Research Topics

Adaptations to subterranean environments

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Adaptations to subterranean environments

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Editorial: Adaptations to subterranean environments

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Editorial on the Research Topic
Adaptations to subterranean environments

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The subterranean realm is a fascinating world that still holds many secrets and wonders (Culver and Pipan, 2019; Ficetola et al., 2019). Species that have successfully colonized caves and other subterranean environments have often had to deal with novel and unique conditions that promoted the appearance (or disappearance) of specific traits (Howarth and Moldovan, 2018; Soares and Niemiller, 2020). Several morphological, behavioral, and physiological traits are considered adaptive; however, an exhaustive list of adaptive traits documented in subterranean species remains to be assembled. Recent studies continue to uncover evidence for the evolution of traits in subterranean environments that were previously unrecognized (e.g. Hesselberg; Lunghi and Bilandžija), especially thanks to new methods that expand our research possibilities (Bierbach et al., 2018; Mammola et al., 2021). Historically, the morphological phenotype was used as an exclusive criterion for recognizing obligate cave species (Christiansen, 1962). Among them, the loss or reduction of eyes, depigmentation, and elongation of appendages have been recognized as defining traits for living in complete darkness (Bilandžija et al., 2012; Gonzalez et al., 2017). Selection pressures imposed by subterranean environments are often so strong that even conspecific individuals can exhibit divergent phenotypes depending on their habitats (Jeffery, 2020; Lunghi and Zhao, 2020), as two studies on fish (Enriquez et al.) and newts (Guillaume) published in this Research Topic also show. Other less conspicuous traits should also be included within the array of adaptations to cave life. For example, behavioral adjustments may be the first changes individuals need to make in a novel subterranean environment (Lunghi et al., 2023). All behaviors based on visual inputs must be replaced by new ones that rely on other sensory stimuli, such as chemical or mechanical cues (Plath et al., 2004; Yoshizawa et al., 2012). Consequently, the nervous system, for example, should undergo specific modifications of its structure and function to adapt to the new conditions. Further, many cave species have modulated their metabolism to better withstand long periods of starvation in generally food-deprived subterranean environments (Hervant, 2012; Lipovšek et al., 2019). This Research Topic on *Adaptations to Subterranean Environments* aimed to assemble some of the latest studies on adaptive traits in obligate and facultative cave species and to highlight new research perspectives (Culver and

Pipan, 2015; Culver et al.). Thirteen papers are included in this Research Topic, including five reviews on specific topics, such as biomimetics (Hesselberg), chemoreception (Berning and Gross), and electroception (Soares et al.). Eight original research articles address various aspects of adaptation to subterranean life, including the genetic mechanisms that enable the establishment of the specific trait in the population (Arcila et al.; Bondareva et al.). Various model species were used for these studies, including less-studied species such as mammals (Luna et al.) and microorganisms (Frumkin et al.). On the other hand, more conventional model species of subterranean invertebrates were used to properly understand the phylogeography of congeneric species (Kováč et al.) and to test whether widely accepted theories such as Island and Rensch's rules can also apply to subterranean species (Herczeg et al.). The array of different studies in this Research Topic not only provide a general overview of subterranean adaptations and demonstrate the growing interest and scope of research on this topic, but also provide new insights into which and how specific adaptive traits evolve in species that have colonized the subterranean realm.

Author contributions

EL: Writing – original draft, Writing – review & editing. MN: Writing – review & editing. HB: Writing – review & editing.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Longevity in Cave Animals

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An extraordinary longevity has been observed in some cave species, and this raised the hypothesis that a longer lifespan may be considered one of the characteristic traits of these animals. However, only a few cave species have been studied thus far, and a firm conclusion remains to be drawn. Here we review the available knowledge on the longevity of subterranean species, point out the limitations of previous studies, and provide suggestions for future studies to answer important questions regarding the longevity in cave animals, its adaptive value and the related promoting factors. We also argue that studying the longevity in cave animals will contribute to the field of aging, especially to understanding the evolution of this phenomenon.

Keywords: adaptation, biospeleology, cave biology, lifespan, senescence, subterranean, troglobite, convergent evolution

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INTRODUCTION

With their unique ecological setting, subterranean environments are theaters of evolutionary processes that lead to fascinating adaptive traits in the species able to colonize them (Mammola, 2019). All subsurface habitats are characterized by a common feature, the absence of light (Culver and Pipan, 2014, 2019). Light can only penetrate and affect the area surrounding their connections to the surface (Lunghi et al., 2015; Culver and Pipan, 2019), limiting the effects of seasonality and the circadian cycle in deeper zones, a condition that contributes to highly stable microclimate characterized by high humidity and constant temperatures (Biswas, 2009; Lunghi et al., 2015). Without sunlight, photosynthetic processes are absent (Culver and Pipan, 2019), what leads to a strong reduction of the available organic matter, and therefore the subterranean food web is largely dependent on inputs of allochthonous organic material from the surface (Schneider et al., 2011; Barzaghi et al., 2017). This scarcity of food resources results in reduced abundance of species at all levels of the food web (Venarsky et al., 2014; Culver and Pipan, 2019; Manenti et al., 2020).

Colonization of subterranean environments by aboveground species is accompanied by numerous changes in their phenotypic traits (Hervant et al., 2001; Bilandžija et al., 2020; Lunghi and Zhao, 2020). The hallmarks of adaptation to subterranean environments are loss of eyes and pigmentation, which are so common in subterranean taxa that they are used as diagnostic traits to assess the degree of adaptation to these environments (Howarth and Moldovan, 2018; but see Lunghi et al., 2014). Some of the other conspicuous adaptive traits in subterranean species include elongation of body appendages, increase in fat stores, overexpression of mechanosensory and chemosensory systems (Jeffery, 2009; Bilandžija et al., 2012; Gonzalez et al., 2017). Although most obvious, morphological adaptations often represent the final chapter in a story that began with the behavioral and physiological changes that accompanied the early stages of cave colonization (Culver et al., 1995; Pigliucci et al., 2006; Bilandžija et al., 2020).

In this review we explore a poorly known aspect of subterranean species: their longevity. Among subterranean taxa, individuals with exceptional longevity have occasionally been reported (e.g., Voituron et al., 2011; Puljas et al., 2014), raising the idea that the dark passages within the earth harbor animals with extended lifespans. Over the years, most studies have aimed to estimate the age and lifespan of cave species (e.g., Trajano, 1991; Voituron et al., 2011; Puljas et al., 2014), but very few in a comparative framework that included a surface relative (Poulson, 1963). Ultimately, it has yet to be proven whether higher longevity is indeed a characterizing trait of subterranean animals, and eventually whether such trait is adaptive. Here, we review what is known about the longevity of subterranean species and provide recommendations for future studies to experimentally test whether these animals show higher longevity compared to their surface relatives and what the potential promoting factors might be.

LONGEVITY IN SUBTERRANEAN SPECIES

The limited information on the lifespan of cave species mostly comes from the few species that have been successfully reared in laboratory facilities (Poulson, 1963; Miaud and Guillaume, 2005; Hinaux et al., 2011; Voituron et al., 2011; Bichuette and Trajano, 2021). Animals kept under controlled conditions enable tracking their growth and development of specific growth rate curves can provide age estimates for wild populations (Brunkow and Collins, 1996; Gallo and Jeffery, 2012). However, controlled conditions may differ from those found in natural environments and may alter growth rates of individuals (Trajano, 1997; Simon et al., 2017). Luckily, growth rates can be estimated also in the wild for species that are marked (e.g., Lunghi and Bruni, 2018; Lunghi et al., 2019) and recaptured over time (Taddei Ruggiero, 2001; Venarsky et al., 2012; Balázs et al., 2020; Lunghi et al., 2022), or when individuals show reliable age marks (Simon et al., 2017; Riddle et al., 2018).

One of the most iconic cave species is the olm, *Proteus anguinus*, an aquatic salamander distributed in the Dinaric karst in the South-Eastern Europe (Sket, 1997; Gorički et al., 2017). Because it is extremely difficult to study *Proteus* in the wild (Buzzacott et al., 2009; Balázs et al., 2020), most of the available knowledge comes from captive observations (Juberthie et al., 1996; Ipsen and Knolle, 2017; Aljančič, 2019). This neotenic salamander has a slow development, becoming sexually mature after 14 years and reproducing every 12.5 for at least 50/60 years (Voituron et al., 2011; Ipsen and Knolle, 2017). The oldest known individual has an age between 48 and 58 years (Voituron et al., 2011). Given its slow growth rate and low reproductive activity, the average lifespan of the olm should be around 70 years, although some individuals can live more than 100 years (Bulog et al., 2000; Voituron et al., 2011). In comparison, the closest aboveground relatives are from the North American genus *Necturus*, and the longevity of *Necturus maculosus* has been estimated at 34 years (Petranka, 1998). Interestingly, the olm's small size, metabolic rate comparable to other salamanders, and lack of increased antioxidant activity are in stark contrast to the predictions of aging theory, which foresee higher lifespan in larger species with low metabolic rate and high antioxidant activity (Blanco and Sherman, 2005; Voituron et al., 2011).

Cavefish are within the best-studied subterranean animals, yet only a few studies on their lifespan exist. For example, the Mexican tetra, *Astyanax mexicanus*, is probably one of the widest used model species in cave biology (Wilkens, 1988; Rétaux and Casane, 2013; Keene et al., 2015; Jeffery, 2020), but reliable information on the potential lifespan of this species is missing. We know from captive breeding that both surface and cave forms of *A. mexicanus* can reach the age of 15 (Riddle et al., 2018). There is a divergence in growth rate and age distribution between several populations of cave and surface forms (Simon et al., 2017). Specifically, the range of estimated ages of fish was greater in cave populations (2–8 years) than in surface populations (2–5 years), and there is variability in age distribution even between cave populations (Simon et al., 2017). In another comparative study, Poulson (1963) used the scale and otolith to age five amblyopsid fishes, two surface species, *Chologaster cornuta* and *Forbesichthys agassizi*, and three subsurface, *Typhlichthys subterraneus*, *Amblyopsis spelaea*, and *A. rosae*. The results showed a higher lifespan in cave fishes, where the age of older individuals ranged from 3 to 7 years, up to three times more than for the surface fishes (Poulson, 1963). A similar age for *A. rosae* (about 4–5 years) has been estimated in another study (Brown and Johnson, 2001). However, more up to date techniques indicate a potential lifespan of 20–30 years for these cavefish (Poulson, 2001). For the Brazilian *Ancistrus cryptophthalmus*, an initial estimate determined a lifespan of 8–10 years (Trajano and Bichuette, 2007), while a subsequent study extended it to more than 15 years and provided evidence that the longest-lived individuals can live up to 20–25 years (Secutti and Trajano, 2009). An estimate of lifespan based on growth rate exists also for some other cavefish from the tropics: *Pimelodella kronei* (10–15 years; Trajano, 1991), *Trichomycterus itacarambiensis* (7 years; Trajano, 1997), *Caecobarbus geertsii* (9–15 years; Heuts, 1952; Proudlove and Romero, 2001), and *Ituglanis passensis* (>10; Secutti and Trajano, 2021). No comparable information on closely related surface species exists for these Brazilian cavefish; however, in comparison with other cyprinids (data from AnAge database; Tacutu et al., 2018) these species are among the longer living ones.

Studies in cave invertebrates also yielded extraordinary estimates of their lifespan. An analysis of the growth line formation in the shells of the cave bivalve *Congeria kusceri* found that the average lifespan was about 30 years. Importantly, authors also showed that growth lines form annually despite animals residing in stable underground waters (Puljas et al., 2014). The oldest individual in this study, a 14.3 mm long male, had an impressive lifespan of 53 years (Puljas et al., 2014). However, other studies have found much larger individuals, so the lifespan of this and possibly other *Congeria* species is likely much higher (Morton, 1969; Bilandžija et al., 2013). The average lifespan of other dreissenid species ranges between 2 and 5 years (Puljas et al., 2014), implying that cave bivalves have evolved lifespans 10 times longer than their surface relatives. As with *Proteus*, this is contrary to predictions which suggest that larger animals have longer lifespans (Blanco and Sherman, 2005), as *Congeria* is one of the smaller dreissenids. However, its slow growth rate and low investment in reproduction (Morton and Puljas, 2013) are consistent with disposable soma theory of aging (Kirkwood, 1977) which suggests a tradeoff between the investment in the reproduction and somatic maintenance; the latter leading to lifespan increase. Similarly, analysis of growth rate of the brachiopod *Neocrania anomala* from marine caves estimated that its lifespan may exceed 40 years (Taddei Ruggiero, 2001). The lifespan of *N. anomala* is about three times higher than *Waltonia inconspicua* and *Terebratalia transversa*, brachiopods that can reach a maximum age of 15 and 13 years, respectively, (Paine, 1969; Rickwood, 1977). Age estimation of an extinct brachiopod, *Gigantoproductus okensis*, indicates that this species could have lived up to 20 years (Angiolini et al., 2012), which is half of the lifespan estimated for the cave *N. anomala* (Taddei Ruggiero, 2001).

A capture-mark-recapture study conducted over 5 years provided important information on the lifespan of the crayfish *Orconectes australis* (Venarsky et al., 2012). Using growth rate data, the authors were able to re-evaluate the lifespan for this species, which was incorrectly estimated to be 100 years, and found out that the species can live over 22 years (Venarsky et al., 2012). In addition, these authors compiled a list of crayfish species for which lifespan estimates exist (see Table 2 in Venarsky et al., 2012), including two additional cave species: *O. inermis* (9–10 years), and *Procambarus erythrops* (>16 years). Of the 10 surface cambarids included in the list, one has an estimated lifespan of 13 years, while the others ≤7 years. Similar results have been obtained for other cave crustaceans. Based on the distribution of body size, Pacioglu et al. (2020) suggested that the lifespan of *Gammarus balcanicus* might exceed that of epigean gammarids, which have lifespan estimates of about 1 year. In another study, Magniez (1975) estimated that the cave *Stenasellus virei* can reach an age of 12–15, a lifespan up to 20 times longer than epigean congeneric species. Furthermore, a 40-year study performed by Carpenter (2021) showed that *Bahalana geracei* has probably the longest known lifespan (ranging from 24.5 to 35 years) among isopods. Capture mark recapture studies showed that the cave beetle *Laemostenus schreibersi* can reach the remarkable age of >6.5 (Rusdea, 1994), almost doubling

TABLE 1 | Summary of the lifespan data for cave species collected in this review.

Species	Average lifespan	Maximum lifespan	References
Vertebrates			
Proteus anguinus	70	>100	Voituron et al., 2011
Speleomantes italicus		25	Lunghi, 2022
Calotriton asper	~12	16–19	Miaud and Guillaume, 2005
Astyanax mexicanus	2–8	15	Simon et al., 2017, Riddle et al., 2018
Typhlichthys subterraneus	1–2	4 (20–30)	Poulson, 1963, Poulson, 2001
Amblyopsis spelaea	2–5	7 (20–30)	Poulson, 1963, 2001
Amblyopsis rosae	2–3	4 (20–30)	Poulson, 1963, 2001
Ancistrus cryptophthalmus	15	20–25	Secutti and Trajano, 2009
Pimelodella kronei	6	10–15	Trajano, 1991
Trichomycterus itacarambiensis	7	7	Trajano, 1997
Ituglanis passensis		>10	Secutti and Trajano, 2021
Caecobarbus geertsii		9–13 (<15)	Heuts, 1952, Proudlove and Romero, 2001
Invertebrates			
Congeria kusceri	30	53	Puljas et al., 2014
Neocrania anomala		>40	Taddei Ruggiero, 2001
Orconectes australis		>22	Venarsky et al., 2012
Orconectes inermis		9–10	Retreived from Venarsky et al. (2012)
Procambarus erythrops		>16	Retreived from Venarsky et al. (2012)
Gammarus balcanicus		>1	Pacioglu et al., 2020
Stenasellus virei		12–15	Magniez, 1975
Bahalana geracei		24.5–35	Carpenter, 2021
Laemostenus schreibersi		>6.5	Rusdea, 1994

Data is shown in years and refer to both observed and predicted species average and maximum lifespan.

the maximum lifespan observed for epigean ground beetles (Lövei and Sunderland, 1996).

All information on cave species lifespan collected in this review are summarized in **Table 1**.

Although bats are not strictly bound to subterranean habitats, as they use caves primarily for sheltering purposes and do not exhibit classic cave-related adaptations (Howarth and Moldovan, 2018; Sadier et al., 2020), it is worth mentioning this animal group in the context of longevity, as they are the longest-lived mammals relative to their mass (Podlutsky et al., 2005; Wilkinson and Adams, 2019). Interestingly, cave use predicts the evolution of longevity in several bat lineages (Wilkinson and South, 2002; Salmon et al., 2009).

CONCLUSION AND FUTURE DIRECTIONS

The information assembled here suggests that longevity can indeed be considered a characteristic of cave animals. However, additional systematic studies encompassing a broader range of animal groups in a comparative framework which includes surface and cave relatives are needed to fortify this conclusion. The main remaining questions are: (1) is longevity adaptive for subterranean lifestyle or did it evolve as a physiological consequence of other adaptive traits, (2) what components of the subterranean environment contribute to the evolution of life extension in resident animals, and (3) what are the molecular and developmental mechanisms that lead to convergent evolution of longevity in cave organisms. Several hypotheses can be made regarding the links of aging with other cave adaptive traits and the characteristics of subterranean environments that may be promoting it. For example, adaptive traits in caves such as slower growth, lower metabolic rate and lower investment in reproduction, have been associated with increased lifespan (Flatt and Schmidt, 2009). Further, many environmental features of caves such as limited food resources (Aspiras et al., 2015), lower extrinsic mortality (lack of predators; Plath and Schlupp, 2008), hypoxia (Boggs and Gross, 2021; van der Weele and Jeffery, 2022) and lack of UV irradiation (Körner et al., 2006), are consistent with known ecological predictors of longevity (Speakman and Selman, 2011; Flament et al., 2013; Omotoso et al., 2021). These nonexclusive hypotheses can be tested using model systems that have closely related species or populations in caves and on the surface, have a reliable method for estimating their age, and can survive in the laboratory to be used for experimental perturbations.

Although important to understand the life history characteristics of subterranean species (e.g., sexual maturity, reproductive potential; Poulson, 1963; Lunghi et al., 2018; Lunghi, 2022), growth rate curves may not be the best method to estimate species ages. First, species generally do not grow indefinitely and their growth rate can only be estimated during a particular phase of their life cycle (Venarsky et al., 2012; Lunghi, 2022). A clear example of this is the aforementioned crayfish *Orconectes australis*, whose lifespan has been reduced by a factor of four using better-tuned methods (Venarsky et al., 2012). Another limitation to using this method stems from the ecological differences between cave and surface populations. For example, low availability of nutrients in subterranean environments (Huntsman et al., 2011; Culver and Pipan, 2019; Pacheco et al., 2020) strongly influences species growth (Simon et al., 2017). Without rearing cave and surface relatives under the same conditions, it is difficult to distinguish intrinsic from extrinsic effects on lifespan.

The use of molecular biomarkers of aging (e.g., SA-β-Gal, 4-HNE, or lipofuscin staining; Nelson et al., 2012; Dimri et al., 1995) may overcome some of these limitations. In addition to allowing reliable assessment of lifespan differences between subterranean and surface species, the use of markers for relevant physiological and molecular processes will enable following the basic processes underlying aging as well as the assessment of variability in aging on population or species levels (Robins et al., 2017).

Studies on cave animals may also provide important insights into the biology of aging, particularly in the areas of evolution and genetics of aging. One of the greatest challenges in the field of aging is to uncover the genes and processes that cause lifespan differences among species (Partridge and Gems, 2006). Because of the known direction of evolution -from surface to cave- and the ability to compare ancestral form with derived form, cave dwellers provide a rich source of information about mechanisms that lead to slowing rates of aging in nature. Knowledge of these mechanisms could be of great benefit and have implications for human welfare. Because of well-defined environmental characteristics and numerous convergently evolved physiological and life-history adaptations, the study of aging in cave dwellers can shed light on how longevity covaries and co-evolves with other organismal and environmental traits.

Aging is a complex phenotype, so it is important to incorporate research on nontraditional and atypical species and integrate approaches from multiple biological disciplines (Cohen, 2018). How and why senescence evolves in natural populations and how to maintain health in old age is still a major issue in contemporary biology. We propose that studies of aging in cave-adapted animals can provide new and original insights into how nature has solved the problem of extending lifespan.

AUTHOR CONTRIBUTIONS

EL drafted the manuscript. Both authors conceived the study and critically revised the manuscript.

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Surface newt *Calotriton asper* acclimation to cave conditions improved their foraging ability in darkness

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Caves in temperate regions are characterized by food scarcity compared to surface habitats. Therefore, hypotheses on adaptation to cave life suggest that many characteristics, such as resistance to starvation, reduced energy demand, and increased food-finding ability, have evolved among cave dwellers to cope with food frugality. To test the hypothesis involving increased food-finding ability, the prey detection performances of three surface and three subterranean populations of the Pyrenean newt, *Calotriton asper*, were compared. First, the rapidity of surface individuals in capturing prey at short distances inside a restricted aquarium under dark and light conditions and their score for capturing prey in a larger aquarium under similar conditions were measured. Simultaneously the scores obtained from individuals in cave populations in darkness were compared. Individuals from one of the surface population were maintained in darkness for approximately five years and retested. The surface *C. asper* individuals captured prey faster at short distances under light conditions than in total darkness; however, the differences were not significant. The scores of the surface *C. asper* individuals in the large aquarium were significantly better in darkness than in light conditions. In addition, the scores of cave populations at short distances in darkness were better than those of epigean populations in light conditions; however, the differences were not significant. Finally, the scores of surface individuals maintained in darkness for five years improved. To explain these results, it should be noted that surface *C. asper* populations forage at night using non-visual cues (e.g., chemical and mechanical cues), further suggesting that vision may somewhat inhibit other senses. This ability to forage at night may be favorable for cave colonization. Moreover, some hypogean populations, when the use of prey-detecting non-visual senses has developed, may start to exhibit adaptivity.

KEYWORDS

newts, food-feeding, detection performance, cave, darkness

Introduction

In temperate regions, caves are characterized by food scarcity, among other features, compared to surface habitats (Juberthie and Decu, 1994; Hüppop, 2000; Culver and Pipan, 2009; Romero, 2009). To cope with frugality, theories on adaptation to cave life suggest that many characteristics have evolved in cave dwellers, such as increased food-finding ability, resistance to starvation, and reduced energy demand (Hervant and Renault, 2002). The Pyrenean newt, *Calotriton asper*, inhabits surface brooks and cave rivers. Previous studies have correlated a fasting adaptation (Issartel et al., 2010), a lower metabolic level in cave populations, and a decreased surface population with their acclimation to cave conditions for years (Guillaume et al., 2020). However, few studies on their food-finding ability have been conclusive. *Calotriton asper* can develop efficient foraging activity in darkness; however, this has only been observed in captivity and not in nature (Uiblein et al., 1992), although the species may exhibit nocturnal activity, as established by some observations reported in the gray literature (for instance Nicol, 1990; Dalibard, 2021). Previous preliminary observations made on some individuals of unknown origin (Uiblein et al., 1992) showed that *C. asper* could detect prey using only chemical and/or mechanical cues but concluded that vision dominates the foraging behavior of *C. asper* at short distances with improved performance in light conditions than in darkness. This study investigated the possible variation in the food-feeding ability between surface and cave populations and its possible improvement when surface *C. asper* populations are maintained in cave conditions for years.

Materials and methods

Animal origin and maintenance

Adult *Calotriton asper* individuals were captured from three surface and three hypogean populations (the locations of these populations are indicated in **Table 1**). They were placed in the "Station d'Ecologie Théorique et Expérimentale, SETE" (Ariège, France) cave in aquaria filled with water from the cave river (10.13 ± 0.19 ppm O_2, 11.5 ± 0.25°C), and marked using Nanotec transponders. Individuals from surface populations were exposed to alternating 12 h day/night photic cycles, whereas those from cave populations were maintained in total darkness. They were fed live blood worms (prey usually found in the diet of *C. asper*) *ad libitum* once a week (Clergue-Gazeau, 1969; Montori, 1991). The first series of tests was performed up to 3 weeks after capture with twenty individuals for each population. The twenty individuals from surface population 1 were maintained in total darkness for five years and retested. These were identified by their transponder so their scores at the first series and the second one five years later could have been individually compared.

Experimental setup

Individuals were subjected to a one-week fast before testing. They were isolated in a small 30 × 15 × 20 cm aquarium filled with 4.5 L water. Following a 2 h acclimatization period, one live blood worm was introduced to the center of the aquarium, and observations were done remotely with a camera connected to a recorder. The time to worm capture was recorded; failure to capture the prey after 2 h was also recorded. Then, the newts were fed *ad libitum* and were replaced with others. Twenty individuals from each population were tested successively. Tests were repeated after with the same individuals identified by their transponders inside a larger 80 × 30 × 20 cm aquarium filled with 22.5 L water. Finally, each individuals from surface populations was tested successively (after a one-week fast between each test) four time (one in a small aquarium in light, one in a small aquarium in darkness, one in a larger aquarium in light, one in a larger aquarium in darkness) except for individuals from the surface population & who were tested again two more time (one in a small aquarium in dark five years later, one in a larger aquarium in dark five years later), and each individuals from cave populations, was tested two times (one in a small aquarium in dark, one in a larger aquarium in dark).For individuals from surface populations, tests under the light condition (spectrum daylight fluorescent lamps Truelight ®Natural Daylight 5500 K/Color Rendering 1 A, Ra > 90) were conducted during the day, and those in darkness were conducted at night. Observations in darkness were made using infrared light [420TVL, 0.5 lux/f2.0, 0, Lux (15m IR ON), 24 LED's IR].

Statistical analysis

Twenty individuals from each population were examined. The variations in time required for prey capture under

TABLE 1 Name and location of the three surface and three cave *C. asper* populations where individuals used in the study were obtained.

	Name of the stream or cave	Coordinates	
		Longitude	**Latitude**
Surface population 1	Cailla	42.805°	2.190°
Surface population 2	Olhadoko	42.990°	−0.947°
Surface population 3	Ribaui	42.790°	1.342°
Cave population 1	Betharram	43.100°	−0.198°
Cave population 2	Labouiche	43.012°	1.342°
Cave population 3	Bernard	42.999°	1.534°

various conditions (aquarium size, in darkness or light conditions) were not normally distributed, and considering the limited sample size, data were thus analyzed using non-parametric tests. The scores of the same identified animals tested under various conditions were compared using the Wilcoxon unilateral test (XLstat software). Differences between populations were analyzed using the Friedman test (XLstat software). $P < 0.05$ were considered statistically significant.

Animal ethics

All the required French permits for animal experimentation on the species used in this study were obtained from the Animal Experimentation Accreditation: n°A09583 for the lab, n° A09-1 (2001)–A09-2 (2007)–A09-3(2011)–A09-5(2013) for the experimenters, and n° 09-19, 09-273, 09-295 for the animal caretakers and handlers for the use for wildlife for scientific purposes.

Field study permissions

All the required French permits (permit no. 2017-04, 2007-11-1342, and 2016-s-01) for the capture, marking, transport, detention, use, and release of protected amphibian species, and animal experimentation were obtained. The project was approved by the National Council for Nature Protection on March 19, 2007, and the Regional Scientific Council for the Natural Heritage of the Region Languedoc-Roussillon-Midi-Pyrénées on April 5, 2016.

Results

For the three surface populations, the time to prey capture in the small aquarium was shorter under light conditions than in darkness, although the differences were not significant (**Figure 1**). In contrast, the time to prey capture in the larger aquarium was significantly longer under light conditions than in darkness for the three surface populations (population 1, $P = 0.32$; population 2, $P = 0.021$; population 3, $P = 0.022$).

The scores of cave populations in the small aquarium in darkness were better than those of the epigean populations in light and darkness; however, in all cases, the differences were not significant.

The scores of the three surface populations in the large aquarium in darkness did not differ from those of the cave populations.

The scores of surface population 1 subjected to darkness for five years improved compared to the previous tests: (i) a significant difference was observed in the small aquarium in darkness ($P = 0.013$), (ii) an insignificant difference was observed in the small aquarium in light conditions, and (iii) an insignificant difference was observed in the large aquarium in darkness.

Discussion

The study findings herein confirmed that surface *C. asper* individuals could develop efficient foraging activity in darkness at night; however, they exhibited an improved speed for prey capture in light at a short distance than in darkness.

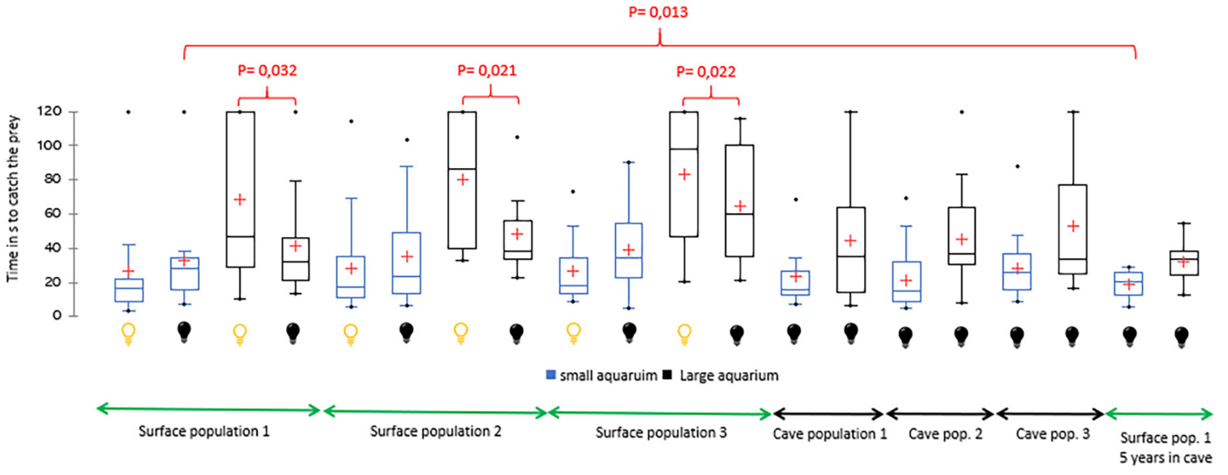

FIGURE 1
Time (in seconds) to prey capture by individuals ($N = 20$) from surface and cave populations in the small aquarium (4.5 L), large aquarium (22.5 L), in light conditions (💡), and darkness (●). The red crosses represent the means. The central horizontal bars represent the medians. The lower and upper limits of the box represent the first and third quartiles, respectively. The minimum is shown at the far bottom of the whisker, and the maximum is shown at the far top. P-values are indicated when significant.

Nevertheless, these values were not higher than those of individuals from cave populations in darkness. Moreover, surface *C. asper* individuals captured prey more rapidly in total darkness at a long distance. In another study with individuals from epigean populations, vision was highly dominant, considerably inhibiting extra-optical prey perception (Uiblein et al., 1995). These results prompted a question: given that multimodal sensory information for foraging is widespread throughout the animal kingdom, can vision blur other senses in *C. asper* and decrease efficiency when combined? Relatively little evidence of such processes exists. For instance, in the pit viper, visual and infrared information is effective in prey targeting; however, interference between the two modalities can occur, causing a reduction in performance (Chen et al., 2012). Moreover, Uiblein et al. (1995) hypothesized that the change in the foraging style of surface *C. asper* was related to the prey encountered in darkness. Similar behavior has been observed in fire salamanders, where larvae from cave-breeding populations have a better predation performance in complete darkness than in light conditions (Manenti and Ficetola, 2013); they exhibit a more active search strategy in cave conditions, giving up the standard sit-and-wait strategy of epigeans, which is less efficient in caves due to prey scarcity and difficulty in detecting preys (Manenti et al., 2013). In surface *C. asper* populations, the shift toward an efficient strategy to forage in the dark may be favored by a predisposition to night foraging. The present study was conducted only with adults, and similar experiments on youngers also could have been interesting to really assess the potential of the species to survive in caves, however, in the majority of the populations, larvae and juveniles are rarely observed, especially among caves populations, and the spontaneous reproduction of this species in captivity is still hard to obtain (Clergue-Gazeau, 1971, 1976; Montori, 1988; Guillaume, 2000). Another important finding in this study was that surface *C. asper* individuals had an improved ability to capture in the dark when maintained in cave conditions for years. The mechanism by which this is achieved is unclear; however, some species, such as zebrafish (*Danio rerio*), have been found to learn to forage in the dark (Carrillo and McHenry, 2016). Foraging plasticity is considered a facilitating trait for colonization and adaptation to new habitats (Crispo, 2008), especially when experiencing challenging pressures, such as in caves (Manenti et al., 2013). Furthermore, plasticity might be a dominant process in the first steps of colonization because it contributes to the persistence of settlers, allowing for rapid adaptive responses, which can, in a subsequent phase, accompany local adaptations (Crispo, 2007). A strong genotypic differentiation exists between the surface and cave *C. asper* populations, suggesting a lack of gene flow between them for a long time and the existence of local adaptive phenotypic divergence for multiple traits (Milá et al., 2010; Valbuena-Ureña et al., 2018; Lucati et al., 2020). However, the contribution of plasticity in the processes of genotypic diversification should be clarified. In particular, the question of whether adaptive evolution proceeds through the genetic fixation of plastic phenotypes must be addressed. Finally, this study supports the hypothesis that surface *C. asper* individuals can colonize caves through plastic traits and that surface and cave populations may have fragmented and diverged during the postglacial climatic warming where conditions in low valleys were unfavorable, except in caves (Guillaume, 2001).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by Ministére de L'Enseignement Supérieur et de la Recherche N°068.

Author contributions

OG conceived and designed the experiments, collected the urodelan species, performed the experiments, analyzed the data, prepared figure and table, authored, and reviewed drafts of the manuscript.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Natural selection versus neutral mutation in the evolution of subterranean life: A false dichotomy?

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Throughout the evolutionary tree, there are gains and losses of morphological features, physiological processes, and behavioral patterns. Losses are perhaps nowhere so prominent as for subterranean organisms, which typically show reductions or losses of eyes and pigment. These losses seem easy to explain without recourse to natural selection. Its most modern form is the accumulation of selectively neutral, structurally reducing mutations. Selectionist explanations include direct selection, often involving metabolic efficiency in resource poor subterranean environments, and pleiotropy, where genes affecting eyes and pigment have other effects, such as increasing extra-optic sensory structures. This dichotomy echoes the debate in evolutionary biology in general about the sufficiency of natural selection as an explanation of evolution, e.g., Kimura's neutral mutation theory. Tests of the two hypotheses have largely been one-sided, with data supporting that one or the other processes is occurring. While these tests have utilized a variety of subterranean organisms, the Mexican cavefish, *Astyanax mexicanus*, which has eyed extant ancestral-like surface fish conspecifics, is easily bred in the lab, and whose whole genome has been sequenced, is the favored experimental organism. However, with few exceptions, tests for selection versus neutral mutations contain limitations or flaws. Notably, these tests are often one sided, testing for the presence of one or the other process. In fact, it is most likely that both processes occur and make a significant contribution to the two most studied traits in cave evolution: eye and pigment reduction. Furthermore, narrow focus on neutral mutation hypothesis versus selection to explain cave-evolved traits often fails, at least in the simplest forms of these hypotheses, to account for aspects that are likely essential for understanding cave evolution: migration or epigenetic effects. Further, epigenetic effects and phenotypic plasticity have been demonstrated to play an important role in cave evolution in recent studies. Phenotypic plasticity does not by itself result in genetic change of course, but plasticity can reveal cryptic genetic variation which then selection can act on. These processes may result in a radical change in our thinking about evolution of subterranean life, especially the speed with which it may occur. Thus, perhaps it is better to ask what role the interaction of genes and environment plays, in addition to natural selection and neutral mutation.

KEYWORDS

natural selection, neutral mutation, troglomorphy, adaptation, *Astyanax*

1. Introduction

For most biologists, the hallmark of subterranean life is the shared morphology of eye and pigment reduction and loss. As Haldane (1933) pointed out, for every case of progressive evolution, there are likely ten cases of regressive evolution (loss or vestigilization). Despite the universal nature of the process of regressive evolution (Fong et al., 1995), it is most apparent in cave and other subterranean organisms. The long-standing interest in the iconic cave dweller, the European olm *Proteus anguinus*, dating to the 17th century (Shaw, 1999), is primarily due to the absence of eyes and pigment in this salamander. This process of vestigilization and loss of eyes and pigment has occurred in hundreds and probably thousands of lineages, including flatworms, arthropods, mollusks, salamanders, and fish (Culver and Pipan, 2019a). Speleobiologists often use the term troglomorphy (Culver and Pipan, 2019b) to highlight the shared morphology of reduced eyes and pigment (as well as elaborated extra-sensory structures).

The obvious morphological losses of subterranean organisms has led many biologists to suggest non-selective, non-adaptational explanations for these losses. This is not only true for Lamarckians (Lamarck, 1984) and neo-Lamarckians (Packard, 1888) but for Darwinians including Darwin himself (Darwin, 1859), some neo-Darwinians (Poulson, 2017), as well as neutral mutationists (Chakraborty and Nei, 1974; Wilkens and Strecker, 2017). On the face of it, natural selection would seem to be unnecessary when relaxation of selection would seem to be a sufficient explanation. Combined with the apparent relict nature of some subterranean lineages (Humphreys, 2000), the prominence of lost features led some French biologists, especially Jeannel (1943) and Vandel (1964) to propose non-Darwinian, non-selectionist theories of the evolution of subterranean life. Rather than selection, Vandel proposed an internal force (deroulement) leading to the death of phyletic lines, just as individuals die. Neo-Darwinians, beginning in the 1960's, mounted a vigorous defense of the centrality of natural selection, not only for elaborated extra-optic sensory structures found in many cave-dwelling organisms but also for eyes and pigment (Christiansen, 1961; Poulson, 1963; Barr, 1968). The centrality of natural selection in the evolution of the subterranean fauna was further emphasized by evolutionary developmental biologists, beginning with the pioneering work of Jeffery on the Mexican cavefish *Astyanax mexicanus* (Jeffery, 2009).

Astyanax mexicanus has emerged as a central model for examining the mechanisms, both proximate and ultimate, underlying cave-evolved traits such as eye regression and pigment loss. This is due to several reasons, including the presence of a cave and surface form of this species, the amenability of *A. mexicanus* to live and breed in the laboratory, and the rich literature on this species dating back over 70 years (Jeffery, 2020). Much of the early work investigating regressive traits in *A. mexicanus* was performed by Charles M. Breder. While not the discoverer of the Mexican cavefish [that honor belongs to Salvador Coronado (Romero, 2001)], Breder, along with Priscilla Rasquin, played a critical role in promoting *Astyanax* as a model system for the study of eye and pigment loss, especially in the laboratory (e.g., Breder and Rasquin, 1950). In particular, they took advantage of the fact that the cave populations interbred in the laboratory with eyed, pigmented surface river populations. Breder and Rasquin were writing before the emergence of the neo-Darwinian synthesis, spearheaded by Dobzhansky, Simpson, and others in the 1950's. The question of whether natural selection or genetic drift was the primary driver of eye and pigment loss was not a question in the Kosswig and Kosswig's (1940). Rather they focused on how cavefish lost eyes, in particular, the physiological mechanisms contributing to eye loss (Breder and Rasquin, 1950). Breder was, however, a keen observer of the ecological conditions under which *Astyanax* cavefish lived firmly held that darkness was the key factor in the evolution of eye regression, which can be the case in either selection or drift models (Breder, 1942; Breder, 1953).

In the last several decades, there has been a heightened interest in the selection/neutrality debate to explain the evolution of regressive cave traits, especially with respect to the work on the Mexican cavefish, made possible not only because it can be bred with its ancestral-like surface counterpart, but also because whole genome sequences are available (McGaugh et al., 2014; Warren et al., 2021) and functional studies can be performed in this species, including manipulation and transplantation of the eye lens in embryos and genetic manipulation (Yamamoto and Jeffery, 2000; Ma et al., 2015; Klaassen et al., 2018). Additionally, there has been the recognition that models of evolution of the eye and pigment in Mexican cavefish and other subterranean species must take into account the likelihood of migration between cave and surface populations in understanding the repeated evolution of cave traits (Herman et al., 2018). Finally, the evidence of the unexpected importance of maternal and epigenetic effects, as well as phenotypic plasticity on morphology (Romero, 2009; Yoshizawa et al., 2012a; Gore et al., 2018; Ma et al., 2018; Bilandžija et al., 2020) calls for an expansion of how the community understands the mechanisms contributing to trait evolution in this species.

The time is propitious for a re-evaluation of this long-standing controversy. We begin with a brief historical review, and then consider epistemological issues surrounding presumed demonstration of the primacy of either selection or neutral mutation. We then consider other ways the previous models were inadequate, and propose a new, broader perspective, one that takes into account how maternal effects and generally how genes and environment interact.

2. Historical background

Both Barr (1968) and Romero (2001) review aspects of the history of ideas about the evolution of cave life, but provide very different perspectives and focus on different time periods. Romero extensively reviews the early history, including the neo-Lamarckian school in North America around the turn of the 20th century, as well as the French school of organicism (roughly, the view that evolution is driven by forces above the individual and population level). Barr focuses on 12 theories for eye and pigment loss, most of which had adherents when he was writing in 1968. We focus on the development of what Trontelj (2019) calls the selectionist school of speleobiology, beginning in the mid-1960's, and subsequent developments.

While the mid-1960's signaled the birth of molecular population genetics, the first efforts at untangling genetic variation of subterranean organisms at the enzymatic level did not occur until the mid-1970's (Laing et al., 1976), and reciprocal eye lens transplantation procedures in *Astyanax* were not available until 2000 (Jeffery and Martasian, 1998; Yamamoto and Jeffery, 2002). Thus, the beginnings of the selectionist school relied primarily on the comparative method, with surface and cave species compared with respect to morphology and behavior. The real triumph of Barr, Christiansen, and Poulson was the demonstration that natural selection was a feasible explanation of observed patterns, not that they "proved" selection.

Christiansen (1961, 1965) in fact did not study the eyes and pigment of Collembola. What he did was demonstrate that both morphology and behavior with respect to the ability to walk on water films and surfaces (a presumed advantage in obtaining food) showed convergent evolution in cave species. While this work was before the rise of cladistics (in fact a phylogeny for the Entomobryinae still does not exist), he demonstrated that this convergence (and hence adaptation) occurred in both European and North American lineages. While it may seem strange in a contemporary context, Christiansen gave evidence for adaptation in cave animals, a point disputed by a number of francophone evolutionary biologists (see Vandel, 1964) who held that the cave fauna represented senescent phyletic lines, that were blind not because they were in caves, but were blind and could not survive outside of caves. Christiansen was very much a part of the comparative school, one that Gould and Lewontin (1979) went on to criticize as consisting of "just so" stories of adaptation.

Poulson (1963) employed a similar epistemology in his study of the North American cave fish in the family Amblyopsidae. He looked at a wide range of morphological and behavioral patterns that were present in the cave dwelling species in the family (which includes both surface and cave dwelling species). Rather than look at convergence in different lineages (it was unclear at the time that there was more than one lineage of amblyopsid fish), he used the degree of eye degeneration as a measure of time isolated in caves, and demonstrated correlated levels of adaptation in traits such as lateral line system development, brain structure, and feeding behavior. His use of the degree of degeneration as a measure of time was a common theme among North American neo-Lamarckians, including Eigenmann (1909) who did the original work on eye degeneration of amblyopsids. Poulson placed this all in the context of adaptation to life in darkness with little food. Unlike Christiansen, he does consider eyes, but only as time keepers. Some decades later, Poulson fully adopts a non-selectionist view of eye loss (Poulson, 2017) while retaining his neo-Darwinian credentials.

More than Christiansen or Poulson, Thomas Barr was immersed in the study of cave science (speleology), not only publishing extensively on cave ecology and evolution, but also on species descriptions (as did Christiansen), and the physical description of caves (Barr, 1961). His research was largely focused on systematics and biogeography, but he provided (Barr, 1968) the most comprehensive review of theories of regressive evolution. He lists 12 theories to explain eye and pigment loss, and divides them into three groups: (1) Lamarckian and neo-Lamarckian; (2) Orthogenesis; and (3) Darwinian and neo-Darwinian. Among the eight Darwinian theories, Barr lists three that involve natural selection as the primary motor of change: direct selection, material compensation (energy conservation), and indirect effects of pleiotropy (Krekeler, 1958). Of these, the last two still have currency. Evidence for material compensation and the attendant competition among parts includes changes in relative sizes of brain lobes in amblyopsid fish (Eigenmann, 1909; Poulson, 1963). Evidence for indirect selection due to pleiotropy has come more recently, and includes a number of genes in the visual and pigment pathways of Mexican cavefish, for example, the *oca2* gene in the melanin pathway and the expansion of *shh* expression at the midline, which have both been implicated in contributing to a number of traits (Yamamoto et al., 2009; Bilandžija et al., 2013; O'Gorman et al., 2021). Interestingly, Barr includes two theories involving neutral mutation (genetic drift and accumulation of random mutations) under the Darwinian rubric. His main critique of these mutation drive hypotheses was that there seemed to have been insufficient time for them to lead to trait loss. In some ways, Barr's summary marks the end of an era. Nearly simultaneously with the publication of his review, Kimura (1968) published a book length exposition on the role of neutral mutation in evolution, Hennig (1965) published the foundation document for cladistics in 1966, and Lewontin and Hubby (1966) ushered in the era of molecular population genetics.

Of course, all of this early work by neo-Darwinists was comparative and correlational. There were no direct tests of hypotheses. The argument for selection had two parts. First, the observed morphological patterns of elaborated characters (such as the claw of Collembola) was elaborated in such a way that was expected in the harsh subterranean environment. Second, losses (eyes and pigment) were unlikely to be the direct result of selection, but energy economy and pleiotropy, both resulting in indirect selection, could explain those losses. Culver et al. (1995) attempted a more rigorous (and falsifiable) test for natural selection in the cave amphipod, *Gammarus minus*, a species that shares with *Astyanax mexicanus* the presence of both surface and cave-dwelling populations that are morphologically distinct. Following Brandon's (1990) rules for the demonstration of natural selection, they demonstrated heritable variation in eye, antennal, and size traits, and that there was significant selection on all three of these components as measured by fecundity and probability of mating. In a later paper, Christman et al. (1997) demonstrated that the differences in eyes, antennae, and body size could not be explained by phylogenetic effects. While the overall result was at least epistemologically appealing, several aspects of the study remained unexplained. First, there appeared to be selection on eye size but no selective factor is known that would cause this. Second, large antennae, expected to be selected against in surface populations, was selected for.

