combination of cave and month. In order to explore the relationships among variables, principal components for each cave were obtained for the same set of variables, in this case ignoring the sampling site effect. Thus, we assumed that the relationships among variables are the same regardless of the cave within which the samples were collected. A mixed model with the interaction of month and cave as a fixed effect and sampling site (drip) within cave as a random effect was run for each physico-chemical variable to assess temporal pattern within each cave. The residuals from each model of the physico-chemical variables were checked to verify that the assumptions of the mixed model were met. In some cases, transformations were required to obtain normality and homogeneous variance while in some of the other variables, the model was modified to allow unequal residual variances among months. Where relevant, pairwise differences of means were tested using the Tukey–Kramer method for controlling the experiment-wise error rate.

Modeling the probability of copepod presence in a sample. We ran an all possible subsets logistic regression analysis in which every possible subset of the physico-chemical variables was used as the predictor set. The models were ordered by number of variables and value of the χ^2 statistic for the overall test of significance. From these the variables identified in the most parsimonious model with the highest χ^2 value, they were chosen for a logistic mixed model for predicting probability of copepod presence with month fixed and sampling site (drip) within cave as a random effect was run.

One concern is that the actual relationship between the explanatory variables and the probability of copepod presence may be different than that assumed for a logistic regression model. In the logistic regression model all variables are assumed to be additive in their effect on the probability of presence, and that the relationship can be described by a logistic curve. In reality, a variable may have a threshold effect such that there is a discontinuous jump in probability at some level of the explanatory variable, or several variables could interact in their effect on the probability. To address these issues, the non-parametric random forest and classification tree approach was used [28–30]. The recursive partitioning approach known as random forests was used to provide individual measures of variable importance [29]. This utilizes a bootstrap method that allows for the detection of the relative importance of co-varying physico-chemical measurements. The basic approach is that sets of five randomly selected variables (out of our total of 12) are used to determine a classification tree. This is repeated many times (in our case 2000). For each tree, the important variables are recorded. This allows variables that may be masked by correlation with other predictor variables to be identified as important predictors. The best predictors from random forests were then used to construct classification trees. The predictor space is recursively partitioned into a set of rectangular areas such that samples with (or without) copepods are grouped. A probability of presence of copepods is predicted within each partition. The process is repeated until no improvement in prediction is possible. Confusion matrices showing the accuracy of prediction of the models were calculated. All computations were done in either R (cran.r-project.org), SAS© v.9.2 (SAS Institute, Cary, NC, USA), or JMP®v9.0.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. What Are the Physical and Chemical Conditions Present in Epikarst Water?

Table 2 summarizes the basic statistical properties of the stratified samples of physico-chemical variables. Overall, the physico-chemical values are typical for carbonate and calcium rich and slightly alkaline waters found in karst regions (Table 2). As is typical of carbonate waters, calcium dominated the cations, and pH was slightly above neutrality. Conductivity was high, a reflection of the relatively high concentrations of Ca^{2+}. The median temperature (8.9 °C) was very similar to the mean annual temperature for the region, approximately 9.0 °C.

Table 2. Basic statistics for measured variables, using stratified estimates (caves and months define the strata). There were 35 strata and 252 observations. All concentrations are in mg/L. Conductivity is in μS/cm. Temperature is in °C.

Statistic	Temperature	Conductivity	pH	Ca^{2+}	K^+	Mg^{2+}	Na^+	NH_4^+	Cl^-	NO_2^-	NO_3^-	SO_4^{2-}
Mean	8.16	356.64	7.79	37.71	0.47	0.93	1.36	0.096	2.34	0.0030	2.84	6.18
S.E. of Mean	0.106	4.641	0.012	0.747	0.013	0.025	0.086	0.005	0.219	0.0004	0.360	0.135
Median	8.91	365.00	7.73	40.60	0.41	0.91	0.95	0.084	1.44	0.000	0.78	5.95
S.E. of Median	0.043	4.91	0.012	1.47	0.013	0.027	0.043	0.002	0.05	0.001	0.083	0.15

Table 3 shows the correlations among the 12 variables. There are four correlations greater than 0.5: K^+ and NO_3^-; Na^+ and NO_3^-; Mg^{2+} and SO_4^{2-}; and Na^+ and Cl^-. The major cations, Ca^{2+}, K^+, Mg^{2+}, and Na^+, are positively correlated, most of them significantly so (Table 3). Overall, 19 of 66 correlations had absolute values greater than 0.25 and 32 of 66 were statistically significant.

Table 3. Pairwise correlations among the variables, using data from all caves and all dates. Values in bold are greater than 0.5 and values marked with an asterisk are statistically significant ($p < 0.05$).

	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	K^+	Ca_2^+	Na^+	Mg^+	Cl^-	°C	pH
NO_3^-	1										
NO_2^-	0.202 *	1									
NH_4^+	0.140 *	0.065	1								
SO_4^{2-}	0.075	0.025	0.061	1							
K^+	**0.500** *	0.316 *	0.327 *	0.261 *	1						
Ca_2^+	0.194 *	−0.034	0.034	0.492 *	0.282 *	1					
Na^+	0.622 *	0.198 *	0.264 *	0.183 *	0.470 *	0.210 *	1				
Mg^{2+}	0.247 *	0.023	0.261 *	**0.588** *	0.420 *	0.461 *	0.406 *	1			
Cl^-	0.480 *	0.030	0.162 *	0.023	0.088	0.157 *	**0.687** *	0.216 *	1		
Temperature	−0.113	0.094	−0.029	0.072	−0.062	−0.271 *	−0.208 *	0.022	−0.258 *	1	
pH	0.121	−0.009	−0.035	−0.074	0.156 *	−0.023	0.058	−0.105	0.021	−0.039	1
Conductivity	0.294 *	0.055	−0.023	−0.228 *	−0.039	0.121	0.005	−0.053	0.085	−0.047	−0.123

To investigate the differences among caves and dates for environmental variables, PCA analysis, by cave, is shown in Figure 2. Three sites (Pivka jama, Postojnska jama, and Škocjanske jame) have no distinct clusters of points but with outliers (Pivka jama with one and Škocjanske jame with two). The other three caves (Črna jama, Dimnice and Županova jama) have two or three clusters of points, but except for Črna jama, there was no clustering of PCA scores by date. In Črna jama values for October and November formed a distinct cluster, along the first principal component. Loadings on the first component of Črna jama were highest for Mg^{2+}, Ca^{2+}, K^{+}, NH_4^{+}, Cl^{-}, and SO_4^{2-}. There was variation in factor loadings among caves. In general, cation and anion loadings were positive for component 1.

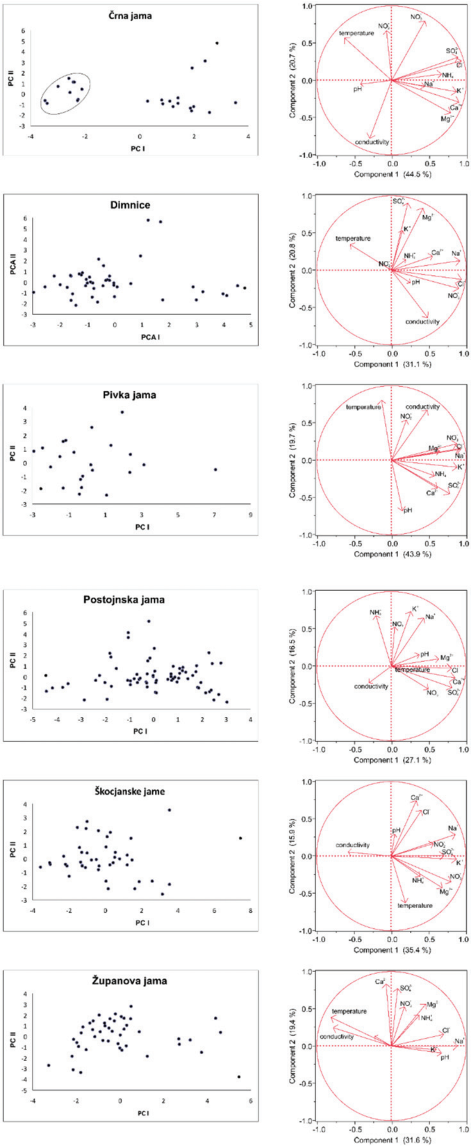

Figure 2. PCA analysis by cave. On the left hand panels, individual samples are shown. For Črna jama, the ellipse encloses all samples taken during October and November. It was the only such cluster of sampling dates for the six caves. See text for details. On the right hand panels the eigenvectors for the physical and chemical variables are shown.