Finally, the work of Konec et al. (2015) follows in the comparative tradition, albeit with closer attention to phylogeny. They measured 62 morphological traits of the isopod *Asellus aquaticus* for two paired populations from Slovenia and Romania. They found that 18 of 62 traits showed convergence, an indication of the importance of the subterranean environment in molding morphology. Of course these traits included both increases and reductions, and some are classic regressive features such as eye reduction. This study, while fascinating, does not help to distinguish selection from neutral mutation.

While Barr considered mutation theories to be part of neo-Darwinism, its main proponent, Horst Wilkens, clearly saw neutral mutation and genetic drift as an alternative, at least to natural selection as the driving force behind eye and pigment loss. Wilkens' seminal paper (1971) elaborated and refined ideas proposed by an earlier generation of mutationists, especially Kosswig and Kosswig (1940), who argued, based in part on their studies of the subterranean populations of the isopod *Asellus aquaticus* in Slovenia, that selectively neutral mutations best explained the high levels of variation in eye and pigment.[1] Relying primary on data on F1 and F2 crosses both between cave and surface, and different cave populations, Wilkens demonstrated polygenic inheritance, independent acquisition of losses, and increased eye variability in darkness. In a career spanning more than 50 years (Wilkens, 1971, 1988; Wilkens and Strecker, 2017), Wilkens has

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[1] The contemporary interpretation of the populations the Kosswigs studied indicates a complex pattern resulting from at least three colonizations of the Postojna Planina Cave System (Slovenia) by *Asellus aquaticus* (Verovnik and Konec, 2019).

consistently argued that eye and pigment loss does not involve natural selection, but is rather the result of genetic drift and accumulation of selectively neutral, morphologically reducing alleles.

There are several unique features to Wilkens' view of the evolution of cavefish. First, his argument has remained largely unchanged over a nearly 50 year time span. Based on ideas of the Kosswigs' about mutation dating back to the 1930's, the pillar of Wilkens' arguments is that the increased variability shown by both hybrids and recent cave colonists indicates a relaxation of selection. That is, he equates increased variability with relaxation of selection. Indeed, relaxation of stabilizing selection, in the absence of other complications, will result in an increased variability of the trait, the result of both migration and mutation. However, other kinds of selection will also increase variability, such as disruptive selection. The presumption is that such kinds of selection are rare in the subterranean domain (but see Culver et al., 1994). Second, he pays scant attention to the development of neutral mutation theory by Kimura (1968) even though it provides opportunities for testing hypotheses and refuting the view that there has been insufficient evolutionary time for gene fixation under neutrality. Several researchers have argued that in fact there has been sufficient time for gene fixation under at least some multi-locus models (Chakraborty and Nei, 1974; Culver, 1982; Nei, 2013). Throughout his writing, Wilkens uses degree of morphological degeneration of the eye as a measure of time, rather than testing it directly. Third, he ignores for the most part constructive traits. This emphasis on eye and pigment loss has led some biologists to mistakenly assume that these are the only differences between cave and surface species (see Romero, 2001). It was not until 2001 that a list of constructive as well as regressive traits for *Astyanax* cavefish (Table 1) was published (Jeffery, 2001). An instructive example of how constructive traits were minimized was the statements both by the Schemmel (1967), the original author of the study and by Wilkens, that the increased number of taste buds in cave populations was probably not significant (even though it was statistically significant) since they showed considerable variation.

TABLE 1 Constructive and regressive changes in *Astyanax* cavefish, modified and simplified from Jeffery (2001).

Trait	Type of change
Cranial neuromasts	Constructive
Egg size	Constructive
Fat content	Constructive
Infraorbital bones	Constructive
Larval jaw	Constructive
Maxillary teeth	Constructive
Taste buds	Constructive
Telencephalon	Constructive
Aggressive behavior	Regressive
Circadian activity	Regressive
Eyes	Regressive
Metabolism	Regressive
Optic tectum	Regressive
Pigmentation	Regressive
Pineal gland	Regressive
Schooling behavior	Regressive
Scales	Regressive
Vertebrae	Regressive

3. The re-emergence of *Astyanax* as a model system and the return of pleiotropy

In the past two decades, a number of advances have been made which allow for experimental manipulation and genetic analysis in *A. mexicanus*. These advances have allowed researchers to further understand the genetic and developmental mechanisms contributing to the evolution of regressive traits in *A. mexicanus* cavefish and to reexamine the role of adaptation in regressive trait evolution in this species.

3.1. Studies of adaptation in *Astyanax*

Two mechanisms for how adaptive evolution *via* energy savings could contribute to eye regression in *A. mexicanus* cavefish have been proposed, and both have some support from experimental evidence.

First, it has been proposed that eye regression is adaptive in cavefish because differences in development or maintenance of the eyes provides energy savings (Jeffery, 2005). Cavefish have evolved a number of metabolic adaptations that suggest they have evolved under selective pressures to conserve energy (reviewed in Rohner, 2018). However, few studies have directly addressed whether there is limited food in the caves inhabited by *A. mexicanus* cavefish. Breder, perhaps the most experienced observer of *Astyanax* in caves, doubted that they were food limited (Breder, 1942, 1953), but current conditions in *Astyanax* caves may not reflect conditions at the time of colonization and isolation (Espinasa and Espinasa, 2016). Pipan and Culver (2012) proposed darkness, not food limitation, as the primary selective force. However, in a sense, darkness imposes a food limitation since it makes the finding of food much more difficult, resulting in a virtual scarcity. Further, darkness in caves is universal, whereas food scarcity is not (Pipan and Culver, 2012). As a convenient shorthand, we refer to both these factors as resulting in food scarcity. The energy savings from loss of eyes and pigment could occur through two mechanisms. First, if an organism does not develop eyes, this could result in energy savings during development. In *A. mexicanus* cavefish, eyes are specified and undergo optic cup morphogenesis during early development, albeit with some changes relative to surface fish counterparts, and then degenerate (reviewed in Jeffery, 2005). Further, while the cavefish retina does not increase in size during larval stages like the surface fish retina, this is not due to loss of cell proliferation, but instead due to increased cell death (Strickler et al., 2002). Thus, because the eye is formed and the cells in the retina proliferate, it is unlikely that the evolutionary changes observed in cavefish eye development would provide energetic savings sufficient to drive adaptive evolution of eye loss in this species (Jeffery, 2005), though it should be noted that whether there is energetic savings in cavefish relative to surface fish during development has not been directly tested.

The second mechanism for energy savings due to eye regression is based on the idea that in a nutrient poor environment, there could be a benefit for not maintaining high energy tissues (Niven and Laughlin, 2008). Neural tissues, including those that make up the visual system, are energetically expensive, and thus, reductions in the size of these tissues could be adaptive in a cave environment (Moran et al., 2022).

Eyes in adult cavefish are highly degenerate, and fish from multiple populations each have a smaller optic tectum compared to surface fish (Soares et al., 2004; Jaggard et al., 2020). The energetic cost of eyes was measured directly in *A. mexicanus* by Moran et al. (2015). Through direct measurements of metabolic rates of neural tissues, they demonstrated loss of eyes in cavefish would provide substantial energy savings (Moran et al., 2022). Thus, energy savings during visual system maintenance, rather than visual system development, may result in eye loss leading to adaptive benefits in the cave environment.

The genetic mechanisms for the evolution of eye and pigment reduction *via* pleiotropy require a linkage of these reduced traits with adaptive traits. The genetic mechanisms for the evolution of reduced traits *via* energy economy do not necessarily require such linkage. Pleiotropy, where the same gene affects multiple traits, has been the focus of nearly all experimental studies of adaptation in *Astyanax* (see Jeffery, 2005). However, it is important to note that there are other genetic mechanisms that could result in co-variance of traits, i.e., physically linked genes or more generally genes that tend to occur together as a result of linkage disequilibrium. A number of studies are consistent with pleiotropy and indirect selection playing a role in the evolution of regressive traits in cavefish. First, regression of eyes has been linked to expansion of constructive traits that may be beneficial in the cave. The expression of the gene *sonic hedgehog* (*shh*) is expanded in cavefish relative to surface fish, with a robust expansion at the midline during early development (Yamamoto et al., 2004) and increased expression in neural tissues at later stages of development (Menuet et al., 2007). Manipulation of *shh* signaling during development revealed that *shh* signaling plays a role in eye development and degeneration in this species (Yamamoto et al., 2004). Further, manipulation of *shh* signaling at early developmental stages also affects traits that are enhanced in cavefish, including number of taste buds and jaw size (Yamamoto et al., 2009). They report an observation that is perhaps the most direct evidence for pleiotropy. They found that a surface-dwelling *Astyanax* individual fish contained a heat sensitive expression plasmid with an *shh* gene that produced with eye degeneration or taste bud magnification, depending on temperature. This is the most direct observation of pleiotropy.

Additionally, quantification of jaw size and taste bud number in cave-surface hybrid fish with large or small eyes revealed a relationship between these traits in hybrid fish, further supporting a developmental relationship between these traits that could be mediated by *shh* signaling (Yamamoto et al., 2009). More recently, expanded *shh* signaling has been associated with brain evolution in *A. mexicanus* (Menuet et al., 2007), suggesting this change during development may impact both eye regression and behavior *via* changes to the brain.

In addition to functional studies, quantitative trait loci (QTL) studies suggest that pleiotropy may play a role in the evolution of cave traits, including eye regression. QTL for different cave-evolved traits cluster together within the genome (Protas et al., 2008; O'Quin and McGaugh, 2015). One explanation proposed for these QTL clusters is pleiotropy: that a gene or gene(s) within these genomic locations affects multiple cave-evolved traits (Protas et al., 2008; Yoshizawa et al., 2012b; O'Quin and McGaugh, 2015). One striking example of QTL clustering was identified by Yoshizawa et al. (2012b). Cavefish from some caves have evolved an adaptation associated with feeding, vibration attraction behavior (VAB), the tendency to swim toward a vibrating object in the water (Yoshizawa et al., 2010). The lateral line is necessary for VAB in cavefish (Yoshizawa et al., 2010), and quantification of VAB, the number of lateral line organs, the superficial neuromasts, within the eye orbit (which are found in cavefish and not surface fish), and eye size revealed correlations between these traits in cave-surface hybrid fish, suggesting these traits may share a genetic basis (Yoshizawa et al., 2012b). This was further supported by QTL analysis: QTL for eye size, VAB and eye orbit neuromast number cluster in two locations in the genome, supporting the hypothesis that the same gene(s) contribute to the evolution of all three of these traits (Yoshizawa et al., 2012b). This is significant, as many of the constructive traits that have evolved in cavefish have not been directly linked to a benefit to these fish within a cave habitat. VAB, however, has been shown to provide an advantage when foraging in the dark: Both cavefish and surface fish with VAB struck more at prey in the dark when directly compared to cavefish and surface fish without VAB (Yoshizawa et al., 2010). Thus, VAB may provide an advantage in a cave habitat for foraging, and the selective advantage of alleles that contribute to increasing VAB and neuromast number and reducing eye size could contribute to indirect selection for eye reduction in this species (Yoshizawa et al., 2010, 2012b).

The most definitive evidence for the role of pleiotropy in cavefish evolution comes from studies of the evolution of albinism in cavefish. Multiple populations of cavefish have evolved albinism, defined as the complete loss of melanin pigmentation (Protas et al., 2006). Genetic analysis of albinism suggests that this trait evolved through a single locus of large effect (Şadoğlu, 1957; Protas et al., 2006). The gene responsible for albinism is *oculocutaneous albinism 2* (*oca2*), which was identified initially through QTL mapping, and confirmed through functional studies, including generation of surface fish with mutations in this gene and finding that the resulting animals lacked melanin pigmentation (Protas et al., 2006; Ma et al., 2015; Klaassen et al., 2018). Distinct coding mutations in *oca2* have been identified in two *A. mexicanus* cave populations, and complementation analysis suggests that regulatory mutations in *oca2* cause albinism in a third population (Protas et al., 2006). In addition to playing a role in pigmentation, recent work demonstrates that *oca2* plays a role in behavioral evolution in *A. mexicanus*. Bilandžija et al. (2013) first suggested that mutations in *oca2* may contribute to the evolution of other traits in *A. mexicanus* cavefish. Cavefish have higher levels of catecholamines compared to their surface fish counterparts (Bilandžija et al., 2013; Elipot et al., 2014), and knockdown of *oca2* using morpholinos increases levels of the catecholamine dopamine in larval surface fish (Bilandžija et al., 2013). Catecholamines play a role in regulating a number of behaviors, including sleep and social behaviors (Saper et al., 2005; Scerbina et al., 2012), raising the possibility that *oca2* plays a role in the evolution of these or other cave-evolved behaviors in albino cavefish populations. Indeed, albino fish behave differently from wild-type surface fish. Both depigmented cavefish and hybrid fish selected to be albino and to have eyes have increased levels of catecholamines, and catecholamine-dependent anesthesia resistance compared to surface fish (Bilandžija et al., 2018). Additionally, surface fish engineered to harbor mutations in the *oca2* gene are albino and sleep less than their pigmented, wild-type siblings, suggesting that *oca2* plays a role in both pigmentation and behavior (O'Gorman et al., 2021). While it has been suggested that reductions in sleep are beneficial to cavefish, possibly providing more time for feeding (Duboué et al., 2011), whether reductions in sleep or other behaviors provides a benefit to fish within caves remains to be demonstrated. However, population genetics analysis of the *oca2* locus suggests *oca2* is under positive selection in multiple cavefish populations (O'Gorman et al., 2021). Together, this work provides the most extensive analysis of pleiotropy in this species to date, and suggests that, at least in the case of pigmentation, pleiotropy plays a role in the evolution of cave-evolved traits.

A major caveat to the pleiotropy and indirect selection argument for regressive traits in cavefish is that other genetic factors could explain many of these results: Closely linked genes that independently affect different traits could explain QTL clustering as well as correlations between traits in hybrid fish (Protas et al., 2008; Yoshizawa et al., 2012b; O'Quin and McGaugh, 2015). Even in the case of *shh*, it is not clear that endogenous levels of *shh* in these fish are sufficient to produce these differences in *shh*-dependent traits. Critical to proving indirect selection *via* pleiotropy is to identify and functionally assess the genes and alleles contributing to the evolution of these traits. Identifying the genes near or within QTL remains a significant challenge, particularly for traits that have evolved through multiple loci of small effect size (reviewed in O'Quin and McGaugh, 2015). The sequencing of the *A. mexicanus* cavefish and surface fish genomes has allowed for the identification of a number of candidate genes located within QTL clusters, at least some of which have expression patterns and functional data from other species that is consistent with pleiotropic effects of these genes (McGaugh et al., 2014; Warren et al., 2021). However, functional assessment of these genes and their cave alleles in *A. mexicanus* is critical for understanding if they play a pleiotropic role in cavefish evolution. Functional analysis of candidate genes is now possible in *A. mexicanus* using methods that allow for targeted gene manipulation, including TALENs and CRISPR/Cas9 (Ma et al., 2015; Kowalko et al., 2016; Klaassen et al., 2018). However, few candidate genes for regressive traits have been assessed at this level (for exceptions, see Klaassen et al., 2018; Ma et al., 2020; O'Gorman et al., 2021; Warren et al., 2021).

3.2. Studies of neutral mutation in *Astyanax*

While the theory of neutral mutation and the related theory of mutation drive evolution is quantitative, and at least in principle, testable, it has been rarely used in actual tests, and rather the general idea of a release of variability following a relaxation of selection has been tested. As we pointed out above, an increase in variability can have different causes.

Chakraborty and Nei (1974) and Nei (2013) estimate, for Pachón Cave in Mexico, whether there has been sufficient time for eye loss to occur. For neutral alleles, the probability of fixation by the t-th generation, ($P(1,t)$), is given by***:

$$P(1,t)=1-(4N\upsilon+1)e^{-\upsilon t},$$

where N is the effective population size and υ the mutation rate per locus per generation. They suggest that υ is of the order 10^{-5} because destructive mutations are selectively neutral, but $N\upsilon$ is effectively 0 since effective population size is likely to be around 200 (or perhaps larger). If the Pachón cave population diverged about 50,000 years ago and generation time is 5 years, then the probability of fixation of destructive mutations is about 0.63 and υt is approximately 1. Both divergence time and generation time numbers are suspect. Generation time in the lab is approximately 5–6 months. While divergence time is likely much greater than 50,000 years, with the most comprehensive analysis to date suggesting between 161,000 and 191,000 generations divergence between cave and surface populations (Herman et al., 2018), one study suggests that divergence may be as little as 20,000 years (Fumey et al., 2018). Reduced generation time makes the probability of fixation more likely while shortened divergence times do the opposite. This, and other similar tests for multiple loci (Culver, 1982), show a concordance with neutral theory, not necessarily a test of its validity.

Using Lande's (1976) work on the rates of phenotypic evolution under genetic drift, Culver (1982) used Wilkens' (1971) data on eye diameters to estimate that effective population sizes could be no larger than $270h^2$ individuals (where h^2 is heritability) for drift to account for the observed changes. If heritability is close to 1, then effective population sizes are in the range of estimates for Pachón Cave, but the mean estimate is about twice as large (Bradic et al., 2012). As Culver (1982) pointed out, there really is not sufficient information for a more rigorous test, and this has not changed in the intervening 40 years.

Certainly the central figure on the neutral evolution side of the debate about the evolution of cave populations of *Astyanax* is the German geneticist, Horst Wilkens, arguing forcefully for the importance of neutral processes for nearly 50 years. In the recent book length treatment of *Astyanax* (Wilkens and Strecker, 2017), he departs from his usual position of pointing out the areas of agreement with neutral theory and includes a critique of selectionist ideas, especially those involving pleiotropy. Theirs is a like a meta-analysis, with arguments taken from a series of papers on pleiotropy. This critique stands alone and they accept precious little of current work on pleiotropy, and it is difficult to judge this objectively. They do raise important points, especially the need to demonstrate pleiotropy with the association of QTLs for constructive traits with major genes of eye development such as *shh*.

3.3. Studies of natural selection and neutral mutation

Cartwright et al. (2017) put forward a general model of the evolution of eye (or pigment) loss in *Astyanax* in which gene frequencies are affected by directional selection (either energy economy or pleiotropy), as well a migration of large-eyed surface dwelling fish. The inclusion of migration is important for *Astyanax* because surface and cave populations are interfertile and in close proximity. This is not universally true for cave-limited species but in the model, migration can be set to zero. Their basic model is as follows:

$q_j=\{(1+s)q^2+(1+hs)q(1-q)\}/\{(1+s)q^2+2(1+hs)q(1-q)+(1-q)^2\}$ selection
$q_a=q_j(1-m)+Qm$ immigration
$q'=q_a+(1-q_a)u$ mutation

q_j = the frequency of the allele that causes blindness as a result of selection; q_a = the frequency of the allele that causes blindness as a result of migration; q' = the frequency of an allele that causes blindness in the cave population in the next generation due to selection, migration and mutation; q = allele frequency of an allele that causes blindness; s = fitness advantage of an allele that causes blindness; h = dominance level of an allele that causes blindness; m = the rate of immigration from the surface population to the cave populations; Q = the allele frequency of an allele that causes blindness in the surface population; u = the mutation rate of an allele that causes sightedness to on that causes blindness.

Even without the complication of multiple alleles and dominance, they show that there can be equilibria at 0, 1, or multiple interior points, depending on the intensity of selection. Among their general conclusions are that immigration rates must be less than mutation rates for neutral processes to predominate; that s must be greater than

48 times the immigration rates for selection to predominate, and that if the population is younger than 1/υ (the inverse of the mutation rate), selection rather than neutral mutation will predominate. In a test with real data, Herman et al. (2018) show that selection coefficients must be 0.01 to drive frequencies of blind alleles toward fixation. These are at least feasible values. Of course, different parameters from the ones Herman et al., used may yield different results. In particular, they posit a recent origin for population splits—approximately 175,000 generations.

Borowsky (2015) claimed that a test based on one developed by Orr (1998) could distinguish between selection and neutral mutation by examining the polarity QTL. In particular, the claim that nearly all QTL had negative polarity (cave alleles were structurally reducing) for eye size and mixed polarity for melanophore number indicates selection was responsible for eye size reduction but neutral mutation was responsible for melanophore reduction, is not necessarily indicative of different processes, but rather the nature of mutations arising in the populations (Culver, 1982). Lande (p. 194 in Wilkens and Strecker, 2017) provides a more general critique of Orr's test, as does (Poulson 2017).

4. Emerging themes

A number of themes emerge from the often fractious literature on the evolution of *Astyanax* in caves.

First, some biological features of *Astyanax* make it an ideal model system for the study of gains and losses in evolution, not just in caves, but in general. The reduction and/or loss of eyes and pigment are among the most obvious examples of losses (regressive evolution) in the animal kingdom. Likewise, the harsh environment of caves, especially the absence of light (which makes food scarce in caves in terms of the ability of organisms to locate it) points to the direction of constructive evolution.

Second, *Astyanax* is one the few cave species that is interfertile with its surface ancestor [the isopod *Asellus aquaticus* is another (Protas and Jeffery, 2012)] allowing for a range of genetic experiments unavailable in other species. In spite of concerns that this makes it atypical (Poulson, 2010), it also makes much work possible.

Third, the kind of neutral evolution modeled by Kimura, and to a lesser extent by Nei, is inevitable. Similarly, directional selection is almost inevitable as well, as long as there is differential fitness of alleles, a fact little disputed for constructive traits, and increasingly demonstrated for features of eye and pigment reduction. The question is whether there has been sufficient time and sufficiently strong selection for either of these evolutionary mechanisms to be quantitatively important.

Fourth, with few exceptions (e.g., Cartwright et al., 2017), there have not been tests of whether either neutral mutation or selection has occurred, but rather whether they are feasible. In particular, models of selection or neutral mutation can, but not inevitably, yield reasonable values of time required.

Fifth, the breadth of the study often determines the outcome. For example, the Hamburg research group headed by Wilkens produced no list of adaptive features—that did not occur until Jeffery did so in 2001. Even when adaptive features were studied by the Wilkens group, their impact was minimized (e.g., Schemmel, 1967). Likewise, studies of pleiotropy ignore neutral mutation, and the very design of experiments on both sides often precludes their joint consideration.

Sixth, there are now a number of studies that demonstrate or at least show the feasibility of both selection and neutral mutation in eye and pigment systems. While interesting, more demonstrations of either process likely will not expand our understanding of the entire evolutionary process.

Seventh, there are processes, especially epigenetic effects, environmental effects, and repeated migration, that have been mostly ignored, yet have proven important (Gore et al., 2018; Herman et al., 2018; Bilandžija et al., 2020). Perhaps the most transformative of these is the potential for *hsp90* to facilitate colonization of caves (Rohner et al., 2013).

5. A new path forward

Astyanax is certainly the most thoroughly studied cave organism in the world. A Pubmed search (accessed 25 October 2022) of "Astyanax" lists 753 publications. However, our knowledge of the species *Astyanax mexicanus* is highly uneven. While there are a large number of papers on genetics and development, there are relatively few papers on ecology, especially differences in environment among caves and population genetics, particularly in a molecular genetic context. We find several areas that are in need of attention and hold promise for new discoveries, outlined below.

First, while a number of loci have been identified for cave-evolved traits in *A. mexicanus* (reviewed in O'Quin and McGaugh, 2015), relatively few causative genes and genetic variants that contribute to these traits have been identified (for exceptions, see: Protas et al., 2006; Gross et al., 2009; Klaassen et al., 2018; Ma et al., 2020). Identification and functional testing of genes contributing to cave-evolved traits is critical for understanding how evolution has shaped cave *A. mexicanus*, and phenotypic analysis of animals harboring mutations in these candidate genes has the potential to illuminate their role in underpinning these traits, as well as whether these genes and alleles have pleiotropic effects (for example, see O'Gorman et al., 2021). Both cave and surface *A. mexicanus* now have published genomes (McGaugh et al., 2014; Warren et al., 2021), and the demonstration that tools for functional genetic analysis, including TALENs, CRISPR-Cas9, and transgenesis, work in this species (Elipot et al., 2014; Ma et al., 2015; Klaassen et al., 2018; Stahl et al., 2019), provide a path forward for this type of analysis.

Except for very early work shortly after the discovery of the species (especially Breder, 1942), little comment has been made about the cave environments where *A. mexicanus* live. Eliott (2015, 2018) provide a detailed description of *A. mexicanus* caves, but this is mostly limited to maps and their interpretation. Most authors have simply assumed that the caves are food poor and without light (Jeffery, 2020). However, Breder (1942, 1953) argues against food limitation, at least in la Cueva Chica, where he did extensive preliminary studies. Few studies have addressed food availability in the caves directly (for exceptions, see Espinasa et al., 2017; Wilson et al., 2021). Even less studied are differences among the caves, which may explain some of the variation seen. There have been hints of interesting environmental variation between localities [e.g., temperature and oxygen (Rohner et al., 2013; Ornelas-García et al., 2018; Tabin et al., 2018; Krishnan et al., 2020)]. Particularly interesting is the suggestion by Rohner et al. (2013) that reduced conductivity in cave waters acts as a trigger to express variation hidden by norm of reaction. Further, differences between microhabitats within caves, such as between different pools within caves, could have ecological and evolutionary significance (for example, Trontelj et al., 2012; Borko et al., 2021).

Understanding the ecological conditions of the cave can provide additional insight into how these environmental conditions affect phenotypic traits. The pioneering work of Jeffery and his colleagues (Gore et al., 2018; Bilandžija et al., 2020) demonstrated the importance of phenotypic plasticity and epigenetic effects in the evolution of cave traits. Somewhat earlier, Romero, 2009 argued that phenotypic plasticity is the key to understanding the colonization of caves in general. Additional studies elucidating the mechanisms underlying this plasticity, as well as response to other environmental factors found in cave habitats, will further our understanding of the role of these processes in the evolution of cave traits.

With a few notable exceptions (Chakraborty and Nei, 1974; Nei, 2013; Borowsky, 2015; Cartwright et al., 2017; Herman et al., 2018), mathematical population genetic models have not been used in the study of evolution of *Astyanax*. These models generally provide tests of the feasibility of particular evolutionary processes in explaining the evolution of morphology and genetics in *Astyanax* populations. Whole genome sequencing of wild-caught fish genomes (as in Herman et al., 2018), is now feasible, and will provide additional information about how cavefish traits have evolved. Further, analysis of *Astyanax* genomes has the potential to provide insight into genome evolution, such as the role of structural variation in the evolution of cavefish (for example, see Warren et al., 2021).

The study of cave life has been plagued by an ever increasing jargon used to describe many aspects of the evolution of cave animals, including convergence, specialization, and distribution (Culver et al., 2023). For example, the phrase "troglomorphic" used to describe characters correlated with subterranean life can easily be replaced by the more general evolutionary term convergence. The effect of this jargon is to diminish the true generality of the results, and appears to directly affect the readership (Martínez and Mammola, 2020). While there has been recent interest in the generality of the *Astyanax* model with respect to human disease related traits (Rohner, 2018; McGaugh et al., 2020), its generality with respect to evolution in extreme environments, such as caves, as not been stressed. This we believe is unfortunate because *Astyanax* holds considerable promise as an exemplar for evolution in extreme environments as well as for understanding basic evolutionary processes.

6. Is *Astyanax* an appropriate model of evolution of cave life?

Ever since its discovery more than 80 years ago, *Astyanax mexicanus* has had a rather ambiguous place in field of speleobiology. It is after all not an iconic cave organism, in the sense that the European cave salamander *Proteus anguinus* is. It does not share with *Proteus* and many other cave dwellers the other-worldly appearance and extremity of specialization. *Proteus* is almost the antithesis of *Astyanax; Proteus* has no known surface ancestors, reaches sexual maturity after decades, and is usually placed in a separate family, the Proteidae. Some of the very features that make *Astyanax* such a useful model system make it unique and therefore suspect. These include its ready hybridization with surface populations (actually not a unique feature) and the possibility of genetic and developmental manipulations. The separation of *Astyanax* research from other speleobiological research is exacerbated by the fact that work on *Astyanax* is laboratory intensive and much other work is field intensive.

The standard view is that *Astyanax* represents an earlier stage of adaptation of subterranean life than the extreme specialists like *Proteus* and the North American cave fish family Amblyopsidae, and that the processes involved (e.g., natural selection) are the same. Indeed, the isopod *Asellus aquaticus* shows many of the pattern of *Astyanax* in caves (Protas and Jeffery, 2012). However, it is possible that old species like *Proteus anguinus* have evolved by different pathways. Perhaps the continued presence of surface ancestors makes for a fundamental difference, or it may be that tropical and temperate cave populations face different challenges and hence different solutions. We think that this is unlikely, but while it is clear that all of the answers about the evolution of cave life cannot be answered with *Astyanax*, we believe many of them can. What phylogenetically old species can add in particular are answers to the following:

1. Does most morphological change occur rapidly followed by a long period of relative stasis?
2. What is the pattern of post-colonization dispersal?
3. Is there evidence of a fundamentally different path to adaptation to subterranean life than that of *Astyanax?*

If *Astyanax* is an appropriate model system, it has huge advantages (Jeffery, 2019)—crossing with surface-dwelling individuals, developmental and genetic accessibility, and the availability of whole genome DNA sequences. However, work on old subterranean groups yields unique insights as well. Only through studying additional cave lineages and other organisms evolving in extreme environments will we understand how generalizable (or unique) what we have learned about the genetic and evolutionary mechanisms that drive evolution of cave populations of *A. mexicanus* are.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An isolated chemolithoautotrophic ecosystem deduced from environmental isotopes: Ayyalon cave (Israel)

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The stable isotopes composition of chemolithoautotrophic cave ecosystems is known to differ from epigenic caves. Here we show that in addition, dead carbon (devoid of ^{14}C), is utilized and transferred throughout this ecosystem, rendering it unsuitable for radiocarbon dating. The connectivity of the Ayyalon Cave ecosystem with the surface is studied, along with its sources of energy and carbon, as well as the interconnections between its constituents. We use isotopic evidence to show that its ancient resilient ecosystem is based on an underground food web depending on rich biomass production by chemolithoautotrophic nutrient supplies, detached from surface photosynthesis. Carbon isotopic values indicate that: (1) the microbial biota use bicarbonate from the groundwater (23.34 pMC [% of modern carbon]) rather than the atmospheric CO_2 above the water (71.36 pMC); (2) the depleted ^{14}C signal is transferred through the entire ecosystem, indicating that the ecosystem is well-adapted and based on the cave biofilm which is in turn based on groundwater-dissolved inorganic carbon. Incubation of Ayyalon biofilm with ^{14}C-labelled bicarbonate indicates uptake of the radio-labeled bicarbonate by sulfur-oxidizing proteobacteria *Beggiatoa*, suggesting that these sulfur-oxidizing microorganisms use the water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. Organic matter in the cave is much lighter in its stable nitrogen and carbon isotopes compared with respective surface values, as expected in chemolithoautotrophic systems. This evidence may be applicative to subsurface voids of ancient Earth environments and extraterrestrial systems.

KEYWORDS

sulfidic cave, arthropod adaptation, radiocarbon, stable isotopes, environmental isotopes, chemolithoautotrophy, cave ecosystem, biofilm

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1. Introduction

Chemoauotrophic micro-organisms use various metabolic strategies to produce biomass by fixing CO_2 at the expense of energy extracted from chemical compounds (Engel, 2007), and support diverse food webs in caves (Culver, 2013). However, most human-accessible caves lack chemoautotrophic production. Chemoautotrophy-based karst ecosystems are often limited not only by energy, but also by the supply of elements such as iron, nitrogen, and sulfur (Sarbu et al., 1996). The dynamics of these elements have a strong influence on the magnitude and location of the energy base of food webs in the cave ecosystems, with zones such as the water-air interface acting as hotspots for productivity. Adaptations in diverse groups of cave-dwelling animals at the physiologic and molecular levels are affected by chemoautotrophy.

Cave chemolithoautotrophic processes were studied mainly since the mid-1990s, in association with studies of deep terrestrial groundwater and deep-sea hydrothermal vents (Stevens and McKinley, 1995). Chemolithoautotrophs have been commonly considered to be inefficient, relative to phototrophs. Therefore, chemolithoautotrophs have been assumed not to be important primary producers. However, under the absence of light and photosynthesis in the subsurface, chemolithoautotrophy can be important, sustaining many ecosystem-level processes (Culver and Pipan, 2019).

Chemolithoautotrophs use compounds present in rocks or groundwater. The energy is obtained by transferring electrons from one chemical compound to another. The electron donors include, for example, reduced sulfur compounds (e.g., hydrogen sulfide, elemental sulfur), molecular hydrogen (H_2), ammonium (NH^+), reduced iron (Fe^{2+}) and manganese (Mn^{2+}).

Along with photosynthesis, chemolithoautotrophy has been crucial in the transformation of chemical energy from one reservoir to another during Earth's history and for the incorporation of inorganic carbon into the biospheric organic carbon. Only members of the domains Bacteria and Archaea are capable of chemolithoautotrophy.

In spite of the importance of chemolithoautotrophy, the knowledge of chemosynthetic systems of caves remains limited. Most chemolithoautotrophic caves with well-developed geomicrobiology ecosystems have sulfidic water (Northup et al., 2001; Hose, 2013; Palmer, 2013).

A good example of such well-developed ecosystem with 37 metazoan endemics is Movile Cave (Brad et al., 2021), which has a sulfidic groundwater pond that is close to the surface, but represents a regional aquifer. The aquifer is accessed not only through the cave, but also through boreholes and wells. The diverse hygropetric fauna of Movile is composed mainly of new species which are congeners of genera present in the epigeic surroundings that penetrated the "dry bells" of that cave system possibly in a relatively recent geological past (Sârbu, 2001). The first use of environmental isotopes to study a chemoautotrophic cave system was performed at Movile by Sarbu et al. (1996).

Frasassi Cave in Italy has 12 endemic species, and was studied using stable isotopes by Sarbu et al. (2000). In the Frasassi Cave, evidence for nitrogen fixation was found in the symbiotic bacterial community of *Niphargus* amphipods, *Beggiatoa*-dominated biofilms, and sulfidic sediment (Desai et al., 2013). Dattagupta et al. (2009) showed in the same cave that *Niphargus ictus* throughout the cave is colonized by a single phylotype of bacteria in the sulfur-oxidizing clade *Thiothri.*

Cueva de Villa Luz (Hose and Pisarowicz, 1999; Palmer, 2013) has several skylights and a large sulfidic water stream coming out from its subsurface voids. Its connection with the surface fauna is thus larger than the above-mentioned caves, sustaining a smaller number of endemism.

In various caves, including anchialine systems, in Europe, America and Australia, chemoautotrophy has been shown to be either aerobic (oxidation of hydrogen, sulfur, iron, manganese, ammonia or methane) or anaerobic (by methanogenesis, acetogenesis, denitrification or the reduction of sulfate, iron or ammonia) with multiple co-occurring chemolithotrophic metabolic processes in most subsurface systems. In some aquifer systems, ^{13}C-depleted biomass indicated the presence of chemoautotrophic pathways through methane-linked carbon cycling, for example in Edwards Aquifer, Texas (Bishop et al., 2014; Hutchins et al., 2016), and Ox Bel Ha, Yucatán peninsula (Pohlman et al., 1997). Based on the prevalence and abundance of ^{13}C-depleted lipids, it has been shown that biofilms in lava caves at Lava Beds National Monument, US, are also fueled by *in situ* chemolithoautotrophy (Selensky et al., 2021). Iron-based metabolism is practiced by reducing microorganisms in iron ore caves (Parker et al., 2018.). However, most of the last-mentioned caves lack Metazoa.

Previous studies have used stable environmental isotopes to study chemoautotrophic cave ecosystems. In Movile and Frasassi caves, organisms with a chemolithoautotrophic diet have $\delta^{13}C$ values ranging from −38 to −46‰ and from −30 to −40‰ respectively (Sarbu et al., 1996, 2000). We hypothesize that such negative values are expected also in other similar chemoautotrophic caves, such as Ayyalon. We therefore test the stable environmental isotopes of Ayyalon, and compare them with their surface counterparts, for testing the dis/connection of the cave ecosystem from above-ground system.

Another hypothesis to be tested here, is that throughout a chemoautotrophic system, the carbon should be mostly 'dead', i.e., devoid of ^{14}C, due to the deep sources of carbon used by the cave ecosystem. This contrasts with epigenic cave systems which rely on carbon produced by photosynthesis using atmospheric CO_2 at the earth surface, i.e., carbon which is rich in ^{14}C. To test our assumption, we measure the ^{14}C content throughout the entire ecosystem, in order to differentiate chemoautotrophic components from associated surface phototrophic ones.

We also use the classic tools of carbon and nitrogen stable isotopes to further understand the ecosystem, from basic inorganic materials to highly-endemic arthropods (Por et al., 2013).

2. Ayyalon cave

2.1. Ayyalon hydrogeology

Ayyalon Cave (34.928157°E, 31.910891°N) is located ~20 km east of the Mediterranean coastline. The natural surface is ~100 m asl (above sea level). The Nesher Ramla quarry breached the natural surface during the second half of the 20th century, reaching Ayyalon Cave and associated watertable in 2006. The 2.7 km long Ayyalon Cave is within late Cretaceous (Turonian) massive limestone (Figure 1C). Ayyalon Cave was geologically covered by a ~100 m thick, almost impermeable chalk. Until its truncation by the quarry it had been effectively isolated from the surface by the impervious rock, which seems to have precluded percolation of water, organic matter and nutrients from photosynthetic surface environments.

A phreatic aquifer is exposed within the cave as a pond, serving as a window into the warm-sulfidic groundwater (Frumkin and Gvirtzman, 2006). The watertable is fluctuating between ~12–24 m asl, thus the water depth of the pond has varied recently between 2 (in 2018) and 9 m (in 2022). The aquifer at Ayyalon vicinity is slightly hydrothermal (also termed 'Ayyalon anomaly') with relatively high salinity and H_2S, compared with the regional groundwater (Frumkin and Gvirtzman, 2006). The 'Ayyalon anomaly' is a unique part of Israel's largest aquifer, Yarkon-Taninim (Weinberger et al., 1994), whose regional freshwater is generally cooler, less saline, and devoid of H_2S. Yarkon-Taninim recharge

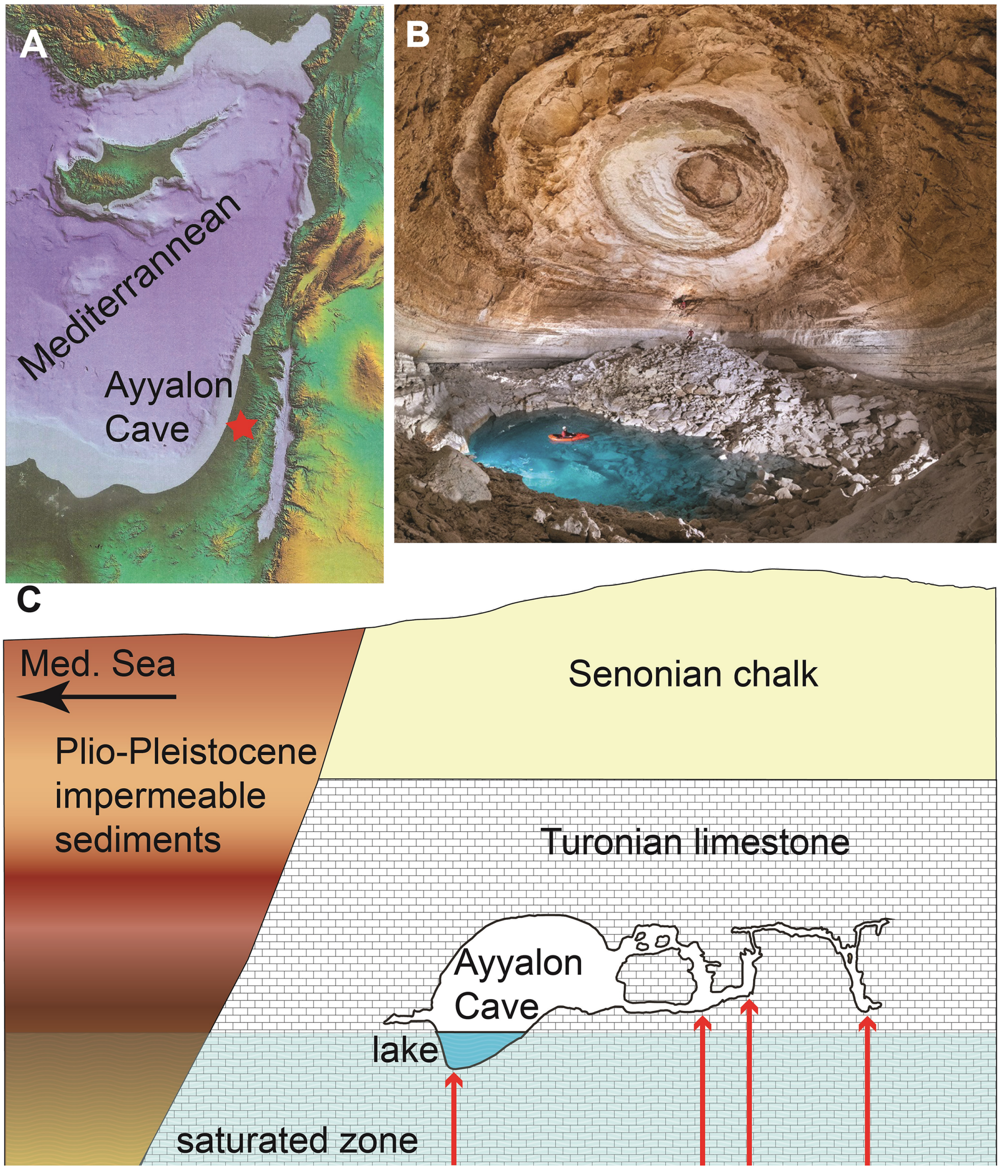

FIGURE 1
Ayyalon Cave configuration. **(A)** location; **(B)** the main chamber and groundwater pond of the cave, acting as the ecosystem focal point (photo: Boaz Langford); **(C)** schematic geological cross section of the cave and associated hydrogeology. For location of section see blue indicators in Figure 2A.

zone extends along the western slopes of Israel's central highland. The western part of the aquifer is confined by Senonian chalks overlain by younger low-conductivity rocks, dominated by the Pliocene Yafo Formation marls, among other late Cenozoic sediments. Ayyalon Cave is located east of the confinement line.

West of the Nesher Ramla quarry, within the Mediterranean Coastal Plain, the facies of the late Cretaceous carbonate rocks changes laterally, becoming impervious to the west, and together with the overlying Yafo Formation clays, these units block westward groundwater flow (Frumkin et al., 2022). Under these confining and damming conditions within the regional aquifer, the artesian hydrothermal flow mixes with fresh water flowing laterally from the recharge zone along the upper aquifer. The water temperature is up to 30°C at the hydrothermal plumes rising from depth through karstic shafts (Frumkin et al., 2015), while the mean surface air temperature is 20°C. Ayyalon Cave groundwater pond temperature ranges from 28.5 to 29.6°C (Table 1). A thermal-infrared survey performed at the flat bottom of the quarry near the Ayyalon Cave showed groundwater temperatures varying spatially by >3°C, in agreement with direct water temperature measurements in boreholes (Frumkin et al., 2015). The geothermal properties of the groundwater thus include 'hot spots' with temperatures around 2–3°C higher than in the surrounding aquifer. The sulfidic water contains no oxygen below the uppermost layer (Table 1). The spatial distribution of this anomalous water may indicate the possible limits of the aquatic ecosystem, and vice versa. Testing the isotopic features of the ecosystem can thus advance the understanding of water-ecosystem interconnection.

The plan and section of Ayyalon Cave (Figures 1C, 2B), show its affinity to the hypogene environment of rising hydrothermal plumes. The ascending groundwater caused karstic dissolution which formed a hypogenic maze, with intricate network of passages, chambers and rising-water shafts. Some of these have collapsed and advanced upwards, until reaching the surface and forming subaerial sinkholes (Frumkin et al., 2015) which serve as traps for surficial sediments, anthropogenic and zooarchaeological remains (Zaidner et al., 2014; Ullman et al., 2022).

2.2. Ayyalon microbial biota

Ayyalon Cave (and some neighboring smaller caves) is probably the only known deep (>100 m below surface) sulfidic cave system with endemic metazoan fauna (Figure 1; Por et al., 2013). The cave ecosystem is closely associated with its groundwater pond whose surface is partly covered by microbial mats. Initial microscopic observations indicate that the microbial mass in the water includes filamentous sulfur bacteria with intracellular sulfur granules, spherical sulfur (?) bacteria, amoeboids and other protozoa. Thus the CO_2 fixation may be performed by several microbial taxa. The microbial study of Ayyalon will be published elsewhere.

Metazoan taxa thrive in the water, on the microbial mats, and around the pond, where stranded relic biomats are observed. The Ayyalon Cave ecosystem demonstrates similar conditions and ecological networks to groundwater in smaller caves and drillings located a few hundred meters away (Frumkin et al., 2022).

TABLE 1 Temperature, oxygen and hydrogen sulfide in Ayyalon Cave groundwater pond at various depths and seasons.

Date (DD-MM-YY)	Depth in water (cm)	Temperature (°C)	O_2 (mg/l)	H_2S (mg/l)
21–05-06	0.1	28.5	3.5	4.7
28–05-06	450	n.d.	0	4.4
14–05-07	10	28.7	1.1	n.d.
19–06-07	10	29	0.11	n.d.
26–07-07	10	29	0.12	n.d.
06–09-07	10	29	0.13	n.d.
25–09-07	10	29.6	0.08	n.d.
01–11-07	10	28.6	0.07	n.d.
31–12-07	10	28.7	0.4	n.d.

The water is thermal (compared to 20°C mean surface temperature), sulfidic, and contains oxygen mainly in the uppermost few mm. n.d. = not determined.

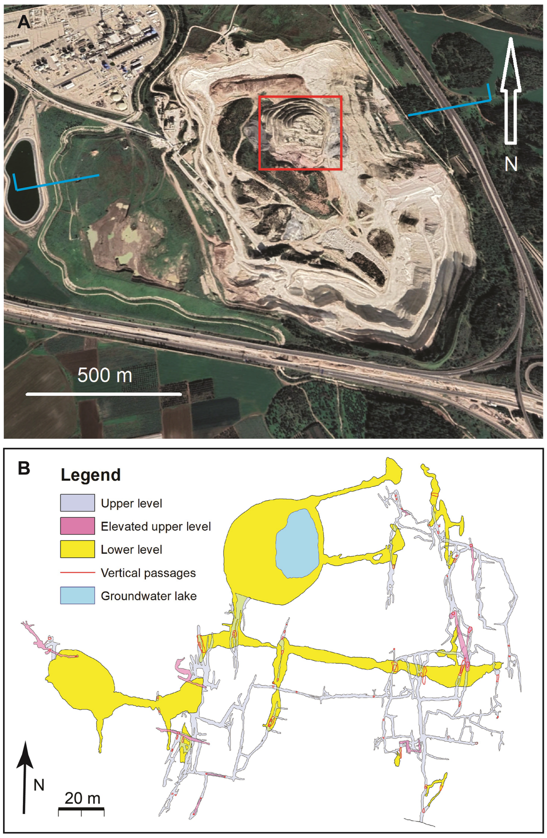

FIGURE 2
Plan view of Ayyalon Cave. **(A)** Google Earth view of Nesher-Ramla quarry with the central quarried 'hill' preserving the cave. Blue tags indicate general location of Figure 1C. **(B)** Ayyalon Cave plan, with 2.7km long passages in three levels, vertical phreatic shafts indicating past rising water, and the groundwater pond where the ecosystem is centered.

2.3. Ayyalon fauna

In recent years, most Ayyalon Cave arthropods have been identified and described (Table 2). The species described up to 2022 include the following detritivores and predators: a copepod, a hot-spring shrimp, a crayfish, a pseudoscorpion, a scorpion, and a collembolan. They include endemic species, endemic genera, tribe and possibly endemic family (Levy, 2007; Ćurčić, 2008; Tsurnamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012).

Ayyalon Cave crustaceans' stomach content has been studied microscopically in the laboratory in order to identify their food source (Tsurnamal, 2008; Wagner, 2012). Phylogenetic studies and molecular comparisons with congeneric relatives were performed on the prawn *Typhlocaris ayyaloni* (Guy-Haim et al., 2018).

Five of the seven arthropod species of the cave are already defined as new and endemic, restricted to the Ayyalon Cave system (Levy, 2007; Ćurčić, 2008; Tsurnamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012). The taxonomic position of two additional arthropod species (*Metacyclops subdolus* and *Troglopedetes* sp.) is still unclear, though probably they are also new endemics (Por et al., 2013).

2.3.1. Adaptive traits of Ayyalon fauna

The arthropod fauna of Ayyalon is partly (four species) stygobiont (obligate aquifer-dwelling), and partly (three species) troglobiont (obligate terrestrial cave-dwelling), documented around the pond in a hygropetric environment. The Ayyalon fauna show typical cave adaptations, such as the loss of eyes and pigmentation of the larger species, *Typhlocaris ayyaloni* (Tsurnamal, 2008) and *Akrav israchanani* (Levy, 2007).

In addition, some adaptations are more specific to Ayyalon fauna and similar cases. The unusual structure of pedipalp fingertips of *Akrav israchanani* may be an adaptive trait useful for preying on aquatic crustaceans abundant in the groundwater of the cave (Fet et al., 2011).

Laboratory observations on living *Typhlocaris ayyaloni* showed that these prawns do not respond to light, but are very sensitive to vibrations or to moving of objects in its vicinity (Tsurnamal, 2008). The prawns reacted by moving their sensory organs, or by getting into an alert position and retreating when an object was moving in the water as far as 15 cm away. Their stomach contents indicate that they feed directly on microbial mats and also on small crustaceans. The lack of egg-bearing females and very small prawns indicate that the groundwater pond of the cave is used by the prawns as a rich feeding ground, while other cavities of the surrounding aquifer may provide environmental conditions, such as cooler water, suitable for reproduction.

TABLE 2 Arthropod species at Ayyalon Cave.

Species	References
Typhlocaris ayyaloni (Tsurnamal, 2008; Crustacea: Decapoda)	Tsurnamal (2008)
Metacyclops longimaxillis (Defaye and Por, 2010; Crustacea: Copepoda)	Defaye and Por (2010)
Metacyclops subdolus Kiefer, 1938 auctorum (Crustacea: Copepoda)	Por et al. (2013)
Tethysbaena ophelicola Wagner, 2012 (Crustacea: Thermosbaenacea)	Wagner (2012)
Akrav israchanani Levy, 2007 (Arachnida: Scorpiones)	Levy (2007)
Ayyalonia dimentmani Ćurčić, 2008 (Pseudoscorpiones: Chthoniidae)	Ćurčić (2008)
Troglopedetes sp. (Collembola: Paronellidae)	Por et al. (2013)

An interesting trait of the cave pond crustaceans is a predominantly upside-down swimming posture of *Tethysbaena ophelicola,* and the occasional back-down swimming at the pool surface of *Typhlocaris ayyaloni*. A similar trait was reported at the sulfurous cave stream in Frasassi, where the amphipod *Niphargus ictus* swims upside-down at the water surface (Flot et al., 2010). Our observation thus supports upside-down swimming to be a general adaptative trait of life for oxygen replenishing in a thin (mm thick) layer of oxygenated water at the top of the groundwater. The life of the sulfidophilic *Tethysbaena* under microaerobic and anaerobic conditions relates to the biochemical and physiological adaptations to anoxia of the whole crustacean order Thermosbaenacea (Por et al., 2013). *Tethysbaena* has developed survival mechanisms to anaerobism, resistance to hydrogen sulfide poisoning, to elevated water temperatures as well as to fluctuating dissolved mineral content. Sulfide is most probably detoxified by intestinal bacteria (Por, 2014).

3. Materials and methods

3.1. Cave measurements and sampling

A geologic-geomorphic field survey of Ayyalon Cave was conducted. The cave was surveyed using the following instruments: Leika™ 'Disto' laser range-meter, inclinometer and compass (Dasher, 2011); plans and cross-sections were drawn to the scale of 1:200 using LimeLight™ cave mapping software. The cave map is presented in Figure 2B. In order to avoid contamination of the cave, the equipment was cleaned and disinfected prior to field studies in the cave.

Aquatic arthropod samples were collected using 60 and 200 μm nets. Terrestrial arthropods were collected by traps and tweezers. Ultraviolet flashlights were used for locating scorpion remains. Inorganic and organic samples collected within the cave were transferred into a cooling reservoir (~5°C) until submission to the relevant laboratory (see below), which occurred within 18 h after sampling. Similar methods were used to collect aquatic and terrestrial arthropods from the surface outside the cave, for isotopic comparison. Three surface winter ponds were sampled at Innaba, (31.9080N 34.9590E). A pond, ~500 meters south-west of the quarry was not used, due to possible contamination from a nearby sewage treatment plant.

Terrestrial arthropods were collected from the vicinity of the ponds.

Biological samples were separated at the Hebrew University and sent for morphological taxonomic identification by specialists of each arthropod group (Levy, 2007; Ćurčić, 2008; Tsurnamal, 2008; Defaye and Por, 2010; Fet et al., 2011, 2017; Wagner, 2012) and for isotopic analysis.

3.2. Carbon and nitrogen isotope analysis

We measured radiocarbon (coupled with $\delta^{13}C$) isotopic compositions which serve as direct indicators for the connectivity, carbon source of the ecosystem, and its food webs adaptations (Rundel et al., 2012). Each organic sample for ^{14}C was pre-treated at the Weizmann Institute of Science. Any foreign carbon material which is not related to the sample and represents therefore a possible source of contamination was visually removed. Using Fourier Transform Infrared Spectroscopy (FTIR), the material was analyzed before and after pre-treatment to check the possible presence and removal of contaminants. Contaminants such as gypsum were removed by ultrasonication in distilled water. The material was then oxidized to CO_2 and then graphitized for Accelerator Mass Spectrometry analysis and determination of radiocarbon content. A fraction of the CO_2 was used for $\delta^{13}C$ analysis. The AMS radiocarbon measurements were carried out on a NEC 1.5SDH-1 0.5MV Pelletron with a 40-sample MC-SNICS ion source. The accuracy of this system is higher than 0.4% and the machine background is lower than 0.03 pMC. Specific accuracy is noted per sample in Table 3. $\delta^{13}C$ is reported by ‰ VPDB (relative to Peedee belemnite) with an accuracy of ±0.15‰. For additional data on preparation and ^{14}C Measurement see Boaretto et al. (2009). Atmospheric isotopic values are given after Hua et al. (2021).

Organic samples were analyzed for $\delta^{13}C$ and $\delta^{15}N$ stable isotopes at the Institute of Earth Sciences, The Hebrew University of Jerusalem. Samples were dried in an oven at 50°C for 24 h. Calcite was removed from biofilms by adding 10% HCl. $\delta^{13}C$, $\delta^{15}N$ and C/N ratios were measured by elemental analyzer / continuous flow isotope ratio mass spectrometry (EA/*CF*-IRMS). The system included: FlashEA 1,112 analyzer coupled with Thermo Finnigan Delta Plus XL isotope ratio mass spectrometer. The results are reported for $\delta^{13}C$ ‰ VPDB (IAEA standard, value of zero 0.01123720) with an error up to 0.15‰, and for $\delta^{15}N$ (‰ relative to air, IAEA-N-1 standard +0.4) with an error up to 0.15‰. Error in %C / %N ratio is up to 2%.

Inorganic samples were collected in clean glass bottles from cave pond water, mud at the pond bottom, limestone bedrock at the cave chamber wall, air above the water, and air in an upper cave passage. For dissolved inorganic $\delta^{13}C$ in water, water was filtered in the field by 0.45 μm filter into a 100 ml glass bottle pre-treated with HgCl. An aliquot of sample was acidified with 5% H_3PO_4 in a gas stripping reactor and $\delta^{13}C$ was measured for the liberated CO_2 with a Thermo Scientific IRMS system with an accuracy of 0.1‰ VPDB. For solid-phase calcite samples, $\delta^{13}C$ was measured by gas bench continuous-flow isotope ratio mass spectrometry (*CF*-IRMS) system including GasBench III connected to Delta Plux XL mass spectrometer with an error up to 0.1‰ (VPDB).

TABLE 3 Carbon isotopes in organic matter of Ayyalon Cave and in possible sources of carbon.

Studied material		pMC	pMC E ±	$\delta^{13}C$ (‰ VPDB)
1	Limestone bedrock	0.00	0.20	−0.21
2	Atmosphere	104.00	0.27	−8.0
3	Air in high passage	97.93	0.43	−12.2
4	Air above water	71.36	0.33	−14.3
5	Mud (pond bottom)	0.72	0.07	−1.5
6	Water (inorganic C)	23.34	0.26	−12.2
7	Live biofilm	29.65	0.20	−41.8
8	Relic, hanging biofilm	25.28	0.27	−43.1
9	*Typhlocaris ayyaloni*	25.85	0.19	−33.5
10	*Akrav israchanani*	24.60	0.19	−32.8

pMC E ± is the error of ^{14}C; $\delta^{13}C$ is given in ‰VPDB, with error of 0.15‰. 1. Turonian limestone bedrock; 2. Atmosphere above the cave; 3. CO_2 in an upper passage of Ayyalon Cave; 4. CO_2 of air above Ayyalon Cave pond, within the cave; 5. Inorganic carbon of carbonate mud at Ayyalon pond bottom; 6. Dissolved inorganic carbon in Ayyalon pond water; 7. Biofilm at Ayyalon water surface; 8. Relic biofilm from previous Ayyalon groundwater surface, hanging today on walls above the water, 26 m asl. 9. *Typhlocaris ayyaloni*, Ayyalon Cave pond; 10. Exoskeleton chitin of dead *Akrav israchanani* specimen from Ayyalon Cave.

To test whether the difference between the cave and surface samples in Ayalon differ significantly in their isotope values we applied two complementary multivariate approaches, namely a parametric MANOVA (Clarke, 1993, using SPSS statistical package) and a non-parametric ANOSIM (Clarke, 1993) modification (using MATLAB software). For the MANOVA analysis we used Pillai's trace parameter defined between zero and one where zero indicates no difference between the groups and a value closer to one indicates a more significant difference. In ANOSIM the R statistic is used, where R is expected to equal zero when groups do not differ and to be greater than zero when they do. The ANOSIM analysis was performed by comparing the observed R_o statistic to 10,000 random values of the statistic, R_e, expected under the null hypothesis. The values of the statistic were calculated using distance matrix based on Euclidean distances. Random statistic values were obtained by randomly shuffling the observations groupings.

Table 4 shows the stable isotopes values, in which each number is averaged between two measurements.

3.3. Water measurement and analysis

Water temperature and oxygen concentration were measured in 10 cm depth of the cave pond using a Solinst Levellogger DIVER 3001. Dissolved oxygen was measured there with a WTW OXI 330 hand-held meter. Temperature, oxygen and hydrogen sulfide of the groundwater pond at various depths and seasons are given in Table 1.

Water from Ayyalon Cave pond was sampled directly from the water surface using a peristaltic pump from 10 to 450 cm depth. Each sample for H_2S concentration was collected in three 1.5 liter bottles. Sulfide was precipitated in the field as ZnS by adding 1–2 ml 1N Zn-acetate to 1 liter sampling bottles prior to sampling. The samples were analyzed at the Geological Survey of Israel. Excess water was removed in the lab, and the remaining deposit and water were reacted in a beaker with KI, HCl, and I_2, the latter in excess, which was then titrated using S_2O_3. For additional details see Gavrieli et al. (2001).

3.4. Primary productivity estimation

The ^{14}C incubation method was used to estimate the uptake of dissolved inorganic carbon (DIC) by microbial mats from Ayyalon groundwater, as was done earlier for other chemoautotrophic caves (Porter et al., 2009). The method is based on the fact that the biological uptake of ^{14}C-labeled DIC is proportional to the biological uptake of ^{12}C-DIC. The incubation was performed in Aharon Oren's laboratory at the Hebrew University according to Oren (2009).

4. Results and discussion

4.1. Radiocarbon

During the study, the atmospheric ^{14}C content of CO_2 outside the cave, in the upper cave levels, and above the groundwater pond were 104.0, 97.93, and 71.36 pMC, respectively, (Table 3; Figure 3A). In contrast, inorganic carbon compounds associated with the cave water and bedrock were highly depleted in ^{14}C: The Cretaceous bedrock, mud (at the bottom of the pond), and inorganic dissolved carbon of the groundwater contained 0.0, 0.72, and 23.34 pMC, respectively. The mud value represents the dominance of old, 'dead' carbon in the inorganic mud, while the dissolved carbon includes a signature of some recent groundwater flowing laterally from the east.

The measured ^{14}C content in the cave organisms was 29.65, 25.28, 25.85, and 24.6 pMC for the living biofilm, stranded relic biofilm (hanging above the pond, associated with previous high watertables), living aquatic prawn *Typhlocaris ayyaloni* and exoskeletons of the scorpion *Akrav israchanani* respectively, representing the highest trophic level of the aquatic and terrestrial Ayyalon ecosystem (Table 3; Figure 3A).

The intake of dissolved carbon from groundwater is further demonstrated by incubation of the biofilm with ^{14}C-labeled bicarbonate (Figure 4). Ayyalon microbiota, sampled from the sulfidic cave pond, was incubated with ^{14}C-labelled bicarbonate. Increase of radioactivity of the microbial lipids during incubation indicates uptake of the radiolabeled bicarbonate by *Beggiatoa*, suggesting that these sulfur-oxidizing microorganisms use water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. The experiment was performed with and without adding sulfide, showing similar results. *Beggiatoa* sp. of the microbial mat was sampled from the top of the water column (upper lines in Figure 4), and showed active CO_2 fixation. Lower lines (Figure 4) indicate no carbon fixation by other bacteria sampled from the water body.

4.2. Stable isotopes

Stable isotopes (δ^{13}C and δ^{15}N) and C/N ratios of the biotic system were measured for terrestrial and aquatic organisms inside the cave, and for comparison, from similar taxonomic groups on the regional surface above the cave (Table 4; Figures 3, 5). The measurements inside the cave included the entire range of organisms, from biofilms through the living aquatic prawn *Typhlocaris ayyaloni* and exoskeletons of the scorpion *Akrav israchanani.*

Cave vs. surface isotopic values fall in distinctively different fields: Organic matter in the cave is lighter isotopically than surface values (for isotopic ranges, see Figure 5). Higher trophic levels generally fall to the right of their food source.

4.3. Inference from isotopic values

Radiocarbon is used here to decipher the ecosystem's connectivity with the surface. Recently recharged groundwater, arriving from rainfall, carry an atmospheric signature. In the Judean hills, the radiocarbon content in the groundwater bicarbonate is ~80 pMC, representing mainly a combination of biogenic soil CO_2 and smaller portion of old bedrock carbonate. At Ayyalon, our measured ^{14}C values of air CO_2 outside the cave, in the upper cave levels, and above the groundwater pond were 104.0, 97.93, and 71.36 pMC, respectively, (Table 3; Figure 3A).

The negligible ^{14}C content of the cave mud (Table 3; Figure 3A) indicates the dominance of decomposed bedrock source. The 71.36 pMC of air above the water indicates the mixing of atmospheric CO_2 from the upper cave levels with depleted carbon from the water. The 23.34 pMC of the cave groundwater inorganic carbon is highly depleted in ^{14}C, in clear contrast with both the young atmosphere above the cave and the regional young groundwater. This indicates that the cave groundwater is mostly old, rising from the depth, after a long-term ^{14}C decay and interaction with carbonate bedrock, in contrast with regional young groundwater, whose ^{14}C content was ~80 pMC (Kroitoru et al., 1992). This verifies the hydrogeologic data, suggesting that Ayyalon groundwater comprises hydrothermal plumes rising from depth (Frumkin and Gvirtzman, 2006; Frumkin et al., 2015).

Cave water oxygen is limited to 0.02–3.5 mg/l, within the uppermost mm-thick groundwater layer, while deeper water demonstrates anaerobic conditions. This redox interface and

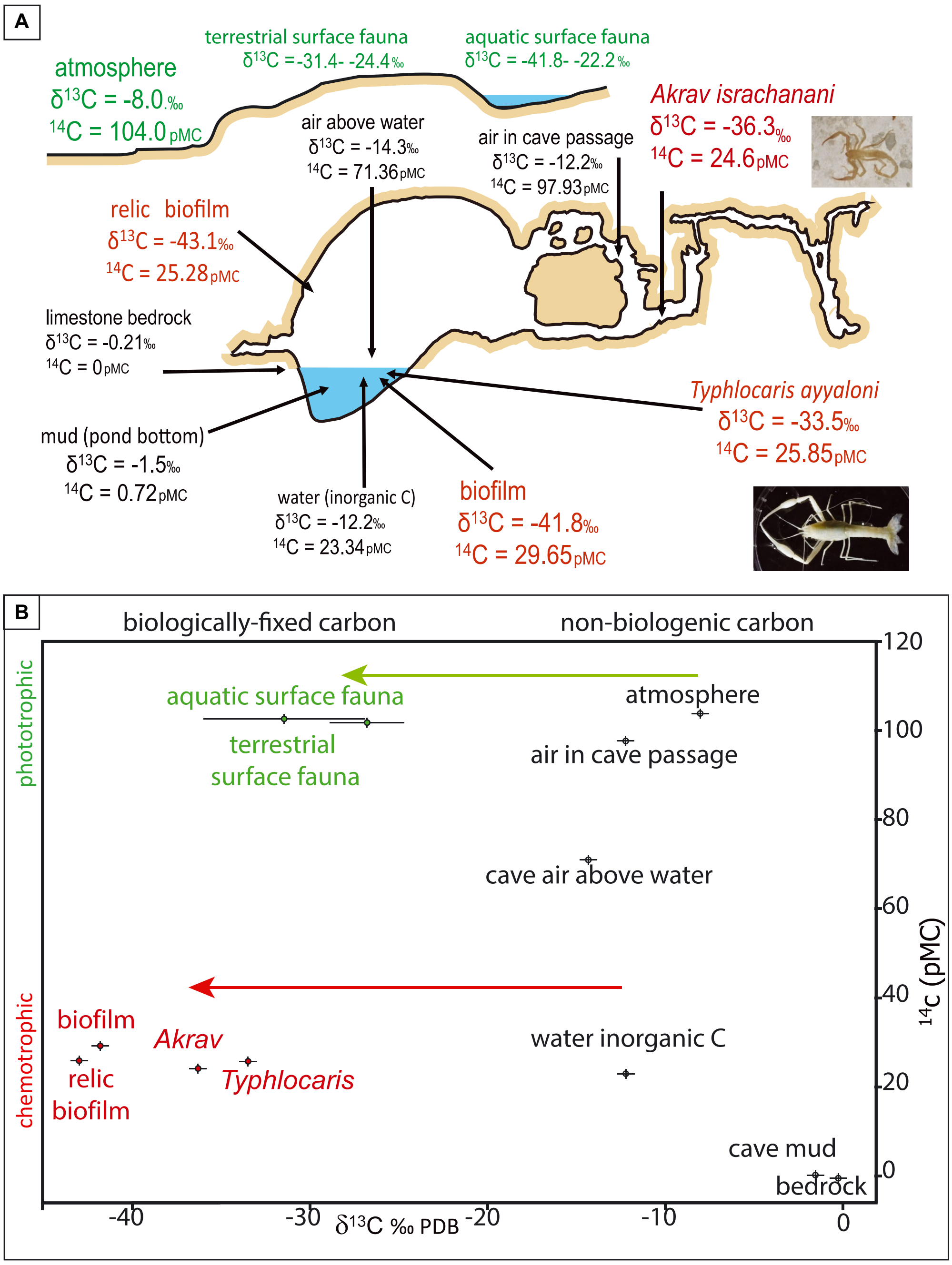

FIGURE 3
Carbon isotopes of the Ayyalon cave and surrounding surface, showing the isotopic difference between the surface phototrophic (green) and cave chemotrophic (red) systems. ^{14}C of surface fauna was assumed to replicate the atmospheric value. **(A)** cartoon indicating the measured values of the samples and their general location. **(B)** the relation between ^{14}C and $\delta^{13}C$ of the system's components. Green arrow represents ~−20‰ fractionation associated with photosynthesis by C_3 plants, subsequently consumed by surface fauna (shown). Red arrow represents fractionation associated with chemoautotrophic sulfur-oxidizing bacteria (Ruby et al., 1987). Individual samples with 1σ laboratory error are shown, except for surface fauna, whose gross diversity is represented by the average values of 15 aquatic species and 11 terrestrial species, with 1σ standard deviation of each group (further details in Tables 3, 4).

associated environment, although poor in oxygen, allows for the rich metazoan life within the groundwater. Specific adaptations to these environmental conditions are discussed above (Section 2.3).

The Ayyalon ^{14}C results (Table 3; Figure 3) indicate that: (1) the microbial biota (29.65 pMC) use mainly bicarbonate from the groundwater (23.34 pMC) with a smaller fraction of atmospheric CO_2 from above the water (71.36 pMC); and (2) the depleted ^{14}C

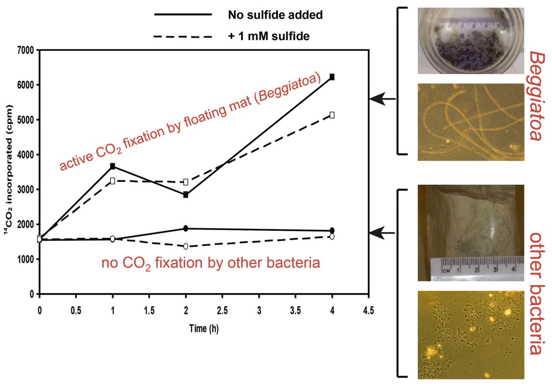

FIGURE 4

Incubation of Ayyalon microbiota, sampled from the sulfidic cave pond, with ^{14}C-labelled bicarbonate. On the right, pictures show bulk samples as sampled within glass bottles, and microscopic view of *Beggiatoa* sp. and other bacteria. On the left, upper lines show active CO_2 fixation by *Beggiatoa* sp. of the microbial mat sampled from the top of the water column. Lower lines indicate no carbon fixation by other bacteria sampled from the water body. *Beggiatoa* vs. other bacteria were differentiated by morphology and sulfur content. The increase of radioactivity of the microbial lipids during incubation indicates uptake of the radiolabeled bicarbonate by *Beggiatoa*, suggesting that these sulfur-oxidizing microorganisms use the water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. The experiment was performed with and without adding sulfide (dashed and solid lines respectively), showing similar results.

signal passes through the entire ecosystem, indicating that the ecosystem is totally based on the biofilm which is in turn based on groundwater-dissolved inorganic carbon.

The increase of radioactivity of the microbial lipids during incubation of the biofilm with ^{14}C-labeled bicarbonate (Figure 4) indicates uptake of the radio-labeled bicarbonate from groundwater, suggesting that the sulfur-oxidizing proteobacteria *Beggiatoa* use the water-dissolved inorganic carbon for chemolithoautotrophic carbon fixation. This is corroborated by the observation that the cave lacks any organic or inorganic material originating from the surface, due to the cave's isolation.

Further insight into Ayyalon ecosystem and its adaptation is obtained from stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of the biotic system (Table 4; Figure 5). Most organisms with a chemolithoautotrophic diet have quite negative $\delta^{13}C$ values (e.g., –38 to −46‰ in Movile; −30 to −40‰ in Frasassi; Sarbu et al., 1996, 2000). This is mainly due to discrimination against ^{13}C, associated with chemolithoautotrophic assimilation of inorganic carbon. Methane-driven chemoautotrophic carbon-fueled ecosystems can have $\delta^{13}C$ values from −30 to −90‰ (Grey, 2016). While $\delta^{13}C$ of dissolved inorganic carbon of Ayyalon Cave water is −12.2‰ VPDB, the $\delta^{13}C$ of the biofilm is −41.8 and −43.1‰ (for live and stranded relic samples respectively, Figure 3), slightly larger than expected for fractionation of stable carbon isotopes during chemolithoautotrophic growth of sulfur-oxidizing bacteria between the inorganic carbon of the water and the chemoautotrophic microbiome of the water (Degens, 1969; Ruby et al., 1987; Galimov, 2006). The reasons causing the difference should be further investigated. The depletion is larger than observed in most photosynthetically-derived organic material. The largest degree of fractionation of carbon isotopes occurs during the initial steps of reduction of inorganic carbon (i.e., carbon dioxide and bicarbonate) and results in a decreased $^{13}C/^{12}C$ isotope ratio in the organic product relative to that of the source carbon. Subsequent utilization of the organic carbon through trophic levels of the food chain leads to small but detectable increases in the $^{13}C/^{12}C$ isotopic ratio (Ruby et al., 1987; Galimov, 2006). Alternatively, isotopically light carbon may rise from the depths as ^{13}C-depleted methane, followed by oxidation to CO_2 and bicarbonate near the water-table, being then metabolized by the biofilm into organic matter (Grey, 2016).

Mean $\delta^{13}C$ of aquatic and terrestrial species in the cave is -35.2 ± 1.5 and -32.7 ± 0.1‰ respectively, both much lower than surface samples averaging −28.65 and -26.8 ± 2.1‰ respectively, which are dominated by the lower carbon fractionation of C_3 photosynthetic vegetation (Figure 3). Within the cave ecosystem, the higher trophic levels show variable isotopic shift relative to their diet (Figure 3B), with a positive shift in both aquatic (−33.0 to −36.3‰, Table 4) and terrestrial fauna (−32.6 to −33.5‰). This is in agreement with the expected isotopic shifts between diets and consumers (Galimov, 2006).

Similarly, the mean $\delta^{15}N$ of Ayyalon aquatic and terrestrial organic samples is -1.0 ± 1.7‰ and -6.5 ± 3.3‰ respectively, both lower than surface samples, whose average measured values are 6.56 ± 2.9 and 3.4 ± 3.6‰ respectively (Figure 5A). The negative trophic shift of N may be indicative of fluid-feeding consumers. McCutchan et al. (2003) showed that estimates of trophic shift for nitrogen differ systematically between fluid-feeding consumers and other consumers.

Both multivariate tests used here (MANOVA and ANOSIM; Clarke, 1993) indicate a highly significant effect of habitat (cave vs. surface) on the isotopic values of the samples. Pillai's trace value was close to unity (0.995) with $F_{(2,22)} = 2{,}402$ and sig. < 0.0001. The ANOSIM analysis produced similar results with the observed statistic, $R_o = 0.0267$, being significantly greater than zero – sig. < 0.0001 (Figure 6).

The combined evidence suggests that the sulfur-oxidizing microbiota obtain the necessary carbon for metabolic processes from bicarbonate in the groundwater, as well as nitrogen from nitrogen compounds in the water. The biofilm covering the cave water is thus the primary producer for the entire food chain in the cave, both inside and outside the water.

The higher trophic levels in Ayyalon are evident also by direct observations: The small crustacean *Tethysbaena ophelicola* has only biofilm cells in its stomach, and is itself found in the stomachs of the other crustaceans (Wagner, 2012), indicating that this species is a primary consumer in the Ayyalon ecosystem. The largest crustacean of Ayyalon, *Typhlocaris ayyaloni*, feeds also on biofilms in addition to predation of other crustaceans (Tsurnamal, 2008). This is in agreement with the isotopic evidence presented above.

The observed isotopic depletion relative to surface values at Ayyalon are in agreement with measurements in other sulfidic

TABLE 4 Carbon and nitrogen stable isotope values of whole-body biogenic materials in Ayyalon Cave and associated surface near the cave.

Sample number	Source	δ^{13}C ‰ VPDB	δ^{15}N ‰ (V. air N_2)	C:N	Taxon
B1	Ayyalon aquatic	−35.8	0.9	5.5	biofilm
A10	Ayyalon aquatic	−33.0	−2.2	4.2	*Cyclopoidae*
A8	Ayyalon aquatic	−36.0	−2.6	4.7	*Tethysbaena*
B5	Ayyalon aquatic	−35.9	−0.2	5.1	*Typhlocaris ayyaloni*
Aquatic average	Ayyalon aquatic	−35.2	−1.0	4.9	
Std. deviation	Ayyalon aquatic	1.5	1.7	0.6	
B7	Ayyalon terrestrial	−32.7	−10.1	5	*Collembola*
B6	Ayyalon terrestrial	−32.6	−3.7	4.4	*Ayyalonia dimentmani*
A12	Ayyalon terrestrial	−32.8	−5.6	5.1	*Akrav israchanani*
Terrestrial average	Ayyalon terrestrial	−32.7	−6.5	4.8	
Std. deviation	Ayyalon terrestrial	0.1	3.3	0.4	
A3	Surface aquatic	−22.2	1.4	9.8	*Ostracoda*
D10	Surface aquatic	−29.6	8.1	4.5	*Corixidae*
C5	Surface aquatic	−29.7	6.5	4.9	*Coleoptera*
C6	Surface aquatic	−31.9	9.1	5.7	*Cyclopoidae*
C8	Surface aquatic	−30.7	10.3	4.4	*Chironomidae*
C9	Surface aquatic	−25.6	6.3	4.2	*Coleoptera*
C10	Surface aquatic	−30.2	7.2	3.6	*Zygoptera*
C11	Surface aquatic	−29.3	3.6	3.7	*Hyla* (tadpole)
Average aquat.ic	Surface aquatic	−28.65	6.56	5.1	
Std. deviation	Surface aquatic	3.17	2.9	2.01	
B9	Surface terrestrial	−28.4	−0.8	3.4	*Acrididae*
D3	Surface terrestrial	−27.4	0.4	3.3	*Grylloidea*
B4	Surface terrestrial	−31.4	9.6	4.3	*Gryllotalpidae*
A4	Surface terrestrial	−27.7	4.7	4.7	*Aranae*
B10	Surface terrestrial	−24.4	−1.3	7	*Chilopoda*
B11	Surface terrestrial	−25.5	5.3	3.7	*Scorpiones*
C1	Surface terrestrial	−27.4	2.7	4.3	*Aranae*
D1	Surface terrestrial	−24.7	1.4	6	*Coleoptera*
D7	Surface terrestrial	−27.4	1.8	6	*Limacus*
D8	Surface terrestrial	−25.5	8.5	4.2	*Aranae*
D9	Surface terrestrial	−24.7	5	5.1	*Coleoptera*
Average terrestrial	Surface terrestrial	−26.8	3.4	4.7	
Std. deviation	Surface terrestrial	2.1	3.6	1.2	

Exceptions are exoskeleton chitin of dead *Akrav israchanani* specimen from Ayyalon Cave (sample A12) which was compared with exoskeleton chitin of a living scorpion specimen from the surface (sample B11). The presented results are averages of two samples per each taxon with an error up to 0.1‰ (VPDB).

caves ecosystems, such as Movile (Sarbu et al., 1996), and Frassasi (Sarbu et al., 2000; Figure 5). All these caves show compatible isotopic differences between the surface ecosystem and the subsurface sulfidic chemolithoautotrophy counterpart (Figure 5B). These offsets of δ^{13}C values of organisms inside the cave vs. outside are −18 to −14‰ at Movile, −8 to −12‰ at Frassasi, and − 4 to −11‰ at Ayyalon Cave. The offsets of δ^{15}N are −7 to −14‰ at Movile, −16 to −17‰ at Frassasi, and − 9 to −10‰ at Ayyalon Cave. This indicates that the biofilms in all these sulfidic environments make similar use of carbon and nitrogen from deep sources rather than from the atmosphere.

However, there are basic differences between the various cave systems. Frassasi Cave has a better connection with the surface, including a natural entrance with open outflow of its subsurface sulfidic stream (Sarbu et al., 2000). The more secluded Movile Cave has a groundwater pond, is located under 20 m-thick caprock

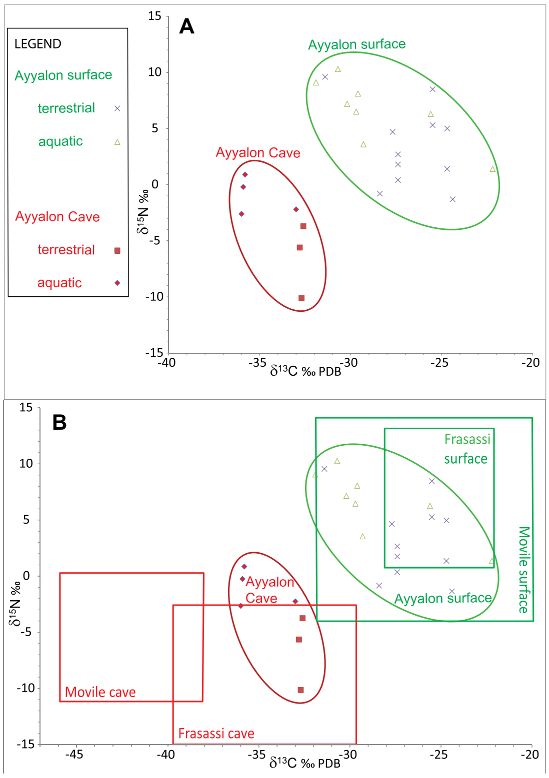

FIGURE 5
Bi-plots of nitrogen vs. carbon isotopes of organic matter at Ayyalon (present study), compared with previously studied caves. **(A)** from Ayyalon cave (red) and surrounding surface (green), divided to terrestrial and aquatic ecosystems. Cave and surface values fall in distinctively different fields: Organic matter in the cave is much lighter isotopically than respective surface values. Higher trophic levels generally fall to the right of their food source. Further details on individual samples are given in Table 4. **(B)** comparison between Ayyalon's data (ellipses) and general fields of similar measurements in two other sulfidic cave ecosystems and their surficial counterparts (rectangles): Movile, Romania (Sarbu et al., 1996) and Frasassi, Italy (Sarbu et al., 2000). The distinctive isotopic depletion is shared by the three caves, suggesting similar controlling factors, with local variations in values.

and soil, and is a rich, ecosystem with many endemic species (Sarbu et al., 1996; Brad et al., 2021). With several faunistic pieces of evidence indicating intermittent connection with the surface, Movile is believed to have been colonized by various species over a much shorter period than Ayyalon, including the late Quaternary. Other known chemolithoautotrophic caves, including anchialine ones, seem to be less isolated than Ayyalon, and commonly have less developed endemic ecosystems (Pohlman et al., 1997; Pohlman, 2011; Hutchins et al., 2016; Engel, 2019).

4.4. Endemism

The taxa of Ayyalon include endemic faunal tribes and/or families (the scorpion *Akrav israchanani* and the pseudoscorpion *Ayyalonia dimentmani*) evolving independently since the Miocene (Levy, 2007; Ćurčić, 2008; Fet et al., 2011). The high-rank endemism and the deep, well-covered setting, suggest that Ayyalon is one of the most secluded metazoan cave ecosystems known today, whose biota may have been virtually disconnected from other ecosystems for millions of years (Ćurčić, 2008; Fet, 2017; Guy-Haim et al., 2018; Frumkin et al., 2022). However, the stable isotopes data from Movile and Frasassi caves indicate that they are more depleted than their associated surface ecosystems compared with the Ayyalon system (Figure 5B). This might be associated with different emissions of deep-sourced gases, such as methane, in each cave.

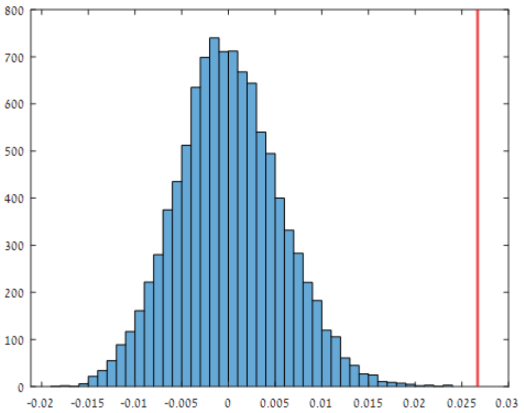

FIGURE 6
Results of analysis of similarity (ANOSIM) analysis with Euclidean distance as a dissimilarity metric between pairs of samples, generated by matrix permutation. A histogram of 10,000 random R_e values is compared with the observed R_o value (R_o =0.0267; red line), using MATLAB. This indicates a highly significant effect of habitat (cave vs. surface) on the isotopic values of the samples.

The high endemism, long-term (millions of years) resilience (Frumkin et al., 2022), and *in situ* evolution of the Ayyalon fauna suggest that similar chemolithoautotrophic ecosystems could be self-sustained for long (geologic scale) periods, allowing significant behavioral and physiological evolution, associated with adaptations to subsurface secluded conditions (section 2.3.1).

5. Conclusion

This study shows that under the lack of epigenic sources of carbon and energy, the Ayyalon Cave ecosystem has adapted by using internal carbon and energy sources. This is demonstrated, for the first time, by the low ^{14}C content of the ecosystem, indicating the uptake of inorganic carbon from old groundwater. The dead carbon used by microbial mats in the cave is recognized throughout all the trophic levels, demonstrating that in such ecosystems dead organisms cannot be dated by ^{14}C.

The ^{14}C evidence is corroborated by the stable isotopic values of the ecosystem, which show a major depletion of C and N isotopes throughout the cave ecosystem. The low values of δ^{13}C and δ^{15}N indicate underground chemolithoautotrophic production, rather than allochthonous import from epigean systems. The high endemism of the ecosystem shows that it has been detached from surface terrestrial and marine environments for a long period, amounting to millions of years. Our initial assumptions of the isotopic values of such an ecosystem thus prove to be true.

Our results also demonstrate the sustainability and adaptation of a deep isolated subsurface ecosystem. This may suggest the possibility of long-lived cave ecosystems early in the evolution of terrestrial life, and may shed light on potential extraterrestrial ecosystems. The subsurface of Mars and other planets is protected from the damaging effects of short-wave UV radiation and the oxidizing nature of the surface, thus providing a promising habitat for life (Chyba and Hand, 2005; Kaltenegger, 2017). In particular, caves and other subsurface environments may be habitable where sufficient geothermal heating permits the existence of liquid water. Such features make subsurface environments on exoplanets seem more plausible for life than their surface counterparts.

The secluded Ayyalon system is in great danger since it was breached by the quarry. The Nature Reserves Authority of Israel is urged to take active measures of conservation in order to avoid disturbance to the fragile system, and keep it as secluded as possible.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

We declare that the research have been conducted in accordance with Frontiers guidelines on study ethics. The study is exempt from ethics approval, due to the following reasons: No vertebrates have been involved in the study. No research on human subjects have been conducted.

Author contributions

AF coordinated the study and wrote the paper. AC ensured biologic validity and edited the text. IN performed the field work as part of his thesis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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How voles adapt to subterranean lifestyle: Insights from RNA-seq

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Life under the earth surface is highly challenging and associated with a number of morphological, physiological and behavioral modifications. Subterranean niche protects animals from predators, fluctuations in environmental parameters, but is characterized by high levels of carbon dioxide and low levels of oxygen and implies high energy requirements associated with burrowing. Moreover, it lacks most of the sensory inputs available above ground. The current study describes results from RNA-seq analysis of four subterranean voles from subfamily Arvicolinae: *Prometheomys schaposchnikowi*, *Ellobius lutescens*, *Terricola subterraneus*, and *Lasiopodomys mandarinus*. Original RNA-seq data were obtained for eight species, for nine species, SRA data were downloaded from the NCBI SRA database. Additionally assembled transcriptomes of Mynomes ochrogaster and Cricetulus griseus were included in the analysis. We searched for the selection signatures and parallel amino acid substitutions in a total of 19 species. Even within this limited data set, we found significant changes of *dN/dS* ratio by free-ratio model analysis for subterranean Arvicolinae. Parallel substitutions were detected in genes *RAD23B* and *PYCR2*. These genes are associated with DNA repair processes and response to oxidative stress. Similar substitutions were discovered in the *RAD23* genes for highly specialized subterranean *Heterocephalus glaber* and *Fukomys damarensis*. The most pronounced signatures of adaptive evolution related to subterranean niche within species of Arvicolinae subfamily were detected for *Ellobius lutescens*. Our results suggest that genomic adaptations can occur very quickly so far as the amount of selection signatures was found to be compliant with the degree of specialization to the subterranean niche and independent from the evolutionary age of the taxon. We found that the number of genomic signatures of selection does not depend on the age of the taxon, but is positively correlated with the degree of specialization to the subterranean niche.

KEYWORDS

selective pressure, protein-coding genes, subterranean voles, subterranean lifestyle, Arvicolinae, RNA-seq

1. Introduction

There are approximately 250 species of rodents that spend most of their lives in burrows (Begall et al., 2007). Underground tunnels protect animals from predators, fluctuations in environmental parameters and extreme conditions. Subterranean rodents live in darkness, lack most of the sensory inputs available above ground, face low food supplies and expend a lot of energy digging. Therefore, this lifestyle is highly challenging and associated with a number of morphological, physiological and behavioral modifications (McNab, 1966; Nevo, 1979, 1999; Buffenstein, 2000; Busch et al., 2000; Begall et al., 2007). That's why they for a long time served as a model for studying adaptive convergence and parallel evolution (Nevo, 1979; Lacey, 2000; Begall et al., 2007).

The molecular studies of adaptation to subterranean lifestyle started with the mitochondrial DNA analyses. First, in analysis of cytochrome *b* (*CYTB*) in subterranean rodent families Geomyidae and Bathergidae, Da Silva et al. (2009) found a significantly higher ratio (*dN/dS*) in each of the subterranean groups with respect to their non-subterranean counterparts. Moreover, the subterranean mole rats and tucu-tucus showed more sites whose amino acid properties may be under positive selection in the *CYTB* gene than their non-subterranean relatives, and some of the sites identified to be under selection exclusively in subterranean taxa were shared among all subterranean taxa (Da Silva et al., 2009). Later on these results were confirmed in analysis of both *CYTB* and cytochrome c oxidase subunit 2 (*COX2*) (Tomasco and Lessa, 2014) and at least three sites under positive selection have been detected in subterranean rodents. Positive selection signals have been detected in various organisms related to shifts in their ecology that imply changes in the metabolic needs (Tomasco and Lessa, 2014). Hence, the variability of mitochondrial genomes may reflect adaptations for demanding lifestyles (Di Rocco et al., 2006; Da Fonseca et al., 2008; Hassanin et al., 2009; Yu et al., 2011; Luo et al., 2013; Wang et al., 2016).

Studies of the mitochondrial genome adaptations were followed by studies of nuclear genes. Results of the *Spalax galili* Nevo, Ivanitskaya, and Beiles, 2001 genome and transcriptome analyses show high rates of ω values in genes associated with RNA/DNA editing, reduced chromosome rearrangements and over-representation of short interspersed elements (SINEs). These may probably be linked to degeneration of vision and progression of photoperiodic perception, tolerance to hypercapnia and hypoxia and resistance to cancer (Fang et al., 2014a). Another comparative analyses including DNA-seq and RNA-seq data of phylogenetically related subterranean species from Bathergidae family revealed possible molecular adaptations for subterranean conditions and factors of extreme longevity, including a divergent insulin peptide, expression of oxygen-carrying globins in the brain, prevention of high $CO2$-induced pain perception, and enhanced ammonia detoxification (Fang et al., 2014b). Large-scale analysis of coding DNA sequences of four representatives of independent subterranean mammal lineages from three superorders indicate positive selection and convergent occurrence of substitutions in the same sites for 53 genes (Davies et al., 2018). While African mole-rats and blind mole-rats have been studied extensively in relation to resistance to oxidative stress, toxin-resistance, and circadian biology (Rado et al., 1991; Oosthuizen et al., 2003; Fang et al., 2014a,b), other groups of subterranean rodents have so far been largely overlooked.

The subfamily Arvicolinae (voles, lemmings, and muskrats) is the evolutionary young rodent taxon that emerged the fastest documented adaptive radiation among recent mammals. The number of extant arvicoline rodents is eight times greater than in the sister subfamily Cricetinae, the most recent common ancestors of both are known in the fossil record from the Late Miocene, ca 10 Ma both in Eurasia and North America (Martin, 2003; Fejfar et al., 2011). Nowadays they occupy a variety of landscapes, habitats, and ecological niches in the Northern Hemisphere, from above ground and even arboreal [e.g., tree voles, *Arborimus* Taylor, 1915 (Corn and Bury, 1988; Swingle and Foreman, 2009) to highly specialized subterranean—*Ellobius* Fischer, 1814 and *Prometheomys* Satunin, 1901 (Ognev, 1950; Kryštufek and Shenbrot, 2022)]. Moreover, the transition to the subterranean lifestyle within this subfamily occurred more than once. Our previous studies showed that selection pressure is relaxed at the mitochondrial *CYTB* gene in particular (Bondareva et al., 2021a), and in mitochondrial genomes for most subterranean Arvicolinae species (Bondareva et al., 2021b). The recent and phylogenetically independent transition of Arvicolinae voles to subterranean lifestyle makes it possible to trace adaptive events at a smaller evolutionary scale, in contrast to the analysis of classical ancient subterranean species such as *Heterocephalus* Rüppell, 1842, *Nannospalax* Palmer, 1903, and *Fukomys* Ogilby, 1838. Thus, this model group may allow insights into the very beginning of the adaptive process at the genome level, comparison with the data obtained in the studies of ancient subterranean taxa may show whether these changes have a convergent character or individual nature in each case.