Loadings on the second component were more variable. As Postojnska jama was sampled at different months (April through October) than Črna jama and Pivka jama (October through February, Table 1), it is interesting to compare these three PCA plots. In Postojnska jama, temperature has a very different loading than in Črna jama and Pivka jama, but this pattern is not repeated for other variables. In Postojnska jama, the first two PCA axes accounted lowest percent of the variance (43.6%) and in Pivka jama, the first two axes account for the highest percent of the variance (65.2%).

Of the 252 samples taken at various times in the six caves, 136 (54.0%) had adult copepods. For none of the six caves were there significant monthly differences in the presence or absence of copepods (Table 4). Therefore, the connection between physico-chemical factors and the presence or absence of copepods can be considered without the confounding effects of cave and time interactions.

Table 4. General linear mixed model type III tests of fixed effect of month/cave combination on presence/absence of copepods and on the chemical and physical variables. NO_2^- was not included because it was not possible to normalize the variable. The list of caves with monthly differences includes all caves with at least one significant difference among months. Some of the results are graphically displayed in Figure 3.

		Covariance Parameter Estimates (SE) for the Random Effects						
Variable	**Transformation**	**Sampling Site within Cave**	**Residual**	**Numerator df**	**Denominator df**	***F*-Value**	***p***	**Caves with Monthly Differences**
Presence of copepods	Binary	2.050 (1.193)	na	37	179	0.74	0.86	None
Temperature	None	1.603 (0.462)	1.070 (0.113)	43	178.1	11.64	<0.0001	All caves except Postojnska jama and Županova jama
Conductivity	None	3595.6 (1026.4)	1935.9 (204.8)	43	177.8	5.22	<0.0001	All caves except Črna jama
pH	None	0.00566 (0.00239)	0.0263 (0.0028)	43	179.4	3.31	<0.0001	Pivka jama, Postojnska jama, Županova jama
Ca^{2+}	None	17.90 (7.37)	*	43	96.5	16.58	<0.0001	All caves
K^+	None	0.00923 (0.00408)	**	43	93.5	3.00	<0.0001	Pivka jama, Postojnska jama, Županova jama
Mg^{2+}	None	0.0838 (0.0257)	0.0772 (0.0082)	43	176.2	5.26	<0.0001	All caves except Županova jama
Na^+	Log	0.232 (0.075)	0.320 (0.035)	43	176.4	4.71	<0.0001	All caves except Županova jama
NH_4^+	None	0	***	43	105.8	2.09	0.0012	Postojnska jama
Cl^-	Square root	0.258 (0.071)	0.0841 (0.0089)	43	177.3	4.66	<0.0001	All caves except Postojnska jama and Županova jama
NO_3^-	Log	0.888 (0.242)	0.232 (0.025)	43	177.1	7.77	<0.0001	All caves
SO_4^{2-}	None	1.449 (0.501)	3.126 (0.301)	43	177.2	9.25	<0.0001	All caves

* Unequal variances among months, with residuals ranging from 8.95 to 338.01; ** Unequal variances among months, with residuals ranging from 0.0064 to 0.0776; *** Unequal variances among months, with residuals ranging from 0.000245 to 0.00370.

In contrast, for at least one cave, average monthly values for every physico-chemical parameter showed at least one significant monthly difference (Table 4, Figure 3). There were differences among caves, and the most prominent of these anomalous patterns was Županova jama. It was unique in showing no temporal differences in either Mg^{2+} or Na^+, and shared with Postojnska jama the lack of temporal differences in temperature and Cl^- (Table 4). Postojnska jama was unique in showing temporal differences in NH_4^+. With the exception of temperature (Figure 3, upper panel), none of the variables showed a yearly cyclical pattern. From April to November, temperature remained near the yearly high for each cave, and then, in all except Županova jama, fell several degrees. In the case of Postojnska jama, there were no data during the winter months. More typical was the pattern shown by conductivity (lower panel of Figure 3), with occasional erratic changes of highs and lows, except for Črna jama which had no significant monthly differences. There was no overall seasonality with respect to the appearance of highs and lows, nor was there any consistent spacing of highs and lows.

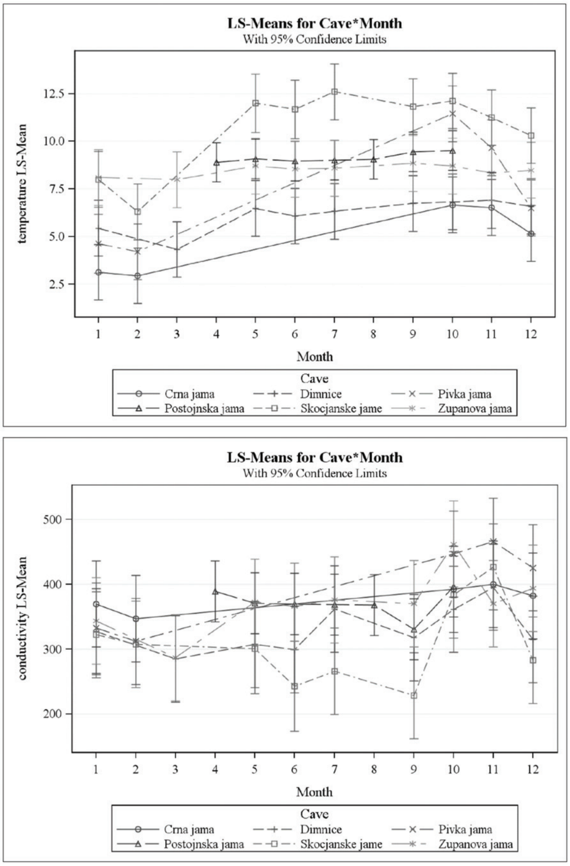

Figure 3. Temporal pattern of temperature (upper panel) and conductivity (lower panel). Vertical bars are 95% confidence intervals. For temperature, Črna jama, Dimnice, Pivka jama, and Škocjanske jame had significant temporal variation. For conductivity, Dimnice, Pivka jama, Postojnska jama, Škocjanske jame, and Županova jama had significant temporal variation.

3.2. Which Variables Could Be Important in Limiting Copepod Distribution?

The results of the all possible subsets regression analysis are shown in Table 5. No model with more than six variables gave a significant improvement in fit over the six variable model which included temperature, conductivity, K^+, NO_2^-, Na^+, and Cl^- (Table 5). When a seventh variable is added (Ca^{2+}), the corresponding increase in χ^2 is less than one. When the random effect of sampling site within a cave was included in the model with these explanatory variables, only conductivity and temperature show a significant effect, both being significant at $p = 0.022$.

Table 5. Best subset of physico-chemical data for predicting presence of copepods for each of k = 1, . . . 7 explanatory variables using logistic regression. The six variable model was chosen for further analysis because addition of an additional variable added less than one to the χ^2 value.