In the current study, we evaluated the selection signals in nuclear genes of the subterranean Palaearctic voles. Similarly to the previously carried mitochondrial genome analysis, we expect to find the magnitude of selective pressure that occurred in the subterranean Arvicolinae lineages using RNA-seq data. We also aimed to test the hypothesis that convergent molecular signatures of selection may occur in unrelated subterranean mammals.

2. Materials and methods

2.1. Taxa selection and samples

In the current study we obtained new material for eight Arvicolinae species (*Alticola lemminus* Miller, 1898, *Chionomys nivalis* Martins, 1842, *Terricola subterraneus* de Selys-Longshamps, 1836, *Dicrostonyx torquatus* Pallas, 1778, *Lemmus sibiricus* Kerr, 1792, *Myopus schisticolor* Lilljeborg, 1844, *Ondatra zibethicus* Linnaeus, 1766, and *Prometheomys schaposchnikowi* Satunin, 1901), see details for sampling localities in Biosamples description. Tissue mix (muscles, liver, heart, lungs, and testes for males) was fixed in an intactRNA buffer (Evrogen, Moscow, Russia) to avoid RNA degradation directly at the moment of catching the animal in the field. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Zoological Institute Russian Academy of Sciences (permission #2-17/14-07-2022).

For nine species, SRA data were downloaded from the NCBI SRA database. Additionally, two already assembled transcriptomes of *Mynomes ochrogaster* Wagner, 1842 and *Cricetulus griseus* Milne-Edwards, 1867 were included in the analysis. Thus, in total, we analyzed transcriptomes of 19 Cricetidae species (see Supplementary Table 1 for details).

We categorized species as either subterranean or above ground based on their behavior and morphological traits by classical description of subterranean rodents (Lacey, 2000; Lessa et al., 2008) as previously (Bondareva et al., 2021b). A species was considered subterranean if it is known to spend the most time underground, perform regular digging activities, and come to the surface only incidentally and within the vicinity of burrow openings (Lessa et al., 2008; Smorkatcheva and Lukhtanov, 2014). Two species fit these definitions in our dataset: *Ellobius lutescens* Thomas, 1897 and *Prometheomys schaposchnikowi*. Following the data available in literature, *Terrícola subterraneus* and *Lasiopodomys mandarinus* Milne-Edwards, 1871 also could be assigned to subterranean or semi-subterranean species (Ognev, 1950; Kryštufek and Shenbrot, 2022). Taking this into account we categorized all four species as subterranean. They are marked by color on Figure 1. We were limited only by these species being unable to consider other subterranean Arvicolinae forms (ex gr. *Microtus pinetorum* Le Conte, 1830) in the analyses due to the lack of data in the SRA database at the period of analysis and the poorly described ecology habits for several potential subterranean species.

2.2. RNA isolation and NGS library preparation

Total RNA was isolated with an RNeasy mini kit (Qiagen) under animal cells/spin protocol. RNA quality was quantified using a Bioanalyzer 2100 (Agilent Genomics) with a minimum of 7.0 for the RNA Integrity Number score. The combined protocol of the NEBNext Poly(A) mRNA Magnetic Isolation Module and the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina[1] was used to isolate polyA-RNA from the total RNA fraction and prepare DNA libraries. Preparation was carried out according to the protocol with the following modifications: (1) sample fragmentation was conducted at 94°C for 10 min; (2) the synthesis of the first chain was hold using the program with the following parameters: 25°C - 10 min, 42°C - 30 min, 70°C - 15 min; (3) DNA purification and concentration was carried out according to the protocol using Ampure XP magnetic particles. Elution was transferred with bidistilled water; (4) the number of cycles in PCR consisted of 10 or 15, depending on the initial RNA concentration.

Sample concentrations were measured on a Qubit fluorometer. The quality of the obtained libraries was checked using the Bioanalyzer 2100 Agilent using the DNA High Sensitivity kit. RNA extraction and library preparation were performed using resources of the Skoltech Genomics Core Facility[2].

2.3. Sequencing and quality assessment of reads

Sequencing was performed on the Illumina HiSeq4000 with read length 75 bp and paired-end tracks. Demultiplexing and conversion of data to the fastq format was performed using the bcl2fastq2 Illumina software. Sequencing was performed using resources of the Skoltech Genomics Core Facility (see text footnote 2).

The quality of the obtained paired readings was assessed by the FastQC (Andrews, 2010). Trimmomatic 0.39 (Bolger et al., 2014) was used to remove low quality reads and Illumina adapters. We took only reads with a quality higher than 28–29.

2.4. Transcriptome assembly and search for orthologous genes

Transcriptomes were assembled with a standard Trinity package (Haas et al., 2013). Base transcriptome statistics were also estimated with Trinity Stats. The ORFs in assembled transcriptomes were predicted by Transdecoder[3]. The resulting sequences were purified from chimeric ones using the DIAMOND program (Buchfink et al., 2015) with the reference from the NCBI protein base (nr). Transcriptome completeness was evaluated by BUSCO (Manni et al., 2021). Orthologous genes were determined using Proteinortho (Lechner et al., 2011). We searched for single-copy orthologs that were present in all analyzed transcriptomes for future analyzes.

2.5. Sequence alignment

Separate orthological genes were aligned using prank (Löytynoja, 2014) with the correction for the protein-coding genes tripletity. Aligned orthologs were concatenated into one meta-alignment with Python-based script.

2.6. Phylogenetic reconstructions

Phylogenetic reconstruction for 19 species was based on found orthological genes and carried out in the IQ-tree[4]. Conserved blocks from multiple alignments were previously removed by Gblocks (Castresana, 2000).

Phylogenetic trees used for tracing signatures of the selection pressure on each subterranean species separately (Figure 2) were obtained with MrBayes 3.2.2 (Ronquist et al., 2012) using seven nuclear genes (6,421 bp), see methods in Bondareva et al. (2021a). The following analysis parameters were: nst = mixed and the distribution of the substitution rates between sites, the dataset was divided into partitions by genes. Each analysis was started with a random tree and had two replicates with four Markov chains (MCMC) and 1 million generations, with the results recorded every hundredth generation. Stationarity and convergence of separate runs were assessed using ESS statistics in Tracer v1.6 (Rambaut et al., 2014). The trees were visualized using the FigTree v1.6 program (accessed on 26 November 2021)[5].

2.7. Detection of parallel amino acids substitution

The evaluation of both convergent and parallel amino acid substitutions was carried out by the ProtParCon program (Yuan et al., 2019) with a further search for amino acids characteristic only for subterranean rodents: we looked for replacements occurring at least in two subterranean taxa using a script on Python 3. The significance of the substitution frequency was estimated with the Evolver program included in the PAML4 (Yang, 2007) using Monte Carlo simulation

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[1] https://international.neb.com

[2] https://www.skoltech.ru/research/en/shared-resources/gcf-2/

[3] https://github.com/TransDecoder/TransDecoder/

[4] http://www.iqtree.org/

[5] http://tree.bio.ed.ac.uk/software/figtree/

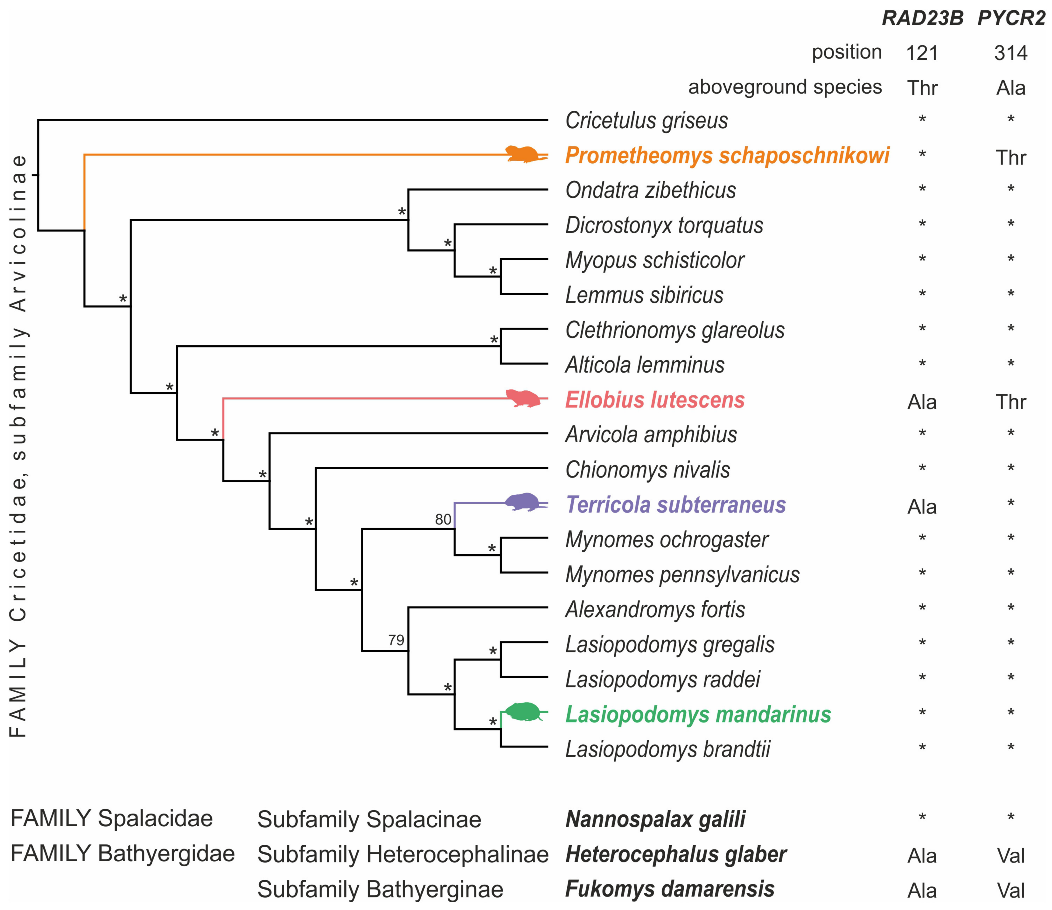

FIGURE 1

Phylogenetic tree of Arvicolinae subfamily based on transcriptome data with observed substitutions unique for subterranean species. Subterranean Arvicolinae species are shown in color. Nodes with 98–100 ML support are marked with an asterisk. On the right, the amino acid substitutions in certain positions are shown. Below the tree, the amino acid substitutions in the same sites in specialized subterranean rodents from other families are shown.

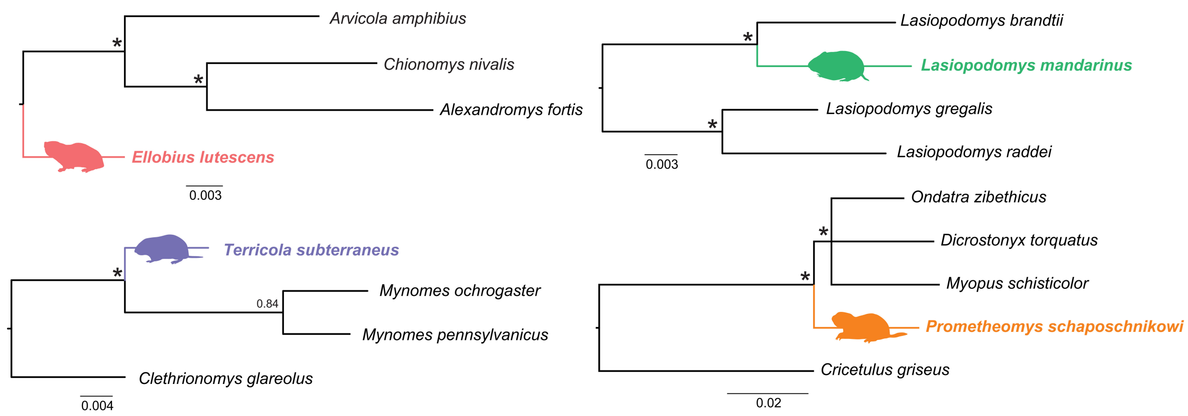

FIGURE 2

Trees used for tracing signatures of the selection pressures on the subterranean vole species. Fifty-percent majority rule consensus trees from Bayesian inference analysis are given. Subterranean species are shown in color. Nodes with 0.99–1.0 BI support are indicated with asterisks.

algorithm to obtain event probability of the substitution in each position. Simulated sequences evolving according to the following parameters: an evolutionary model, the branching pattern and branch lengths of the tree, amino acid frequencies and sequence length. Additionally Holm multiple adjustment used to obtain *p*-value in R software v.3.4.4 (R Core Team, 2020). Detected significant amino acid substitutions were subsequently checked for influencing protein structure by the Sift program[6] and modeled in SWISS-MODEL (Waterhouse et al., 2018). Visualization and comparison of modeled

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[6] https://sift.bii.a-star.edu.sg/

structures was carried out in PyMol 2.2.0 (Schrödinger and DeLano, 2020).

To test for possible convergent occurrence of the detected substitutions in other subterranean rodents, we used protein sequences of homologous genes from NCBI Protein database (*Heterocephalus glaber*—XP_004848222.1, XP_021097910.1; *Nannospalax galili*—XP_008820604.1, XP_008852875.1; *Fukomys damarensis*—XP_010611512.1, XP_010637758.1).

2.8. Analysis of selective pressure

Selective pressure was estimated as variation in the levels of non-synonymous (*dN*) and synonymous (*dS*) substitutions, as well as ω (*dN/dS* ratio) using several approaches. We applied the classic codeml program (Yang et al., 2000; Yang, 2007) with the comparisons between free-branch and M0, and free-branch and neutral-branch models with the ete-toolkit interface (Huerta-Cepas et al., 2016).

A branch models assumes significant differences between ω values for marked branches (foreground or ωfrg) and the rest branches of the tree (background branches or ωbkg). For branch analysis the total set of species was subdivided into several subsets so that each subset contained a subterranean species and its phylogenetically closest or sister aboveground species (Figure 2). For each subterranean species analysis was implemented with free-branch model (b_free, where ωfrg, and ωbkg are free), neutral-branch model (b_neut, where ωfrg is fixed to one) and M0 model, where all branches evolve at the same rate. The comparison between free-branch and M0 shows whether foreground branches have ω significantly different from the rest of the tree. The comparison between free-branch and neutral-branch models detects if the ωfrg value is significantly higher than 1. The values 999 and 0.001 were regarded as errors. The calculated *p*-values of all likelihood-ratio tests (LRTs) were corrected using Holm multiple adjustment in R software v.3.4.4.

In addition, we calculate a selection level for each species by free-ratio method. Free-ratio method does not imply the selection of branches and calculates across the whole three giving a unique value for each species. The Wilcoxon test was used to compare the differences between the mean values of *dN/dS* ratio for subterranean and terrestrial species groups. Test was conducted using R v.3.4.4.

3. Results

3.1. Transcriptome assembly and search of single-copy orthologs

Newly obtained raw sequence RNA-data for nine Arvicolinae species listed above (see "section 2 Materials and Methods," Supplementary Table 1) are available at the NCBI Sequence Read Archive: SRR21996854—SRR21996864, SRR21996867—SRR21996869 (BioProject No. PRJNA590630).

After the *de novo* assembly, from 34,750 to 371,688 "Trinity genes" per species were generated (Supplementary Table 2). The N50 length was 1,644–3,571 bp, whereas the mean contig length was about 525 bp. Complete BUSCO values (C in Supplementary Table 2) varied from 48 to 80.1% except already assembled *Mynomes ochrogaster* and *Cricetulus griseus* with mean values 98 and 93%, respectively. Since we did not make a functional comparison of transcriptomes, even such a low percentage (48%) of complete BUSCO for several species was sufficient for further analysis.

The search for orthologs by Proteinortho resulted in more than 1,000 orthologous genes for the studied species. But only 112 single-copy orthologs were present in all analyzed transcriptomes. They were used in further analyzes. Such a small number of intersections is explained by our assembly's incompleteness and limited amount of initial raw data. Since the already assembled and published transcriptomes of *Mynomes* and *Cricetulus* were also included in the analysis, we were confident that detected universal single-copy genes were not chimeric or erroneously generated by Trinity annotation.

3.2. Phylogeny reconstruction and estimates of natural selection

The total length of the concatenated single-orthologs alignment was 214,696 nucleotides. The 98,595 nucleotides remained after cleaning with the Gblocks (45% of the original alignment). The topology of trees for nuclear (Figure 1) and previously published mitochondrial gene trees (Abramson et al., 2021) practically does not contradict each other. The only difference concerns the position of the taxa in the first basal radiation. According to the data on nuclear genes the earliest split is represented by the *Prometheomys schaposchnikowi*, whereas the tribe Lemmini is the earliest derivative according to mitochondrial genes (Abramson et al., 2021).

The calculation of free *dN/dS* ratio model values across the whole tree for each taxa was carried out for each of 112 single-copy orthologs independently. Free *dN/dS* ratio model values across all genes showed that median *dN/dS* meanings turned out to be significantly (p-value = 0.002) higher for subterranean rodents as compared to above ground forms: 0.078 and 0.037, respectively.

To test for branch-specific positive selection in each of the four subterranean rodents, we ran a branch model for each of these species independently in comparison only with phylogenetically close terrestrial taxa (Figure 2). For each of 112 single-copy orthologs we failed to find any genes with significant changes in selection level. Despite the observed trend of higher *dN/dS* values for subterranean species and possible selection relaxation for these genes (Supplementary Table 3), all LRT *p*-values were insignificant after Holm correction. This tendency is especially pronounced for *Ellobius lutescens*, where for several genes *p*-values were significant before Holm correction, in contrast to other subterranean Arvicolinae analysis.

3.3. Search for parallel amino acid substitutions

We compared respective sets of substitutions generated by ProtParCon on 112 single-copy orthologous protein sequences in search of parallel molecular selective signatures among the four subterranean species. Parallel amino acid substitutions shared in at least two subterranean species were found in several genes: *ERP29, RAD23B, HIKESHI, ZADH2, MRPS14, PYCR2, CCDC86, GTPBP2, SNAPC2,* and *TTLL12* (Table 1). We additionally checked manually that there was no variability in these positions for above ground rodents sequences. Subsequent verification by the Evolver simulation process showed the significance of non-random occurrence of substitutions in the *RAD23* and *PYCR2* genes. These genes are associated with DNA repair processes (Pohjoismäki et al., 2012) and response to oxidative stress (Kuo et al., 2016), respectively.

Substitution Ala314Thr in *PYCR2* gene was predicted as affecting the protein function with a significant score of 0.00 by Sift program, while Thr143Ala substitution in *RAD23* gene was shown as tolerated with a score of 0.81. The *RAD23* tertiary structure modeling showed that the replacement site in the protein is located at a non-structural fragment and therefore does not have visible effect on the protein (Figure 3). It was not possible performing such modeling for the *PYCR2* gene, because the length of the template turned out to be less than the length of our original sequence. By coincidence, the site of interest was located in the lacking part of the template. Other templates offered by the SWISS-MODEL program were much worse and could not be used.

Search of convergent protein substitution across ancient and highly specialized subterranean species revealed substitution in *RAD23* gene of *Heterocephalus glaber* and *Fukomys damarensis*. For the same species, we found a substitution in the position 314 of the *PYCR2* gene, but with an amino acid different from those found in subterranean Arvicolinae: Val instead of Thr, respectively. We were unable to find similar substitutions in another subterranean rodent subfamily Spalacidae—*Nannospalax galii.* Amino acids in the studied positions coincided for this species with those in terrestrial Arvicolinae rodents.

4. Discussion

In the current study, we test for molecular adaptations signs in protein-coding nuclear genes across the subterranean lineages of vole species. Specifically we aimed to check whether the tendencies revealed in studies on mitochondrial genes (Bondareva et al., 2021b) may also be observed across transcriptome data.

4.1. Detected parallel amino acids substitutions

Within our transcriptome-based data set we uncover several genes with parallel amino acid substitutions. Verification showed the reliable substitutions in two genes: the *RAD23B* (Nucleotide Excision Repair Protein) and *PYCR2* (pyrroline-5-carboxylate reductase 2). These substitutions were found in both genes in the genome of *Ellobius lutescens,* in *PYCR2* in *Prometheomys schaposchnikowi* and in the *RAD23B* in *Terricola subterraneus* respectively (Table 1).

The found genes were not previously reported in studies of molecular adaptations of subterranean rodents. At the present stage it is impossible directly to link the functional impact of these substitutions to adaptive processes. However, the biochemical pathways and processes in which these genes are involved may play a certain role in the adaptation to subterranean lifestyle. The active work of the mitochondria potentially causes the formation of reactive oxygen forms that represent a damaging factor for the cell (Turrens, 2003). The *RAD23B* and *PYCR2* genes are associated with DNA repair processes (Pohjoismäki et al., 2012) and response to oxidative stress (Kuo et al., 2016), respectively. The *RAD23* homolog gene was found in the study of drought adaptation in plants, since clear signs of positive selection were observed (Zhang et al., 2013). The change in expression level of this gene was also revealed in the analysis of cold tolerance of *Thinopyrum intermedium* (Jaikumar et al., 2020). Moreover, the presence of the same amino acid substitutions in highly specialized subterranean rodent species from the family Bathyergidae may also indicate the importance of these substitutions in the adaptive processes.

The small number of genes with parallel substitutions typical for subterranean forms is not surprising. Even the wide scale study of Kalina Davies revealed (Davies et al., 2018) only 35 genes with parallel substitutions in all analyzed species which was less than 1% of the total number of analyzed genes. Another wide-scale study performed on subterranean rodents (Fang, 2015) discovered parallel substitutions for *Heterocephalus glaber* and *Fukomys damarensis* only in two genes: *ARG1* and *Na(V)1.7* across the whole-genome comparison. However, interesting to note that in the analysis of *CYTB* gene sequences across more than 60 Arvicolinae species we identify only three parallel substitutions occurring in subterranean forms (Bondareva et al., 2021a).

4.2. Signs of selection in subterranean vole species in nuclear protein-coding genes

The previously observed tendency of the selection relaxation in mitochondrial genomes was not fully confirmed for nuclear data. The median *dN/dS* value is significantly higher across all analyzed genes for subterranean voles compared with above ground using free ratio model. However, branch-model analysis failed to detect genes with significant changes in selection level for individual subterranean lineage compared to its sister one at the tree. Opposite to this in the analogous branch-model analysis of mitochondrial genomes we observed dramatic differences in a number of genes with clear selection signatures in subterranean lineages compared to sister above ground ones (Bondareva et al., 2021b).

This difference between mitochondrial and nuclear genes may be explained both by the slower rate of nuclear genes evolution (Lin and Danforth, 2004) compared to mitochondrial ones and the insufficient number of analyzed genes. It is worth mentioning that Arvicolinae is the youngest group among order Rodentia and even its oldest subterranean lineage, *Prometheomys schaposchnikowi*, is not older than 7 Ma (Abramson et al., 2021). However even in the very old and highly specialized subterranean groups from different mammal superorders signs of positive selection were revealed only in 10% out of all protein-coding nuclear genes studied (over 8,000) (Davies et al., 2018).

Our screening of nuclear genes showed despite the small number of them in the analysis we nevertheless may see not strong but clear adaptive signs. These signs can be seen in the increase of *dN/dS* ratio in the free ratio analysis and in the presence of parallel substitutions in subterranean forms. These may from the first look indicate adaptive signals to subterranean life, but we cannot exclude any other explanation for these findings. Analogous results detected in other subterranean rodents were linked to a variation in metabolic rate, body mass, population size, and generation time among lineages (Martin and Palumbi, 1993; Martin, 1995; Bromham et al., 1996). However, we assume that in case of decrease of effective population size, that is characteristic for most subterranean forms, we would observe, in the first turn, the decrease of overall genetic diversity

TABLE 1 Obtained genes with parallel amino acid substitutions shared in at least two subterranean species.

	ERP29	*RAD23B*	*HIKESHI*	*ZADH2*	*MRPS14*	*PYCR2*	*CGR1*	*GTPBP2*	*SNAPC2*	*TTLL12*
Position	255	**121**	189	162	5	**314**	125	30	204	91
Terrestrial species	Ala	Thr	Ala	Ala	Val	Ala	His	Val	Glu	Gln
Prometheomys schaposchnikowi	Val	–	Thr	Val	–	**Thr**	–	Met	Gly	–
Ellobius lutescens	Thr	**Ala**	–	Thr	Met	**Thr**	Pro	Met	Gly	Arg
Lasiopodomys mandarinus	–	–	–	–	–	–	Arg	–	–	–
Terricola subterraneus	–	**Ala**	Thr	–	Met	–	–	–	–	Lys

Significant substitutions indicated by bold.

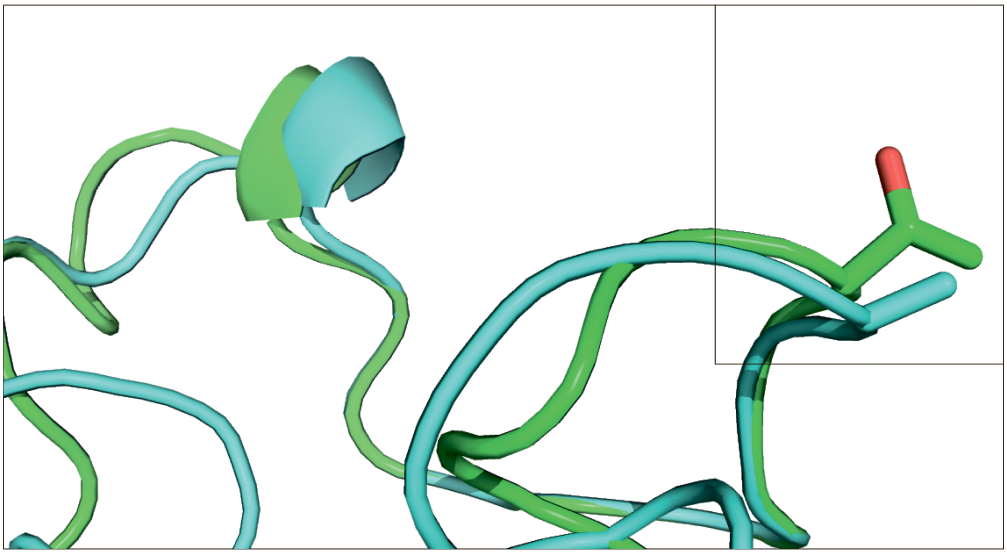

FIGURE 3
Structural model of substitutions in the *RAD23* gene. *E. lutescens* (subterranean) model is green, *L. gregalis* Pallas, 1779 (terrestrial) is cyan. Position 143 is framed.

but not the increase of non-synonymous substitutions as we see in our case. Similarly, the other factors listed above may explain the difference in overall genetic diversity between subterranean forms and above ground ones, but could not be responsible for the significant increase in non-synonymous substitutions.

4.3. Convergent evolution across Arvicolinae voles and other subterranean mammals

We aimed first to test the hypothesis on convergent molecular signatures of selection in several phylogenetically distant lineages of subterranean Arvicolinae and second, compare if found these signs with published data on other subterranean mammals. According to published data, we can identify two main trends: selection relaxation and occurrence of parallel amino acid substitutions. Combined results of analysis of mitochondrial genomes and transcriptomes showed both these tendencies for subterranean Arvicolinae (Figure 4).

Despite the limited number of nuclear genes in the analysis we have found parallel substitutions for subterranean forms in *RAD23B* and *PYCR2* genes in the current work. Studies performed on representatives of the families Bathyergidae and Spalacidae revealed parallel substitutions in completely different genes, e.g., *Na(V)1.7* and *ARG1* (Fang et al., 2014b), *EPAS1* and *AJUBA* (Shao et al., 2015). Thirty-five loci with parallel substitutions were common across

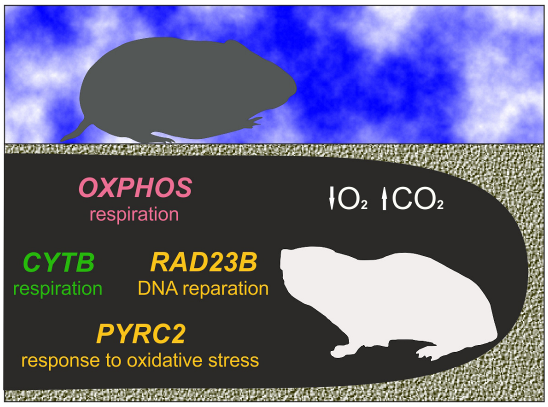

FIGURE 4
Graphic representation of possible molecular adaptations in response to subterranean life. Genes are colored as follows: Red—under positive selection in at least one subterranean lineage; yellow—with parallel substitution in at least one pair of subterranean taxa; green—both.

subterranean pairwise comparisons (Golden moles, African mole-rats, blind mole rats, Star-nosed mole) by Davies et al. (2018). They include *FREM1*, *LAMA3*, *MFI2*, and *PIEZO2* (Safran et al., 2010). Additionally, at least three of the loci (*SLC26A8*, *TDRD6*, and *TEX15*) have roles in sperm development, and at least eight (e.g., *C3*, *CLCA1*, and *TLR4*) are involved with an immune response (Safran et al., 2010). As mentioned above, *RAD23B* and *PYCR2* genes were not listed in previous studies, but their function could be associated with adaptive processes. Our own analysis of the protein sequences of these genes in highly specialized *Heterocephalus glaber* and *Fukomys damarensis* from Bathyergidae family shows convergent substitution in the same position with Arvicolinae subterranean rodents.

Several previous studies have investigated convergent molecular evolution based on *dN/dS* ratio estimation in subterranean mammals. Da Silva et al. (2009) found a significant difference for its values in *CYTB* sequences of subterranean rodents from families Ctenomyidae, Geomyidae and Bathyergidae compared to their aboveground closely related species. Later, signatures of selection were found in several mitochondrial genes of subterranean rodents (Tomasco and Lessa, 2011, 2014; Tavares and Seuánez, 2018). These have revealed convergent evolution in genes relating to hypoxia between plateau zokors (Spalacidae) and naked mole-rats (Bathyergidae), and comparisons of subterranean and closely related terrestrial rodents found significant differences in substitution rates of protein-coding genes between the two groups (Shao et al., 2015). Additionally, two genome-wide screens for changes in evolutionary rates identified enrichment in vision- and skin-related loci (Prudent et al., 2016; Partha et al., 2017). Our previous studies on the Arvicolinae subfamily also showed the relaxation of selection strength in *CYTB* and mitochondrial protein-coding genes reflecting an increased ω ratio in subterranean lineages compared to aboveground ones (Bondareva et al., 2021a,b). In this work we observed the trend to selection relaxation by free-ratio analysis, but didn't find individual genes with significant changes in selection level. We can associate it with the fact that Arvicolinae is the youngest group among order Rodentia and even its oldest subterranean lineage, *Prometheomys schaposchnikowi*, is not older than 7 Ma (Abramson et al., 2021). It is quite likely that this time span is insufficient for the pronounced selection change in slowly evolving nuclear genes. In addition, our sample dataset was limited, which could also affect the result of sensitive branch-based analysis.

The greatest number of selection signs within Arvicolinae subterranean species was found in the genus *Ellobius* both in mitochondrial genes (Bondareva et al., 2021a,b) and in the nuclear protein-coding genes. This genus is younger than the oldest representative of the subfamily, subterranean *P. schaposchnikowi*, by at least ca. 2 Ma. The oldest fossil remains of the true *Ellobius* are known from the Late Pliocene (Lytchev and Savinov, 1974; Zazhigin, 1988; Tesakov, 2004) but the morphological features in all species of the genus are very similar with those observed in the "model" subterranean rodents of the families Bathyergidae and Spalacidae: protruding incisors, very small eyes, and isolation of the mouth region by lips (Gromov and Polyakov, 1977).

So, on RNA-seq data we additionally confirmed our previous conclusion that the number of selection signatures is independent of the evolutionary age of the lineage but fits the degree of specialization to the subterranean niche and adaptive mechanisms to the subterranean lifestyle are convergent subterranean rodents from different families.

5. Conclusion

We indicate the signs of positive selection in nuclear genes for voles during colonization of the subterranean environment. Also we found two genes with parallel substitutions: *RAD23B* and *PYCR2*. Similar substitutions were found in representatives of the highly specialized family Bathyergidae. Our combined results of mitochondrial and nuclear genes analyses show that the number of adaptive traits does not depend on the evolutionary age of the lineage, but coincides well with the degree of specialization to the subterranean niche. The obtained data suggests that these signatures are convergent across distant subterranean rodents and mammals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, SRR21996854—SRR21996864 and SRR21996867—SRR21996869.

Ethics statement

This animal study was reviewed and approved by Ethics Committee of Zoological Institute Russian Academy of Sciences (permission #1-17, April 07, 2022).

Author contributions

OB: methodology, software, investigation, formal analysis, data curation, writing—original draft, and writing—review and editing. TP: data curation, formal analysis, and visualization. SB: data curation and writing—review and editing. MG: data curation. AS: data curation and writing—original draft. NA: conceptualization, data curation, methodology, resources, writing—review and editing, project administration, and funding acquisition. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1085993/full#supplementary-material

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Evidence for rapid divergence of sensory systems between Texas populations of the Mexican tetra (*Astyanax mexicanus*)

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Population divergence is often quantified using phenotypic variation. However, because sensory abilities are more difficult to discern, we have little information on the plasticity and rate of sensory change between different environments. The Mexican tetra (*Astyanax mexicanus*) is a fish distributed throughout Southern Texas and Northern Mexico and has evolved troglomorphic phenotypes, such as vestigial eyes and reduced pigmentation, when surface ancestors invaded caves in the past several hundred thousand years. In the early 1900s, surface *A. mexicanus* were introduced to the karstic Edwards-Trinity Aquifer in Texas. Subsequent cave colonization of subterranean environments resulted in fish with phenotypic and behavioral divergence from their surface counterparts, allowing examination of how new environments lead to sensory changes. We hypothesized that recently introduced cave populations would be more sensitive to light and sound when compared to their surface counterparts. We quantified divergence using auditory evoked potentials (AEPs) and particle acceleration levels (PALs) to measure differences in sound sensitivity, and electroretinography (ERGs) to measure light sensitivity. We also compared these results to measurements taken from native populations and lab-born individuals of the introduced populations. Honey Creek Cave fish were significantly more sensitive than proximate Honey Creek surface fish to sound pressure levels between 0.6 and 0.8kHz and particle acceleration levels between 0.4 and 0.8kHz. Pairwise differences were found between San Antonio Zoo surface and the facultative subterranean San Pedro Springs and Blue Hole populations, which exhibited more sensitivity to particle acceleration levels between 0.5 and 0.7kHz. Electroretinography results indicate no significant differences between populations, although Honey Creek Cave fish may be trending toward reduced visual sensitivity. Auditory thresholds between wild-caught and lab-raised populations of recently invaded fish show significant differences in sensitivity, suggesting that these traits are plastic. Collectively, while these results may point to the rapid divergence of *A. mexicanus* in cave habitats, it also highlights the responsive plasticity of *A. mexicanus* auditory system to disparate environments.

KEYWORDS

***Astyanax mexicanus*, auditory, vision, divergence, phenotype, environment, sensory ecology**

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1. Introduction

Recent studies have documented rapid phenotypic evolution in organisms, detailing significant changes over time scales of a few generations rather than millions of years. Several studies have demonstrated rapid evolution in fishes, including color pattern selection based on predator presence over 15 generations in Trinidadian guppies (Endler, 1980), morphometric changes in South American cichlids within decades following river damming (Gilbert et al., 2020), and ecotype divergence in three-spine stickleback within a single generation (Laurentino et al., 2020). The sensory systems of organisms can evolve to match environmental conditions (Endler, 1980; Endler and Basolo, 1998) but it remains unclear how quickly sensory systems and their sensitivity to stimuli might adapt to changing conditions (Zakon, 2015; Dunlop et al., 2018) or may diverge between individuals within a population, resulting in speciation (Seehausen et al., 2008; Puechmaille et al., 2011; Tait et al., 2021).

Cave organisms offer a robust framework to study divergence, plasticity, and evolution. Due to the similarities of characteristics among cave systems, such as perpetual darkness, limited nutrient availability, and the general absence of predators, there is a convergence of phenotypic attributes observed in cave animals (Jeffery, 2001; Pipan and Culver, 2012; Bradic et al., 2013; Herman et al., 2018; Xiong et al, 2018; Recknagel and Trontelj, 2022). Globally, animal lineages have evolved to inhabit cave ecosystems in a process known as troglomorphic adaptation. Troglomorphy describes the phenotypic features associated with cave organisms and includes lack of pigmentation, tolerance to low nutrient availability, vision loss, and the enhancement of non-visual sensory systems to navigate dark environments (Yoshizawa et al, 2010; Protas and Jeffery, 2012; O'Quin et al., 2013; Yoshizawa, 2016). While comparisons between cave and surface species offer insight into how similar organisms adapt to extreme environments (Porter et al., 2007), little is known about how quickly these traits can evolve.

The Mexican tetra, *Astyanax Mexicanus*, provides a unique model for examining troglomorphic sensory adaptation, since it is found in two distinct environments: surface, abundant in many rivers in Northern Mexico and parts of Texas, United States, and subterranean, with approximately 30 known cave populations (Espinasa et al., 2018) in Mexico. The Mexican cave morphotype of this species has troglomorphic features, including lack of pigmentation and loss of eyes. However, they can interbreed with the surface morphotype and hybridize in the wild (Herman et al., 2018; Jeffery, 2020).

Surface *A. mexicanus* were introduced into the San Antonio River in Central Texas between 1908 and 1940 (Brown, 1953). Several populations were later established in both cave and surface waterways in the Edwards-Trinity Aquifer system, with *A. mexicanus* first observed in the Guadalupe River in 1953 (Constable et al., 2010). Thus, the populations in Central Texas likely diverged from proximate Rio Grande (Rio Bravo) surface populations within the last century. Although Mexican *A. mexicanus* have acquired troglomorphic characteristics over several hundred thousand years (Herman et al., 2018), a recent study indicated morphological and behavioral changes between the two ecotypes in Honey Creek and Honey Creek Cave, suggesting rapid divergence between cave and surface populations (McGaugh et al., 2020).

The cave environment differs from the surface streams and rivers in light availability and environmental soundscapes (Niemiller and Soares, 2015). Caves have been characterized using light availability and temperatures found within different cave zones (Pipan and Culver, 2012), but the soundscapes within caves are not well studied. The aquatic soundscapes of caves often lack atmospheric input, such as wind and rain, that are major contributors to marine (Parks et al., 2014) and freshwater surface environments (Putland and Mensinger, 2020), which may affect auditory sensitivity. In the karst cave systems inhabited by *A. mexicanus*, lower ambient sound is attributed to fewer abiotic and biotic sound sources, sound absorption by porous limestone, and dissipation by irregular surfaces within the caves (i.e., stalactites, stalagmites, concavities, tunnels; Iannace and Trematerra, 2014; Carvalho and Sousa, 2015; Badino and Chignola, 2019). The absence of light in cave systems has predictably resulted in the degradation of visual systems in troglomorphic fish (Wilkens, 1988; Wilkens and Strecker, 2017; Soares and Niemiller, 2020). This loss of vision is offset by increased sensitivity in other sensory modalities, such as increased hair cell density of the lateral line, which may lead to increased lateral line sensitivity (Yoshizawa et al., 2014; Lloyd et al., 2018). However, it is unclear if newly colonized cave populations, prior to undergoing visual degradation, may initially be under selection for increased retinal or auditory sensitivity (Krishnan and Rohner, 2017). Selection for increased retinal sensitivity and other traits is expected in troglophilic populations that occupy spring sites and other areas of resurgence (re-emergence of karst groundwater to surface areas).

Several studies have compared sensory systems between cave and surface ecotypes of *A. mexicanus* (Yoshizawa, 2016). Early studies of *A. mexicanus* found no significant difference in auditory sensitivity between Mexican cave and surface populations using classical conditioning approaches (Popper, 1970). More recent works have indicated that cave *A. mexicanus* have increased olfactory sensitivity (Protas et al., 2008; Blin et al., 2020) and increased number of tastebuds when compared to surface counterparts (Varatharasan et al., 2009; Yamamoto et al., 2009). Several studies also reveal increased wall following behavior (Sharma et al., 2009; Patton et al., 2010), which may be related to reliance on the lateral line. A recent study has even characterized sound production behaviors in both native cave and surface fish (Hyacinthe et al., 2019). However, these studies have been confined to Mexican populations that have diverged over hundreds of thousands of generations. The study of relatively recent colonization events in Texas, in which cave and surface populations remain similar in external morphology, allows investigation into potential early divergence of sensory systems. The goal of this study is to compare auditory and visual sensitivity between populations from recently invaded subterranean environments and surface populations of *A. mexicanus* in Texas.

The Mexican tetra is a member of the Characiae family, in the superorder Ostariophysi (Nakatani et al., 2011; Gross, 2012) which possess Weberian ossicles that connect the swim bladder to the inner ear, allowing for greater detection of sound pressure (Schulz-Mirbach et al., 2020). The increased auditory sensitivity and enhanced range of ostariophysans such as *A. mexicanus* provides an excellent model for investigating any changes in auditory thresholds. We hypothesize that the change in environmental pressures from surface systems to underwater caves have selected for increased auditory and visual sensitivity in recently introduced populations of *A. mexicanus* and expect to see these changes when comparing auditory and visual thresholds of surface, subterranean, and facultatively subterranean populations. To provide context to the direction of changes observed in the newly invaded populations, we also compare the auditory sensitivity of these recently introduced populations to lab-raised fish from the native range. We employ measurements of sound pressure *via* the auditory brainstem response (also known as the auditory evoked potential, or AEP) and measurements of particle acceleration of the presented sound stimuli to capture the two metrics of auditory sensitivity found in ostariophysan fish. We additionally measure visual sensitivity using electroretinography, a methodology that records the rapid electrical response of light-sensitive cells in the eye. Furthermore, to determine if these traits have a heritable (rather than plastic) mechanism, wild-caught and lab-raised fish from the recently introduced *A. mexicanus* populations are compared.

2. Methods

2.1. Population sampling

Previous work compared Honey Creek Cave fish to Honey Creek surface fish (McGaugh et al., 2020). Here, we examine this comparison, in addition to fish from a second river drainage within Texas. For this additional comparison, we collected fish from San Pedro Springs, Blue Hole, and the San Antonio Zoo. San Pedro Springs and Blue Hole fish reside underground during low aquifer conditions while San Antonio Zoo fish are exclusively surface dwellers in a portion of the river maintained by a groundwater well. *A. mexicanus* specimens were collected from five locations (Figure 1). Fish were obtained in Honey Creek which feeds into the nearby Guadalupe River in Comal Country, Texas, with cave fish obtained <100 m inside of the spring entrance of Honey Creek Cave (the source of Honey Creek) during June 2019 and February 2020, and surface fish were obtained from Honey Creek approximately 1,500 m downstream of the cave entrance in February 2020. San Pedro Spring fish were collected June 2019 from springs emerging from a bedrock headwall in San Pedro Springs Park, San Antonio, TX, which is a public recreational area with springs feeding into an outdoor pool. Blue Hole fish were collected June 2019 from the Blue Hole, a (formerly artesian) modified open spring that feeds into the nearby San Antonio River on the University of the Incarnate Word campus in San Antonio, TX. The Headwaters Sanctuary at University of the Incarnate Word houses the headwaters of the San Antonio River, with the Blue Hole serving as the centerpiece of the sanctuary. The spring is ephemeral: during periods of low aquifer levels, the population retreats to the subterranean portion of the spring. Above 204 asl, the spring flows into the San Antonio River *via* a short spring run. Fish do not appear to colonize the spring pool *via* the river, but rather from deeper in the spring system (A. Gluesenkamp, pers. observation).

In addition to collection, a SoundTrap STH 300 hydrophone (Ocean Instruments NZ; Warkworth, Aukland, NZ) was deployed for 24 h at each collection site. San Antonio Zoo surface fish were collected in June 2019 from a headwater branch of the San Antonio River that originates on San Antonio Zoo grounds. San Antonio Zoo, San Pedro Springs and Blue Hole are within 7 km from one another and connected by the San Antonio River. Individuals from all five populations were transported to the University of Minnesota (St. Paul Campus) within 7 days of collection *via* a direct flight by Delta Cargo. Fish were maintained at the University of Minnesota, St. Paul Campus between 3 months to 1 year before transport to the University of Minnesota Duluth. Aquariums at the St. Paul campus were kept between 21 and 23°C and on a 14:10 light cycle. All fish were fed frozen bloodworms, brine shrimp, or Tetra Cichlid flakes (Spectrum Brands Pet, LLC, Blacksburg, VA, United States) 1–2 times a day, *ad libitum*. Fish were transported to the University of Minnesota-Duluth in buckets filled with water conditioned using 0.5% API Stress Coat Plus (Mars Fishcare Inc., Chalfront, PA, United States) that were equipped with battery-operated air pumps for water circulation.

To test the lab-raised descendants of wild-caught fish, wild-caught Blue Hole, Honey Creek Cave, and Honey Creek Surface fish were bred in the lab and raised to at least 6 months post-fertilization

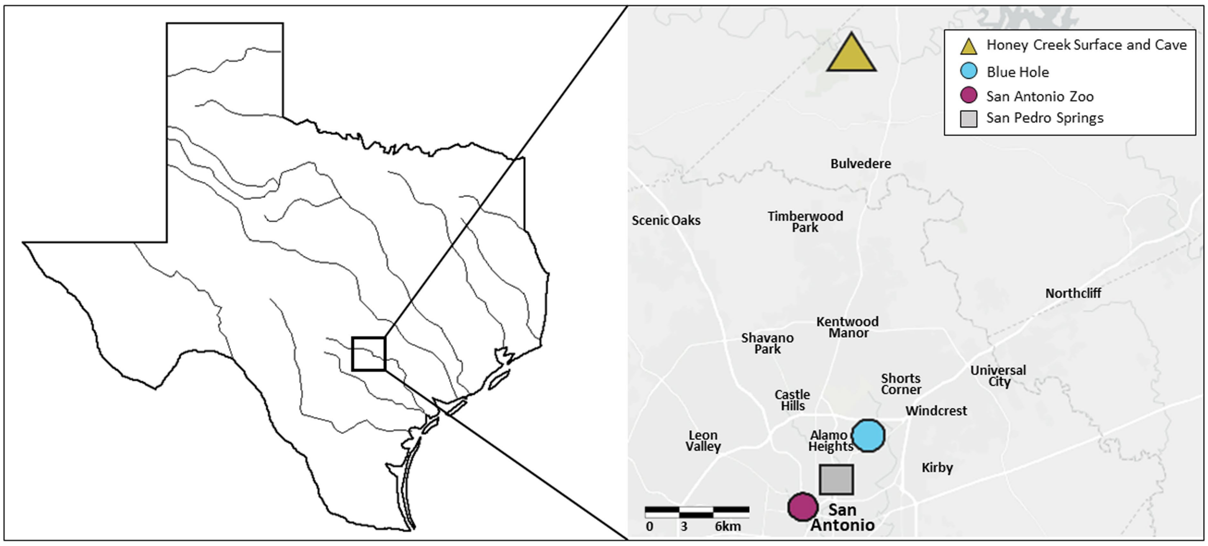

FIGURE 1
Geographic locations of recently invaded population sampling. Five recently invaded populations of *Astyanax mexicanus* found in the Edwards-Trinity Aquifier system were sampled for this study, with collection points indicated on the map. The triangle shape indicates both a surface and cave environment. Circles indicate intermediate environments, while a square indicates a surface environment.

prior to transportation to the University of Minnesota Duluth. Fish were bred following an adapted protocol from Borowsky (2008): larval fish were fed Hikari First Bites Fish Food (Hikari Sales USA, Inc., Hayward, CA, United States) for the first 30 days post-fertilization in small aquaria kept at room temperature. At approximately 30 days post-fertilization, larval fish were switched to a diet of crushed flake, frozen brine shrimp, and frozen bloodworms in heated (26°C) fully-filtered tanks at a density of one fish per 9–18 L. Heat was removed 6 months post-fertilization, and lab-born fish were treated as adults. Animal care and housing practices were carried out in accordance with all IACUC guidelines (UMN IACUC protocol 2002-37827A).