Number of Variables	χ^2	Variables
1	12.978	Temperature
2	18.265	Temperature, Conductivity
3	22.426	Temperature, Conductivity, K^+
4	24.350	Temperature, Conductivity, K^+, NO_2^-
5	26.117	Temperature, Conductivity, K^+, NO_2^-, Na^+
6	27.782	Temperature, Conductivity, K^+, NO_2^-, Na^+, Cl^-
7	28.441	Temperature, Conductivity, K^+, NO_2^-, Na^+, Cl^-, Ca^{2+}

A more general and realistic approach, which assumes neither independence or monotonicity of predictor variables is a random forest, showing the factors affecting the presence or absence of epikarst copepods is a random forest (Figure 4). As expected, temperature and conductivity are the most important, as was the case for the logistic regression. However, four other variables seem likely to play a role in determining copepod distribution, listed in decreasing order of importance: Cl^-, NO_3^-, Ca^{2+}, and Mg^{2+}. How these factors influence presence or absence of epikarst copepods can be determined by a classification tree using these six factors. Three of the six variables are shared with all possible subsets regression (Table 5)—temperature, conductivity, and Cl^-.

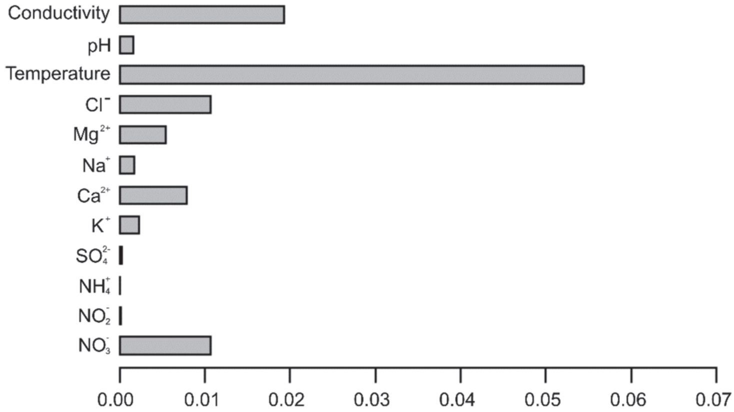

Figure 4. Bar graph of the relative importance of different variables in generating classification trees, when five variables are repeatedly drawn at random.

The best model from the classification tree (Table 6) is shown in Figure 5 and summarized in Table 6. Overall, the tree classified 192 of 252 samples (76%) correctly with regard to the presence or absence of copepods, and had a generalized R^2 of 0.45. The presence of copepods was correctly predicted 69% (94 of 136) of the time and the absence of copepods was correctly predicted 84% (98 of 116) of the time. The first branch was for temperature and 57 of 136 occurrences of copepods could be predicted solely on the basis of temperature being less than 8.2 °C (Table 6). There was no further branching on the left but for temperatures greater than 8.2 °C, Ca^{2+} concentration was the next partition with high concentrations also being associated with the presence of copepods. The remainder of the partitions (Figure 5) involved Cl^- (twice), NO_3^-, Mg^{2+}, and Ca^{2+} (again). Of the eight terminal leafs, three were associated with absence, and the most important of these (81 of 116 samples without copepods) were samples with high temperature, intermediate Ca^{2+}, low Cl^-, low Mg^{2+}, and high NO_3^- (Table 6).

Table 6. Terminal "leafs" of classification tree (see Figure 5). Conductivity differences were not in the optimal classification tree. Blanks indicate that no dichotomy for that variable exists for that leaf. Concentrations are in mg/L and temperature is in °C.

Temperature	Ca^{2+}	Cl^-	NO_3^-	Mg^{2+}	Frequency with Copepods	Frequency without Copepods	*n*
<8.2					0.668	0.332	85
≥8.2	≥57.5				0.758	0.242	22
≥8.2	<15.3	≥2.11			0.843	0.157	9
≥8.2	<15.3	<2.11			0.410	0.590	22
≥8.2	<57.5 and ≥15.3		<0.21		0.587	0.413	15
≥8.2	<57.5 and ≥15.3		≥0.21	≥1.25	0.447	0.553	11
≥8.2	<57.5 and ≥15.3	≥2.85	≥0.21	<1.25	0.556	0.444	5
≥8.2	<57.5 and ≥15.3	<2.85	≥0.21	<1.25	0.052	0.948	83

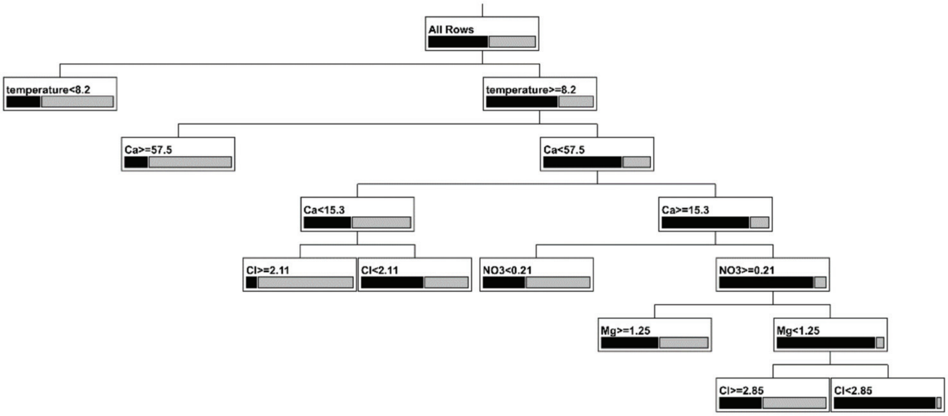

Figure 5. Classification tree for presence or absence of epikarst copepods. The size of the black rectangle is the relative number of empty samples and the size of the gray rectangle is the relative number of samples with copepods. See Table 6 for details.

4. Discussion

4.1. Patterns of Variation of Physico-Chemical Factors

The overall physico-chemical characteristics of epikarst drip water are that of water in contact with carbonate rock, with resulting high levels of conductivity, a predominance of Ca^{2+} cations, slightly basic pH, and temperatures close to the mean annual air temperature (Table 2) [31].

The differences among samples were caused by a combination of location (Figure 2), seasonality (Table 4), and water retention time. We did not measure residence time of water in this study, but previous studies in Postojnska jama [32], found that residence time of water varied from 2.5 months to over a year. Similarly Kluge et al. [33] found retention times of one to three years in three German caves. Three caves—Dimnice, Postojnska jama, and Škocjanske jame—have relatively thick ceilings [20,26], so that, all things being equal, residence time of water may be greater in these caves. If residence time is correlated with presence (and abundance) of copepods, then differences in residence time will result in differences among the caves. The underlying geology of the caves is nearly identical, with all caves formed in Jurassic limestones, although there may be some dolomite in Županova jama [34].

There are factors that vary on the scale of the projected area of a cave onto the surface that influence the physico-chemical variables that we measured. For example, sites in Pivka jama are high in NO_3^- [20]. We suspect, but cannot demonstrate, that this is the result of the presence of a campground and associated structures [35]. Other potentially anthropogenically causes include high Cl^- in Dimnice [20], which is also correlated with NO_3^- (Table 3), and may indicate water from wells with high concentrations of both [36]. There is no commercial activity and minimal agricultural activity in the area, but elevated levels of Cl^- nonetheless suggest anthropogenic inputs because of the relative rarity of naturally occurring Cl^-. High Cl^- concentrations may be from animal waste, human wastewater, or other sources such as salt licks.

Given variation in residence time of water underground, it is not surprising that there is almost no seasonality, but high temporal variability with respect to physico-chemical variables. One manifestation of the variability of residence time is the response to the flow rate of drips to rainfall events, according to Kogovšek [32]. After a dry period or drought, the flow rate of drips does not increase after a rainfall event, but once the epikarst layer is saturated with water, the increase of flow can be in a matter of hours. Of course, the water exiting the drip is not the precipitation water itself, but some of the water previously stored. That is, rainfall has a piston effect on the water in epikarst [37,38].