In addition to recently established populations, we tested descendants of wild-caught cave and surface fish from their native range. Fish were sourced from the Stowers Institute for Medical Research (Kansas City, MO) and included lab lineages of two cave populations (Pachón and Molino) and one surface population (Mexican surface river). These three populations were transported to the University of Minnesota *via* FedEx overnight shipment.

2.2. Animal husbandry

A total of eight separate populations were used in this study: Honey Creek Cave ($n = 7$), Honey Creek surface ($n = 10$), San Pedro Springs ($n = 7$), Blue Hole ($n = 7$), San Antonio Zoo surface ($n = 11$), lab-lineage Molino Cave ($n = 5$), lab-lineage Pachón Cave ($n = 5$) and lab-lineage Mexican surface ($n = 5$). In addition, we also tested lab-raised Blue Hole ($n = 10$), lab-raised Honey Creek Cave ($n = 6$), and lab-raised Honey Creek Surface fish ($n = 5$). Fish were maintained indoors at the University of Minnesota Duluth. Each population was housed separately in 75.7 L glass tanks filled with buffered pond water (0.56 g KCl, 0.44 g NaCl, and 2.6 g $CaCl_2$ per 75.7 L, pH = 7.0) and equipped with mechanical, chemical, and biological filters. Water temperature was maintained between 20 and 22°C. Illumination was provided by a Marineland LED Light Hood (Spectrum Brands Pet, LLC., Blacksburg, VA, United States) on top of each tank containing surface fish on a 9 h light:15 h dark cycle. The cave tanks were not illuminated and separated from the surface tanks by opaque dividers but still received dim indirect light from the surface tanks. All populations were fed Tetra Cichlid Flakes daily. Prior to each experiment, standard length (SL, mm), total length (TL, mm) and wet weight (M, g) were recorded.

2.3. Auditory evoked potentials

Auditory evoked potentials (AEP) testing was conducted in a 375 L cylindrical fiberglass tank (88 cm inner diameter, 62 cm height, 57 cm water depth) seated on a 1 cm thick rubber mat on cinderblocks (41 × 20 × 10 cm) to reduce noise (Figure 2A). The experimental tank was housed within a galvanized angle iron frame (110 × 125 × 182 cm) surrounded on the top and three sides with FOAMULAR Insulation Sheathing 2.5 cm thick (Owens Corning; Toledo, OH, United States) to reduce background sound and prevent the fish from seeing the experimenter.

Prior to electrode implantation, fish were anesthetized for 5 min using phosphate buffered tricaine methanesulfonate (MS-222) at a concentration of 0.005%; (Western Chemical Inc., Ferndale, WA, United States) and then were suspended in a mesh sling using an adjustable Omano Microscope arm boom stand (Microscope LLC.; Roanoke, VA, United States) within a smaller plastic anesthetic chamber (26.5 × 18.5 × 19 cm) containing the anesthetic solution to maintain quiescence throughout the experiment. The dorsal surface of the fish was maintained 4 cm below the surface of the water and 42 cm above an underwater speaker Model UW-30 (Electro-Voice; Burnsville, MN, United States).

Stainless steel electrodes (Rochester Electro-Medical Inc.; Tampa, FL, United States) were insulated with acrylic paint to within 2 mm of the electrode tip and implanted subcutaneously. The recording electrode was positioned above the brainstem and placed medially on the dorsal surface of the head approximately 2 mm posterior to an imaginary line drawn between the anterior margins of the opercula. A reference electrode was placed medially between the nares (Figure 2A). AEP signals were amplified using a headstage (gain = 10×) connected to a Model EX1 extracellular differential amplifier with a gain of 100× (Dagan Corporation; Minneapolis, MN, United States) with a 0.02 kHz high-pass filter and a 5.0 kHz low-pass filter. A Micro-3 model 1401 data acquisition system (Cambridge Electronic Design [CED], Milton, Cambridge, United Kingdom) and Spike2 (Version 8, CED) script (file "fishabr v1.20.s2s")[1] were used to set sound signal parameters, calibrate sound pressure level (SPL) attenuation, and digitize the received AEP signals. A programmable attenuator (Model 3505, CED) and Model AS-35 amplifier (Accusonic Corp., Markham, Ontario, Canada) controlled the SPL of the presented signals. The attenuator and amplifier were calibrated using a Model 8103 hydrophone (Brüel & Kjaer; Virum, Denmark) placed in the same position as the experimental fish. The hydrophone was connected to a Nexus Model 2609-01s Conditioning Amplifier (Brüel & Kjaer; Virum, Denmark). Pure tone signals were attenuated in 3 dB 1 µPa SPL_{rms} steps.

Auditory thresholds for 15 frequencies between 0.1 and 4.0 kHz were tested. For stimulus presentation, pure tone bursts for each frequency were broadcast (50 ms; 500 repetitions; 3 ms delay) and responses were collected and averaged using the same Spike2 script as mentioned above. The presence of AEPs were verified by two means: (1) through the observation of the characteristic wave visible above the background noise (≥0.001 mV) at the second harmonic of the stimulation frequency (Supplementary Figure S1) and (2) by fast Fourier transform power spectrum analysis (FFT, Hanning window = 1,024) (Supplementary Figure S2) to calculate power spectra of the average waveforms at two times the stimulus frequency (Vetter et al., 2018). Visual AEPs with FFT peaks above the background noise (≥0.001 mV) at the second harmonic of the stimulation frequency were considered evoked potentials. The auditory threshold at each tested frequency was defined as the minimum SPL that elicited an observable AEP response and a FFT peak at the second harmonic of the stimulus frequency. Threshold measurements were conducted by gradually increasing SPL_{rms} until AEPs were detected or the maximum output of the speaker at a given frequency was reached.

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[1] https://ced.co.uk/downloads/scriptspkexpr

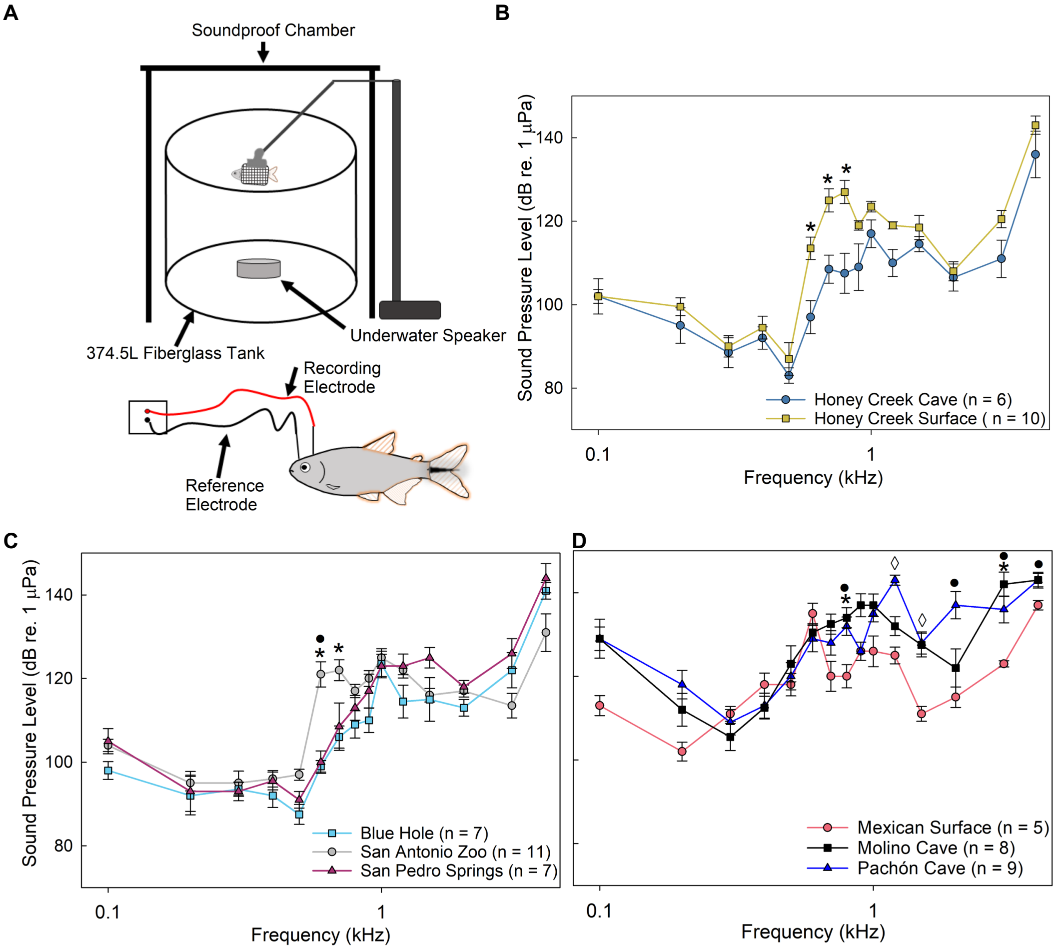

FIGURE 2

Auditory evoked potentials. **(A)** Schematic of auditory evoked potential (AEP) experimental setup. **(B–D)** Median auditory sound pressure level (±SE) sensitivity compared between populations. **(B)** Comparison between Honey Creek Cave (blue circle) and Honey Creek Surface (yellow square). Asterisks (*) indicate a significant difference (Mann-whitney *U* test, $p<0.05$) between populations. **(C)** Comparison between San Antonio Zoo (grey circle), Blue Hole (cyan square) and San Pedro Springs (magnenta triangle). Asterisks (*) indicate a significant (Kruskal-Wallis with *post-hoc* pairwise Wilcox w/Holm correction, $p<0.05$) difference between San Antonio Zoo and Blue Hole. A black circle (•) indicates a significant difference between San Antonio Zoo and San Pedro Springs. **(D)** Comparison between Mexican Surface (red circle), Molino Cave (black square) and Pachon Cave (blue traingle). A significant difference is defined as $p<0.05$ using a Kruskal-Wallis test with post-hoc pairwise Wilcox w/ Holm adjustment. Asterisks (*) indicate a significant difference between Mexican surface and Pachon Cave. A black circle (•) indicates a significant difference between Mexican surface and Molino Cave. A diamond (◊) indicates significant differences between all pairwise population comparisons.

2.4. Particle acceleration thresholds

Particle acceleration level (PAL) thresholds were determined by running a playback experiment which used the experimentally determined sound pressure threshold levels to then measure and calculate particle acceleration thresholds for each fish tested. PALs were calculated using a Model W356A12/NC (PCB Piezotronics Inc.; Depew, NY, United States) triaxial accelerometer (sensitivity, $x = 10.47\,\mathrm{mVms^{-2}}$, $y = 10.35\,\mathrm{mVms^{-2}}$, $z = 10.29\,\mathrm{mVms^{-2}}$) modified to be neutrally buoyant and connected to a signal conditioner (482C15; Piezotronics Inc.) and positioned within the AEP experimental tank in the same position as the fish head. For each frequency, corresponding PAL measurements were made for each SPL throughout the attenuation range. The accelerometer was positioned with its x-axis in the rostral-caudal, the y-axis in the lateral, and the z-axis to the dorsal-ventral planes of the fish. To calculate the PAL, the V_{rms} was determined for each axis (x, y, and z) and then converted into individual magnitude vectors. The following equation was used to calculate PAL thresholds:

$$dB\,re.1\,ms^{-2} = 20\log\left(\sqrt{x^2 + y^2 + z^2}\right).$$

2.5. Electroretinography

All electroretinography (ERG) testing was conducted in a room illuminated by dim red light (15 W light bulbs with Kodak GBX-2 dark red safelight filter). Each fish was anesthetized with MS-222 solution between 0.0075 and 0.0085%, buffered with sodium bicarbonate to a pH of 7.0, for 5 min prior to placement in experimental chamber. The fish was then placed on a moist sponge in the acrylic experimental tank (13 × 18 × 8 cm) and covered with a wet Kimwipe (Kimberly-Clark Professional; Roswell, GA, United States). The experimental tank was housed within an opaque metal faraday cage (77 × 67 × 97 cm) to prevent equipment light from reaching the fish (Supplementary Figure S3). The buffered MS-222 solution was delivered to the fish *via* a gravity-fed tube placed in the buccal cavity of the fish to maintain the surgical plane of anesthesia.

A small incision through the limbus of the eye was made with a 0.3 mm 15° stab knife (Surgical Specialties; Westwood, MA, United States). A 0.64 mm diameter silver-silver chloride electrode was inserted into the incision, with the reference electrode placed within the nostril of the ipsilateral side. ERG waveforms were amplified using a DAM50 (World Precision, Inc.; Sarasota, FL, United States) bioamplifier (1,000× gain; 1 Hz high pass, 3 kHz low pass), filtered with a digital 60 Hz notch filter to reduce electrical noise in the system, and recorded with a Powerlab 4SP (AD Instruments, Inc.; Colorado Springs, CO, United States) using Lab Chart7 Software (AD Instruments) on a Dell laptop.

A 100 W quartz tungsten-halogen Model 6333 lamp (Newport Corp.; Stratford, CT, United States) powered by a constant current power supply (Model 68938; Newport) produced the light stimulus. Stimulus duration (200 ms) was regulated with an electronic shutter (Model 76994; Newport) and controller (Model 76995; Newport). A dual filter wheel (Model 7736; Newport) containing neutral density filters from 0.1 to 3.0 regulated light intensity, with wavelength controlled by a monochromator (Model 77250; Newport). Light intensity was determined using a radiant power energy meter (Model 70260; Newport) and probe (Model 70268; Newport). A fiber optic light pipe (Model 77632; Newport) transmitted the light from the monochromator to the eye, completely illuminating it.

All fish were dark adapted for 30–60 min. Test flashes were initiated at the 30 min mark to determine if the retina was dark adapted, which was defined as the absence of a negative inflection (also known as the "a-wave") prior to the positive inflection ("b-wave," see Supplementary Figure S4) in the ERG response waveform. If the a-wave remained, the fish was allowed to dark adapt for an additional 10 min, and the process was repeated until the a-wave was undetectable. Wavelengths between 425 and 700 nm were presented to the fish in random order with flash duration of 200 ms and interflash interval of 10 to 30 s. Experiments were conducted during the fish's light cycle to avoid circadian rhythm effects.

The response criterion was set as the b-wave amplitude (baseline to peak) at 425 nm and averaged approximately 30 mV (Supplementary Figure S4). Other wavelengths were reduced in intensity using neutral density filters until the response equaled to the b-wave amplitude at 425 nm. Upon completion of the ERG, fish were revived by delivering buffered pondwater to the fish *via* a separate gravity-fed tube until gilling resumed, and fish were returned to home aquaria.

2.6. Statistics

To determine if Honey Creek Cave fish were more sensitive to auditory and visual stimuli than Honey Creek surface fish, the SPL, PAL and ERG threshold data were analyzed using a T-test. Because the SPL, PAL and ERG data were not normally distributed (Shapiro–Wilk test: SPL data [$W = 0.99$, $p < 0.001$], PAL [$W = 0.98$, $p < 0.001$], ERG [$W = 0.81$, $p < 0.001$]), non-parametric tests were used. Similarly, lab-raised populations were compared to wild-caught fish using a non-parametric T-test. If the dataset had equal variance as determined by Levene's test, a Welch's T test was conducted; otherwise, a Mann–Whitney *U* test was used.

To test for differences in auditory and visual sensitivity between the surface San Antonio Zoo population and the facultative Blue Hole and San Pedro Springs populations, the SPL, PAL and ERG threshold data were analyzed *via* a Kruskal-Wallis H. To determine if native cave populations were more sensitive to auditory stimuli than native surface populations, the SPL and PAL values of lab-raised fish derived from wild caught fish sampled from Pachón Cave, Molino Cave and a Mexican surface site were also compared using a Kruskal-Wallis H test. If significant differences ($p < 0.05$) were found between groups, *post hoc* pairwise Wilcoxon Rank-Sum tests with Holm adjustment were conducted."

Statistical analysis was completed using R Version 4.2.2 "Innocent and Trusting" (R Core Team, 2022). Graphs were created in both R and SigmaPlot (Version 15.0). Data are reported as median ± SE.

3. Results

3.1. Sound pressure thresholds

All populations responded to frequencies between 0.1 and 4.0 kHz, with the highest sensitivity detected between 0.2 and 0.5 kHz. Honey Creek Cave populations displayed significantly more sensitive sound pressure thresholds (Mann–Whitney *U* test, $p < 0.05$) than surface conspecifics between 0.6 and 0.8 kHz (Figure 2B). Blue Hole and San Pedro Springs populations were significantly more sensitive (Pairwise Wilcox with Holm Adj., $p < 0.05$) than San Antonio Zoo surface fish at 0.6–0.7 kHz and 0.6 kHz, respectfully (Figure 2C). Mexican surface fish were most sensitive of all the native populations at 0.2 kHz while Molino Cave and Pachón Cave were more sensitive between 0.3 and 0.4 kHz (Figure 2D), although these thresholds are not significantly different from Mexican surface fish.

Sound pressure sensitivity also differed between wild-caught and lab raised fish. Only Honey Creek Surface exhibited similar thresholds between wild-caught and lab-raised fish at 0.3–0.7 kHz ($p < 0.05$) and 1.2–3.0 kHz ($p < 0.01$) (Figure 3A). Wild-caught fish were significantly more sensitive to sound pressure than lab-raised descendants at all frequencies except 3.0–4.0 kHz in Honey Creek Cave (Welch's *T* test; $p < 0.05$) and Blue Hole (Mann–Whitney *U* test, $p < 0.05$) population fish (Figures 3B,C). While it appears that sound pressure threshold is highly dependent on developmental environment, higher sensitivity in wild-caught fish suggests that the soundscape of these recently colonized cave-like areas hold ecological information relevant to *A. mexicanus*.

Native cave and surface populations exhibited overall less sensitivity to sound pressure than the recently invaded populations (Figure 2D). Fish inhabiting subterranean environments appear to consistently detect frequencies between 600 and 700 kHz at lower sound pressure levels when compared to surface fish, indicating increased auditory sensitivity.

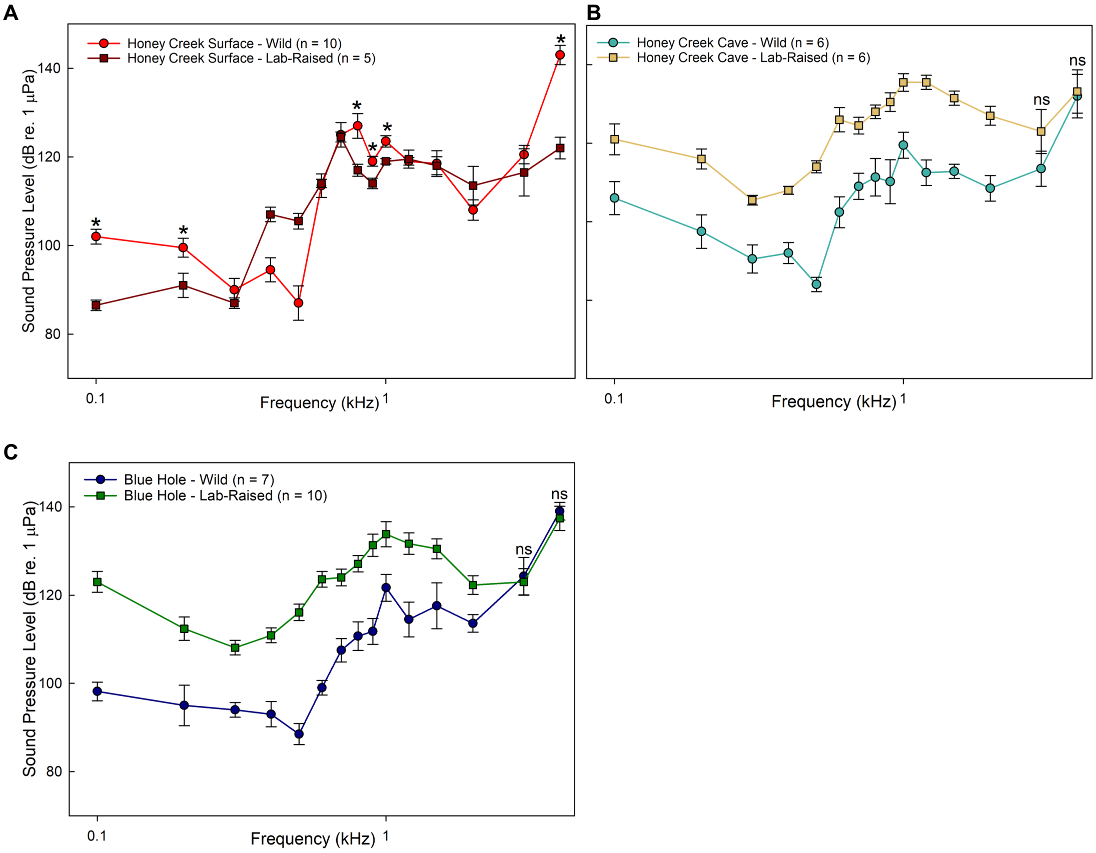

FIGURE 3
Wild caught and lab-raised AEP Thresholds. **(A–C)** Median auditory sound pressure level (±SE) sensitivity compared between populations. **(A)** Comparison between wild-caught (red circle) and lab-raised (brown square) Honey Creek Surface fish. Asterisks (*) indicates significant (Mann-whitney *U* test, $p<0.05$) difference between populations. **(B-C)** All frequencies tested show significant differences (Welch's *T* test, $p<0.05$) between populations unless marked with "*ns*", indicating no significant difference. **(B)** Comparison between wild-caught (light blue) and lab-raised (beige square) Honey Creek Cave fish. **(C)** Comparison between wild-caught (dark blue circle) and lab-raised (green square) Blue Hole fish.

3.2. Particle acceleration thresholds

The threshold values determined from playback particle acceleration experiments (Figure 4A) revealed additional differences between populations. Honey Creek Cave fish were significantly more sensitive (Mann–Whitney *U* test, $p < 0.05$) to particle acceleration than Honey Creek surface fish between 0.4–0.8 kHz and at 3.0–4.0 kHz (Figure 4B). Wild-caught Blue Hole and San Pedro Springs fish both had significantly lower particle acceleration thresholds than wild-caught San Antonio Zoo surface fish between 0.5–0.7 (Pairwise Wilcox w/ Holm Adj., $p < 0.05$) (Figure 4C). In addition to this range, among wild-caught fish, Blue Hole fish were significantly more sensitive than San Antonio Zoo at 1.2 kHz (Pairwise Wilcox w/ Holm adjustment, $p < 0.01$), and San Pedro Springs fish were significantly more sensitive than San Antonio Zoo at 3.0 kHz (Pairwise Wilcox w/ Holm adjustment, $p < 0.01$).

Similar to the results seen in sound pressure level thresholds, all native Mexican cave and surface fish exhibited less overall particle acceleration level sensitivity than the recently invaded fish from Texas. While both Molino and Pachón cave fish demonstrated qualitatively higher sensitivities at lower frequencies (0.3–0.4 kHz), though this difference was not statistically significant from surface fish (Figure 4D). However, Molino cave fish were significantly more sensitive to particle acceleration at 0.8 kHz than Mexican surface (Pairwise Wilcox w/ Holm adjustment, $p < 0.05$), while Pachón cave fish were more sensitive than Mexican surface fish at 4.0 kHz (Pairwise Wilcox w/ Holm adjustment, $p < 0.05$).

When compared to wild-caught populations, lab-raised fish were less sensitive than wild-caught fish, especially in the Honey Creek Surface (Figure 5A) and Honey Creek Cave (Figure 5B) comparisons. At most frequencies wild-caught Blue Hole fish were more sensitive than the lab-raised individuals; however, lab-raised Blue Hole fish were significantly more sensitive than wild-caught fish at 4.0 kHz (Mann–Whitney *U* test, $p < 0.05$) (Figure 5C).

Taken holistically, it appears that wild-caught fish from subterranean environments were more sensitive to particle acceleration detection than wild-caught fish from surface habitats or those raised in the lab. The pattern of greater sensitivity among wild-caught cave-dwelling individuals than surface conspecifics is upheld both when comparing both sound pressure sensitivity in auditory evoked potentials thresholds and particle acceleration sensitivity in particle acceleration level thresholds. However, this sensitivity appears to be highly dependent on developmental environment.

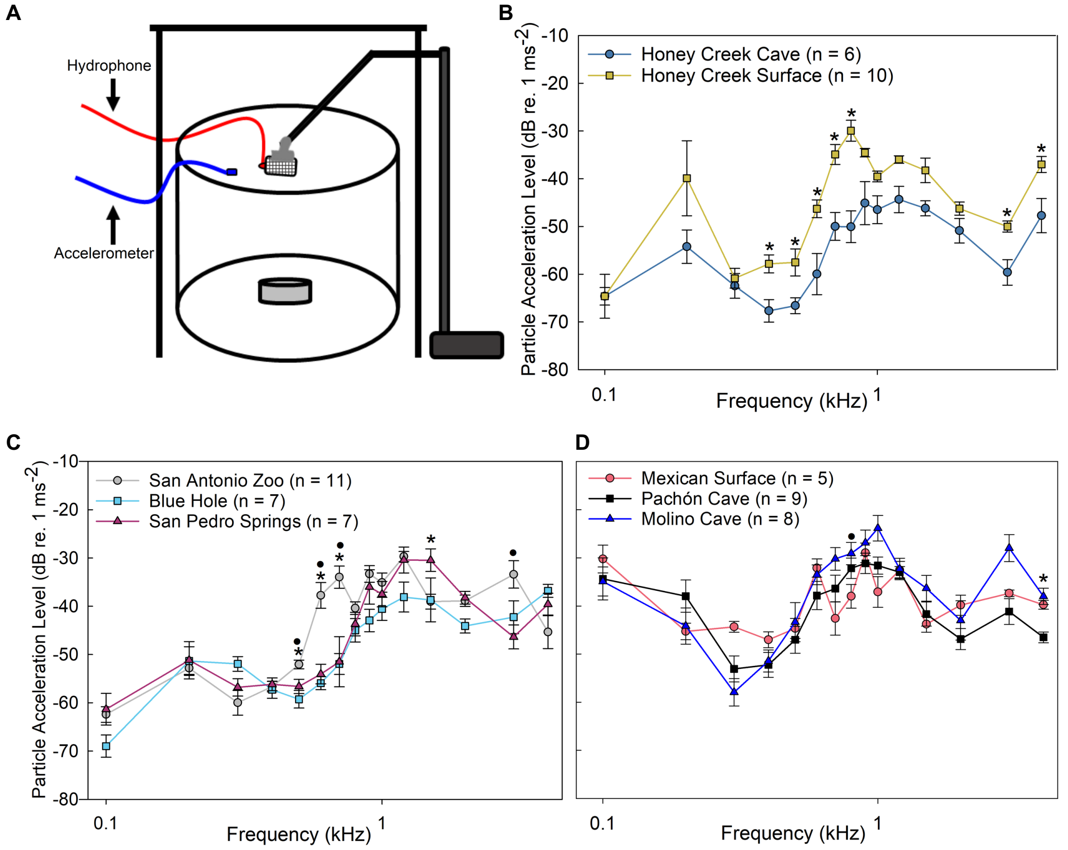

FIGURE 4

Particle acceleration levels. **(A)** Schematic of particle acceleration level (PAL) experimental setup. **(B–D)** Median auditory particle acceleration level (±SE) sensitivity compared between populations. **(B)** Comparison between Honey Creek Cave (dark blue circle) and Honey Creek surface (yellow square); asterisks (*) indicates a significant difference (Mann-Whitney *U* Test, $p<0.05$) between populations. **(C)** Comparison between San Antonio Zoo (grey circle), Blue Hole (cyan square) and San Pedro Springs (magnenta triangle); A significant difference is defined as $p<0.05$ using a Kruskal-Wallis test with post-hoc pairwise Wilcox w/ Holm Adjustment. Asterisks (*) indicate a significant difference between San Antonio Zoo and Blue Hole. A black circle (•) indicates a significant difference between San Antonio Zoo and San Pedro Springs. **(D)** Comparison between Mexican Surface (red circle), Molino Cave (black square) and Pachon Cave (blue traingle). A significant difference is defined as $p<0.05$ using a Kruskal-Wallis test with post-hoc pairwise Wilcox w/ Holm adjustment. Asterisks (*) indicate a significant difference between Mexican surface and Pachon Cave. A black circle (•) indicates a significant difference between Mexican surface and Molino Cave.

3.3. Electroretinography thresholds

All populations were maximally sensitive to wavelengths between 500 and 600 nm. There were no significant differences between Honey Creek Cave and surface populations. There were also no significant differences in spectral sensitivity found between San Antonio Zoo surface, San Pedro Springs, and Blue Hole populations. We had expected cave-dwelling fish to be the less sensitive to light levels, which is maintained in the Honey Creek Cave and Honey Creek surface sensitivity curve comparison at 530 and 560 nm, albeit not at a significant level (Mann–Whitney *U* test, $p = 0.054$ at 530 nm, $p = 0.055$ at 560 nm; Supplementary Figure S5). However, comparisons between the surface population of San Antonio Zoo and the intermediate populations of San Pedro Springs and Blue Hole suggest that cave-dwelling fish were more sensitive than those living in surface habitats, as sensitivity curves (Supplementary Figure S5) seem to indicate that the San Pedro Springs population is the most sensitive to all wavelengths of light, followed by Blue Hole population, and ending with San Antonio Zoo population as the least sensitive of the three. Lab-raised populations were not tested since these wild-caught population differences were not significant.

4. Discussion

Phenotypic divergence is often contextualized using morphological features as indicators of change (Kozak et al., 2011). However, few studies examine changes in sensory thresholds due to the difficulty in quantifying the phenotypic features underlying sensory systems (Partan, 2017; Kelley et al., 2018). Recently diverged populations allow us to examine both precursors to evolution as well as phenotypic plasticity of sensory systems in response to environmental pressures. Here, we show clear auditory sensory divergence between two fish populations: Honey Creek Cave fish are significantly more sensitive to several frequencies of sound than Honey Creek surface fish. The two facultatively subterranean populations (San Pedro Springs and Blue Hole) were also more sensitive to certain frequencies than San Antonio Zoo surface fish. However, when comparing wild-caught fish to lab-raised progeny, it was evident that these traits are non-heritable and that auditory sensitivity is likely a plastic trait influenced by developmental environment. While most comparisons of retinal sensitivity were not statistically significant, qualitative examination of the ERG curves showed both recently introduced and partially subterranean

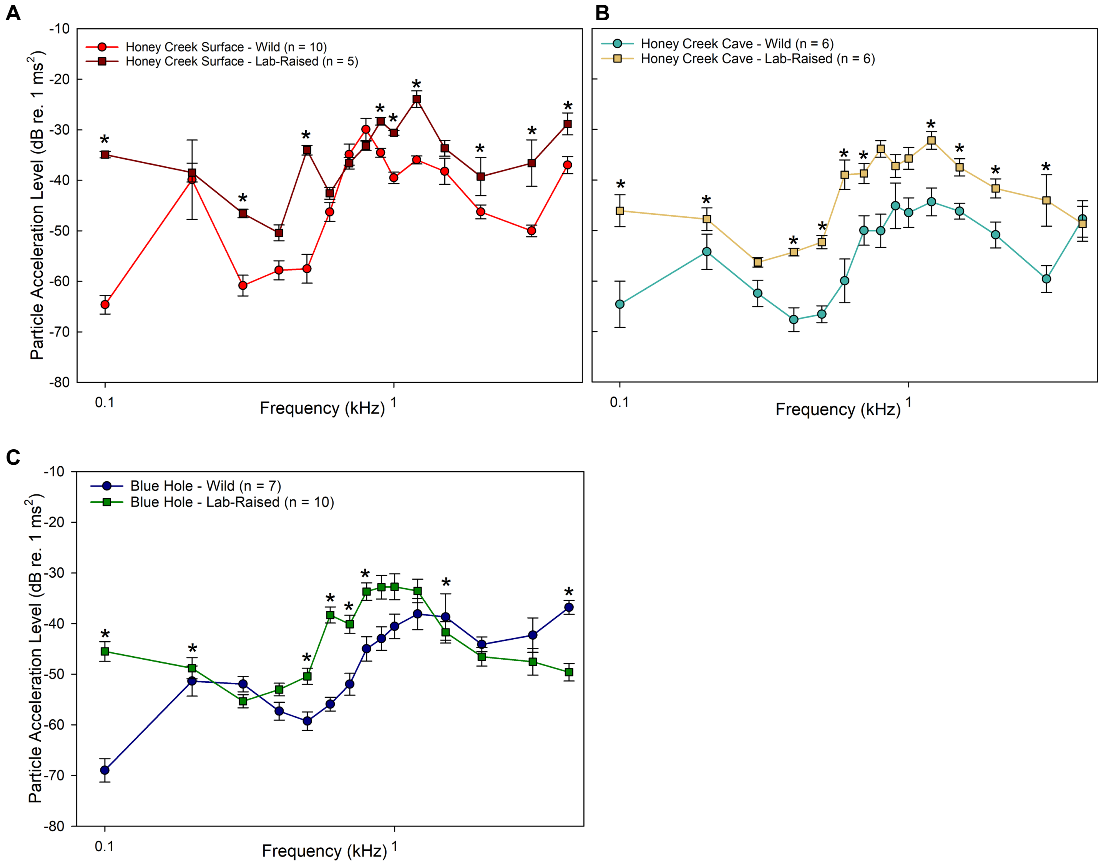

FIGURE 5
Wild-caught and lab-raised PAL thresholds. **(A–C)** Median auditory particle acceleration sensitivity (±SE) between wild-caught and lab-raised populations. Asterisks (*) indicate significant (Mann–Whitney *U* Test, $p<0.05$) differences between populations. **(A)** Comparison between wild-caught (red circle) and lab-raised (brown square) Honey Creek Surface fish. **(B)** Comparison between wild-caught (light blue) and lab-raised (beige square) Honey Creek Cave fish. **(C)** Comparison between wild-caught (dark blue circle) and lab-raised (green square) Blue Hole Fish.

populations trended toward increased sensitivity compared to surface fish. Taken together, these results delineate a relationship between sensory thresholds and environmental pressures in these recently established *A. mexicanus* populations.

4.1. Sound pressure and particle acceleration sensitivity

Few studies have been conducted on auditory sensitivities of cavefishes (Soares et al., 2016), and previous investigations examining auditory sensitivity between cave and surface fish have not found many physiological differences. For example, a previous study using avoidance conditioning techniques to determine sound thresholds indicated no significant differences in auditory sensitivities between cave and surface populations of Mexican *A. mexicanus* (Popper, 1970). Although a separate study between cave and surface populations of the Atlantic Molly (*Poecilia mexicana*) showed differences in otolith morphology, AEP experiments were unable to detect any differences in auditory sensitivity (Schulz-Mirbach et al., 2010). However, while both cave and surface amblyopsid fish exhibit similar auditory sensitivity to pure tones up to 800 Hz, only surface fish were able to detect frequencies >800 Hz, suggesting cave amblyopsids may have lost higher frequency sensitivity in response to high frequency noise in caves (Niemiller et al., 2013). Our data corroborates and provides additional evidence of this low-frequency sensitivity, while also showing significant differences between cave and subterranean dwelling fish.

While many previous AEP experiments only reported sound pressure measurements, it is imperative to report particle motion as all fish can detect the particle motion component of sound (Ladich and Fay, 2013), therefore both methods are included here to allow comparison with previous studies. Our results indicate that wild-caught cave populations are more sensitive to particle acceleration than sound pressure when compared to surface conspecifics. The only previous study which has measured cave fish particle motion sensitivity was done comparing cave and surface ecotypes of *P. mexicana* and found no significant difference in particle motion sensitivity between the two ecotypes (Schulz-Mirbach et al., 2010). Further examination of particle motion sensitivity should be pursued to better understand the relevancy of this modality of sound detection in cave soundscapes. While we acknowledge the limitations of AEPs since physiological responses do not necessarily equate to active perception or match with thresholds found in behavioral studies (Popper et al., 2019), we used this methodology because it is minimally invasive and can still provide important insights on sensory perception (Ladich and Fay, 2013).

When comparing auditory sensitivity, we found that wild-caught fish were generally more sensitive to particle acceleration and sound pressure level than their lab-raised descendants, with the exception being that both lab-raised and wild-caught Honey Creek Surface population fish had similar sound pressure level sensitivity thresholds. However, we observed no consistent patterns when comparing lab-raised and wild-caught populations. We suggest that auditory sensitivity is a trait that exhibits phenotypic plasticity, and that the differences observed among populations in this study are not likely the result of genetic differences. Behavioral and phenotypic plasticity has been suggested as an evolutionary adaptation to environmental variation (Sommer, 2020). Adaptive plasticity is well studied across several organisms in response to both sensory deprivation and sensory stimulation in lab environments (Bharmauria et al., 2022). Additionally, previous studies have demonstrated that both *A. mexicanus* surface and cave ecotypes express rapid behavioral and morphological responses to environmental conditions (Bilandžija et al., 2020; Espinasa et al., 2021). While the results presented do not indicate that auditory sensitivity is an adaptive phenotype in recently established populations, highly plastic organisms may be better suited to colonize novel selective landscapes and sensory plasticity may play a role in convergent troglomorphic traits.

It should be noted that this data is presented with the caveat that individual fish were not of the same age during data collection. Wild-caught fish were maintained in the lab for over a year prior to testing and aging of these individuals was prohibitive due to the large sample sizes needed for accurate age validation (Campana, 2001).The age of experimental organisms can critically affect results when collecting neurological data (McCutcheon and Marinelli, 2009), therefore we encourage future iterations of this work to control for age between tested individuals.

4.2. Visual sensitivity trends

Honey Creek Cave fish showed no significant difference in sensitivity when compared to Honey Creek surface fish, although Honey Creek Cave fish did have readings that indicated less visual sensitivity. While the Blue Hole population, San Pedro Springs populations, and San Antonio Zoo surface fish have remarkably similar ERG curves, at wavelengths greater than 575 nm, San Pedro Springs were most sensitive to light stimuli, followed by Blue Hole, then San Antonio Zoo. The genetic underpinnings of vision in *A.mexicanus* has been heavily studied in order to trace back the eyeless phenotype seen in cave ecotype fish (Dowling et al., 2002; O'Quin et al., 2013; McGaugh et al., 2014; Krishnan and Rohner, 2017). However, such studies have not explored the plasticity of the visual system. In general, there exists a gap in the literature exploring candidate genes that control plastic traits. The authors propose that methods using genomic sequencing and QTL mapping to characterize traits such as metabolic function (Carlson et al., 2018; Riddle et al., 2021) can be similarly applied to questions regarding sensory trait plasticity.

While newly invaded cave populations have not previously been examined for eye function, deep water fish similarly inhabit low-light environments. Unlike cave organisms, who have lost use of their visual system in dark cave environments, deep water oceanic fish have evolved elaborate retinal specialization to detect downwelling light (Collin and Partridge, 1996; Warrant and Locket, 2004) and bioluminescent organisms (Locket, 1970). Similarly, the dim light, found in the transition (twilight) zone between the cave mouth and perpetually dark recesses may select initially for increased scotopic vision.

4.3. Environmental factors of sensory divergence

The geology of Honey Creek Cave may play a large role in the divergence seen between Honey Creek Cave fish and their surface counterparts. Honey Creek Cave is the longest cave system in Texas (Veni, 1994) and its current surveyed length exceeds 30 km, with extensive subterranean stream habitat (Reddell, 1964), although *A. mexicanus* have only been observed in the first 200 m of stream passage (A. Gluesenkamp, pers. observation). Our results suggest that there are changes in both auditory and visual sensitivity between Honey Creek surface and Honey Creek Cave populations. The amount of change in sensitivity may be tempered by both the recent colonization of this environment and potential continual gene flow between cave and surface populations. While surface individuals were collected 1,500 m from the cave mouth, the cave population was sampled within 100 m of the cave entrance. Further studies on the migration of cave and surface fish between environments need to be conducted. Additionally, while Honey Creek Cave and Honey Creek surface populations may experience physical barriers to gene flow between surface and subterranean habitats, populations at the other sites enjoy ephemeral to perennial access to subterranean and surface environments. Therefore, it is not surprising that San Pedro Springs and Blue Hole populations displayed intermediate auditory sensitivities compared to Honey Creek cave and San Antonio surface fish.

The recent establishment of *A. mexicanus* populations in Central Texas provides a unique opportunity to observe potential rapid sensory divergence occurring over a period of less than a century. Our data suggests that divergence is primarily influenced by environment and is more pronounced in auditory sensitivity rather than vision, with increased auditory sensitivity in subterranean populations compared to surface conspecifics. However, further genetic and genomic work will be necessary to exclude the genetic determination of such traits and better characterize the plasticity of the adaptable *A.mexicanus*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the University of Minnesota the Institutional Animal Care and Use Committee (IACUC).

Author contributions

AM, SM, RP, and ME contributed to the conception and design of the study. RP, AM, and AG significantly contributed to the methodology. AG, NS, and SM conducted specimen collection. ME and TT performed data collection and analysis. ME wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1085975/full#supplementary-material

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Growing underground: Development of thermogenesis in pups of the fossorial rodent *Ctenomys talarum*

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In mammals, during the pup's development and adult life, integrated requirements of all activities of the individual must conform to a sustained rate of metabolism. Thus, partitioning the available energy according to short-term priorities at a specific moment allows animals to survive and optimize long-term reproductive success. In altricial rodents, thermal balance is a key factor for survival. When no exogenous source of heat is present, altricial pups rapidly lose heat, reaching ambient temperature (T_a). Fossorial rodents showed a strong dependence on burrows, where T_a remains relatively stable within narrow ranges. Pups of the fossorial rodent *Ctenomys talarum* are altricial, making them an excellent model to evaluate the development of thermogenic capacity. In this study, the ontogeny of the thermogenic capacity of pups of *C. talarum* was evaluated. Using respirometry techniques, non-shivering thermogenesis (NST), total thermogenic capacity (cold-induced maximum metabolic rate, MMR), and resting metabolic rate (RMR) in pups until post-weaning age (day 60) were analyzed. No NST was present in pups until day 60 despite the presence of molecular markers for NST in brown adipose tissue deposits, which became functional in adults. Although pups are altricial at birth, they maintain their thermal balance behaviorally during lactation. Total thermogenic capacity became fixed at an early age, indicating an improvement in shivering thermogenesis (ST) efficiency after day 10, which might be related to the development of musculature related to digging. Before the aboveground dispersal period (~day 60), pups gradually reached adult T_b by improving ST and thermal isolation, allowing them to confront climatic fluctuations on the surface.

KEYWORDS

thermoregulation, non-shivering thermogenesis, postnatal development, maximum metabolism, *Ctenomys*

Introduction

The energy budget, that is, a sum of processes associated with energy intake and expenditure, has enormous relevance to individual fitness since it integrates all aspects of the life of organisms. The changes in the energy budget during the lifespan are related to requirements and the use of time and space (Withers et al., 2016). Thus, partitioning the available energy according to short-term priorities at a specific moment allows animals to survive and optimizes long-term reproductive success (Wade and Schneider, 1992). In endotherms, the development of thermoregulatory mechanisms after birth is the determinant of the adult's fitness. The development of functional mechanisms that ensure thermal homeostasis is one of the main requirements that must be achieved before parental care independence (Thompson, 1992). Defined by their physiological maturity, different strategies for the development of endotherm pups have been proposed to exist between altriciality and precociality extremes (Hill, 1992). The distinction between altricial and precocial development is related to the time at which pups become furred, possess capable eyesight, coordinate their locomotor abilities, or, particularly, reach endothermy (McClure and Randolph, 1980). Precocial pups can use physiological thermoregulation at an early stage, whereas altricial pups show a low capacity to control their body temperature (T_b) due to the lack of functional thermogenic mechanisms, losing heat rapidly when they face an ambient temperature (T_a) below thermoneutral zone (TNZ; Hill, 1992). In the case of altricial pups, the requirement for warmth might be provided by the exogenous source of heat. Direct physical contact of pups with their parents might help to overcome the thermal imbalance. Thus, when physiological thermogenic capacity is limited, behavioral thermoregulation could restrict heat loss, for example, either by huddling with other members of the litter (Geiser and Kenagy, 1990), by behavioral thermotaxis (Farrell and Alberts, 2007), or by remaining in the nest (Antinuchi and Busch, 2001). Total thermogenic capacity is defined as the maximum amount of heat that can be produced to maintain a constant T_b and can be approximated by cold-induced maximum metabolic rate (MMR). The components of total thermogenic capacity are shivering. Shivering thermogenesis involves rapid, involuntary contractions of skeletal muscles to produce heat, related to biochemical-mechanical processes at the cellular level, such as inefficiency of biochemical pathways or friction among myofibrils (refer to Hohtola, 2004). Unlike ST, NST has specialized tissues to produce heat without any muscle contraction. Brown adipose tissue (BAT) is the main source of NST (Janský, 1973; Foster and Frydman, 1979; but see Nowack et al., 2017 for a discussion about muscle-mediated NST). BAT is a highly vascularized tissue with a large number of mitochondria and respiratory enzymes and is found in discrete areas in the body, mostly surrounded by the main organs (Cannon and Nedergaard, 2004). The activation of BAT depends on the release of norepinephrine (NE) by the sympathetic nervous system, and its functioning is related to uncoupling the oxidative phosphorylation in BAT's mitochondria. The presence of uncoupling proteins (UCP), particularly UCP1, short-circuits the electron transport chain circuit, producing heat (Oelkrug et al., 2015; Chouchani et al., 2019).