Temperature showed a rather different pattern than the chemical variables (Figure 3). The differences in temperature among caves are likely the result of details of the differences in sampling times (Table 1), climate differences (especially with respect to Škocjanske jame, which is in region of more Mediterranean climate than the others), and vertical distance from the surface. Except for Postojnska jama, for which there were no winter samples (Table 1), all caves showed a seasonal pattern of reduced temperatures from January to April (Figure 3, upper panel). In some cases, this difference was small. In Županova jama, the monthly least squares means (see Figure 3), varied less than 1 °C, but the coldest months were between January and March. While these differences were not statistically significant, the seasonal pattern in Črna jama, Dimnice, Pivka jama, and Škocjanske jame was statistically significant, and the temperature differences were greater. Kogovšek [32] also found a seasonal pattern in temperature for two drips in Postojnska jama, although the range was less than 1 °C. The relationship between temperature and residence time is a complex one (see [39–41]) for a more detailed discussion, but the temperature of resident water eventually approaches the mean annual temperature [39]. Temperature also reflects season, as seen in this study. The lower temperatures in winter are indicative of other events, such as changes in evapo-transpiration and precipitation.

4.2. The Epikarst Copepod Physico-Chemical Niche

A useful beginning point of the analysis is to see which individual physico-chemical factors can account for presence or absence of epikarst copepods in a sample. If the effect of cave is included as a covariate, only temperature and conductivity were significant predictors of the presence or absence of copepods in a sample. However, when a non-parametric multivariate approach, using variables shown to be important in a random forest analysis (Figure 4) in a classification tree (Figure 5, Table 6), was employed, the most important correlates of copepod presence were:

- Temperature,
- Ca^{2+},
- Mg^{2+},
- Cl^-,
- NO_3^-.

Conductivity itself was unimportant in the classification tree, presumably because some its major components, especially Ca^{2+} and Mg^{2+}, were exposed by the random forest analysis, which teased apart correlated variables (see Table 3). Correlation analysis indicated that conductivity was not pairwise correlated with any of the cations (Table 3). The results of the classification tree argue that the relationship of conductivity with the other variables is more complicated than simple pairwise relationships. In fact, a regression tree (not shown) using conductivity as the response variable and the other five variables as predictors indicated that conductivity is explained in order by Ca^{2+}, Cl^{-}, NO_3^{-}, and Mg^{2+} which is the same order that these variables enter the tree for predicting presence of copepods.

Copepods tend to be in samples with lower temperatures (Table 6), and so the connection between temperature and copepod presence is likely a seasonal one. It is very likely that the relationship between occurrence probability and temperature is driven by copepods getting washed out of epikarst in greater numbers in winter because flow rates are greater. We did not measure flow rates but Kogovšek [32] continuously monitored discharge from two epikarst drips in Postojnska jama for a period of more than two years, and demonstrated that discharge rates were highest in the winter. Rouch [42] observed a similar pattern of copepod drift from a karst spring.

Of the other variables shown to be important in the classification tree, Ca^{2+} has a strong connection with the biology of copepods. It is critical in the molting process, and some subterranean crustacean species, like the amphipod *Gammarus minus*, are limited to carbonate springs [43]. Additionally, of interest is that copepods tend not to be found in water with temperature greater than 8.2 °C and Ca^{2+} concentrations greater than 57.5 mg/L. Water in epikarst can be supersaturated with respect to Ca^{2+} (part of the mechanism of deposition of $CaCO_3$ in caves (e.g., stalactites)) and this may cause physiological problems for animals in this water. While carbonate geochemists have long focused on the Ca^{2+} -HCO_3^{-} system, we suggest it deserves more attention from biologists working in the same systems. Mg^{2+} is also a critical nutrient [44], and it is possible that it is limiting in some contexts.

While Mg^{2+} concentration may or may not be biologically significant, it seems likely that the correlation of epikarst copepod abundance and Cl^{-} is due to some other unmeasured variable, one that varies at the scale of cave. Cl^{-} concentrations in Dimnice are twice as high (5.54 ± 1.12 mg/L) as in any other cave [26]. We suspect that it is not Cl^{-} that is important but some other unmeasured factor.

The correlation with NO_3^{-} is perhaps also the result of some other unmeasured variable, but nitrate is also biologically important. It is a frequent contaminant of karst aquifers, resulting from agricultural runoff, septic tanks, and perhaps atmospheric deposition [45]. There are few studies of the nitrogen cycle in caves or epikarst, but available evidence suggests that it is not a limiting nutrient [15,46]. However, there are still a number of puzzling aspects of the nitrogen cycle (see, [45]), such as whether nitrogen fixation occurs in caves. If not, Barton [47] points out it is likely to be limiting in some circumstances.

If we take classification trees as the most general approach to the understanding of the connections of copepod occurrence to physico-chemical parameters, then we have the following factors, listed in order of importance:

1. Temperature, which is likely a reflection of flow velocities rather than community structure.
2. Calcium and perhaps magnesium ions, which are important, both as essential nutrients and in molting.
3. Anthropogenically augmented ions—Cl^{-} and perhaps NO_3^{-}—may indicate contamination from upgradient well water, or they may be surrogates for particular epikarst sites, where some unmeasured variable is important.

The multi-faceted statistical approach, combined with an emphasis on the overall community rather than individual species, has made some sense of the complex patterns of variation of physico-chemical variables.

4.3. The Relationship between Community Physico-Chemical Niche and Individual Physico-Chemical Niches

Pipan [20,26] and Pipan et al. [27] analyzed the same data from a different perspective, one that emphasized niche separation among the epikarst copepod species. Using the same variables with the addition of ceiling thickness, they found that the following parameters were significant factors in distinguishing individual species in a Canonical Correspondence Analysis (CCA): ceiling thickness;

NO_3^-; K^+; and Na^+. Of these, only NO_3^- was important on a community-wide basis. Thus, the parameters by which the species are separated are, for the most part, distinct from the parameters that predict the presence or absence of one or more species (see Figure 5).

An example of individual niche analysis for NO_3^- is shown in Figure 6. This is reflected in the presence of some species, especially *Brycocamptus dacicus*, *Bryocamptus* n.sp., *Moraria varica*, and *Maraenobiotus brucei*, only in high NO_3^- concentrations and only in Pivka jama (Figure 6). Whatever the source, it points to nitrate as an important factor in organizing communities (see [44]). What is also apparent in Figure 6 is the difficulty in separating or even characterizing the physico-chemical niches of the different species. Of the 27 species found in drips, there were data on more than 100 individuals for only three species, and only an additional four species had more than 10 individuals for which nitrate data were available.

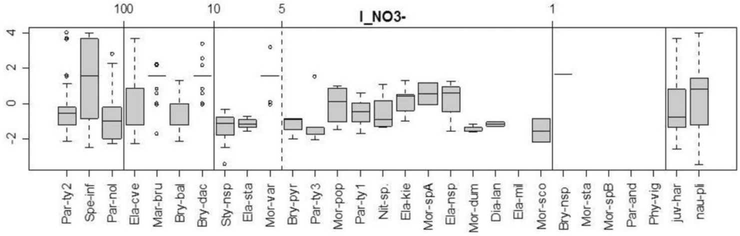

Figure 6. Box and whiskers plot of the log NO_3^- concentrations (mg/L) for different copepod species, which are arranged by their abundance (indicated by the vertical lines). The rectangles enclose the middle 50% of the data, the line across each rectangle is the group median, the "whiskers" are the minimum and maximum values. Species names are Par-ty2: *Parastenocaris* sp.2; Spe-inf: *Speocyclopes infernus;* Par-nol: *Parastenocaris nolli alpine;* Ela-cve: *Elaphoidella cvetkae;* Mar-bru: *Maraenobiotus brucei;* Bry-bal: *Brycamptus balcanicus;* Bry-dac: *Brycamptus dacicus;* Sty-nsp: *Stygepactophanes* n.sp.; Ela-sta: *Elaphoidella stammeri;* Mor-var: *Moraria varica;* Bry-pyr: *Bryocamptus pyrenaicus;* Par-ty3: *Parastenocaris* sp. 3; Mar-pop: *Moraria poppei;* Par-ty1: *Parastenocaris* sp. 1; Nit-sp: *Nitocrella* n.sp.; Ela-kie: *Elaphoidella kieferi;* Mor-spA: *Moraria* sp. B; Ela-nsp: *Elaphoidella* n.sp.; Mor-dum: *Morarioipsis dumonti;* Dia-lan: *Diacyclops languidoides;* Ela-mil: *Elaphoidella millennii;* Mor-sco: *Morariopsis scontenophila;* Bry-nsp: *Bryocamptus* n.sp.; Mor-sta: *Moraria stankovitchi;* Mor-spB: *Moraria* sp. B; Par-and: *Parastenocaris andreji;* Phy-vig: *Phyllognathopus viguieri.*