Depending on the pup's development strategy, the onset of functional total thermogenic capacity can vary (Brück and Hinckel, 1996). While thermogenic mechanisms in precocial pups are well developed right after birth, complete development takes days in altricial pups (Berthon et al., 1994; Farrell and Alberts, 2007; Canals et al., 2009; Lezama-García et al., 2022). For example, in rats, behavioral control of T_b is important during the first days of life; by the second week, with the increase of body mass and thermal insulation, the appearance of ST enhances thermogenic capacity (Sant Anna and Mortola, 2003). In the white-footed mouse *Peromyscus leucopus*, NST appears by day 10, whereas ST was observed around 2 weeks after birth (Hill, 1976; Robertson et al., 2019). Deer mice *Peromyscus maniculatus* from a low-altitude population can shiver at day 14, but only NST is observed in individuals from a high-altitude population at this age (Robertson and McClelland, 2019). Given the variation between altricial species, it is difficult to establish a temporal pattern of postnatal development of different thermogenic mechanisms, but it is generally recognized that, in mammal pups, shivering is not functional at early stages, but NST tends to be active. Deposits of BAT are well developed and matured only a few days after birth, contrary to muscles, which show a slow development during ontogeny (Lagerspetz, 1966; Robertson and McClelland, 2019).

In this general framework, inhabiting a relatively thermally stable environment can diminish the cost of thermoregulation. Some mammalian species live in underground burrows that can buffer cold external T_as and its fluctuation. In particular, subterranean rodents occupy almost all available habitats, from cold to tropical, from mountain slopes to deserts, inhabiting tunnel systems parallel to the surface. Although subterranean burrows buffer T_a and protect against predators, they are stressful in most cases because they are humid, dark, stagnant, and lack most clues available for orientation (Burda et al., 2007). Among rodents, different degrees of adaptation to the use of burrows are present (Begall et al., 2007), with some species adapted to digging and living almost exclusively underground (i.e., subterranean) and some other species also adapted to digging but with extensive use of surface (i.e., fossorial). Either subterranean or fossorial rodents, underground ecotope had determined the observed convergence in morpho-physiological features observed between both groups of rodents (e.g., low mass-specific minimal metabolic rate, a wide range of thermoneutrality, high thermal conductance, and low Tb; McNab, 1966; Vleck, 1979). Different studies have been carried out for evaluating the total thermogenic capacity, ST, and NST in adults of subterranean and fossorial species, finding significant variability in the type and proportion of the mechanisms on total thermogenic capacity. While in some South American Ctenomyid species, a combination of NST and ST is observed (e.g., *C. porteusi*), in other species, only ST is present (*C. tuconax*; Luna et al., 2019). Similarly, in African bathyergids, the cost of NST varies depending on the body mass and sociality (Luna et al., 2021). However, no studies have been performed to evaluate the ontogeny of total thermogenic capacity and the nature of thermogenic mechanisms involved in pups of subterranean or fossorial species.

The genus *Ctenomys* is distributed in several environments in the southern cone of South America, being one of the most speciose genera among subterranean and fossorial rodents, including species from ~100 g (*C. pundti*) to more than 1,000 g (*C. conoveri*; Reig et al., 1990). Particularly, *Ctenomys talarum* is a solitary fossorial rodent that lives in the coastal grasslands of Argentina (Antenucci and Busch, 1992). Similar to other *Ctenomys* species, tunnel systems show a linear pattern with no differences in total burrow length between sexes, feeding mainly on aerial plant parts near the burrow openings (Antenucci and Busch, 1992; Comparatore et al., 1995). Males of *C. talarum* established a dominance hierarchy among them, monopolizing mating activity, and consequently, dominant male burrows occupied central distribution surrounded by several female burrows (Busch et al., 1989; Antenucci and Busch, 1992). The reproductive season lasted ~9 months, beginning in June–July (austral winter) and continuing until February (austral summer). Birth takes place from August through early December after a long gestation period (~100 days; Malizia and Busch, 1991; Fanjul et al., 2006). Females produced as many as two litters per reproductive season, with a mean litter size of 4 (Busch et al., 1989). For *C. talarum*, the cost of acceding to new food patches made through the construction of new tunnel systems is relatively high (Luna et al., 2002).

During the ontogeny, *C. talarum* pups show an initial altricial phase, represented by a period of high thermal vulnerability. During lactation (~10 days), pups are not capable of maintaining T_b on their own, depending, therefore, on their mother to thermoregulate. After this period, pups enhance their thermoregulatory capacity and start eating solid food. At approximately 30–40 days after birth, pups reach the T_b of adults and become behaviorally and physiologically independent. At this age, the weaning period starts, and pups show active exploratory behavior. Around day 60, solitary pups disperse aboveground from their maternal burrow and construct their burrow (Malizia et al., 1995; Zenuto et al., 2002; Antenucci et al., 2003; Cutrera et al., 2003; Baldo et al., 2014). In this context, the aim of this study was to evaluate the thermogenic capacity development of pups of the fossorial rodent *Ctenomys talarum*. We also evaluated the presence of BAT during ontogeny and the timing of mitochondrial uncoupling proteins related to BAT-mediated NST. We hypothesized that no thermogenic capacity is attained during the altricial phase of their development (~day 10), increasing their thermogenic capacity after weaning, before the dispersal period. Furthermore, we expect that NST is completely functional after day 10, reaching the value of the adult during this period.

Materials and methods

Animal capture and husbandry

Pregnant females were live-trapped in Mar de Cobo (37°45’S, 57°56’W, Buenos Aires Province, Argentina). In the laboratory, females were housed in individual cages (0.30 × 0.40 × 0.25 m), with wood shavings as nesting material. A total of 27 pregnant females were used in this study. The animal room was kept at 25 ± 1°C, and the photoperiod was LD 12:12 (lights on at 7.00 a.m.). Animals were fed with mixed native grasses, carrots, lettuce, corn, alfalfa, and sunflower seeds *ad lib.* As *C. talarum* does not drink free water, it was not provided. Pregnant females were monitored daily until pups were born. After birth, pups were kept in the same cage with their mother and siblings until day 60, after which the remaining pups were placed in individual boxes until they were released in the field. On days 2, 6, 10, 15, 30, and 60 after parturition, 1 pup from a randomly assigned mother was weighed, and metabolic variables and T_b were measured. To avoid possible maternal effects during the experiment, only one pup of each litter on each day was used in NST_{max} and MMR trials. Due to the difficulty in maintaining pregnant females and obtaining parturition in the laboratory, we used 4 pups from 4 different females that delivered more than 4 pups. In this case, 1 pup was used between days 2 and 6, and another between days 30 and 60. A total of 31 pups were used to estimate the pattern of NST_{max} and MMR. The sex of the pups in the experimental design was not considered, using both males and females independently, because sex cannot be stablished externally in the early stages of development. Given that our primary focus was thermogenic capacity, we used another set of pups (7 pups from different litters) for the RMR experiment. This set of pups was maintained in the same husbandry conditions and was related to an undergoing study on thermoregulatory physiology.

Resting metabolic rate and non-shivering thermogenesis of pups

Oxygen consumption was measured using a computerized positive pressure open-flow respirometry system (Sable System, United States). A cylindrical chamber (volume 500 mL) was used to estimate O_2 consumption. Chamber was placed inside an incubator (Simedix, model I-501, Ingelab, Argentina). Ambient temperature (T_a) was maintained with an accuracy of 0.2°C. The chamber received dry and CO_2-free air ranging from 300 mL min^{-1} to 600 mL min^{-1}, depending on the body mass of the pups, from a mass-flowmeter (Side-Trak Sierra, model 830/840, Sierra Instruments, United States). To scrub CO_2 and water, the air was passed through a CO_2 absorbent (self-indicating soda lime, Laboratorios IQB, Argentina) and water scrubber (Drierite, W. A. Hammond Drierite Company, United States) before and after passing through the chamber. Excurrent air from the chamber was subsampled at 80 ± 10 mL min^{-1}, and oxygen consumption was obtained from an Oxygen Analyzer FC-1B every 1 s by an EXPEDATA data acquisition program (Sable System, United States). Rates of oxygen consumption were calculated using equation 4a of Withers (1977). All metabolic trials were performed between 9:00 and 17:00 h. The body mass of individuals (M) was measured before each metabolic trial using an electronic balance (model FX-3000, ± 0.01 g, A&D Company Limited, United States), and the pharyngeal temperature was recorded at the beginning and the end of each experiment with a YSI probe (model 93 k73545-402) connected to a Cole-Parmer thermistor meter (model 8,402–10, ± 0.1°C, Cole-Parmer Instrument Company, United States).

Resting metabolic rate was estimated as the lowest 5–8 min steady-state O_2 consumption of a total trial period of 35–40 min. A total trial period was chosen because *C. talarum* pups are highly dependent, during the early stage of their development, on mother and sibling contact (Zenuto et al., 2002), showing signs of hypothermia if pups are alone for more than 30 min (Cutrera et al., 2003). However, brief isolation did not affect the development of thermoregulatory physiology (Baldo et al., 2014). In addition, total chamber volume and air flow allowed a short equilibration period of the chamber (2–4 min to achieve 90% of equilibration, depending on the air flow; Lasiewski et al., 1966). After being placed in the metabolic chamber, pups showed an exploratory behavior, which was performed during the period in which the metabolic chamber was equilibrating (~5 min).

Maximal non-shivering thermogenesis (NST_{max}) was estimated using the same chamber and protocol used to estimate RMR, but after intramuscular injection of norephinefrine (NE). In eutherians, the O_2 consumption in response to NE occurs 10 min after the injection and lasts at least 5–10 min (Feist and Rosenmann, 1976). Doses of NE were estimated according to Wunder and Gettinger (1996) and described as NE (mg Kg^{-1}) doses = 2.53 $M^{-0.4}$. Doses of NE and procedures were validated for adults of this species (Luna et al., 2012). The maximum 10-min steady-state oxygen consumption after the injection of NE was considered to be NST_{max}, which includes both RMR and thermoregulatory NST (Wunder and Gettinger, 1996). To avoid unnecessary long-time exposition in pups, we did not assess the effect of injection on pups. Values of O_2 consumption of adults between the control group and after saline solution injection were similar (Luna et al., 2012). For RMR and NST_{max} estimations, the metabolic chamber was maintained at T_a of 25°C, which is within the thermoneutral zone of adults for this species (Busch, 1989).

Cold-induced maximum metabolic rate

Maximum thermogenic metabolism was estimated in a HELOX atmosphere according to the procedure described by Rosenmann and Morrison (1974). A mixture of He (79%) and O_2 (21%) was passed through a mass flowmeter before entering the metabolic chamber (Side-Trak, Sierra model 830/840, Sierra Instruments, United States). Before MMR estimation, the flow rate was corrected for the He-O_2 gas mixture. As described before, the mixture was passed through a CO_2-absorbent and water scrubber before and after passing through the chamber. Oxygen consumption of pups was recorded for a 30–40 min period at an ambient temperature of 19°C. This T_a was chosen based on previous studies that determined the lowest T_a within burrows during the reproductive period when pups are born (Cutrera and Antinuchi, 2004) and because pups fall into deep hypothermia even when there are huddling (Cutrera et al., 2003).

Shivering thermogenesis

Shivering thermogenesis (ST) was estimated for each pup according to the equation proposed for eutherian mammals ($MMR = BMR + NST + ST$; Wunder and Gettinger, 1996). Experimentally, ST was estimated as O_2 consumption after cold-induced minus NST_{max}.

Measurement of UCP1 and COXII content and COX activity

After NST_{max} and MMR, pups were euthanized, and interscapular brown adipose tissue (iBAT) was carefully dissected, frozen in liquid nitrogen, and stored at −80°C. All other main organs were also frozen and stored at −80°C for further analysis. We adhered to the 2012 Revised International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS) and the International Council for Laboratory Animal Science (ICLAS). Pups used in RMR experiments were released at the capture site after day 120. Interscapular BAT was homogenized with a manual homogenizer in STE buffer (250 mM sucrose, 5 mM Tris–HCl, 2 mM EDTA, pH 7.4) with protease and phosphatase inhibitors (10 μM Leupeptin, 10 μM Pepstatin, 0.2 mM PMSF, and 0.2 mM Orthovanadate) in a proportion of 10 mL of buffer per gram of tissue and filtered through a layer of inert gauze. Total protein content was determined using the Bradford method (Bradford, 1976). Samples of iBAT were denatured, and 40 μg of proteins per line were loaded and run in an SDS-PAGE (3% stacking gel and 12% running gel), according to Laemmli (1970), and electrotransferred onto a nitrocellulose filter, as described by Puigserver et al. (1991), using a Trans-Blot® Turbo™ Transfer System (Bio-Rad, United States). After that, membranes were blocked in 5% nonfat powdered milk in TBS-Tween and incubated with the corresponding antibodies (UCP1, COXII antibodies, Alpha Diagnostics, United States). Protein bands on the nitrocellulose filters were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad, United States) using the Western blotting detection systems. The chemiluminiscence signal was captured using a Chemidoc XRS densitometer (Bio-Rad, United States) and analyzed with Quantity One Software (Bio-Rad, United States). COX activity was measured using a spectrophotometric method (Wharton and Tzagoloff, 1967; Chrzanowska-Lightowlers et al., 1993). Briefly, aliquots of the iBAT homogenates were incubated in 0.1 M $NaPO_4H_2$, pH 7.0, in the presence of 2 μg/mL Catalase and 5 mM substrate DAB (3, 3′ diaminebenzidine-tetrachloride). After 30 s, 100 μM reduced cytochrome c was added to start the reaction, and the absorbance variation was recorded for 20 min at 450 nm.

Statistics

Physiological variables (NST_{max}, MMR, and RMR) were analyzed by linear mixed-effects model with the R package "nlme" (Pinheiro et al., 2022) in R (R Core Team, 2021) inside RStudio (R Studio Team, 2020). To account for repeated measurements, individual IDs were included as a random factor when comparing NST_{max} and MMR. Body mass was included as a covariate in the model. Assumptions were verified using residual plots and tested for normality of residuals by the Shapiro–Wilk test. To evaluate each fixed factor (treatment and day), we used hypothesis tests with a significance level set to $\alpha = 0.05$ for all analyses. To explore changes in metabolic rate, the variance correction function varIdent was used. We performed a general linear mixed model with heterogeneous variance for the absolute T_b obtained before metabolism measurement (cold-induced or NE injection) with the same fixed and random effects as the models detailed above, without using body mass as a covariate. To compute *p*-values for multiple comparisons, the R package "emmeans" (Lenth, 2022) was used when we found significant differences between means. For ΔT_b, we used linear models to test for differences between days in each experimental condition (cold-induced or after NE injection). In addition, linear models were used to evaluate differences in the enzymatic activity of COX between days. Finally, we used the Kruskal-Wallis test for differences in protein content (UCP1 and COXII) in iBAT. Linear models and the Kruskal-Wallis tests were performed in the R package "R stats" in R (R Core Team, 2021) inside R Studio (R Studio Team, 2020).

Results

When RMR and NST_{max} were compared, MR was affected by age ($F_{5,66} = 108.28$, $p < 0.001$) and treatment ($F_{1,66} = 33.95$, $p < 0.001$). However, no significant effects of the interaction between age and treatment ($F_{5,66} = 1.92$, $p = 0.1$) were found (Figure 1; Table 1). There is an increment in MR from day 2 until day 10 [$P_{(day\ 2–day\ 10)} = 0.008$, $P_{(day\ 6–day\ 10)} < 0.001$]. When pups were older than day 10, age affected MR [$P_{(day\ 10–day\ 30)} < 0.005$, $P_{(day\ 15–day\ 30)} = 0.01$]. Moreover, differences in MR were found between day 6 and day 30 [$P_{(day\ 6–day\ 30)} < 0.005$]. When MMR and NST_{max} were compared, we found age differences

($F_{5,31} = 93.21$, $p < 0.001$) and treatment ($F_{1,30} = 36.01$, $p < 0.001$). The interactions between age and treatment were also different ($F_{5,30} = 11.55$, $p < 0.001$, Figure 1; Table 1). There is an increment in MMR from day 2 to day 10 [$P_{(\text{day 2–day 10})} = 0.01$, $P_{(\text{day 6–day 10})} = 0.006$], with values of MMR between day 2 and day 6 being similar ($p = 0.99$). After day 10, MMR was different from the following days [$P_{(\text{day 10–day 15})} = 0.99$, $P_{(\text{day 10–day 30})} = 0.99$, $P_{(\text{day 10–day 60})} = 0.81$]. After day 15, due to the relatively lower value at day 30, MMR was different only from day 60 [$P_{(\text{day 15–day 30})} = 0.99$, $P_{(\text{day 15–day 60})} = 0.004$, $P_{(\text{day 30–day 60})} = 0.002$]. When comparing MMR with NST_{max}, differences between days were observed. In this sense, MMR was higher than NST_{max} at day 15 ($p = 0.002$), day 30 ($p = 0.049$), and day 60 ($p < 0.001$). Similarly, as no differences were observed between RMR and NST_{max}, MMR was different from RMR between days ($F_{5,66} = 168.05$, $p < 0.001$), treatment ($F_{1,66} = 124.13$, $p < 0.001$), and the interaction between days and treatment ($F_{5,66} = 45.48$, $p < 0.001$, Figure 1; Table 1). MMR was higher than RMR at day 15 ($p = 0.002$), day 30 ($p = 0.049$), and day 60 ($p < 0.001$). For comparative purposes, in Figure 2A, physiological variables were expressed in mass-specific MR.

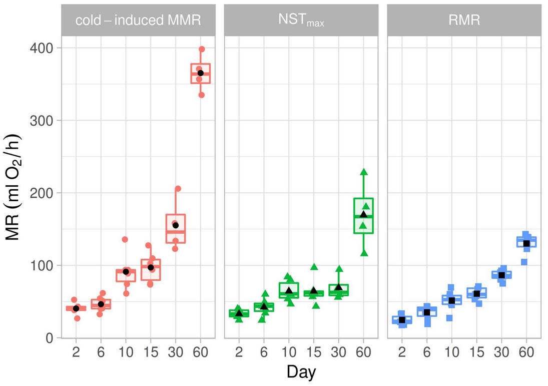

FIGURE 1
A box plot of metabolic rate in different experimental conditions during postnatal development in the fossorial rodent *Ctenomys talarum*. The lower and upper boxes are the 25th and 75th percentiles, respectively. The line inside the box is the median, and the black symbol is mean. The lower and upper error lines are the 10th and 90th percentiles, respectively.

When comparing T_b before the exposure to cold conditions to estimate MMR or NE injection, neither the exposure alone nor their interaction with age was different ($F_{1,31} = 1.49$, $p = 0.23$ and $F_{5,31} = 1.52$, $p = 0.21$, respectively), whereas T_b between days was different ($F_{1,31} = 17.64$, $p < 0.001$; Table 2). Body temperature at day 60 was higher than those observed at days 2, 6, 10, and 15 (all, $p < 0.001$) and also T_b at day 30 compared to day 2 ($p = 0.01$) and day 6 ($p = 0.03$). After cold exposure, ΔT_b was similar between days ($F_{5,31} = 0.88$, $p = 0.51$). After NE injection, ΔT_b on day 2 was lower than ΔT_b on all other days ($F_{5,31} = 8.58$, $p < 0.001$; Table 2). Interscapular BAT mass increased linearly with body mass. The relationship of iBAT mass with body mass was iBAT mass $= 0.009\,M - 0.011$ ($R^2 = 0.89$, $F_{1,39} = 293.25$, $p < 0.001$, Figure 3). UCP1 and COXII content in iBAT were similar between days ($T = 5.88$, df $= 5$, $p = 0.32$ and $T = 8.96$, df $= 5$, $p = 0.11$, respectively; Table 3). The activity of the COX complex in iBAT was similar at all ages ($F_{5,32} = 1.12$, $p = 0.37$, Table 3).

Discussion

To attain thermal independence before the dispersal period, pups must fully acquire thermoregulatory capacity (Hill, 1992). The main objective of this study was to determine the thermogenic development of pups of the fossorial *C. talarum*, stablishing the onset of thermogenic capacity and its components. To the best of our knowledge, this is the first study to establish the postnatal development of non-shivering thermogenesis (NST) and cold-induced maximum metabolic rate (MMR) in a fossorial species. Pups of *C. talarum* showed a biphasic pattern of mass-specific RMR during their development. Mass-specific RMR increases until day 10, with a subsequent decline to day 60, confirming the pattern found by Zenuto et al. (2002). In this species, acquisition of thermoregulatory capacity takes days, since the onset of homeothermy is observed around day 30, a period in which pups showed T_b of adults (Zenuto et al., 2002; Cutrera et al., 2003; Baldo et al., 2014). Notably, no thermogenic mechanism is observed until day 10, and NST_{max} does not differ from RMR up to day 60.

In altricial species, it has been described that the first thermogenic mechanism to appear is NST (Robertson and McClelland, 2019; Lezama-García et al., 2022), involving the maturation of BAT a few days after birth (Robertson et al., 2019). In pups of the golden hamster

TABLE 1 Resting metabolic rate (RMR), maximal non-shivering thermogenesis (NST_{max}), shivering thermogenesis (ST), and cold-induced maximum metabolic rate (MMR) during postnatal development in the fossorial rodent *Ctenomys talarum*.

	Day						
	2	6	10	15	30	60	Adult*
RMR (mL O_2/h)	24.7 ± 2.5^{a*}	35.1 ± 3.5^{a*}	51.2 ± 5.0^{a*}	60.8 ± 3.4^{a*}	86.4 ± 2.7^{a*}	130.1 ± 5.0^{a*}	162.9 ± 9.7
	(9.6 ± 1.0)	(11.4 ± 0.8)	(15.6 ± 1.6)	(21.2 ± 1.8)	(44.7 ± 2.0)	(82.1 ± 3.7)	
NST_{max} (mL O_2/h)	33.02 ± 2.50^{a1}	42.29 ± 4.31^{a1}	64.28 ± 6.05^{a1}	64.27 ± 7.22^{a1}	68.89 ± 8.71^{a1}	169.4 ± 23.5^{a1}	245.1 ± 10.1
	(12.2 ± 0.6)	(12.6 ± 1.3)	(18.5 ± 2.1)	(20.94 ± 2.6)	(36.61 ± 4.4)	(80.66 ± 5.8)	(182.0 ± 19.1)
MMR (mL O_2/h)	40.4 ± 5.4^{1*}	46.4 ± 3.7^{1*}	91.1 ± 10.3^{1*}	97.0 ± 8.5^{2†}	155.1 ± 18.4^{2†}	365.2 ± 13.3^{2†}	786.0 ± 30.7
	(12.8 ± 0.7)	(13.23 ± 1.4)	(19.5 ± 2.0)	(22.0 ± 2.7)	(37.3 ± 3.8)	(82.0 ± 5.2)	(180.0 ± 6.2)
ST (mL O_2/h)	11.6 ± 5.4	4.1 ± 4.1	26.9 ± 7.2	32.7 ± 4.8	86.2 ± 18.2	195.8 ± 31.6	378.1 ± 22.4

*Data from Luna et al. (2012). Body mass in parentheses. Similar letters indicate no differences between RMR and NST_{max}. Similar small numbers indicate no differences between NST_{max} and MMR each day (see Results). Similar symbols indicate no differences between RMR and MMR each day (see Results). All data are means ± SE.

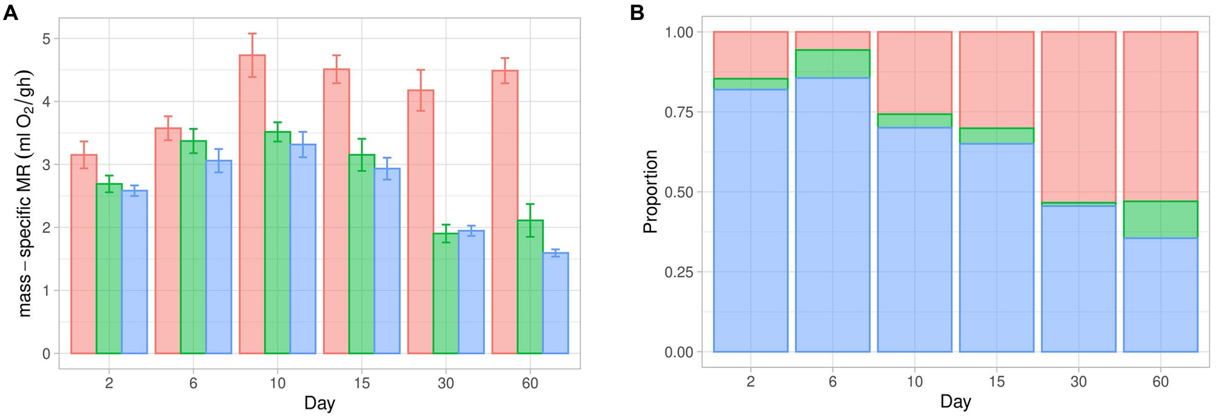

FIGURE 2
Thermogenic capacity during postnatal development in the fossorial rodent *Ctenomys talarum*. **(A)** Mass-specific metabolic rate in different experimental conditions. Red: cold-induced maximum metabolic rate (MMR). Green: maximal non-shivering thermogenesis (NST_{max}). Blue: resting metabolic rate (RMR). **(B)** Relative contribution of non-shivering thermogenesis (NST), shivering thermogenesis (ST), and resting metabolic rate (RMR) to cold-induced maximum metabolic rate (MMR).

TABLE 2 Body temperature (T_b) and ΔT_b after norepinephrine (NE) injection after cold exposure during postnatal development in the fossorial rodent *Ctenomys talarum*.

	Day					
	2	6	10	15	30	60
NE injection						
T_bi (°C)	32.5 ± 0.5^{a}	32.6 ± 0.5^{a}	33.5 ± 0.4^{a}	33.5 ± 0.5^{a}	34.0 ± 0.9^{bc}	36.0 ± 0.3^{c}
ΔT_b (°C)	−2.8 ± 0.4	−0.3 ± 0.4	−0.1 ± 0.4	−0.2 ± 0.5	0.4 ± 0.5	1.2 ± 0.4
Cold exposure						
T_bi (°C)	32.4 ± 0.6^{a}	33.0 ± 0.5^{a}	33.2 ± 0.4^{a}	33.2 ± 0.3^{a}	34.7 ± 0.3^{bc}	36.3 ± 0.1^{c}
ΔT_b (°C)	−7.1 ± 0.8	−5.7 ± 1.2	−4.8 ± 1.0	−2.9 ± 1.0	−3.6 ± 0.5	−5.0 ± 1.9

Similar letters indicate no differences between days. All data are means ± SE.

Mesocricetus auratus, the onset of NST occurs around 14 days after birth, whereas in the Djungarian hamster *Phodopus campbelli*, the onset of NST occurs after day 9 (Newkirk et al., 1995). Despite the high metabolic activity of BAT, the common rat showed a weak NST on day 5 after birth (Spiers and Adair, 1986). In the golden Syrian hamster *Mesocricetus auratus*, thermogenic capacity develops within a week after birth and is determined by qualitative changes in mitochondrial function related to the initiation of UCP synthesis (Houštěk et al., 1990). In newborn rats, NST capacity appears after weaning, but UCP1 is detectable in iBAT just after birth, reaching its maximum value on day 10 (Porras et al., 1990). Interestingly, *C. talarum* pups showed a complete absence of NST until day 60, but this does not imply the complete absence of BAT. Interscapular BAT patch is directly recognizable, increasing in size with age but never exceeding 1% of the body mass. Neonatal exposure to cold can establish modifications in NST capacity, induced by changes in neuronal stimulation and content of UCP in BAT (Morrison et al., 2000; Sant Anna and Mortola, 2003). However, even though pups of *C. talarum* can be exposed to cold when isolated from the mother and siblings, UCP1 content does not vary between days. In the same way, different molecular markers of the oxidative capacity of BAT ensure NST (Klingenspor, 2003) does not vary between days. For example, the activity of cytochrome c oxidase (COX), a marker enzyme for the mitochondrial membrane, and the content of COXII, commonly used to estimate respiratory capacity in BAT mitochondria (Klaus et al., 1988; Klingenspor et al., 1996), remain similar between days. Although unlikely, given the relative thermal stability of burrows, it would be worth establishing whether acclimatization to low T_as by mothers might affect the magnitude of pup's thermogenesis, as has been observed in the surface-dwelling altricial leaf-eared mouse *Phyllotis darwini* during the first days of life (Canals et al., 2009).

Day 60 corresponds to the beginning of the period of dispersion of this species, which occurs on the surface (Malizia et al., 1995). Interestingly, the size of iBAT and the amount of UCP1, as well as markers of the oxidative capacity of BAT in adults, are similar to that in juveniles (Luna et al., 2012). Due to the complete maturation of endocrine and neural pathways (Symonds et al., 2012; Symonds, 2013; Lezama-García et al., 2022), changes in the efficiency of BAT-related NST might establish the functional NST after day 60. Complete maturation of neuro-endocrine pathways would allow an increased capacity for fatty acid oxidation and tricarboxylic acid cycle activity to uncouple oxidative phosphorylation to produce heat (Chouchani et al., 2019). For example, in *P. maniculatus*, a delay in the onset of NST in pups reared at high altitude was associated with a delay in the maturation of nervous and O_2 supply systems (Robertson et al., 2019; Robertson and McClelland, 2019; Velotta et al., 2020).

As proposed, ST matures later than NST because of the slow development of muscles (Robertson and McClelland, 2019; Lezama-García et al., 2022). In *C. talarum* pups, shivering was characteristically observed over the first days (Baldo et al., 2014) but appeared to be fully functional after day 15. Aerobic-resistant muscles that generate repeated contractions could be mainly recruited to produce heat; thus, the proportion of this type of muscle can establish ST potential (Hohtola, 2004). Muscles of altricial rodents are relatively immature at birth, and they mature due to changes in muscle fiber structure and hypertrophy (White et al., 2010). From day 10, offspring of *C. talarum* start to show exploratory behaviors, wandering the tunnel systems and beginning to scratch and push the soil (Vassallo, 2006; Echeverría et al., 2016). The progression of digging behavior has particular energetic requirements to loosen, remove, and break down the soil to construct a new burrow during dispersal. Similar to ST, aerobic-resistant muscles are used during digging activities (Alvarez et al., 2004). Thus, muscular work performed during digging could also be exploited as a source of heat via ST (Luna and Antinuchi, 2007). The onset of functional ST overlaps with the beginning of the exploration of tunnels near the thermally stable nest, tunnels that usually show T_as below TNZ (Cutrera and Antinuchi, 2004). In this context, even though it is not clear how muscle maturation occurs during the first days of life, the capacity for ST can be associated with an increase in digging capacity. Furthermore, cold acclimation in adult rodents was proposed to lead to muscle training and an increase in ST capacity (Shefer and Talan, 1998). As digging becomes more efficient, ST might also do so. Interestingly, from day 15 until dispersal, total thermogenic capacity relies entirely on ST, increasing its specific energetic cost due to the progressive decrease in mass-specific RMR and the constancy of total thermogenic capacity.

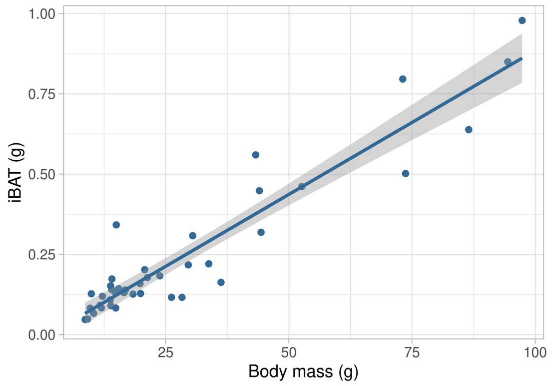

FIGURE 3
Relationship between the mass of interscapular brown adipose tissue patch (iBAT) and body mass during postnatal development in the fossorial *Ctenomys talarum*. The solid line represents the regression line.

The total thermogenic capacity of pups from day 10 is similar to those observed in adults (Luna et al., 2012). The consistency of mass-specific MMR over the lifespan of this species addresses important questions about the mechanistic meaning of physiological adjustments within upper and lower metabolic limits. A fixed metabolic ceiling determines that any energetic modification must be within the aerobic scope. The net aerobic scope represents the capacity of aerobic processes above minimal metabolism (see Nespolo et al., 2017 for a discussion). For example, unlike juveniles, adults of *C. talarum* show a functional NST that represents ~40% of the total thermogenic capacity (Luna et al., 2012). Thus, from day 10, when the metabolic ceiling is established, there is an increase in the net aerobic scope given the progressive decrease in mass-specific RMR. Any energy input intended for other processes, in addition to growth, must be within metabolic limits. In fact, the part of the energy cost of NST in adults could be related to the difference between mass-specific RMR at day 60 (1.75 mL O_2/gh; this study) and in adults (0.89 mL O_2/gh; Luna et al., 2012). Such metabolic trade-offs between different energy-consuming mechanisms might ensure a balanced daily energy expenditure, hence survival. Maximal metabolism is an ecologically relevant trait (Bozinovic and Rosenmann, 1989), and although it appears to be infrequently reached during life, maximal metabolism can be affected by natural selection (Rezende et al., 2004). It is not the focus of this study to evaluate which factor determines mass-specific MMR constancy, but it has been proposed that there would have been an evolutionary limitation on reaching high MMR values (Luna et al., 2012, 2019) because of limitations in respiratory and cardiovascular systems imposed by burrow's atmosphere (i.e., low O_2 and high CO_2). Therefore, the value of maximal metabolism might be independent of the physiological mechanism that elicits it. In adults of *C. talarum*, cold-induced MMR also does not vary after acclimation to cold, being similar to exercise-induced MMR (see Luna et al., 2012). As proposed, further studies are needed to assess the consequences of fixed ceilings and net aerobic scope in subterranean and fossorial species when compared to surface-dwelling species (Luna et al., 2015, 2019).

Heat balance in endotherms results from the integration of a coordinated system of heat production and loss (Naya et al., 2013). One of the main characteristics of underground rodents is their high

TABLE 3 Content of uncoupling protein I (UCP1) and cytochrome c oxidase II (COXII) and activity of COX in interscapular brown adipose tissue (iBAT) during postnatal development in the fossorial rodent *Ctenomys talarum*.

	Day						
	2	6	10	15	30	60	Adult*
iBAT mass (g)	0.13 ± 0.01	0.14 ± 0.04	0.14 ± 0.01	0.16 ± 0.03	0.31 ± 0.07	0.71 ± 0.08	0.83 ± 0.11
UCP1 (*au*)	37.9 ± 15.4	26.7 ± 17.8	39.4 ± 10.5	39.2 ± 18.0	26.9 ± 4.4	31.6 ± 6. 5	23.1 ± 10.0
COXII (*au*)	57.6 ± 7.0	63.1 ± 8.6	45.7 ± 17.1	48.8 ± 9.9	39.1 ± 7.4	26.0 ± 10.9	55.6 ± 14.0
COX activity (Nkat/μg prot)	0.7 ± 0.2	1.0 ± 0.9	0.7 ± 0.4	1.2 ± 0.7	0.8 ± 0.3	0.8 ± 0.5	28.9 ± 5.9

*Data from Luna et al. (2012). *au*, arbitrary units. nKat (nanokatal), defined as a unit of catalytic activity. All data are means ± SE.

thermal conductance (see McNab, 1966). Thus, not only changes in thermogenic capacity but also thermal insulation are important to maintaining thermal homeostasis (Withers et al., 2016). Early development in altricial pups represents a stage of dependence on external thermal sources (MacArthur and Humphries, 1999; Antinuchi and Busch, 2001; Zepeda et al., 2018), which mostly depends on thermal resources provided by the mother and behavioral thermoregulation, such as huddling with their siblings (Zenuto et al., 2002). The effectiveness of huddling depends not only on the reduction of the individual's exposed surface but also on the augmented heat transfer between littermates and the mother (Webb et al., 1990; Gilbert et al., 2007). In the altricial *P. darwini*, a strong maternal effect on the thermoregulatory development of pups was observed until day 7 (Canals et al., 2009). McClure and Randolph (1980) found in the eastern wood rat *Neotoma floridana* pups that poor insulation after birth is related to the maximization of heat transfer. In *C. talarum*, as no thermogenic mechanisms are observed until day 10, behavior can alleviate the need for these energetically costly mechanisms. Until day 10, hairless pups spend most of the time in contact with their mother and siblings (Cutrera et al., 2003; Baldo et al., 2014). Zenuto et al. (2002) established that the increase in mass-specific RMR until day 10 was related to the increase in fur coverage and the increase in the intake of solid food after day 7. After day 10, pups remained huddled for shorter periods of time due to the increment of agonistic aggressive behavior between them (Cutrera et al., 2003; Baldo et al., 2014). Thus, the onset of ST also coincides with the time that pups start to spend less time huddled and become furrier, increasing their insulation. Therefore, thermoregulatory benefits associated with the underground environment might allow a short period where behavioral strategies are important to avoid thermogenic costs. During lactation, pups might invest energy to accelerate the production of new tissue and its maintenance (Hill, 1992), without compromising their thermal balance. The thermally stable environment of the nest and behavior might lead to a delay in the onset of ST, hence independent thermoregulation, until the period in which pups find new thermal environments by exploring and digging nearby tunnels.

In conclusion, although pups of *C. talarum* were altricial at birth (Zenuto et al., 2002; Cutrera et al., 2003; Baldo et al., 2014), they maintained behaviorally their thermal balance during lactation. Until day 10, pups are dependent on their mother to obtain heat, remaining in the nest where they commonly huddle with their littermates (Cutrera et al., 2003; Baldo et al., 2014). During this period, resting metabolism increases, but no thermogenic mechanisms were observed. As most of the energy is destined for growth, pups at this age showed impossibility to maintain T_b when confronted with T_as below adults TNZ. From day 10, mass-specific RMR showed a progressive decrease and MMR became fixed, establishing a specific increment of ST until day 60. Postnatal development of musculature related to digging could be employed as a heat source when pups start to explore and dig in the surrounding of the nest, but it appears to be inefficient until day 30 when pups achieved adult T_b. No functional NST was observed in pups until day 60 besides the presence of UCP1 and other molecular markers (e.g., COX activity) of NST in BAT. Attaining complete thermal independence is critical after day 60 since juveniles disperse at this age (Malizia et al., 1995). During their aboveground dispersal, juveniles must dig a new burrow or eventually find an empty burrow, so they are exposed to aboveground climatic fluctuations. Interestingly, in adults, NST complies with around 40% of the total thermogenic capacity (Luna et al., 2012), so, at some point during or after dispersal, NST becomes functional.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Committee for the Care and Use of Laboratory Animals (number 2555-06-14 RD141/15; CICUAL), Facultad de Ciencias Exactas y Naturales (FCEYN), and Universidad Nacional de Mar del Plata (UNMDP).

Author contributions

FL designed the study. CDA supported the respirometric experimental procedures. JS-S and JO supported the molecular experimental procedures. FL and CDA analyzed the data, prepared the manuscript, and created figures and tables. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Island and Rensch's rules do not apply to cave vs. surface populations of *Asellus aquaticus*

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Body size is a trait of fundamental ecological and evolutionary importance that is often different between males and females (sexual size dimorphism; SSD). The island rule predicts that small-bodied species tend to evolve larger following a release from interspecific competition and predation in insular environments. According to Rensch's rule, male body size relative to female body size increases with increasing mean body size. This allometric body size – SSD scaling is explained by male-driven body size evolution. These ecogeographical rules are rarely tested within species, and has not been addressed in a cave–surface context, even though caves represent insular environments (small and isolated with simple communities). By analyzing six cave and nine surface populations of the widespread, primarily surface-dwelling freshwater isopod *Asellus aquaticus* with male-biased SSD, we tested whether cave populations evolved larger and showed higher SSD than the surface populations. We found extensive between-population variation in body size (maximum divergence being 74%) and SSD (males being 15%–50% larger than females). However, habitat type did not explain the body size and SSD variation and we could not reject isometry in the male–female body size relationship. Hence, we found no support for the island or Rensch's rules. We conclude that local selective forces stemming from environmental factors other than island vs. mainland or the general surface vs. cave characteristics are responsible for the reported population variation.

KEYWORDS

competition, body size, isopod, predation, sexual size dimorphism, adaptation, evolution

1. Introduction

Body size correlates to various physiological and behavioral traits and it is often directly linked to fitness, underscoring the evolutionary and ecological importance of this quantitative trait (Peters, 1983; Roff, 1992; Stearns, 1992). The evolution of body size is shaped by different, often opposite forces of natural selection. For instance, fecundity selection, inter- and intrasexual selection usually favors large body size (Shine, 1989; Andersson, 1994). However, organisms do not constantly evolve larger (Stanley, 1973; Blanckenhorn, 2000). The two main selective forces acting against evolving larger are predation and interspecific competition. This is because prey-size-unlimited predators actively prefer larger prey and the increased behavioral activity needed for achieving and maintaining large body size increases general predation risk, while larger bodied competitors select against an evolutionary increase in body size (Wilson, 1975; Lomolino, 1985; Blanckenhorn, 2000; Simberloff et al., 2000; Herczeg et al., 2009). Considering the above detailed selective forces, one would expect the release from the selective pressures acting against large body size when small-bodied species colonize islands, which are typically isolated and characterized by simple communities. Such mechanism would result in gigantism of small-bodied species evolving under insular conditions. On the other hand, resource limitation combined with reduced prey-size-limited predation pressure on islands could result in the dwarfism of large-bodied species evolving under insular conditions. The evolution of gigantism of small species and dwarfism of large species on islands was observed long ago and referred as the island rule (Foster, 1964; Van Valen, 1973a,b; Lomolino, 1985), which later received further support (e.g., Lomolino, 2005; Benítez-López et al., 2021). This rule, originally proposed in an interspecific context was also supported in intraspecific population comparisons (Herczeg et al., 2009; MacColl et al., 2013; Runemark et al., 2015).

Adult body size also varies within population. A major source of this variation is sexual size dimorphism (SSD). It is widespread among animals (Fairbairn, 1997). In ectothermic vertebrates and invertebrates, typically females, while among endothermic vertebrates, males are larger (e.g., Fairbairn and Preziosi, 1994; Fairbairn, 1997; Blanckenhorn, 2005). The degree of SSD often scales allometrically with body size across species (Fairbairn, 1997). Rensch's rule states that in species with female-biased SSD, the level of SSD will decrease (hypo-allometry), while in species with male-biased SSD, the level of SSD will increase (hyper-allometry) with increasing body size (Rensch, 1950, 1959). To emphasize that hypo- and hyper-allometry are two parts of the same continuum, the rule was simplified (with the same meaning) stating that male body size relative to female body size increases with increasing mean body size (Meiri and Liang, 2021). Rensch's rule was supported in many taxa (mainly with male-biased SSD; e.g., Fairbairn, 1997; Székely et al., 2004; Fairbairn et al., 2007). However, in taxa with female-biased SSD, the rule has been questioned (Webb and Freckleton, 2007; Liang et al., 2022) and there are even examples of inverse Rensch' rule (see Fairbairn, 1997). Opposite trends have been observed even between closely related taxa (Piross et al., 2019). Patterns following Rensch's rule or the inverse of Rensch's rule are both accommodated by Fairbairn's (1997) correlational selection hypothesis. It states that allometric SSD patterns are resulting from directional selection on body size acting primarily on one sex (e.g., sexual selection on males or fecundity selection on females) followed by correlational selection occurring in the other. In a wider sense, if body size of one sex shows a stronger response to selection stemming from geographic or ecological factors than the other, the intraspecific level of SSD will vary (Blanckenhorn et al., 2006). Similarly to the island rule, Rensch's rule was originally proposed for interspecific comparisons, but intraspecific tests have also started to accumulate with mixed results (Herczeg et al., 2010; Wu et al., 2014; Liao et al., 2015; Yu et al., 2022).

Caves and other subterranean habitats are widespread in every continent, yet they have some important general characteristics. Organisms living in caves are typically faced with total darkness, limited food supply, highly stable daily, seasonal, and annual environmental conditions, and live in simple communities (Culver et al., 1995; Culver and Pipan, 2009; Romero, 2009, 2011). The latter results in low or negligible predation and interspecific competition, which jointly with the typical high isolation from surface habitats and other caves make caves insular habitats (e.g., Poulson and White, 1969; Mammola, 2019). Adaptations to cave environment have attracted considerable scientific interest ever since (Darwin, 1859). Morphological adaptations observed across independent caves and phylogenetically distant cave-adapted taxa were recognized early and referred as troglomorphism (Christiansen, 1962). This term has been since broadened to include any kind of cave-related adaptations. Classic examples for cave adaptations are loss of pigmentation, eye reduction, appendage elongation, decreased egg number/increased egg volume, increased longevity, and loss of circadian rhythm (e.g., Voituron et al., 2011; Pipan and Culver, 2012; Beale et al., 2013; Howarth and Maoldovan, 2018; Lunghi and Bilandžija, 2022). Even though cave–surface species or population pairs are rarely studied in the island–mainland framework, perhaps because the general environmental differences like the presence/absence of light seem more important than the insular characteristics of caves, some results can be interpreted from this aspect. For instance, Fišer et al. (2013) showed that females of cave-dwelling *Niphargus* (freshwater amphipod crustacean) species are generally larger than those of surface-dwelling species and Mojaddidi et al. (2018) showed in a common garden experiment that a cave *Asellus aquaticus* (freshwater isopod crustacean) population has larger hatchlings than two surface populations. These results are consistent with the predictions drawn from the island rule. However, we are not aware of an intraspecific study on the same effect with repeated sampling of cave vs. surface habitats or any studies testing predictions of the Rensch's rule, or even simply comparing SSD between cave and surface species or population pairs.

In the present paper, we performed an intraspecific test of the predictions drawn from the island and Rensch's rules, using cave vs. surface habitats in an island–mainland context. We studied six cave and nine surface populations of the common waterlouse, *A. aquaticus*, species complex. This widespread surface-dwelling freshwater isopod successfully colonized European aquatic cave habitats on several independent and relatively recent occasions (Verovnik et al., 2003, 2004, 2009). Cave-adapted populations show troglomorph characteristics, like eye reduction and depigmentation (Verovnik and Konec, 2019). Body size is heritable in this species (Thompson, 1986), and male-biased SSD has been recognized (Ridley and Thompson, 1979; Adams et al., 1985; Bertin and Cezilly, 2003). We tested two predictions drawn from the island and Rensch's rules. First and in-line with the island rule, we predicted that *A. aquaticus* will evolve larger in the low predation, simple communities of caves. Second, assuming that the first prediction holds, we predicted that patterns in SSD will follow the Rensch's rule, resulting in larger male-biased SSD in caves than in surface populations.

2. Materials and methods

2.1. Study system

The taxonomic status of distinct cave and surface *A. aquaticus* populations has not yet been resolved (Verovnik and Konec, 2019). Cave populations typically show troglomorphic traits and genetic isolation from each other and from adjacent surface populations (e.g., Verovnik et al., 2009; Pérez-Moreno et al., 2017). They have been described as a new species, new subspecies or simply as an isolated population (Turk-Prevorčnik and Blejec, 1998; Verovnik et al., 2004, 2009; Pérez-Moreno et al., 2017; Simčič and Sket, 2019). Similarly, some surface populations have been given a subspecies status due to differences in morphology (Sket, 1994). In the absence of comparative genetic and phenotypic data to clarify the whole situation, we treat each population used in this study as a sample from the "*A. aquaticus* species complex" and refer to them as *A. aquaticus* populations for simplicity.

The six caves sampled were not similar in their ecological characteristics. Four of them are "typical" caves, in which meteoric water and sinking rivers formed the subterranean passages (epigenic speleogenesis), whereas Movile aquifer and Molnár János Cave are hydrothermal cave systems, in which hypogean thermal water is responsible for cave formation (hypogenic speleogenesis). The temperature of a "typical" cave corresponds approximately to the average annual temperature of the respective region, in our case between 11°C and 12°C. In these caves, food is transported from the surface by sinking rivers and is of limited quantity and, more importantly, low quality (Poulson and Lavoie, 2000). The temperature of a hydrothermal cave depends on the temperature of the thermal water forming the cave and approximates 21–23°C in both Movile aquifer and Molnár János Cave. The ecosystems in the studied hydrothermal caves are highly isolated from the surface and likely rely on the endogenous production of organic matter by chemoautotrophic bacteria (Sarbu et al., 1996; Herczeg et al., 2020, 2022). It is assumed that the amount of available food is higher in hydrothermal caves than in epigenic caves (Poulson and Lavoie, 2000).

2.2. Sampling and measurements

We collected 358 adult male and 298 adult female *A. aquaticus* from six cave and nine surface populations spanning through Slovenia, Italy, Hungary and Romania (Table 1; Figure 1). Sampling was done with hand nets in all but the Molnár János Cave population, where cave diving was necessary. The Movile aquifer was sampled at the Dimitru Ana 9. well. According to Hasu et al., (2007) and Bloor (2010), individuals larger than 3–3.5 mm can already be considered as adults, however, we used a more conservative 4 mm threshold. Sex was determined via visual inspection of gonopod morphology under stereomicroscope.

Collected individuals were conserved in RNAlater (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States). For measuring body size, digital images were taken with a Canon 600D (Canon Inc. Tokyo, Japan) camera in a standardized setup and a millimeter scale bar for reference. We used body length (distance between the end of the pleotelson to the apical line of the head) as a proxy for body size and measured it from the digital images with the TpsUtil 1.74 and TpsDig2 2.30 softwares.[1] We performed two independent body length measurements for each individual and the average value of the two measurements was used in the analysis.

2.3. Statistical analyses

Asellus aquaticus undergo indeterminate growth (e.g., Lafuente et al., 2021) and thus using mean body size to compare populations can be biased by variation in the relative contribution of different age groups in the sample. Therefore, we analyzed the five largest males and females from every population to correct for the potential bias (e.g., Stamps and Andrews, 1992; Kratochwíl and Frynta, 2002). We note that analyzing the complete dataset revealed qualitatively similar results (data not shown). We also note that our approach could not control for age effects directly, since the age of the sampled individuals was unknown. Hence, there is a possibility that in the low predation cave habitats the largest individuals were older, than the largest conspecifics in surface habitats.

For testing habitat-dependent body size and SSD trends, we ran a Linear Mixed Model (LMM) with body length as the dependent variable, habitat, sex and their interaction as fixed effects and population nested in habitat as a random effect. We checked model residual distribution via Q–Q plots. This model revealed no significant habitat effect, but a highly significant population effect (see Results). Therefore, to compare populations directly, we also ran a Linear Model (LM) with body length as the dependent variable and population, sex and their interaction as fixed effects. We judged differences between groups based on 95% confidence intervals. This is a conservative approach, because according to Payton et al. (2003), the lack of overlap between 83% and 84% confidence intervals is analogous to $p < 0.05$.

We note that Rensch's rule could be present in our sample irrespective of the validity of island rule. With other words, an allometric SSD–body size relationship could be present in the absence of habitat-dependent body size or SSD patterns (i.e., the outcome of the previous models). For testing Rensch's rule directly (see Herczeg et al., 2010), we calculated the mean size for each sex in every population and then regressed $\log_{10}$ male body length against $\log_{10}$ female body length. This allowed us to test whether the slope differed from zero (i.e., the size of the sexes is not independent) and from one (i.e., there is a departure from isometry, supporting Rensch's rule or its inverse). Because in such case neither variable is fixed, or with other words, both variables are measured with error, ordinary least squares (Model I) regression would be statistically incorrect and thus major axis (Model II) regression is recommended (Fairbairn, 1997). However, this view was challenged recently, and the parallel usage of Model I and II regressions were recommended (Meiri and Liang, 2021; Liang et al., 2022).

All analyses were done in the R 4.0.3 statistical environment (R Core Team, 2020). We used the lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), emmeans (Lenth, 2020), and lmodel2 (Legendre, 2018) packages.

3. Results

The LMM revealed a significant sex effect, but no habitat or habitat × sex effects (habitat: $F_{1,13} = 2.17$, $p = 0.16$; sex: $F_{1,133} = 468.59$, $p < 0.001$; habitat × sex: $F_{1,133} = 0.32$, $p = 0.57$). SSD was male-biased (least squares means and 95% confidence intervals in mm; male: 8.95 [8.27–9.63]; female: 6.81 [6.13–7.49]). The lack of habitat effect rejected the island rule, because there was no sign of larger size in the

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[1] https://life.bio.sunysb.edu/morph/index.html

TABLE 1 Sampled *Asellus aquaticus* populations and sample sizes.

Population ID	Population locality	Habitat	Country	GPS coordinates	Female (*N*)	Male (*N*)
MJ	Molnár János Cave	Cave	Hungary	47.518° N, 19.03608° E	17	26
ML	Malom Lake	Surface	Hungary	47.518277° N, 19.035999° E	18	22
CS	Csömör Stream	Surface	Hungary	47°35′35.03″N 19°07′21.78″E	14	19
DL	Dunakeszi Lake	Surface	Hungary	47°36′23.15″N 19°07′24.63″E	16	20
DA	Dimitru Ana well	Cave	Romania	43°49′23.59″N, 28°34′01.45″E	17	27
KO	Kara-Oban Lake	Surface	Romania	43°50′46.0″N 28°33′59.1″E	9	11
TB	Turkish bath spring	Surface	Romania	43°49′12.15″N 28°29′28.26″E	11	13
PL	Planina Polje	Surface	Slovenia	45°49′56.2″N 14°15′30.0″E	28	28
PI	Pivka Channel of Planina Cave	Cave	Slovenia	45°49′11.6″N 14°14′44.4″E	27	24
CE	Cerknica Polje	Surface	Slovenia	45°46′23.0″N 14°19′31.2″E	29	29
ZE	Zelše Cave	Cave	Slovenia	45°47′26.4″N 14°18′12.6″E	24	28
LJ	Ljubljana Marsh	Surface	Slovenia	45°58′02.9″N 14°32′52.0″E	28	28
KR	Krška Cave	Cave	Slovenia	45°53′24.0″N 14°46′16.5″E	24	26
TI	Timavo Spring	Surface	Italy	45°47′15.8″N 13°35′28.7″E	26	28
LA	Labodnica Cave (Grotta di Trebiciano)	Cave	Italy	45°41′04.1″N 13°49′42.9″E	10	29
Total					298	358

insular cave environment than in surface habitats. The lack of habitat × sex interaction revealed no systematic difference in SSD between cave and surface habitats. The random effect of population nested in habitat was highly significant (likelihood ratio test: $\chi^2 = 171.14$, df = 1, $p < 0.001$), indicating high between-population variation in body size.

Our LM revealed significant population, sex and population × sex effects (population: $F_{14,120} = 70.61$, $p < 0.001$; sex: $F_{1,120} = 784.18$, $p < 0.001$; population × sex: $F_{14,120} = 6.89$, $p < 0.001$). Between-population variation in body size was large, reaching 74% difference between the smallest and largest population (Molnár János Cave vs. Movile aquifer; Figure 2). Male-biased SSD was present in every population, showing large variation from 15% (Molnár János Cave) to 50% (Movile aquifer; Figure 2).

The Model I and II regressions revealed a significant relationship between male and female body size across populations ($R^2 = 0.77$; p (for both regressions) < 0.001). However, the slope did not differ from one (Model I: $\beta = 0.96$; 95% confidence interval [CI] = 0.65–1.27; Model II: $\beta = 1.1$; CI = 0.79–1.54), indicating no violation of isometry (Figure 3). With other words, the level of SSD was unrelated to size, and thus our data reject the Rensch's (or inverse Rensch's) rule in our system.

4. Discussion

The most salient finding of our study is that there is high variation in body size and the level of SSD across the studied 15 *A. aquaticus* populations. However, habitat type (cave vs. surface) did not explain a significant proportion of the variation and the female–male body size relationship across populations did not diverge from isometry. Therefore, we found no support for the island and Rensch's rules in our study system.

We expected an increase in body size in cave habitats based on the predictions of the island rule (e.g., Lomolino, 1985, 2005; Benítez-López et al., 2021). According to this rule, the relaxation of predation and interspecific competition in insular environments (McNab, 1994; Lomolino, 2005) would allow for an evolutionary increase in body size due to its advantages in fecundity, and inter- and intrasexual selection in small-bodied species (Clutton-Brock et al., 1982; Andersson, 1994). Our data rejected this expectation. While the two populations with the largest body size were indeed from caves, the population with the smallest body size was also a cave population and the rest of the cave populations were within the (large) range covered by surface populations. The lack of habitat effect is definitely not because the cave populations had no time to adapt. The estimated divergence times between cave and surface populations exceeds 60,000 years (Verovnik et al., 2003, 2004; Konec et al., 2015; Pérez-Moreno et al., 2017) and the typical troglomorphic adaptations, like eye degeneration and depigmentation, are observed in all cave populations studied (Verovnik et al., 2009; Pérez-Moreno et al., 2017; Balázs et al., 2021). Adaptive variation in body size can be the result of various environmental factors other than predation and interspecific competition. For instance, resource levels, prey size, and intraspecific competition can be important sources of selection on body size (e.g., Case, 1978; Forsman, 1991; Clegg and Owens, 2002; Wu et al., 2006; Herczeg et al., 2012), and even random events might be responsible for it (Wasserzug et al., 1979; Biddick and Burns, 2021). Obviously, there are other characteristics of caves that are not related to their insularity, such as the absence of light or scarcity of food (Culver and Pipan, 2009;

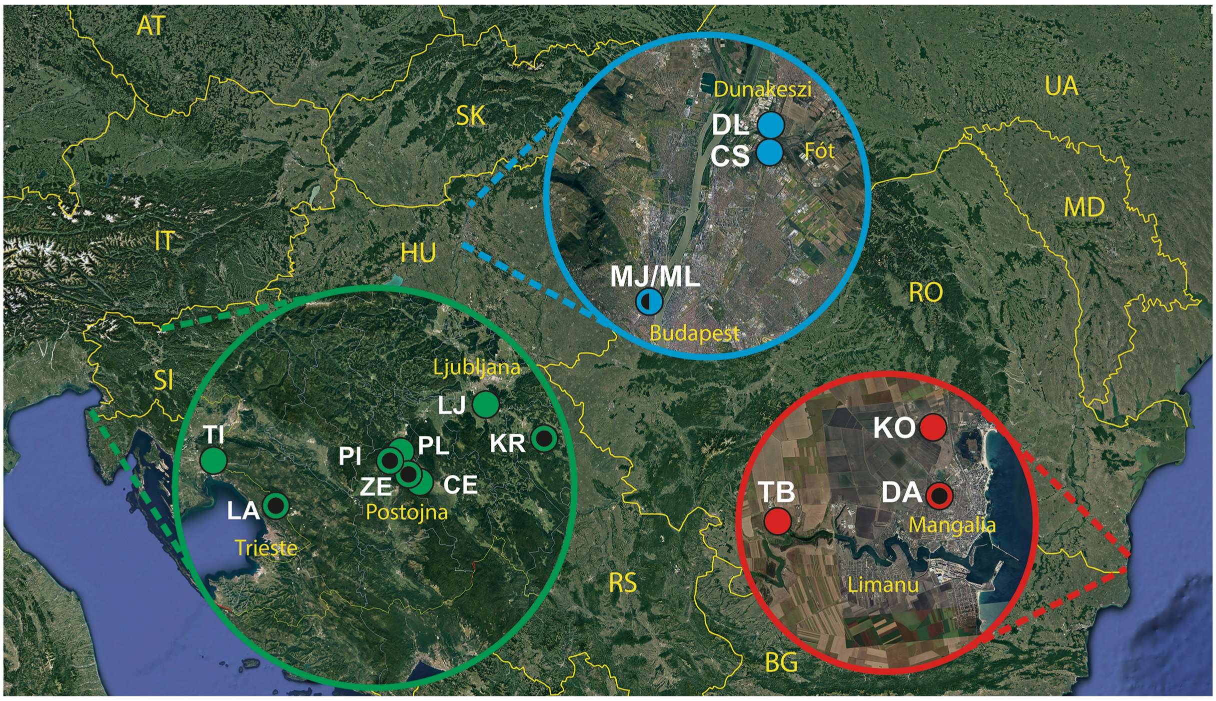

FIGURE 1
Map showing the geographical locations of sample sites. Black dots in the middle of colored circles denotes cave populations. For the population abbreviations, see Table 1. Note that MJ (cave) and ML (surface) populations are very close to each other, hence, they are represented by the same circle.

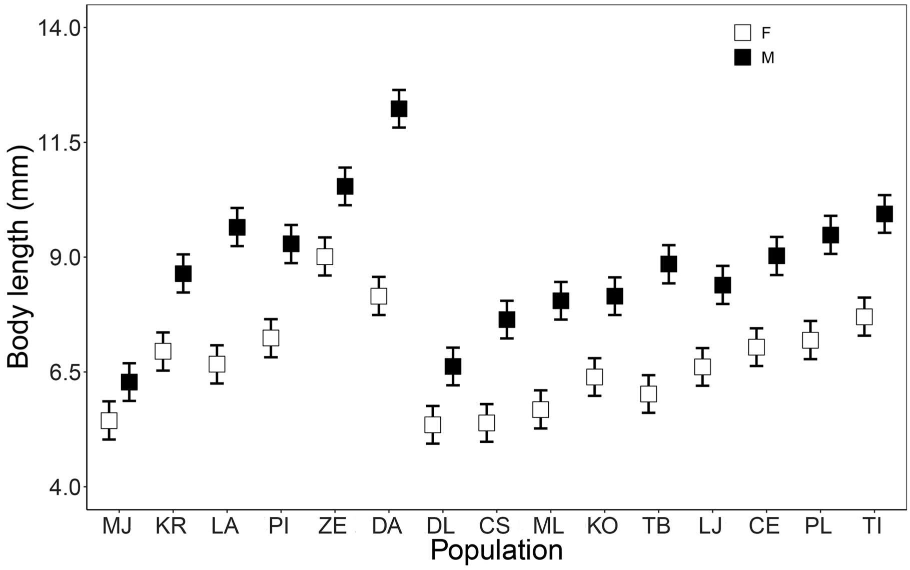

FIGURE 2
Body length variation of female (F) and male (M) *Asellus aquaticus* from the cave (MJ, KR, LA, PI, ZE, DA) and surface (DL, CS, ML, KO, TB, LJ, CE, PL, TI) populations. Estimated marginal means and 95% confidence intervals are shown. The population codes are explained in Table 1.

Romero, 2009). For instance, food scarcity, especially in caves that rely on exogenous organic material, might have led to smaller body size in caves (Fišer, 2019). It is conceivable that this factor counterbalanced the above detailed selective forces stemming from the caves' insular characteristics and thus constrained body size evolution. Sexual or fecundity selection also acts differently between cave and surface *A. aquaticus* populations (Balázs et al., 2021), but their effects on body size might be different from that predicted by the island rule. For instance, the generally lower population densities in caves (Mammola et al., 2021) might relax selection for traits used in male–male aggression

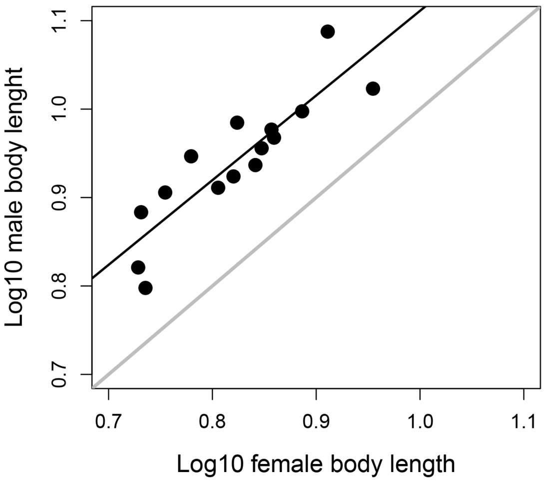

FIGURE 3
Lack of deviation from isometry in male-biased sexual size dimorphism in *Asellus aquaticus*. Ordinary Least Squares (Model I) regression line (β=0.96) and its 95% confidence interval (shaded area) are shown. The thick gray line depicts isometry (β=1) without sexual size dimorphism.

(e.g., body size) and target traits aiding mate finding (e.g., sensory systems; Bertin and Cézilly, 2005; Thiel and Duffy, 2007; Elipot et al., 2013; Balázs et al., 2021). At any rate, body size is not a conservative trait in the studied *A. aquaticus* species complex: body size divergence reached up to 74% in cave (Molnár János Cave vs. Movile aquifer, the two hydrothermal cave systems) and 47% in surface populations (Dunakeszi Marsh vs. Timavo Spring). It seems that variation among cave populations is considerably larger than among surface populations, suggesting that divergent selective forces acting on body size are stronger in caves, which are intuitively more similar to each other than surface environments. Speculating about cave colonizations might also invoke founder effects, bottlenecks and subsequent genetic drift as an evolutionary driver. Therefore, understanding the striking body size variation in *A. aquaticus*, especially among its cave populations, warrants future studies.

Various selective forces can result in SSD, for instance, sexual selection acting on males, fecundity selection acting on females or divergent ecological selection acting on both sexes (e.g., Shine, 1989; Andersson, 1994). If the relationship between SSD and body size was allometric (as predicted by Rensch's rule or the inverse of Rensch's rule), size of one sex shows greater variation than size of the other sex (Fairbairn and Preziosi, 1994; Fairbairn, 1997). The correlational selection framework (i.e., establishing which sex shows higher body size variation; Fairbairn, 1997) allows researchers to see that body size divergence in a certain system is a result of selection acting mainly on males or females. In our study, we detected large variation in male-biased SSD (from 15% to 50% difference), but found no allometric SSD–body size relationship. Here, the conclusion drawn from the correlational selection hypothesis would be that there is no general environmental trend resulting in selection acting mainly on one sex's body size, but rather locally variable selective forces acting on males, females, or both sexes are responsible for the pattern. This is an attractive explanation, especially considering that even such an extreme environmental difference as the one seen between cave and surface habitats did not explain a significant proportion of variation in SSD. Since body size is a trait that might contribute to fitness in various ways in both sexes and be constrained by a variety of factors (resource levels, competition and predation), environmental variation within cave or surface habitats might cause the lack of clear habitat divergence in SSD, in contrary to certain morphological traits with marked habitat-dependent sexual dimorphism (Balázs et al., 2021). All we can say is that the male-biased SSD in our system is a sign that sexual selection on male body size is a stronger force than fecundity selection on female body size. This seems plausible when considering the importance of precopulatory mate guarding in male reproductive success (Ridley and Thompson, 1979; Adams et al., 1985; Bertin et al., 2002). It is interesting to note that in the Molnár János Cave population (with which we have been working intensively in the last years both in the field and in the laboratory: Herczeg et al., 2020, 2022; Balázs et al., 2021; Horváth et al., 2021, 2023; Berisha et al., 2022), which is the smallest in size and has the lowest SSD, precopulatory mate guarding was never observed (personal observation).

We must note that the body size range utilized in the present study is narrower (but not by magnitudes—note that we use body length here, while many studies use body mass) compared to the ranges in interspecific studies on Rensch's rule (e.g., Colwell, 2000; Kratochwíl and Frynta, 2002; Székely et al., 2004; Webb and Freckleton, 2007). On the contrary, similar ranges in intraspecific comparisons were sufficient to detect significant allometry in other species (e.g., Herczeg et al., 2010). Further, the sample size from some populations might not guarantee that even by using only the largest individuals from the population sample we could avoid biased sampling of different age groups, especially that the samples could not been collected at the same time of the year. However, we are working with the studied populations for more than a decade now and based on our experience, the sample representation is adequate. Hence, we think that the conclusion regarding locally variable selective forces being responsible for the body size and SSD variation in the studied *A. aquaticus* system is plausible. One potential problem is that even though cave communities are indeed simpler than surface communities in the typical *A. aquaticus* habitats, some of the studied caves can be considered harboring diverse communities by subterranean standards. For example, the Postojna-Planina Cave System harbors 50+ aquatic species including predators, and can be considered as a hotspot for subterranean biodiversity (Zagmajster et al., 2021). The Movile Cave, which is one window of access to the Movile aquifer, harbors 20+ aquatic species, also including aquatic predators (Brad et al., 2021). However, hand-dug wells (including our sampling site), providing further windows of access to the Movile aquifer in and around the town Mangalia, are predator-free and harbor lower diversity (Serban Sarbu, pers. comm.). It is possible that in an intraspecific context, only insular environments with extremely simple communities will induce body size evolution in line with the island rule. For instance, in the case of the nine-spined stickleback, *Pungitius pungitius*, a single larger-bodied competitor species could inhibit the evolution of gigantism in insular environments (Herczeg et al., 2009).

We note that two of the studied cave populations were not typical in the sense that they were not cold-water epigenic caves relying on organic material of exogenous origin. The Molnár János Cave in Hungary and the Movile aquifer in Romania are both hypogenic hydrothermal cave systems with no exogenous organic material being present, with communities relying on endogenous bacteria of chemoautotroph origin (proven for Movile aquifer: Sarbu et al., 1996;

proposed for Molnár János Cave: Herczeg et al., 2020, 2022). Despite this fact, *A. aquaticus* from these two caves showed the highest body size and SSD divergence among all possible population pairs (Movile aquifer > Molnár János Cave for both body size and SSD). We can only speculate about the environmental factors that led to this divergence. The water temperature is similar in the caves (between 21°C and 23°C) and both caves are characterized by stable and low flow velocity. Water chemistry is intuitively harsher in Movile aquifer with extremely low oxygen and high sulfur (Sarbu et al., 1996; Brad et al., 2021). Still, the Movile aquifer population had the largest body size among all populations tested. The endogenous bacterial mats in Molnár János Cave are likely to represent low quality food source (Herczeg et al., 2020, 2022), which might explain that this population have the smallest body size of all studied populations, but the extremely large body size in Movile aquifer definitely requires further studies.

Taken together, even though we found large population variation in body size and SSD, we found no support for the island rule, habitat-dependent SSD or Rensch's rule in our system of six cave and nine surface *A. aquaticus* populations. We conclude that the marked general environmental differences between cave and surface habitats, like lack of light or differences in food sources and food availability, might override differences stemming from the island–mainland characteristics. Further, local selective forces unexplained by the major environmental divergence between cave and surface habitats govern variation in both body size and SSD, without one of the sexes being the driver of body size evolution in the study system. For a better understanding of body size evolution in cave and surface *A. aquaticus*, common garden experiments are warranted to separate the genetic and environmental contributions to body size variation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

GH, SK-F, and CF contributed to the conception and design of the study. GB, AB, and ŽF organized the database. GH performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1155261/full#supplementary-material

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Transcriptomic analysis of the Brazilian blind characid, *Stygichthys typhlops*, reveals convergent selection with *Astyanax mexicanus* and other cavefishes

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Molecular studies have shown that Neotropical fishes of the order Characiformes have undergone two independent events of cave colonization. Among these fishes are the Mexican blind cavefish (*Astyanax mexicanus*), a well-studied model system for cave adaptation, and the lesser-known Brazilian blind characid (*Stygichthys typhlops*). Although various genomic and transcriptomic approaches have been used to identify genes responsible for cave adaptation in *A. mexicanus*, these genetic factors have not been explored in an evolutionary comparative framework in cave-adapted characiforms. To address this gap, we assembled a *de novo* transcriptome for the Brazilian blind characid, identifying 27,845 assembled unigenes, of which 22,580 were assigned as putative one-to-one orthologs to the Mexican cavefish. We then used the package RELAX to analyze 789 genes in cavefishes, identifying 311 genes under intensified or relaxed selection. Our analysis revealed 26 genes with signatures of convergent, relaxed selection linked to vision, circadian cycles, pigmentation, and hematopoiesis processes. Additionally, we conducted differential gene expression analyzes between the snout region and a control tissue sample (muscle), identifying 96 differentially expressed genes associated with cell-surface-bound and calcium-binding proteins. Our study offers insights into the genetic mechanisms underlying cave adaptation in characiform fishes, particularly the Brazilian blind characid. Moreover, our transcriptome dataset and list of genes under convergent, relaxed, and intensified selection serve as a valuable resource for future functional studies of genes involved in cave adaptation. Our work highlights the importance of examining genetic adaptations in multiple independent lineages to better understand the evolutionary processes underlying cave adaptation.

KEYWORDS

Teleostei, Characidae, cavefishes, RNA-seq, differential gene expression

Introduction

Fishes are among the few evolutionary groups that have undergone multiple independent origins of adaptation to caves, one of the most adverse environments on earth. Together, cavefishes include over 298 species, demonstrating high evolutionary success in their adaption to life in caves (Proudlove, 2004; Protas et al., 2006; Soares and Niemiller, 2013; Proudlove, 2022). Habitat shifts from surface to cave systems are often accompanied by extreme morphological phenotypes, including loss of eyes, lack of pigmentation, and enhanced non-visual sensory structures, providing an excellent system to investigate the role of morphological and genomic convergence associated with this ecological transition. The Mexican blind cave tetra, *Astyanax mexicanus*, is the most popular model organism for studying cave adaptive evolution (Borowsky, 2008b), with several studies deploying transcriptomic and genomic data in an effort to understand the evolutionary and genetic mechanisms linked to the morphological, physiological, and genetic evolution of cave-obligate forms (Protas et al., 2006; Borowsky, 2008b; Bernardi et al., 2012; Beale et al., 2013; Yoshizawa, 2015; Krishnan and Rohner, 2017; Gross, 2018).

In the last decade, researchers in Southeastern Brazil collected over 20 wild specimens of the enigmatic subterranean characid, *Stygichthys typhlops*, from the phreatic zone of the Middle Sao Francisco basin (Moreira et al., 2010). This characid is associated with small subterranean drainages and is restricted to the Jaiba region in Minas Gerais (Bichuette and Gallão, 2021). Due to the rarity of its characteristic habitat, the conservation of *S. typhlops* is of significant concern, and it is currently listed as an endangered species (Romero and McLeran, 2000; Bichuette and Gallão, 2021).

While both *Stygichthys typhlops* and *Astyanax mexicanus* belong to the family Characidae (order Characiformes), a diverse group of Neotropical fishes that also includes the popular aquarium "tetras," phylogenetic evidence indicates that cave adaptation occurred independently in these two species (Figure 1; Oliveira et al., 2011; Arcila et al., 2017; Betancur-R et al., 2019). *Stygichthys typhlops* has no known surface relatives while *A. mexicanus* has both cave and surface populations or morphs (Borowsky, 2008a; Moreira et al., 2010; Gross et al., 2013). Like the cave morph of the Mexican blind cave tetra, the Brazilian blind cave characid is cave obligate; as such, the fish exemplifies troglomorphism with no external eye characters (lost circumorbital bones vs. partially fragmented in *A. mexicanus*) and pigment loss (Moreira et al., 2010). Furthermore, studies on the circadian rhythms of *S. typhlops* have demonstrated that the species does not exhibit any significant circadian or ultradian rhythms (Moreira et al., 2010; Sampaio et al., 2012).

The close but non-sister relationship between *Stygichthys typhlops* and *Astyanax mexicanus* provides a remarkable system to investigate the genetic bases of convergent adaptation to life in caves at the molecular level. Here, we assemble the first transcriptome of the Brazilian blind cave characid, *S. typhlops*, and compare it to both surface and blind morphs of *A. mexicanus*. In addition, we analyzed a set of 789 genes to investigate whether there are signatures of relaxed or intensified selection related to cave phenotypic convergence across other closely related ostariophysian lineages. We also compared tissue-specificity between the snout region (olfactory organs) and muscle by performing differential gene expression analysis for *S. typhlops*.

Methods

Fish sampling, RNA extraction and sequencing

The samples used in this study were collected in November 2010 from phreatic water outcrops at "Poço do Mandioqueu," Jaíba municipality, Minas Gerais state, Brazil (collecting permits ICMBio/SISBIO 28992; financial support for collections: FAPESP). The fish were kept in captivity for 30 days before RNA extraction. Three individuals were sacrificed using an MS-222 overdose and subsequently preserved in RNAlater (Ambion). The samples were frozen at-80C until RNA was extracted. Four different types of tissue samples (muscle, mouth, snout, and eye region) were collected from the right side of each individual. The main purpose was to capture tissue regions that might reveal differential gene expression. However, due to potential concerns with tissue sampling overlapping (e.g., snout vs. mouth or muscle vs. eye region), only the muscle and snout tissues were used for downstream differential gene expression analyzes. All procedures followed the UFAW Handbook on the care and management of laboratory animals.[1] Total RNA was extracted using the timing and centrifugation steps specified in the TRIzol (ThermoFisher) protocol. After mRNA purification, samples were treated with Ambion turbo DNA-free DNase to remove residual genomic and rRNA contaminants. Quantity and quality (purity and integrity) of mRNA were assessed using the Agilent 2,200 TapeStation (Agilent Technologies, Santa Clara, CA).

The libraries were prepared using the Illumina TruSeq RNA Sample Preparation kit. The mRNA was purified using poly-T oligo-attached magnetic beads to pull down the poly-A mRNA. After purification, the mRNA was fragmented and copied into first strand cDNA using reverse transcriptase. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase. The concentration of the cDNA libraries was measured with the qubit dsDNA high-sensitivity (HS) assay kit using the qubit fluorometer (Invitrogen). Concentrations of sequencing runs were normalized based on final concentrations of the fragmented cDNA. Next-generation sequencing (NGS) was carried out using the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA) at the University of Chicago Genomics Facility. The 150 bp paired-end raw reads were submitted to the NCBI's Sequence Read Archive (SRA) database: BioProject PRJNA891276.

De novo transcriptome assembly

The raw read quality was visualized with FastQC v 0.11.5 (Andrews, 2010). Initial removal of low-quality reads and TruSeq multiplex index adaptor sequences (Illumina) was performed using Trimmomatic v 0.32 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP:2:30:7, HEADCROP:13, SLIDINGWINDOW:7:15, MINLEN:25. *De novo* assembly for the three specimens of *S. typhlops* was performed using Trinity v 2.8.3 (Haas et al., 2013) with the strand-specific parameter option on. The resulting transcriptome was filtered for ribosomal RNA and microbial contaminants using SortMeRNA

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[1] http://www.ufaw.org.uk

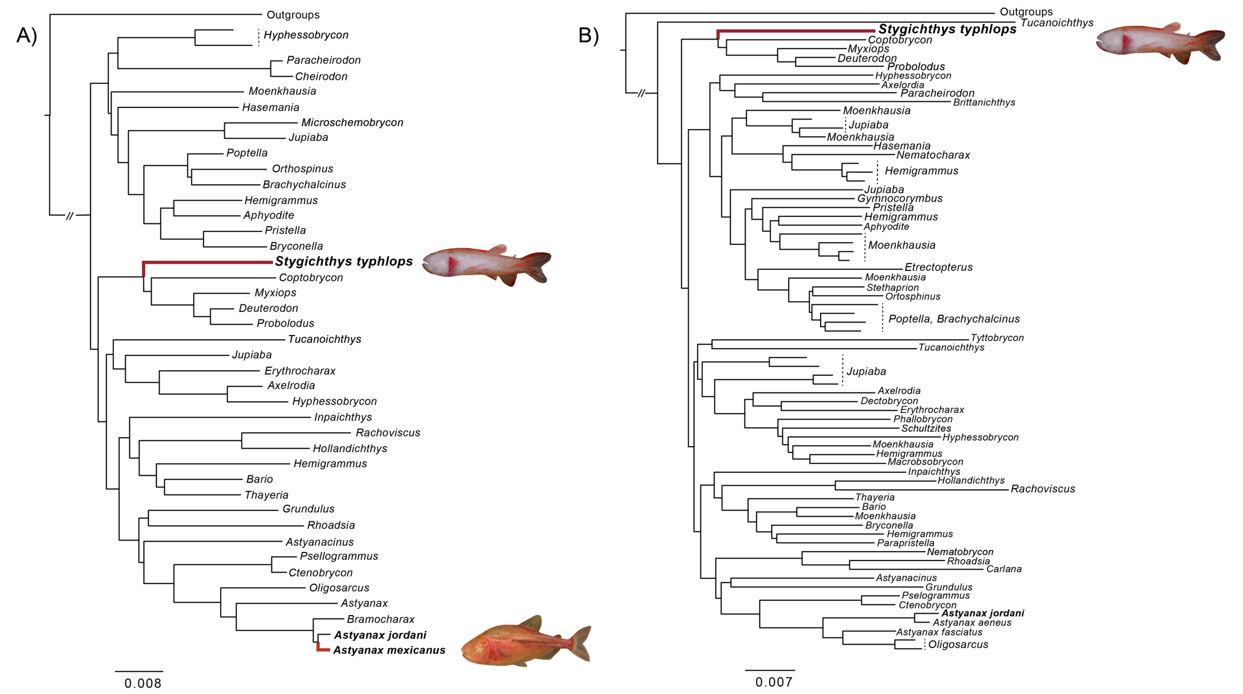


FIGURE 1
Summary of phylogenomic hypotheses indicating the evolutionary relationships of *Stygichthys typhlops* and the origins of cave adaptation in the family Characidae. Previous hypotheses based on **(A)** 1,051 exons markers sequenced for 93 characid species (Betancur-R et al., 2019), and **(B)** 1,141 ultraconserved markers (UCE) sequenced for 162 characid species (Melo et al., 2021).

v 2.1 (Kopylova and Touzet, 2012) against a non-redundant database compiled from coding-protein genes. TransDecoder v 3.0.0 was used to identify all likely coding regions within our assembled transcripts and filtered by selecting the single best open reading frame (ORFs) per transcript. Only contigs longer than 100 bp were kept for downstream analyzes. We reduced the number of redundant transcripts in the final assembly by clustering highly similar sequences using an amino acid sequence identity threshold of 1.0 with CD-Hit v 4.6.6 (Fu et al., 2012). Quantification for the abundance of transcripts was performed using the pseudo-aligner Kallisto (Bray et al., 2016) in no strand-specific paired-end mode.

After clustering was complete, annotation and mapping were performed within OmicsBox using the BLAST2GO functional annotation pipeline (Conesa et al., 2005). The ORFs were BLASTed against the Ostariophysi nr v5 protein sequences database with a hit filter of 1.0E-6. A reference-based transcriptome was also assembled using *A. mexicanus* (PRJNA89115) model. This transcriptome was annotated in OmicsBox following the same procedure for *de novo* annotation. BUSCO v 5.4.3 (Benchmarking Universal Single Copy Orthologs) was used to assess the completeness of both assemblies using the Eukaryota databases as reference (Simao et al., 2015). The *de novo* assembly was found to be significantly more complete than the *A. mexicanus*-based assembly for both databases (see Results), and thus the former was used for all downstream analyzes.

Differential gene expression analyzes

For the identification of differentially expressed genes (DEGs) between the nostril and muscle regions, we used the TMM (Robinson and Oshlack, 2010) normalization counts at gene level with EdgeR in Trinity v 2.8.3. For this method, genes with an FDR (false discovery rate) <0.1 were considered to be differentially expressed. Using the Fisher's exact test implemented in software BLAST2GO with an FDR threshold of 0.05, a GO term enrichment analysis was performed to identify functional categories enriched in DEGs between the nostril and control group. We conducted further Gene Ontology enrichment analyzes using differentially upregulated and downregulated genes from both treatments using ShinyGO v 0.76.2 (Ge et al., 2020).

In silico capture of troglomorphic candidate genes

We retrieved genomes or transcriptomes of 14 Ostariophysian species, including both surface and Pachón morphs of *Astyanax mexicanus*, from the NCBI database (Supplementary Table S1). We initially mined a set of 1,051 single copy nuclear exons previously identified and optimized for phylogenetic analyzes of ostariophysan fishes (Arcila et al., 2017). Sequences for each exon were aligned with MACSE v 2.03 (Ranwez et al., 2011) after cleaning out potentially non-homologous fragments with the -cleanNonHomologousSequences option. Next, we concatenated all genes alignment using the python package AMAS (Borowiec, 2016). Maximum Likelihood (ML) analyzes were performed in RAxML v 8.2.9 (Stamatakis, 2014) using a by-codon-position partitioning scheme and 30 independent search replicates. The best scoring tree across searches was selected using the GTRGAMMA model. Branch support was assessed using the rapid bootstrap algorithm with 1,000 replicates; the collection of bootstrapped trees was used to draw bipartition frequencies onto the optimal tree.

We identified an extra set of 458 candidate genes from previous studies related to vision, pigmentation, and circadian cycles in cavefishes and subterranean mammals (Emerling and Springer, 2014; McGaugh et al., 2014; Aardema et al., 2021; Policarpo et al., 2021; Appendix 1). From the cavefishes dataset, we first retrieved 404 (of 458) candidate genes using reference alignments for seven ray-finned fishes, which represented species from the orders Cypriniformes, Ophidiiformes, Cichliformes and Percopsiformes (Policarpo et al., 2021), and 14 additional genes using reference sequences from *Danio*, *Astyanax*, *Sinocyclocheilus*, and *Lamprologus* (Pottin et al., 2011; Meng et al., 2013; McGaugh et al., 2014; Yang et al., 2016; Aardema et al., 2021). From the subterranean mammal dataset, we identified three to five ortholog sequences for ray-finned fishes for 40 additional candidates (Emerling and Springer, 2014) using the NCBI Orthologs database.

We also examined an additional set of 481 genes that were extracted using BUSCO and the Actinopterygii Single-Copy Orthologs library (with 3,640 genes), for a total of 939 genes (458 candidates plus 481 BUSCOs). As the species in the genus *Sinocyclocheilus* are tetraploids while the other species are diploids, BUSCO analyzes also allowed us to accurately identify orthologous sequences from all species. We then used the hidden Markov model (HMM) approach available in HMMER v 3.2.1 (Wheeler and Eddy, 2013) to extract the 918 orthologous sequences from the 13 ostariophysian genomes and the *S. typhlops* transcriptome.

The candidate gene sequences extracted were aligned using MACSE v2.03 03 (Ranwez et al., 2011), which takes into account the codon structure of protein-coding nucleotide sequences. The alignments were visually verified using Geneious Prime® v2022.1. We excluded 125 gene alignments that contained four or fewer ostariophysian species. Additionally, we merged four genes that were common to both the Emerling and Springer (2014) and Policarpo et al. (2021) studies, resulting in a total of 789 candidate genes retained for downstream analysis.

Assessments of relaxed and intensified selection

We used the RELAX model (Wertheim et al., 2015) implemented in the package HyPhy (Pond et al., 2005) to investigate whether the genes associated with vision, pigmentation, circadian cycles, hematopoiesis, and others, show any signatures of intensified or relaxed selection in foreground branches (test and reference) of cavefishes relative to background (non-cavefish) branches, based on estimates of the selection strength parameter K (using a p value = 0.05). A value of $K < 1$ indicates that test branch was under significant relaxed selection, whereas a value of $K > 1$ suggests intensified selection on the test branches. Two alternative schemes were used, each employing test, reference, and background branches. The first scheme included test branches comprising only obligate cavefish species (*A. mexicanus* cave, *Sinocyclocheilus anshuiensis*, and *S. typhlops*), reference branches consisting of facultative cavefish species (*Sinocyclocheilus rhinocerous* and *A. mexicanus* surface), and background branches representing the remaining non-cave species in the tree. The second scheme involved *S. typhlops* as the test branch, the remaining obligate and facultative cavefish species represented as reference branches, and non-cavefish species as background branches. Multiple testing was accounted for with false-discovery rate (FDR) correction (Benjamini and Hochberg, 1995) in *R*.

Results

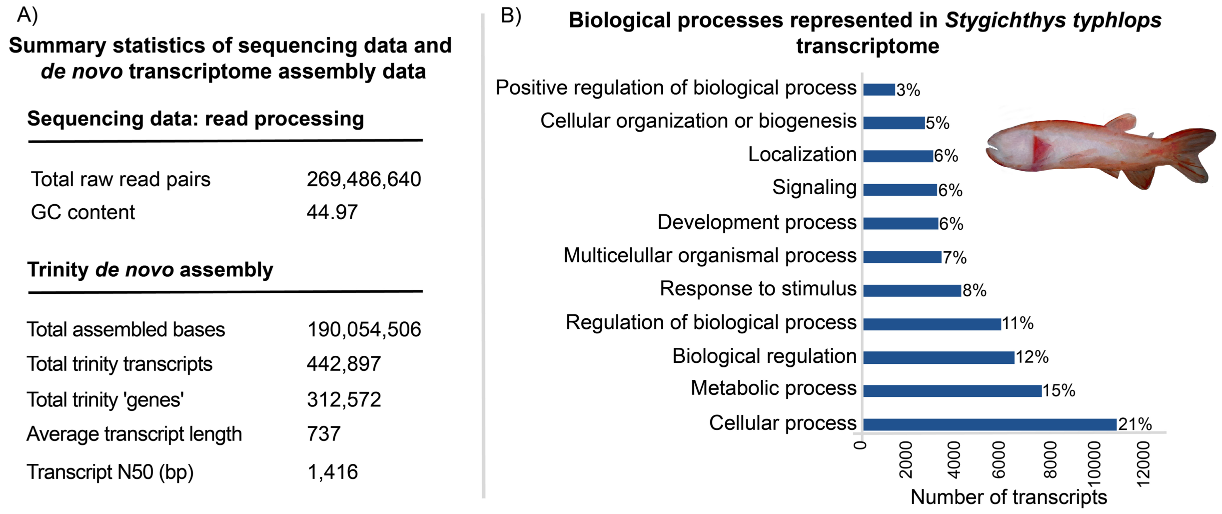
Sequencing and transcriptome assembly

Illumina HiSeq 2000 pair-end reads obtained ranged from 13 to 32 million pairs per tissue sample. All biological replicates yielded a total of 190 million paired end reads that were combined to generate a single reference transcriptome assembly. Preliminary analyzes mapping the *S. typhlops* raw reads to the closest available reference genome, *Astyanax mexicanus*, resulted in a low percentage of accurately mapped reads (<40%). Thus, we utilized a *de novo* approach to assemble the transcriptome of *S. typhlops.* Raw reads were first quality filtered and assembled in Trinity, resulting in 442,897 transcripts, with an N50 length of 1,416 bp (vs. N50 length of 1,572 bp in *A. mexicanus* Pachón cavefish and 1,521 bp in *A. mexicanus* surface morpho; Gross et al., 2013). To reduce the number of potentially spurious contigs, a filter was applied based on the abundance and quality of the assembly. For abundance, the isoform expression level and redundance was used as estimated using the Kallisto method, with only highly expressed isoforms being retained. After filtering the nonredundant transcripts using cd-hit-est, the total number of transcripts was reduced to 52,151 contigs ranging from 297 to 6,981 bases and identified as putatively homologs based on BLAST alignment against the NR, InterPro, and GO databases. To further refine the assembly, we considered transcript expression and identified 20,652 transcripts that represented 90% of the total expression data (Ex90), producing an N50 length of 1,027 bp (Ex90N50). Detailed statistics are shown in Figure 2.

When comparing the transcripts with the data available for the *A. mexicanus* genome, a total of 22,528 predicted proteins were aligned to one of *S. typhlops* transcripts. In addition to the N50 and ExN50 values, transcriptome completeness was assessed in terms of gene content, by searching the transcriptome for the presence or absence of conserved orthologous genes in the Eukaryota and Actinopterygii BUSCO libraries. The *S. typhlops* assembly was 97.6% complete with only 2.0% fragmented reads, when compared to the Eukaryota ortholog library which includes a total of 255 BUSCO groups. Furthermore, when compared to the Actinopterygii ortholog library, which includes 3,640 BUSCO groups, the assembly was 76% complete with 5.3% fragmented reads.

Identification of differential gene expression

Significantly DEGs between the snout and muscle regions were detected for all analyzed replicates using edgeR. All three replicate libraries for each treatment showed a high correlation (Pearson r = 0.89–0.99; Supplementary Figure S1) and were used for expression analysis. A total of 96 unigenes were found to be differentially expressed among the snout (olfactory) and muscle tissue samples. Using the snout region as a reference, 65 and 31 unigenes were upregulated and downregulated, respectively. The majority of the DEGs were expressed with fold change values > −2.0 and < 6.0 (Figures 3A,B). Over 25% of



A)

Summary statistics of sequencing data and *de novo* transcriptome assembly data

Sequencing data: read processing

Total raw read pairs	269,486,640
GC content	44.97

Trinity *de novo* assembly

Total assembled bases	190,054,506
Total trinity transcripts	442,897
Total trinity 'genes'	312,572
Average transcript length	737
Transcript N50 (bp)	1,416

FIGURE 2
Summary of *Stygichthys typhlops* transcriptome. **(A)** Summary statistics of sequencing data and the combined *de novo* transcriptome assembly. **(B)** Histogram of transcript classifications of biological processes. The BUSCO library of Eukaryota and Actinopterygii orthologs indicated that the transcriptome assembly is 97.6 and 76% complete, respectively.

the genes were uncharacterized or incompletely annotated, matching only genes associated with unknown functions. A total of 20 GO terms were associated with the DEG. The most significant GO terms for each set of DEGs across the treatments are shown in Figure 3C. Interestingly, genes related to the Ephrin (cell-surface-bound proteins) and Parvalbumin (calcium binding proteins) were upregulated in the snout tissue samples. Genes related to myosin tail and cardiac muscle contraction were downregulated in the muscle tissue.