The data we used were not originally collected for the purpose of elucidating physico-chemical niches, and this is the case for many ecological and bioinventory studies. In many of these studies data collected on basic water chemistry remains unconnected and often unanalyzed with respect to the organisms being studied. The results of our study suggest that a careful field study exploring the impact of variation in the physical and chemical characteristics of water on the likelihood of copepods being present may yield additional insights into the forces that control aquatic community structure and dynamics. These impacts could be implicit such as when variables act as surrogates for other factors (such as temperature) or explicit, i.e., with direct effects (such as is likely the case with Ca^{2+}). There are other constraints on the epikarst copepod community, especially the lack of light and low levels of organic carbon, that are a formidable barrier for not just copepods, but any species to survive in epikarst. Thus, the physico-chemical constraints suggested by the classification tree (Figure 5), are not absolute, but constraints in the context of no light and little organic carbon, among the extreme conditions that characterize epikarst in general.

5. Conclusions

A focus on general community occupancy of extreme habitats may ultimately yield insights into environmental constraints on even groups that seem ubiquitous and unconstrained by environmental factors, like copepods. There were physico-chemical differences between samples with fauna and without fauna. The physico-chemical factors that constrained epikarst copepod communities in this study were, with the exception of NO_3^-, different than those that separated the physico-chemical conditions in which different species were found. Some of the community constraints were likely surrogates for age of the epikarst water; and some, like NO_3^- and Ca^{2+}, may be important biological requirements themselves. In general, the study of such community constraints is made difficult by co-variation of explanatory parameters, and the technique of random forests and classification trees is a useful way out of this dilemma. The study of community constraints in extreme habitats is a fruitful field for further inquiry.

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Review

Reduced Oxygen as an Environmental Pressure in the Evolution of the Blind Mexican Cavefish

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Abstract: Extreme environmental features can drive the evolution of extreme phenotypes. Over the course of evolution, certain environmental changes may be so drastic that they lead to extinction. Conversely, if an organism adapts to harsh environmental changes, the adaptations may permit expansion of a novel niche. The interaction between environmental stressors and adaptive changes is well-illustrated by the blind Mexican cavefish, *Astyanax mexicanus*, which has recurrently adapted to the stark subterranean environment. The transition from terrestrial rivers and streams (occupied by extant surface morphs of the same species) to the cave has been accompanied by the resorption of eyes, diminished pigmentation and reduced metabolism in cave-dwelling morphs. The principal features of caves most often associated with evolution of these common cave features are the absence of light and limited nutrition. However, a putatively essential cave feature that has received less attention is the frequently low concentration of oxygen within natural karst environments. Here, we review the potential role of limited oxygen as a critical environmental feature of caves in the Sierra de El Abra. Additionally, we review evidence that *Astyanax* cavefish may have evolved adaptive features enabling them to thrive in lower oxygen compared to their surface-dwelling counterparts.

Keywords: cavefish; dissolved oxygen; cave evolution; *Astyanax mexicanus*; Sierra de El Abra

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1. Introduction

Extreme environmental changes can have dramatic consequences on organismal form and physiology. The freshwater teleost species *Astyanax mexicanus* is a ”natural” model system that has provided novel insight to interactions between environment and phenotype. This species exists as two distinct morphotypes—an extant ”ancestral surrogate” surface form and an obligate cave-dwelling form (Figure 1b,c). Over the course of several hundred thousand years, multiple discrete cave invasions occurred in which surface morphs of this species colonized the limestone caves of the Sierra de El Abra region in northeastern Mexico (Figure 1a [1]). Numerous common troglobitic traits accompanied this surface-to-cave transition, including complete eye regression [2,3], lack of pigmentation [4,5] and reduced metabolism [6,7]. These divergent phenotypes are often linked to common features of the cave microenvironment, including complete darkness and scarce nutritional resources [8,9]. Interestingly, however, few investigations in this system have focused on the putatively low levels of oxygen (hypoxia) present within caves as an important environmental feature.

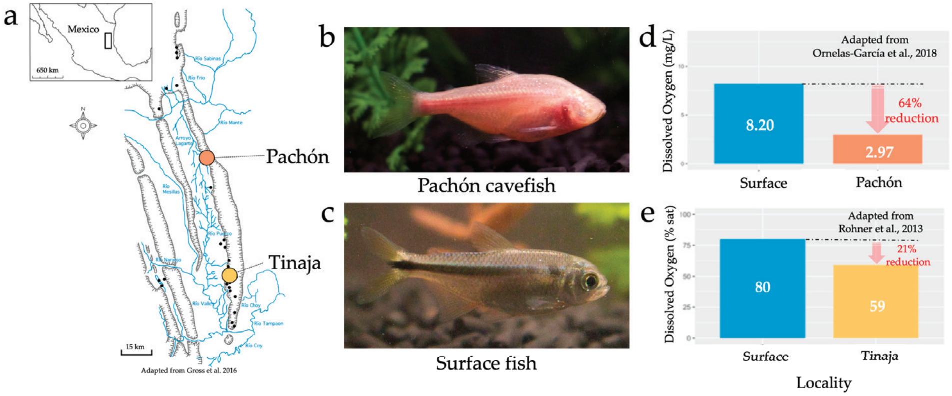

Figure 1. The numerous caves, fish and oxygen measurements found in the Sierra de El Abra and surrounding regions. 30 named caves are located in this region of northeastern Mexico (**a**). The species *Astyanax mexicanus* is composed of two morphotypes, the cavefish (**b**) and surface fish (**c**). Oxygen measurements taken with the Pachón cave (**d**) and Tinaja cave (**e**) show reduced levels of oxygen compared to surrounding surface environments.

The number of aquatic environments with reduced concentrations of oxygen are growing, likely as a consequence of climate change. For instance, recent studies indicate that hypoxic zones in both freshwater and marine environments are expanding globally [10–12]. The dramatic switch from normoxia (well-oxygenated) to hypoxia can be devastating for native fauna and has already decimated aquatic populations around the world [13–16]. Further, subterranean environments have long been appreciated as having less oxygen compared to the above-ground terrestrial environment [17]. The question of how certain aquatic species have adapted to low oxygen has been examined in certain contexts. However, the cavefish model system, *Astyanax mexicanus*, presents the opportunity to examine interspecific differences in oxygen metabolism in two morphs of the same species. This powerful comparative paradigm has enabled broad insights to the developmental, genetic and morphological differences that underlie adaptation to the cave environment. Here, we argue that despite having received less attention in the literature, reduced oxygen has likely had a meaningful impact on the evolutionary history of the cave morphotype. Since numerous ecological factors differ across the many caves of the Sierra de El Abra, this model system may provide improved resolution for understanding how differences in oxygen levels across similar cave networks explain subtle differences in oxygen metabolism and respiratory physiology. This review has two principal aims: First, we examine the available evidence of low oxygen in the caves of the Sierra de El Abra. Second, we assess whether *Astyanax mexicanus* cavefish exhibit phenotypes consistent with "hypoxia-tolerant" species. In sum, we propose that reduced oxygen is a critical driver of cave-associated phenotypes, and that *Astyanax* cavefish have adapted to the low oxygen concentrations in the cave environment.

2. Determinants of Dissolved Oxygen Concentration and Their Status in Subterranean Environments

The concentration of dissolved oxygen in a body of water is influenced by a number of factors. Thus, the dissolved oxygen concentration can vary according to the magnitude of these diverse features. For instance, if the environmental conditions surrounding a body of water vary, so too will the concentration of dissolved oxygen within that body of water. Alternatively, if those factors remain stable, so too will the concentration of dissolved oxygen. Here, we review key factors determining dissolved oxygen concentrations in natural bodies of water, including how these factors may differentially influence the cave and surface environments of the Sierra de El Abra. We note that the level of reduced oxygen in caves likely varies over both time and space, and therefore should be regarded as a dynamic feature. Moreover, karstic systems are known to be quite dynamic, rendering one's ability to generalize from other cave complexes (e.g., in temperate regions) quite challenging. Nonetheless, the caves of the Sierra de El Abra have likely experienced reduced level(s) of oxygen, in a manner consistently lower than terrestrial environments, across vast geologic and evolutionary timescales.

2.1. Low Dissolved Oxygen in Subterranean Environments and the Sierra de El Abra

Considerable research has been conducted on hypoxia tolerance in a variety of species, owing to increasingly common environmental oxygen limitations [18–20]. Additionally, studies relating to dissolved oxygen in subterranean environments (including caves) have revealed the presence of low dissolved oxygen compared to surface waters [21]. These observations have mostly been recorded since the early 1970s, when trends of reduced dissolved oxygen in subterranean environments were first appreciated by numerous ecologists [17]. Similarly, direct measurements of reduced oxygen have been recorded in the Sierra de El Abra. For example, Rohner et al. [22] reported less oxygen in the Tinaja cave compared to the surrounding surface waters (Figure 1e, 80% air saturation in surface waters, 59% air saturation in the Tinaja cave). Additionally, Ornelas-García et al. [23] reported substantially less oxygen in Pachón cave pools (2.97 mg/L), compared to two river locations: Micos River (4.43 mg/L) and Rascón (8.2 mg/L; Figure 1d). Remarkably, one historical measurement in the Sierra de El Abra found dissolved oxygen concentrations as low as 0.9 mg/L [24]; however, it should be noted that cavefish are not believed to be present at this locality. Interestingly, a recent measurement reported by Krishnan et al. [25] found much higher dissolved oxygen concentrations in the Pachón cave (~6.9 mg/L on average) compared to previous measurements. We believe this finding provides evidence that dissolved oxygen levels may fluctuate across time and space within the dynamic karst environment. It is difficult to say whether stable reductions in oxygen, or long-term fluctuations, are more stressful depending on how physiological stress is defined. Regardless of ″reduced″ versus ″variable″ oxygen levels, we would argue that either factor will have been at play over extensive geological periods, and therefore either feature (but hypoxia more saliently) represents a challenging environmental factor likely driving changes in oxygen metabolism. Further, although direct measurements of dissolved oxygen in the region are limited, environmental features that contribute to low levels of oxygen are frequently reported in caves. Although less is known of the specific factors contributing to hypoxia (e.g., water depth of different cave pools), the following sections examine those environmental features that most likely influence oxygen levels in the El Abra cave complex.

2.2. Atmospheric Composition and Mixing

A key determinant of oxygen concentration within a body of water is the amount of oxygen in the surrounding atmosphere. Exchange occurs at the surface of the water, where oxygen can enter or leave depending on the concentration in either substrate. In principle, oxygen in a body of water and in the atmosphere will either be at equilibrium or moving towards equilibrium [26]. If a body of water is in complete equilibrium with the ambient atmosphere, then the body of water has 100% air saturation. However it is important to note that if the ambient atmosphere has limited levels of oxygen, the body of water will likely also have limited levels of oxygen (even at 100% air saturation). Many factors impact the efficiency of this exchange, such as temperature, atmospheric pressure, water pressure and salinity. Further, diffusion of oxygen is also impacted by turbulence of the water body. Accordingly, a greater flow velocity (increased turbulence) increases the diffusion rate [27]. Thus, stagnant water mixes poorly with the ambient atmosphere, leading to a slower oxygen diffusion into the body of water. Not surprisingly, many pools within the caves of the Sierra de El Abra are reportedly stagnant [28,29], and therefore likely do not mix efficiently with atmospheric oxygen. We note that many of these features likely differ across the El Abra cave complex, even for cave localities that are geographically close to one another.

Beyond stagnation of the water, some cave environments in the Sierra de El Abra are characterized by ”bad air” [30]. Even if a body of water is able to maintain 100% air saturation, if the level of oxygen in the ambient atmosphere is low, the level of oxygen in the local body of water will also be low. The pioneering El Abra caver William Elliott recounted an anecdote relating to low oxygen in the air of the Tinaja cave, which created a dangerous environment for a group of explorers. In 1972, a group of technical cavers entered the Tinaja cave with the hope of finding an underground connection to a nearby cave, Sótano del Arroyo. During this expedition, the only diving gear brought along was a rope and flashlight—no scuba or diving gear. Don Broussard dove into a sump and found a pocket of air into which he swam. There, after an estimated four deep breaths, the other explorers saw the light coming from Don’s flashlight begin to sink in the cave pool—a sign that he was unconscious. The group frantically pulled him from the water and discovered he was not breathing. Following CPR to remove the water from his lungs, he regained consciousness [31].

”Bad air” is frequently present in caves [30]. Many cavers utilize the ”Bic” test as an empirical way to sample atmospheric oxygen in an unknown cave. If a Bic brand handheld lighter ignites properly and retains its flame, there is enough oxygen to breathe. However, if the lighter cannot light or maintain its flame, one could be in a dangerous spot with little oxygen. Elliott [30] recounted some of his experiences and mentioned that ”normal people” may be turned off by the fact that frequent cavers develop a ”body expectation” to the bad air of caves. He continued, “Maybe it also causes minor brain damage and that’s why we continue to go caving . . . hmmm. Maybe it also causes minor brain damage and that’s why we continue to go caving . . . Wait, I already said that. That’s all for now.”

2.3. Photosynthesis

One primary input of dissolved oxygen into a body of water is photosynthesis [26]. Vegetation, phytoplankton and other algae within a body of water utilize natural light to produce energy, and oxygen is released as a byproduct. Thus, the presence of these species in a natural environment is normally crucial for maintaining sufficient oxygen for aquatic organisms. Since natural light is necessary for photosynthesis, daily fluctuations occur in most terrestrial bodies of water, with the concentration of oxygen being highest near the end of the day, and lowest just before sunrise [32]. However, these fluctuations are typically not sufficient to cause hypoxia around sunrise in typical terrestrial bodies of water [32].

Given that the Sierra de El Abra and surrounding regions are filled with dense vegetation, robust photosynthesis occurs within this over-ground environment. In fact, vegetation is so dense in some areas that it has caused cavers to get lost [33]. However, caves experience limited light beyond the entrance, and therefore are essentially devoid of photosynthetic activity. This lack of photosynthesis is described many times in the *Astyanax* literature; however, few published works link this commonly reported cave feature to low levels of dissolved oxygen. Some even discuss the benefit of seasonal flooding for cavefish as the main influx of energy [2,7,34–36]. We propose that these floods may also provide an essential input of oxygen into the cave pools. In this context, we also note that it will be interesting to determine if cave morphs demonstrate direct genetic or physiological changes such as differences in mitochondrial function and/or DNA repair mechanisms in cavefish compared to surface morphs.

2.4. Respiration and Organic Matter

Just as photosynthesis is the major driver of oxygen input in water, aerobic respiration is regarded as the principal consumer of oxygen in a body of water [26]. Many caves and most surface aquatic environments of the Sierra de El Abra harbor organisms that consume oxygen. Further, oxygen is consumed by microbes that respire during decomposition of organic material [17]. Organic material is present within the caves of the El Abra, even when actively flowing water is absent. For instance, many caves harbor sizable bat populations providing bat guano, as well as deceased bats, to the cave waters below [31].

These substrates undergo decomposition through a process that ultimately serves to further reduce oxygen levels within the cave.

An additional factor affecting the cave environment is methane gas. Methane is known to displace oxygen in water; thus, concentrations of methane and other substances should be considered when determining levels of oxygen deficiency [37]. Elliott [31] recalled a striking anecdote relating to methane in the Arroyo cave of the Sierra de El Abra. After navigating through "Strickland's Bad Air Passage" and "The Wallow," cave explorers will come upon "Methane Passage," so named for the bubbles of methane that can be seen floating up from the muddy sediment of the cave pools. The concentration of methane in these bubbles is so high that they can be ignited, producing blueish and yellowish flames. A video of "tourist" cavers in Arroyo posted to YouTube in 2008 illustrated the combustible nature of methane gas. At first, small bubbles only produced a small, brief flame. However, later in the video, enough methane was released above the surface of the water that a dangerous amount of fire was produced. Instinctively, the person in the water panicked and tried to exit the water as quickly as possible. This only caused more methane to be released, increasing the surface area of the flame. Luckily, the cavers were not harmed. This remarkably high amount of methane in and near the cave pools clearly impacts oxygen concentration within the Arroyo cave pools. At present, it remains unclear if the concentration of methane is similar across all cave environments, or between different pools within a single cave locality.

3. Adaptation to Low Oxygen across Teleost Fish Taxa

Many aquatic animals encounter hypoxic water and must emigrate or adapt to the reduced levels of oxygen. However, certain species are able to tolerate low oxygen better than others. These animals are regarded as hypoxia-tolerant, -resistant or -acclimated (particularly in controlled experiments). Many adaptations have evolved to permit survival in low oxygen conditions [38]. Here, we discuss adaptive phenotypes that may be relevant to cave-dwelling organisms, such as *Astyanax mexicanus*.

3.1. Metabolism

A well-known response of hypoxia-acclimated fish is the suppression of metabolic rate. Typically, the metabolic rate is established experimentally via respiration. Therefore, respirometric methods are used to determine metrics as a proxy for metabolic change. These metrics include maximum metabolic rate (MMR), standard metabolic rate (SMR) and routine metabolic rate (RMR). Additionally, the critical oxygen tension (P_{crit}) metric is often reported in hypoxia resistance studies, which is the minimum amount of oxygen required to maintain the standard metabolic rate [39]. Fish with lower P_{crit} values are considered to be more hypoxia-resistant compared to individuals with higher P_{crit} values [40]. To determine the extent to which hypoxia-resistant goldfish (*Carassius auratus*) could suppress their standard metabolic rate, Fu et al. [41] determined the critical oxygen tension of this species. Juvenile fish were commercially bought and reared under normoxic conditions. Prior to the start of the experiment, half the population was exposed to hypoxia. After 48 h of exposure to extreme hypoxia (0.3 mg/L) the critical oxygen tension was calculated. The critical oxygen tension of hypoxia-acclimated goldfish was nearly half that of the control group, illustrating how the standard metabolic rate can be suppressed as an adaptation to lower oxygen environments.

Beyond suppression of aerobic metabolic rate, some teleost species are capable of utilizing anaerobic metabolism to survive in low-oxygen conditions [42,43]; however, this phenomenon is less understood. An example of "paradoxical anaerobism" is observed in the desert pupfish [44]. Following the last ice age, the groundwater table began receding in the Mojave desert, trapping this species in several distinct springs. These springs are located below the surface of the desert and are characterized by limited light during winters, resulting in limited primary production and scarce food sources. During testing, anaerobic metabolisms could not be activated "on-demand" and even occurred randomly in environments with ample ambient oxygen—hence ”paradoxical.“ One individual went over two hours consuming negligible amounts of oxygen. Ultimately however, the authors could not rule out the possibility that ”paradoxical anaerobism” provides any real benefit and that it may simply be the indirect consequence, or component, of another underlying physiological mechanism. Additional studies of potential adaptations to hypoxia in animals living in extreme aquatic environments will provide further insight to the underlying mechanisms of this unusual respiratory feature.

3.2. Gill Morphology

Fish may also adapt to low oxygen through alterations in gill morphology. Gills are the primary surfaces of gas exchange in fish, and morphological alterations to the structure of gill organs can impact oxygen capture from the environment. Some examples of alterations to gill morphology are quite astounding. For example, the crucian carp (*Carassius carassius*), a hypoxia-tolerant species, dramatically changes the morphology of its gills in response to hypoxic stress [45]. Interestingly, this species displays a unique gill morphology trait even in normoxic conditions—the lack of protruding lamella. Teleost fish gills are composed of many gill arches. Attached to these gill arches are fingerlike projections known as gill filaments. Each gill filament has numerous disklike protrusions called lamella. The surfaces of these lamella are the sites of gas exchange. Thus, fish with expanded lamella have greater overall surface area available for respiration and are able to capture more oxygen. Although during normoxic conditions the crucian carp has lower-than-average lamella surface area, during hypoxic conditions the crucian carp increases the surface area of its lamella via apoptosis of the interlamellar cell mass (ILCM). Strikingly, these changes can be visualized via scanning electron microscopy within 24 h of low-oxygen exposure. Additionally, this feature of gill remodeling is reversible. Upon returning the carp to well-oxygenated water, lamella reverted to an intact ILCM within seven days. This study provided evidence of a remarkable morphological adaptation to low oxygen, but also indicates that adaptations to hypoxia may persist in a population even if hypoxia is no longer present.

3.3. Hemoglobin

The ability of teleosts to inhabit such a wide range of environmental conditions may be attributed, in part, to the teleost-specific whole genome duplication event that occurred ~350 million years ago [46]. This whole genome duplication event also impacted syntenic regions of the genome housing the hemoglobin superfamily of genes. Beyond a few anomalies [47], all teleost hemoglobin genes are organized into two distinct genomic regions named for the genes that flank them [48]. The MN region (flanking genes: *mpg* and *nprl3*) is generally larger and contains the majority of hemoglobin genes, compared to the LA cluster (flanking genes: *lcmt1* and *aqp8*).

An important adaptation to life in hypoxic environments involves changes to the structure and function of the heterotetramer hemoglobin protein. In principle, modifications can also impact the genetic sequence, organization and regulation of the *hemoglobin* gene repertoire. For instance, a survey of multiple Gadiformes (codfish) species revealed that the number of *hemoglobin* genes can vary among even closely related species [49]. This discrepancy was largely explained by certain aquatic features, namely the water depth at which each species resides. Phylogenetically older species, living in deeper waters, possessed fewer *hemoglobin* genes than the phylogenetically younger species living in shallower waters. The authors explained this finding based on the characteristic that the deep ocean is “an extreme, yet stable environment” and that environmental conditions are more variable near the surface. Therefore, an expanded *hemoglobin* repertoire, capable of producing a more diverse set of proteins, could be advantageous for animals that live in fluctuating oxygen conditions.

Simply having a diverse repertoire of *hemoglobin* genes does not confer hypoxic tolerance. Conceivably, efficient regulation of the repertoire is vital for producing the most effective hemoglobin protein(s) for a given environmental condition. The phenomena of hemoglobin ″switching″ as a response to hypoxia was fortuitously discovered by Rutjes et al. [50] in African cichlids. Three species were utilized in multiple split-brood style experiments. Half of each brood was reared under either normoxic or hypoxic conditions for ~15 months. Through the use of isoelectric focusing, diverse isoforms of hemoglobin protein within each blood sample were separated on a polyacrylamide gel using electrophoresis. This approach enables one to identify and quantify the number of isoforms and their relative abundance. Of the three species examined, two displayed no differences in hemoglobin isoform diversity in response to varying oxygen concentration. However, the third species, *Haplochromis ishmaeli*, showed robust differences in both identity and relative abundance of hemoglobin isoforms in response to diverse oxygen conditions.

It was noted in this study that one defining characteristic of *H. ishmaeli* is that it is native to Lake Victoria in southeastern Africa while the others, although closely related, are native to different regions [51]. The authors argued that the adaptation of hemoglobin ″switching″ may have originated in the ancestors of these fish during an extreme desiccation event ~15,000 years ago. To test this notion, van den Thillart et al. [51] conducted isoelectric focusing on three species of African cichlids all native to Lake Victoria. These species were characterized by varying likelihood of experiencing hypoxia in the wild, as well as having diverse nutritional requirements. Interestingly, all of the examined species exhibited hemoglobin switching when reared under hypoxic conditions in captivity. They also found that the hemoglobin molecules produced by *H. ishmaeli* had a higher oxygen binding affinity at lower concentrations of ambient oxygen. In sum, changes to *hemoglobin* genomic organization may provide robust paths towards adaptation in hypoxic environments. These changes could be accomplished through alterations in splicing that enable the production of hemoglobin molecules with improved binding efficiency, and/or through changes in the structure and expression of *hemoglobin* family members.

4. Evidence of Adaptation to Low Oxygen in *Astyanax mexicanus*

Teleosts have evolved a number of interesting adaptations to endure the challenge of low oxygen. To this point, few studies on hypoxia tolerance have been conducted in *Astyanax mexicanus*. Here, we review prior work that may indicate differences in respiration and/or hypoxic tolerance in the blind Mexican cavefish.

4.1. Metabolism

Several respirometric studies have examined metabolic differences between *Astyanax* cave and surface morphs [6,36,52,53]. These studies measured the rate at which oxygen was consumed per hour. Although the experimental design of each study varied, a consistent finding across different studies revealed that cave morphs consume far less oxygen compared to surface fish. Hüppop [6] found that for both routine consumption (average consumption over 24 h) and standard consumption (i.e., the minimum consumption recorded), Pachón cavefish consumed significantly less oxygen compared to their surface fish counterparts. Although the Micos and Chica populations did not differ significantly from the surface population, qualitatively they consumed less oxygen as well. Salin et al. [52] largely confirmed these results several years later. Over the course of a two-month fast, cavefish consumed roughly half the oxygen of surface morphs. Moran et al. [53] similarly investigated metabolic differences; however, they also examined photic condition as an environmental cue during measurements. They discovered surface fish increase mean oxygen consumption during the day, but cavefish retain stable consumption across the 24 h day. As a result, they concluded that cavefish use ~27% less energy than surface fish per day.

Respiration has also been measured across early development in *Astyanax mexicanus*. Bilandžija et al. [36] investigated differences in respiration rates of Pachón cavefish and surface fish when reared on a 12:12h light dark/cycle versus being reared in complete darkness. Regardless of lighting conditions at 7.5 days post-fertilization, Pachón cavefish consumed less oxygen than light/dark-reared surface fish. Interestingly, however, surface fish reared in constant darkness consumed less oxygen and did not significantly differ from Pachón cavefish. In sum, these studies provide direct evidence that cavefish likely consume less oxygen than surface fish, and these alterations were likely driven by life in complete darkness.

4.2. Gill Morphology

Few studies have examined differences in gill morphology between *Astyanax mexicanus* morphs. However, Moran et al. [54] reported a greater percentage of body weight was appropriated to the gills of Pachón cavefish compared to surface fish. Specifically, the gills of the Pachón cavefish, on average, accounted for 3.1% of wet body weight, while surface fish gills accounted for only 2.0% of wet body weight. Further, the gills of Pachón x surface F_2 hybrids accounted for an intermediate (mean) percentage of 2.6%. Although the authors found this difference in gills, they did not find similar results in other organs (excluding eyes and brain). Accordingly, this unique difference in wet weight proportion could be the result of selection for larger gills as a consequence of lower oxygen concentrations in caves. This increased gill size in cavefish most likely provides greater surface area to augment the efficiency of gas exchange during respiration.

4.3. Hemoglobin

Recently, two independent studies examined differences in *hemoglobin* expression in *Astyanax* cavefish [55,56]. To our knowledge, these studies represent the only published works examining *hemoglobin* in this model system. Sears et al. [55] examined global changes to the transcriptome using RNA sequencing of adult surface and Pachón *Astyanax* morphs reared under different lighting conditions (i.e., 12:12 h light/dark conditions and constant darkness). When comparing gene expression patterns under natural photic conditions (light/dark for surface, total darkness for cavefish), several *hemoglobin* genes demonstrated significantly higher expression values in cavefish compared to surface fish. One gene, *hemoglobin subunit adult alpha 1* (*hbaa1*), demonstrated the largest fold-change difference within this transcriptomic comparison, expressed 20× higher in cave compared to surface morphs. Further, a Gene Ontology (GO) enrichment analysis of highly expressed genes in dark-reared cavefish revealed terms such as “oxygen transport”, “oxygen binding” and “hemoglobin complex”. The functional and adaptive significance of this upregulation remains unclear, but it is intriguing to consider that *hemoglobin* gene expression may be influenced by photic conditions.

Additionally, van der Weele and Jeffery [56] measured expression of two *hemoglobin* genes in developing Pachón cavefish and surface fish (~10–84 h post-fertilization) under normoxic conditions, using qPCR and whole-mount in situ hybridization. They found that embryonic Pachón cavefish have increased expression of these two *hemoglobin* genes under normoxic conditions. Further, based on in situ gene expression, developing cavefish have more mature red blood cells (erythrocytes) compared to surface fish. The authors noted a stark contrast in the response of multiple other genes to hypoxia. Many genes associated with hypoxic pathways, and metabolic processes impacted by hypoxia, increased expression in both morphotypes compared to normoxic rearing. Interestingly, the extent of this increased expression was significantly higher in Pachón cavefish than in surface fish. These recent studies suggest that cavefish have evolved resistance to hypoxia, and these changes can be observed in both development and adulthood.

5. Conclusions

Over the last few decades, the blind Mexican cavefish *Astyanax mexicanus* has been utilized to investigate diverse adaptive phenotypes, such as vision loss, sleep reduction and changes in nutrient metabolism evolving in cave organisms [57]. Certain cave features such as total darkness and limited food are regarded as having clear regressive impacts at the genetic, developmental and morphological levels. However, one environmental feature that has received less attention is the oxygen level within the caves inhabited by these fish. Low oxygen has commonly been reported in other temperate cave and subterranean environments around the globe [17]. Hypoxia is not limited to hypogean habitats, and the number of affected habitats is increasing as a consequence of climate change [10]. Therefore, in order to anticipate potential impacts on natural populations, it is essential that we improve our understanding of how certain taxa have evolved strategies to cope with limited oxygen. We propose that this question can be robustly examined using the natural model system, *Astyanax mexicanus*. Given the powerful paradigm of intra-specific comparisons, prior work reveals that this system has evolved hypoxic adaptations as a consequence of life in a low-oxygen environment. Future work, designed to parse the adaptations to low oxygen as a selective pressure, position *Astyanax* as an emerging model system that could reveal key insights that enable them to flourish in hypoxic environments.

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