Relaxed and intensified selection on troglomorphic candidate genes

Using RELAX with two alternative schemes, we analyzed 789 genes and identified 311 genes under intensified or relaxed selection after assessing their statistical significance using the *FDR* correction. In the first scheme, we used the obligate cavefish species as the test branches, and the facultative cavefish species as the reference branches. The second scheme employed *S. typhlops* as the test branch, with other obligate and facultative cavefish species as the reference branches (Figure 4). Our results revealed a consistent pattern across both schemes, with a higher number of genes under relaxed selection in test branches compared to the reference branches (107 vs. 101 for scheme 1, 79 vs. 49 for scheme 2, and 38 vs. 35 for genes shared between schemes), and a lower number of genes under intensified selection in the test branches compared to the reference branches (74 vs. 67 for scheme 1, 88 vs. 62 for scheme 2, and 22 vs. 19 for genes shared between schemes).

We examined signatures of selection on the 311 genes, of which 174 had a significant p value ($p < 0.05$) for scheme 1, including 107 genes under relaxed selection and 67 under intensified selection. For scheme 2, 137 genes had a significant p value, with 79 being under relaxed selection and 58 under intensified selection. Of these, 73 genes were identified in both schemes, with 38 genes being under relaxed selection and 19 under intensified selection on the test branches for both schemes (Figure 4). However, we found that 16 out of the 73 shared genes exhibited different signatures of selection. Specifically, seven genes were under relaxed selection and nine genes were under intensified selection for scheme 1. When treating *S. typhlops* as the sole test branch, nine genes were under relaxed selection and seven genes were under intensified selection.

We annotated the 311 genes examined and found that 103 (32.5%) of them were associated with hematopoiesis processes. Among these, 68 (21.5%) showed evidence of relaxed selection (36 for scheme 1; 32 for scheme 2) and 35 (11.0%) showed intensified selection (17 for scheme 1; 18 for scheme 2). We also identified 67 (21%) genes related to vision, with 33 (10.4%) under relaxed selection (21 for scheme 1; 12 for scheme 2) and 34 (10.7%) under intensified selection (18 for scheme 1; 16 for scheme 2). Pigmentation-related genes accounted for 49 (15.5%) of the total genes, with 22 and 6 under relaxed selection (e.g., *gpc3* and *atp6p1*), and 11 and 6 under intensified selection (e.g., *bnc2* and *zic2b*) for schemes 1 and 2, respectively. Genes associated with the circadian cycle constituted 22 (6.9%) of the total, with 6 and 4 under relaxed selection (e.g., *nr1d1* and *rorcb*), and 7 and 5 under intensified selection (e.g., *bmrl1b* and *cry2b*), for schemes 1 and 2, respectively. We also identified a smaller number of genes related to DNA repair, hypoxia, metabolism, morphology, and the olfactory system that exhibited signatures of selection. Furthermore, out of the 311 genes under selection, 47 genes were classified as unknown due to a lack of available annotations (Appendix 2). Interestingly, we identified only two gene families (*B3GNT* and *Rab GTPases*) that overlapped between the 96 genes showing differential expression and the 311 genes under selection.

Discussion

The annotated transcriptome of *Stygichthys typhlops*

The generation of *de novo* transcriptome data for non-model organisms has established itself as a powerful approach to identifying

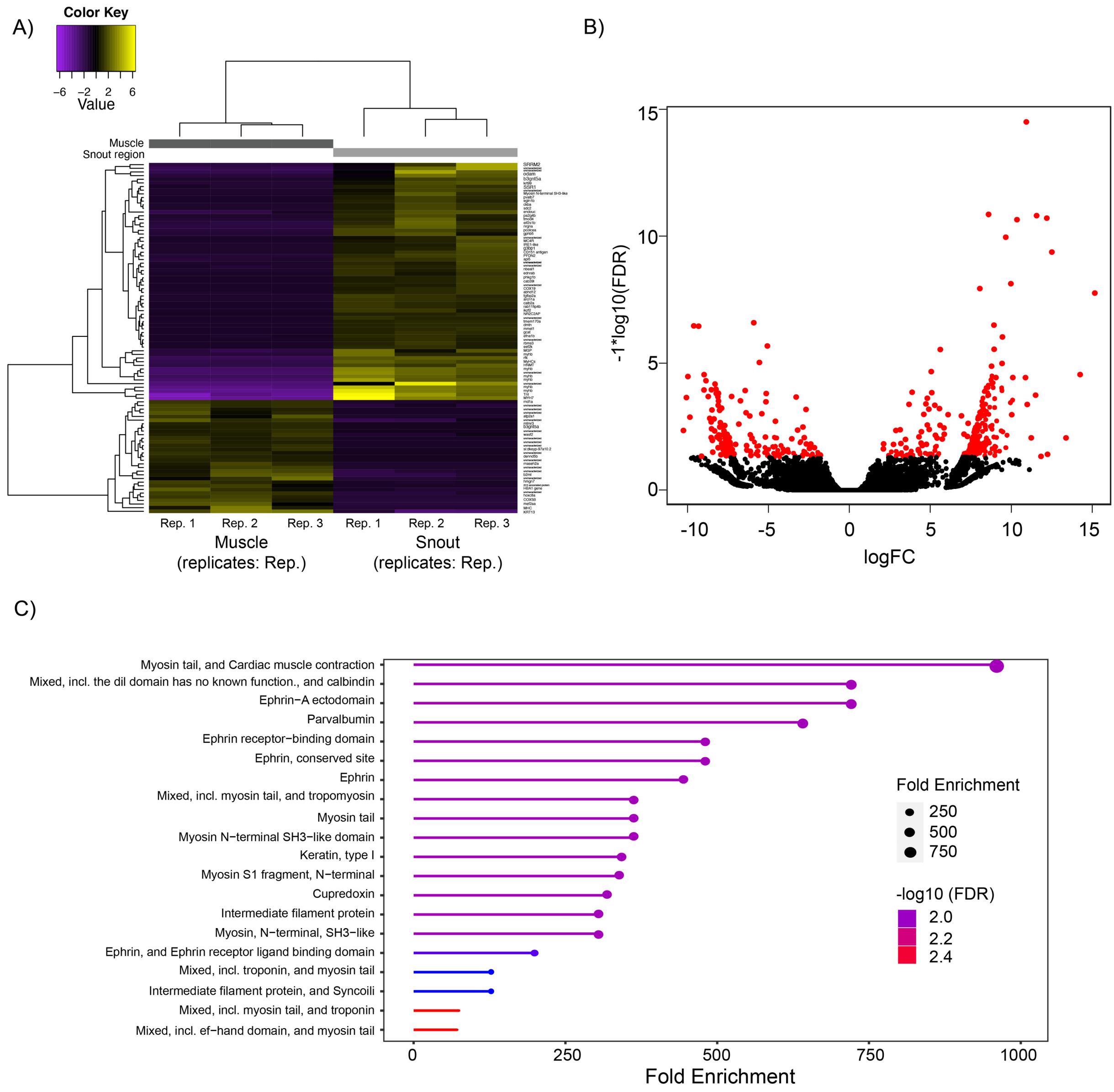


FIGURE 3
Summary of differentially expressed genes (DEGs). **(A)** Heatmap of DEGs in *Stygichthys typhlops*. Both upregulated (yellow) and downregulated (purple) genes with logFC values of 0.3 were clustered. Rows represent single transcripts, and columns represent the replicates for each treatment. Genes were clustered based on expression similarity (Euclidean distance). A complete list of genes DEG is included in Supplementary Figure S1. **(B)** Volcano plot visualization of DEGs from the two main treatments (muscle vs. snout) using the edgeR method. **(C)** Gene ontology enrichment analysis for set of DEGs, including the top of 20 GO terms with the highest enrichment values for each pairwise comparison.

novel transcripts and differentially expressed genes. However, among vertebrates, transcriptomic resources for fishes remain relatively underrepresented in public databases and mainly restricted to model species (Sun et al., 2016; Yang et al., 2020). Additionally, transcriptomes of cave-obligate fishes continue to surprise researchers as the molecular pathways related to troglomorphism show unique molecular signatures in each species (Gross et al., 2013; Meng et al., 2013; Casane and Retaux, 2016). We conducted a *de novo* assembly transcriptome of the Brazilian cavefish, *S. typhlops*, to provide additional insights into the extreme phenotypic adaptations to cave systems. The lack of a reference genome or transcriptome resulted in the generation of partial and fragmented transcripts. However, the fraction of annotated transcripts in relation to the Mexican cavefish, *A. mexicanus*, was remarkably high (27,845 transcripts for the cave morpho and 22,090 transcripts for the surface morpho).

Differentially expressed genes

A total of 96 genes were differentially expressed between the snout (olfactory) and muscle regions in *Stygichthys typhlops*. We observed that for the GO analysis, upregulated genes were enriched with more functional categories, including the presence of Ephrin A receptors considered to play a critical role for establishing topographic projections in the olfactory, auditory and somatosensory systems (Prakash et al., 2000; Serizawa et al., 2006; Cramer and Gabriele,

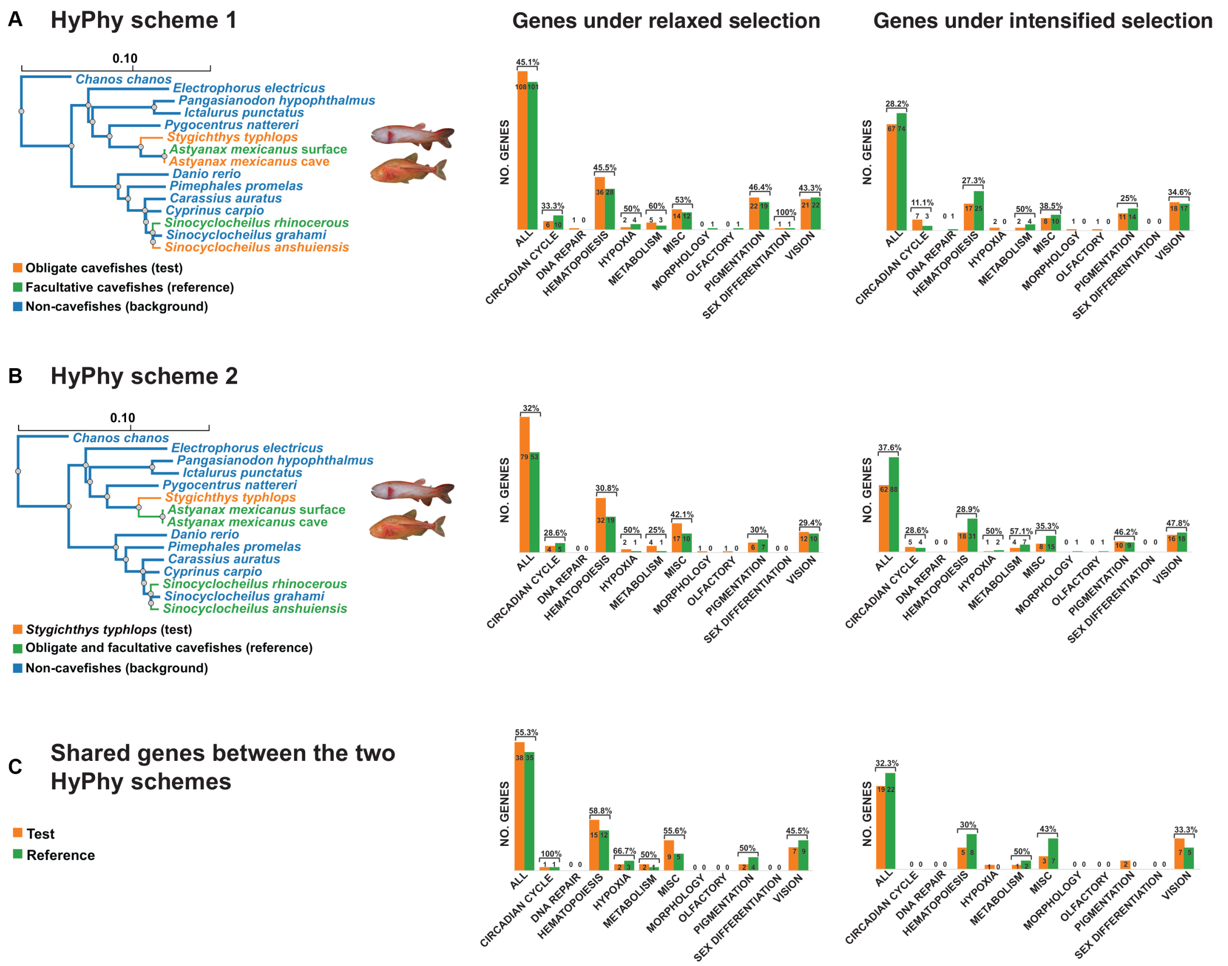


FIGURE 4
Selection dynamics using RELAX and two alternative schemes in HyPhy for relaxed ($K<1$) and intensified ($K>1$) selection ($p=0.05$). **(A)** Scheme 1 represents obligate cavefishes as test branches (orange), facultative cavefishes as reference branches (green), and non-cavefishes (blue) as background branches. **(B)** Scheme 2 shows *Stygichthys typhlops* as the test branch (orange), other obligate and facultative cavefishes as reference branches (green), and non-cave species as background branches (blue). **(C)** Genes shared among schemes 1 and 2.

2014). Additional coding protein genes found included the calcium-binding calbindin, parvalbumin, and myosin usually involved in sensory organs of the ear, nose, and throat, which have shown to play an important role in spatial navigation, memory processes, and social interactions in guinea pigs and rats (Yamagishi et al., 1993; Bjerke et al., 2021). Olfaction by way of self-odor has also been shown to be an important part of spatial awareness in the blind cave cyprinid *Phreatichthys adruzzii* (Annalisa et al., 2006).

We also found the *hoxc8a* gene upregulated in the muscle region, a candidate gene previously associated with the evolution of rib axial variability in *A. mexicanus*, resulting in fewer ribs in the cave versus the surface morphs (Gross et al., 2008). The decrease in the number bones and bone density is considered a regressive (reductive) trait in troglomorphic organisms (Wilkens, 1988; Protas and Jeffery, 2012). The upregulation of *hoxc8a* in *S. typhlops* could indicate changes in bone structure.

Another differentially expressed gene is *cox5b*, which encodes for a hypoxia-inducible isoform of COX subunit recently identified in the pike icefish *Champsocephalus esox* (Rivera-Colón et al., 2022). The absence of primary productivity decreases the oxygen content in caves and cave streams creating hypoxic conditions (Malard and Hervant, 1999; Boggs and Gross, 2021). The heterogeneity of dissolved oxygen content in groundwater has even been proposed as a selective pressure on low metabolic rates in cave-obligate organisms, in lieu of low food supply (Malard and Hervant, 1999). The increased expression of *cox5b* could indicate an adaptation in *S. typhlops* to hypoxic conditions.

Additionally, downregulation of myosin tail and cardiac muscle contraction in *Stygichthys typhlops* muscle tissue may make sense in light of hypoxic conditions. Many fishes in hypoxic conditions have adapted the unique cardiac reaction of a slower heartbeat (i.e., hypoxic bradycardia; Farrell, 2007). Hypoxic bradycardia may have significant benefits to fishes in these conditions, including a slower beating heart generating more force, maintaining cardiac stroke volume (Shiels et al., 2002; Farrell, 2007). Thus, downregulation of genes relating to cardiac muscle contraction may indicate *S. typhlops*' cardiac adaptations to low-oxygen groundwater systems.

While all tissues carry out common processes, tissues are distinguished by gene expression dynamics, implying that distinct regulatory programs control tissue specificity. Although we found a higher number of genes differentially expressed in the muscle compared to the snout region in *Stygichthys*, this result may indicate that an undetermined number of genes can remain undetected due to low expression levels (García-Ortega and Martínez, 2015). Sensory organs vary significantly among cave-adapted species suggesting adaptive responses to the microenvironment. Our results provide new insights to understand the role of differential gene expression among cave organisms as well as additional genomic resources for further transcriptome-wide association studies to test the association between traits and genetically predicted gene expression levels (Hinaux et al., 2016).

Selection dynamics of troglomorphic candidate genes

Our study adds to the growing evidence that many genes associated with vision, circadian cycles, hematopoiesis, and pigmentation have undergone genome-wide relaxation in cavefish evolution (Hinaux et al., 2013; Calderoni et al., 2016; Mack et al., 2021; Policarpo et al., 2021; Van der Weele and Jeffery, 2022; Zhao et al., 2022). Consistent with previous studies, we identified 26 genes that exhibited signatures of convergent, relaxed selection and 10 genes with convergent, intensified selection on all obligate cavefishes (*S. typhlops*, *A. mexicanus*, and *S. anshuiensis*, scheme 1), as well as in *S. typhlops* alone (scheme 2). These results provide further evidence that relaxed selection have played a significant role in the adaptation of multiple cavefish lineages to cave environments.

Seven genes associated with vision exhibited convergent, relaxed selection, including *TELO2*, which has been implicated in cortical visual impairment and cataracts (Albokhari et al., 2023), and *PCIF1*, which has been associated with binocular phenotypes in vertebrates (Bosten et al., 2015). Additionally, in fishes and other vertebrates, the circadian system plays a crucial role in regulating important genes in metabolic pathways, creating a two-way relationship between circadian clocks and metabolic signals (Froland and Whitmore, 2019). One of the genes that displayed convergent, relaxed selection was Sirtuin 6 (*SIRT6*), which regulates circadian chromatin recruitment physiology and cellular metabolism in mammals (Masri et al., 2014). However, additional research is needed to understand the role of *SIRT6* in modulating the brain and peripheral clocks in cavefish (Delgado et al., 2017). Additionally, we identified under relaxed selection, two previously identified clock genes in zebrafish, *nr1d* (scheme 1) and *rorcb* (scheme 2) genes (Policarpo et al., 2021).

In recent transcriptomic studies in *A. mexicanus*, Sears et al. (2020) identified at least eight genes with reduced level of differential expression associated with blood physiology. In our study, we identified 17 additional genes linked to hematopoiesis processes that were under relaxed selection in both schemes used. Among these genes, we found *EGF1*, which plays a significant role in blood coagulation in vertebrates (Davidson et al., 2003), and *ASXL2*, which is a critical regulator in the formation of blood cellular components (Micol et al., 2017). Furthermore, we identified two genes associated with pigmentation in vertebrates that exhibited convergent, relaxed selection in obligated cavefishes. *VAMP7* is required for the production of melanin pigments and has been linked to hypopigmentation in mice (Jani et al., 2015; Dennis et al., 2016), while *LRP11* is involved in carotenoid metabolism and has been significantly linked to the skin coloration in fishes (Wu et al., 2023).

The genes that displayed signatures of convergent, intensified selection in the test and reference branches analyzed (schemes 1 and 2) were primarily associated with hematopoietic and metabolic processes. Among these genes were *MVD*, which is necessary for cell survival (Wong et al., 2021); *SCARA5*, previously identified to exclusively express on epithelial cells in vertebrates (Yap et al., 2015); *SLC4A1AP*, an ion transporter gene identified in crabs (Shih et al., 2023); *STARD7*, which is thought to play an essential role in morphogenesis and respiration in mammals (Horibata et al., 2020); and *PDK4*, previously found to have increased levels of expression in fasting in chickens and mice (Connaughton et al., 2009). The identification of intensified selection on genes associated with hematopoietic and metabolic processes in our dataset may suggest that selection exerts ongoing pressures on a limited number of genes that are crucial for ensuring the survival of fishes in cave environments characterized by limited food availability (Poulson and Lavoie, 2000). This finding underscores the importance of physiological adaptation, such as slow metabolism and fasting, as strategies for coping with food scarcity (Langecker and Longley, 1993). In future studies, it will be interesting to determine the role of genes involved in hematopoiesis processes in the evolution of "constructive" adaptive traits in cavefishes.

Our study identified an intriguing result regarding the *PIK3CG* gene, which maintains epithelial integrity. We observed that this gene was under relaxed selection in scheme 1, including all obligate cavefishes, but under intensified selection in *S. typhlops* as the sole test foreground branch in scheme 2. This finding is particularly interesting because recent research has identified *PIK3CG* as a regulator and responder to multiple physiological processes and suggested that it may contribute to the ability of deep-sea fishes to resist pressure (Bo et al., 2022). The karst aquifer where *S. typhlops* is found typically has an average depth of 20 to 30 meters, but the thickness of the aquifer can easily exceed 200 meters or more (P. Pessoa *personal comm.*). It is possible that *PIK3CG* might have undergone adaptive evolution in *S. typhlops*, enabling it to overcome physiological challenges common to the deep sea and cave environments of the Middle Sao Francisco basin. In addition, we also identified genes related to the *B3GNT* and *Rab GTPases* gene families under intensified selection and upregulated in *S. typhlops.* The role of these genes families in fishes remain unknown.

The investigation of genes responsible for phenotypic adaptations in obligate cavefishes (*A. mexicanus*, *S. typhlops*, and *S. anshuiensis*) has revealed that different genetic mechanisms can lead to "many ways to build a cavefish," as suggested in previous studies (Sears et al., 2020; Policarpo et al., 2021; Van der Weele and Jeffery, 2022). However, the identification of 73 shared genes between *S. typhlops* and other cavefishes suggests that molecular convergence also plays a significant role in cave adaptation, as previously observed in the *Oca2* gene associated with the albinism phenotype in vertebrates (Protas et al., 2006); note that we were unable to retrieve *Oca2* from *S. typhlops*.

Phylogenetic relationships of *Stygichthys typhlops*

Previous molecular studies investigating the evolutionary relationships of the diverse family Characidae consistently identified *Stygichthys* as most closely related to the genera *Coptobrycon*, *Myxiops*, *Deuterodon*, and *Probolodus*, and as part of the so called "Clade C" (Figure 1). Two alternative placements for *Stygichthys* have been proposed, either as the sister of the monotypic genus *Coptobrycon*, based on two mitochondrial and three nuclear markers (Oliveira et al., 2011), or as sister to a clade including *Coptobrycon*, *Myxiops*, *Deuterodon*, and *Probolodus*, based on exonic and ultraconserved markers (Betancur-R et al., 2019; Melo et al., 2021).

A recent total evidence analysis, based on 520 morphological characters and nine molecular markers for *Astyanax* and allies, resolved *Stygichthys* as most closely related to *Phycocharax rasbora*, *Cyanogaster noctivaga*, "*Hyphessobrycon*" *elachys* and *Coptobrycon* (Terán et al., 2020). While the sister-group relationships of *Stygichthys* remain contentious, it is clear that the Mexican and the Brazilian cavefish belong in different clades within "Clade C" of Characidae. The lack of phylogenetic resolution among the early branching lineages of "Clade C," which encompasses over 500 species and includes some of the most taxonomically challenging genera in the family, suggest that future phylogenetic analyzes aimed at elucidating the sister-group relationships of *Stygichthys* will require dense taxonomic and genetic sampling to provide new insights into its evolutionary history.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA891276.

Ethics statement

The animal study was reviewed and approved by ICMBio/SISBIO 28992.

Author contributions

DA, RB-R, and MB conceived the original idea. WH, MR-S, RB-R, VG, and DA analyzed the data. MB collected samples. VG and DA performed lab work. DA prepared the manuscript. All authors discussed the results, implications, and commented on the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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The constructive evolution of taste in *Astyanax* cavefish: A review

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Animals inhabiting subterranean environments tend to evolve a constellation of 'regressive' and 'constructive' features. Regressive traits like vision and pigmentation are reduced or lost in derived organisms. In contrast, constructive traits like non-visual sensation, are commonly augmented and evolving under strong selection. Numerous studies have examined the genetic, developmental and molecular bases for regressive traits, while constructive traits have received less attention. A key constructive sensory feature in cave animals is the gustatory system which is likely useful for animals living in complete darkness, given the need to secure food for survival. Interestingly, despite having been studied for decades in the Mexican tetra, *Astyanax mexicanus*, much remains unknown regarding the biological basis, and adaptive relevance, of taste system evolution in cave morphs. Here, we present a brief review of taste system research in this system, conducted over the past ~90 years. We underscore key differences in gustation between cave and surface fish that reside at the levels of anatomy, perception and behavior. From this review, we sought to identify key knowledge gaps in our understanding of constructive taste system evolution. Future studies will provide further insights to the nature of constructive trait evolution by determining if constructive and regressive traits evolve through similar or different genetic and developmental mechanisms, and provide an essential case study for examining convergence of constructive traits across geographically distinct populations.

KEYWORDS
gustatory, taste buds, taste receptor cells, chemical sensation, subterranean

Introduction

Fish live in an immersive aquatic environment where their sensory systems are continuously in contact with a variety of chemical agents (Hara, 2012). Generally, chemoreception facilitates diverse behaviors in fish including obtaining food, defense from predators, sex identification, sensing carbon dioxide, and discriminating conspecifics, among others (Hara, 1971; Jonz et al., 2015). In fish, chemoreception is governed by broad cell and organ types including taste buds, olfactory epithelia, neuroepithelial cells, and solitary chemosensory cells (Reutter, 1986; Jonz et al., 2015). Two major chemosensory systems commonly examined in cave animals are taste (gustation) and smell (olfaction) – which are frequently augmented in the absence of visual input. Olfaction in fish is detected by neurons directly exposed to the environment, and are responsive to four chemical classes: amino acids, bile salts and acids, prostaglandins and sex steroids (Hara, 1994). Gustation is detected by taste receptor cells housed within taste buds which are responsive to nucleotides, salts, sugars, organic acids and amino acids. While the modalities sensed by these two systems are similar with respect to their detection of chemicals (e.g., amino acids), signals are transmitted to different regions of the brain and mediate different behavioral outputs (Hara, 1994).

As a consequence of life in perpetual darkness, olfaction and gustation have undergone diverse changes in a variety of cavefish systems. For instance, species of the Chinese cavefish genus, *Sinocyclocheilus*, frequently harbor an expanded number and wider distribution of taste buds, as well as gene duplications of taste receptor genes (Ma et al., 2019). These features imply a 'highly developed sense of taste' in this lineage (Yang et al., 2016). Additionally, *Sinocyclocheilus* are regarded as having more sensitive olfaction as a function of more anteriorly-directed, and larger, nostrils (Ma et al., 2019). Interestingly, in Amblyopsid cavefish such as the Spring Cavefish (*Chologaster agassizi*), feeding behavior appears to rely heavily on taste, but less so on olfaction (Hill, 1969). In other hypogean species, including *Phreatichthys andruzzi* (Dezfuli et al., 2009), *Astrolebus pholeter* (Haspel et al., 2012), and *Poecilia mexicana* (Parzefall, 2001) a large number of taste buds are found distributed, sometimes among mechanosensitive neuromasts, on the head and jaw.

The taste system in particular, appears to be especially labile – capable of adapting to different life history modes and feeding ecologies (Kasumyan, 2019). Here, we focus our attention to the gustatory system of a well-characterized cave dwelling fish, the Mexican tetra (*Astyanax mexicanus*). This species has long served as a natural model for examining adaptive change in response to extreme environmental pressures. With the rise of this remarkable animal system has come substantial interest in the developmental and genetic regulation of regressive traits. While the underpinnings of regressed traits have been advanced through integrative studies (Moran et al., 2023), the biological bases for constructive traits remain less well understood. Here we focus on an example of constructive evolution – the augmented taste system in *Astyanax*.

Studies of the gustatory system in blind Mexican cavefish stretch nearly as far back as their discovery ~90 years ago (Breder and Rasquin, 1943). We begin with a review of the genetic basis for peripheral expansion of taste buds in cavefish, both in number and anatomical distribution (Figure 1). We then summarize recognized differences in tastant perception, as well as current knowledge of the developmental basis for taste system expansion. We finish with a summary of recent studies taking a genome-level approach, examining bitter taste receptor family members and their putative expansion within the *Astyanax* lineage (Shiriagin and Korsching, 2019). Throughout, we revisit hypotheses seeking to explain the adaptive significance and drivers of taste system expansion, e.g., as a "compensatory" mechanism for survival in the absence of vision (Boudriot and Reutter, 2001). At the close of this review, we share our assessment of important yet unresolved aspects of taste biology in *Astyanax*, and propose future areas of focus.

A key finding from our review is that gustation is enhanced in cavefish compared to surface fish at multiple levels of analysis (i.e., anatomical, morphological, developmental, physiological, behavioral and genetic; Figure 2A). The characterization of these differences in the literature, without an overarching context, has rendered it difficult to understand the adaptive relevance and inter-relationship between features. In the past several decades, research has mostly capitalized on a comparative paradigm wherein cavefish represent the derived morph and surface fish represent the (surrogate) ancestral morph – and the polarity of evolutionary change is clear (Jeffery, 2001). Notably, however, early classical studies did not utilize this paradigm, because cave morphs were regarded as members of their own, separate genus *Anoptichthys* (Breder and Rasquin, 1943), and contrasts were illustrated by comparison to closely related, or well-characterized, teleost species (e.g., see Humbach, 1960).

Among the gustatory differences setting cavefish apart from other fish taxa include *genetic differences* focused on different features of relevance to gustation including threshold sensitivity and feeding behavior, *perceptual differences* principally based on heightened sensitivity (reduction in threshold sensitivity), *anatomical differences* which are mainly reflected in the numerical and distributional expansion of extraoral taste buds (see schematic; Figure 2B), and *developmental differences* relating to the timing of appearance (and number of taste buds) in cave and surface morphs. More recently, *genomic differences*, has been examined in the context of taste receptor genes between morphs, which may tie together some of the previously-listed categorical differences. The following short review of the historical and contemporary findings examines these categorical differences between *Astyanax* morphs.

Genetic analyses of the numerical and distributional expansion of taste buds

Schemmel (1967) was the first to report a profound expansion in the distribution of extraoral taste buds in cavefish (Figure 1). Although this work examined fish from two (of the ~30) natural cavefish populations, Pachón cavefish harbored substantially more than Sabinos cavefish (Schemmel, 1967; Rétaux et al., 2016). This work *implied* a conceptual link between the numerical expansion of peripheral taste buds and a heightened sensitivity to tastants. However, it should be noted that such a causative link between higher numbers of extraoral taste buds and a lower threshold of sensitivity has not been experimentally proven. Moreover, as suggested by Rétaux et al. (2016), these studies also did not discriminate between the probable contribution of olfaction to the enhanced detection sensitivity of cave morphs.

A later study, however, sought to connect the expansion of extraoral taste buds to food-finding behavior. Schemmel (1980) reported an angular difference in feeding between cave and surface morphs. Cavefish approach the bottom substrate at a ~55° angle, while surface fish (in the dark) tended to approach the substrate at a ~ 80° angle (Figure 2C). Using a classical genetics approach, this work concluded that the distributional expansion of taste buds and feeding angle are most likely unlinked, and mediated by complex (polygenic) genetic factors (Schemmel, 1980).

Studies by Wilkens (1988) expanded this classical genetic approach, reporting estimates of the number of genes implicated in taste bud expansion. This work revealed some diversity in the number and (qualitative) pattern of extraoral taste bud expansion among different cavefish populations (Wilkens, 1988). Using the approach of Lande (1981), the estimated number of genes implicated in extraoral expansion of taste buds was ~11–12, with the suspicion being that Pachón cavefish are homozygous for the genes involved in expansion (Schemmel, 1974; Wilkens 1988). This study, however, may have overestimated the number of taste buds by scoring solitary chemosensory cells alongside taste buds, which would likely inflate the number of estimated genes (see Wilkens et al., 2017). By at least one estimate, the number of loci involved in numerical expansion of taste buds may be closer to three, as reported by Protas et al. (2008).

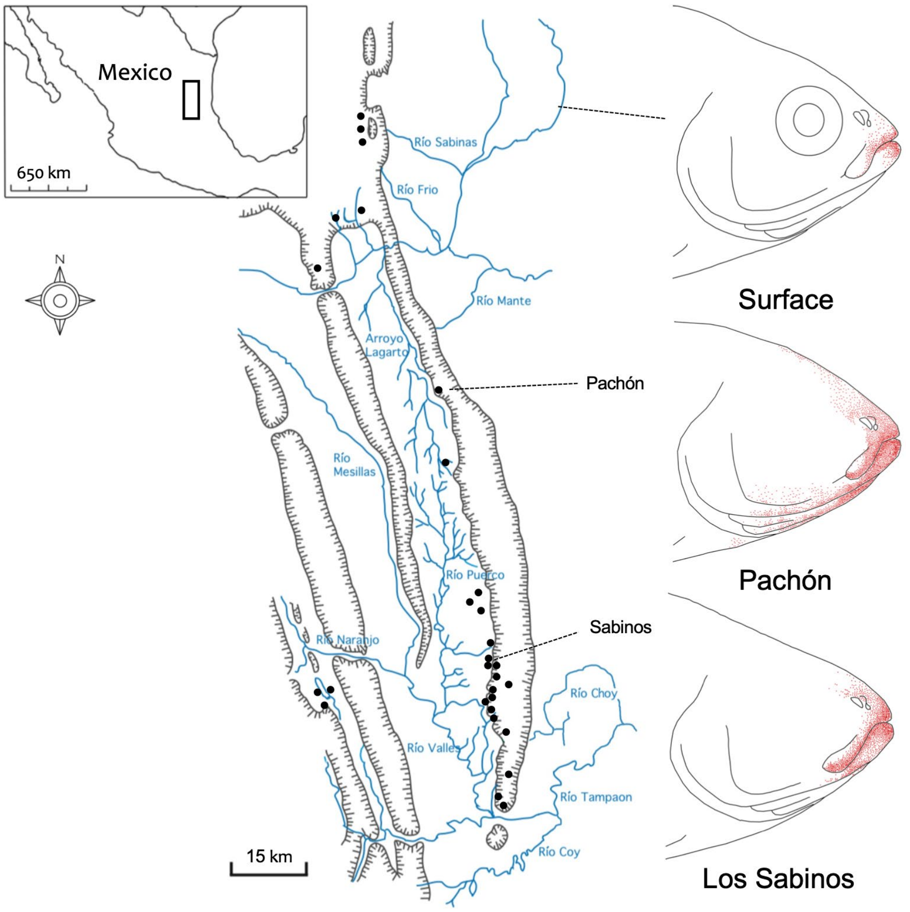

FIGURE 1
The distribution of extraoral taste buds in three populations of Mexican tetra. Surface-dwelling morphs of *Astyanax mexicanus* (top) demonstrate a modest distribution of taste buds (red dots) that reside close to the oral opening. Pachón cavefish (middle) have the most significant numbers, and largest distribution, of extraoral taste buds. Cavefish from the Sabinos (bottom) cave locality (see Sierra de El Abra map, left) harbor an intermediate distribution of extraoral taste buds that clearly exceeds those in surface fish. Map scale=15 km; redrawn from Schemmel (1967).

Wilkens (1988) reported that the largest number of taste buds among studied cave populations was Pachón, with the number of taste buds on the ventral jaw exceeding those in the mouth by ~3- to 4-fold. Interestingly, the genetic architecture (i.e., estimate of the number of 'genetic factors') for Sabinos cavefish was comparable to Pachón cavefish, despite those fish harboring fewer taste buds (Wilkens, 1988). Cavefish derived from the more western Micos caves have taste bud numbers roughly intermediate between Pachón and Sabinos cavefish. An interesting feature described by Wilkens was that the larger the area of ventral head covered, the higher the density of taste buds. At present, the biological basis for this feature remains unexplained. In sum, Wilkens (1988) concluded that extraoral taste bud expansion is a cave-associated feature demonstrating an additively polygenic basis.

More recently, taste buds were scored as part of a larger genetic analysis examining the role for peripheral taste reception in feeding behavior. This study revisited postural differences in substrate approach by surface and cavefish, examining this feature using a quantitative trait locus (QTL) mapping approach. Kowalko et al. (2013) formally tested whether postural differences in feeding angle were associated with craniofacial morphology, body depth, and numerical expansion of taste buds. The authors examined correlations using experimental F_2 individuals derived from both the Tinaja and Pachón caves (each crossed to surface fish), as well as examining co-localization of QTL in association mapping studies (Kowalko et al., 2013).

This work scored taste buds in members of an F_2 pedigree, but did not identify a significant association between feeding posture and taste bud numbers. Interestingly, one score – ventral taste bud number – was positively correlated with feeding posture angle, but this association did not reach statistical significance ($p = 0.051$). Further, this finding did not conform to the prediction that an expanded number of taste buds would yield a more acute feeding angle. Rather, ventral expansion was associated with an increase in feeding angle (reminiscent of surface fish feeding postures). The authors concluded that the derived feeding posture in cavefish is unlikely related, at least directly, to taste bud expansion. Interestingly, this study revealed QTL positions associated with extraoral taste bud numbers were different for Pachón and Tinaja cavefish (Kowalko et al., 2013). Thus, extraoral taste bud expansion has likely evolved independently in these two cave populations, through non-overlapping regions of the genome.

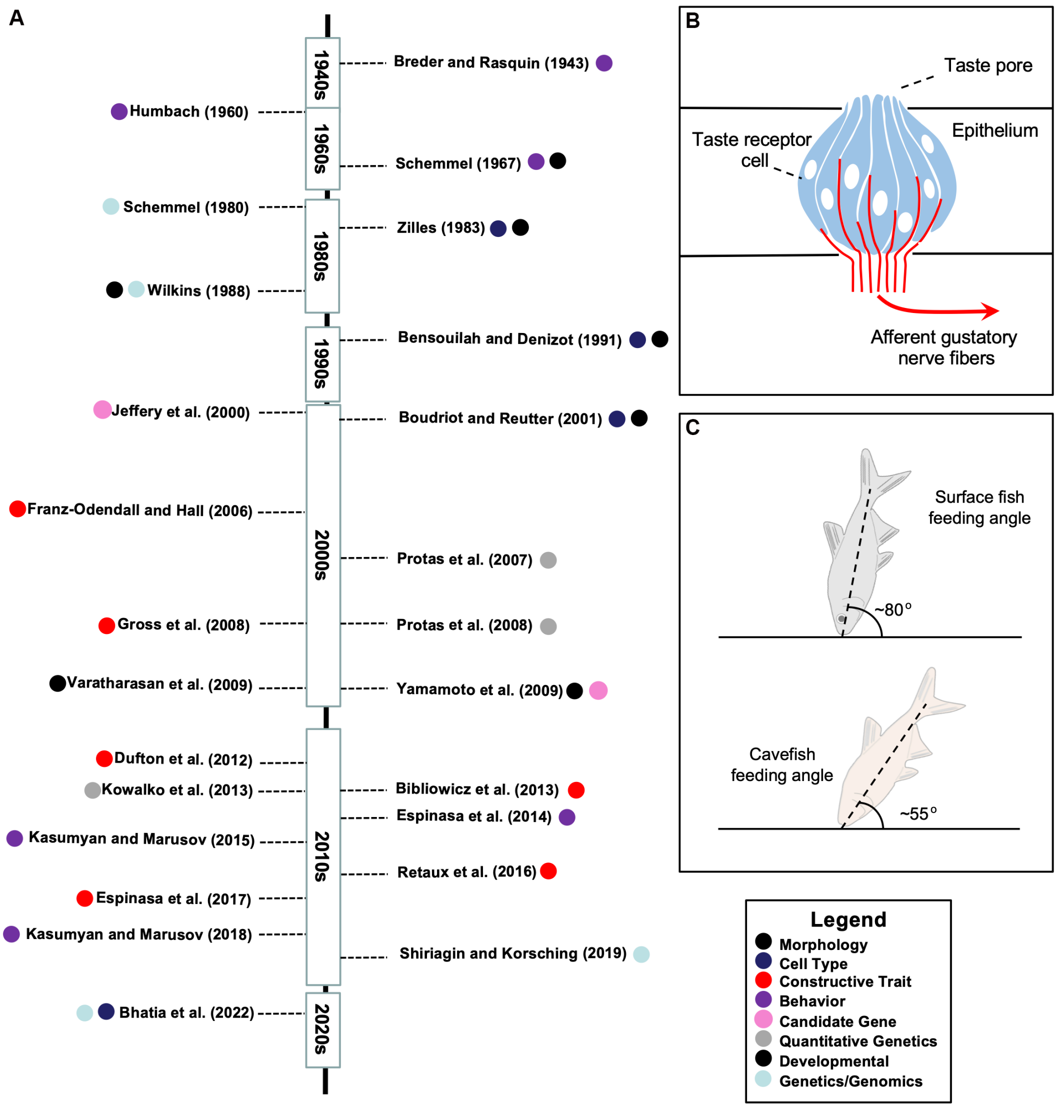

FIGURE 2
A timeline of research investigations and features of constructive taste evolution in the Mexican tetra. **(A)** A summary of research articles addressing taste system research across roughly seven decades. The topic of each study is indicated by one of eight principal categories determined by the authors (see Legend, bottom right). **(B)** A schematic representation of an extraoral taste bud labeled to show the apical taste pore, fusiform taste receptor cells (blue), and afferent gustatory nerve fibers (red). Note that taste buds can harbor between 50 and 150 cells, and include additional cell types (e.g., basal cells) not represented in this schematic. **(C)** A schematic representation of the different feeding postures of surface (top) and cave (bottom) morphs, which were reported to be ~80° and ~55°, respectively (see Schemmel, 1980).

The unclear source of perceptual differences in gustation between surface and cavefish

One of the first studies of the taste system in *Astyanax* appeared in the early 1940s focusing on heightened tastant detection in cave-dwelling morphs. Breder and Rasquin (1943) examined "chemosensory reactions" by comparing responses of river (i.e., "surface") morphs and cave morphs to chemical "repellents." Their anatomical observations revealed larger nasal pits in cave morphs, but they reported no differences in the number or morphology of taste organs (i.e., "taste buds") between surface- and cave-dwelling morphs (presumably referring strictly to those taste buds in the oral cavity, see above). This study also did not formally discriminate between olfaction and taste, since chemical "repellents" included acetic and citric acid, as well as ammonium carbonate (Breder and Rasquin, 1943). Although these acids are predicted to stimulate sour perception - one of five canonical taste modalities, it is unclear if ammonia would excite bitter taste receptors, olfactory receptors, or both. Interestingly, they noted that acetic acid elicited repulsive behavior but citric acid did not (Breder and Rasquin, 1943). Although the avoidance reaction was comparable between morphotypes, cave morphs were regarded as having a "stronger" reaction, i.e., a reduced threshold of sensitivity for tastant detection. Individuals rendered anosmic lost their reaction to these chemicals, indicating (at least a partial) reliance on olfaction for chemical detection in their assay. In sum, this work provided early evidence for enhanced sensitivity to tastants in cavefish. Over a decade later, however, a similar study by

Humbach (1960) reinforced the notion of enhanced taste sensitivity, showing that the bitter modality was ~300x more acute, and salty/acid/sweet were ~2000–4000× more acute, in cavefish compared to the common minnow (*Phoxinus*; Humbach, 1960).

Studies exploring taste sensitivity differences in Mexican tetra were first scored based on behavioral responses to different chemicals (Breder and Rasquin, 1943). A contemporary QTL mapping study similarly scored threshold sensitivity to "chemical sense." Accordingly, amino acids dissolved in system water were detected at a much lower concentration in Pachón cavefish compared to surface fish (Protas et al., 2008). A potential mediator of this response could be the savory taste receptor, T1R1, which binds glutamate and mediates the canonical umami taste modality (Oike et al., 2007). However, since several amino acids were examined in this study, the potential participation of olfaction in this enhanced sensitivity cannot be ruled out.

Although the biological basis for this lower threshold of sensitivity has not yet been determined, Schemmel (1967) suggested it may be explained, at least in part, by a richer supply of neurotransmitters and/or neuromodulators in the taste receptors themselves. A study by Bensouilah and Denizot (1991) revealed that Substance P was present in cavefish taste buds, but not surface fish, suggesting this neurotransmitter may be involved in the elevated gustatory sensitivity in cave morphs. A later study by Jeffery (2001) identified expression of another molecule in taste buds using immunohistochemistry, Prox1, however this protein was expressed transiently during development. Interestingly, Prox1 expression persisted in cavefish taste buds until ~14 dpf, while its expression in mechanosensory neuromasts continued much longer. At present, the role of Prox1 in cavefish taste buds is unclear. However, rather than playing a role in signal transmission, this protein may be involved in early taste bud specification, as in mouse taste papillae where it is co-expressed during papillae formation alongside *Shh* (Nakayama et al., 2008).

Enhanced taste sensitivity may be due to the larger number of peripheral (extraoral) taste buds in cavefish. Humans with more taste papillae (i.e., epithelial specializations housing taste buds) and taste pores have a lower threshold for tastant detection (Miller, 1986; Segovia et al., 2002). Although this mechanism explaining heightened sensitivity is intuitive, it remains to be rigorously tested. Additionally, it is unclear how the numerical expansion of extraoral taste buds integrates with other characterized anatomical, molecular and genetic differences between morphotypes.

Anatomical differences of taste buds and beyond

Using scanning electron microscopy, Zilles et al. (1983) reported no obvious anatomical differences in the surface structure of taste buds between cavefish, surface fish and cave x surface hybrids (Zilles et al., 1983). Similarly, a study by Boudriot and Reutter (2001) using transmission electron microscopy found no obvious ultrastructural differences between taste buds of cave and surface morphs. Notably, however, this group did report that the afferent innervation of types II and III taste receptor cells in cavefish have a higher density of axons in cavefish compared to surface fish (Boudriot and Reutter, 2001). A later qualitative assessment of afferent nerves associated with taste buds during development supports the notion that cavefish taste buds have more axons compared to surface fish (Varatharasan et al., 2009). However, in contrast to findings from ultrastructural analyses, Varatharasan et al. (2009) reported more taste receptor cells in the taste buds of cavefish compared to surface fish. This analysis was restricted to taste buds within the oral cavity, so it is unknown if cavefish extraoral taste buds harbor similar taste receptor cell densities, or cell types, as those in the cavefish oral cavity.

In addition to differences at the periphery, it is known that in cavefish the telencephalon (which processes chemoreceptive modalities of taste and olfaction) is ~40% larger than surface fish (Peters et al., 1993; Franz-Odendaal and Hall, 2006). The extent to which this change impacts taste signaling alone, as opposed to both taste and olfaction, is currently unknown. In sum, although perceptual differences exist, the causative explanation for these differences between morphs remains unknown. A lower threshold for detection of tastants, in principle, could be a function of the increased numbers of taste buds in cavefish, the extraoral distribution of those taste buds, or molecular differences in cavefish taste buds that impact chemoreception. The perceptual differences for tastants between cave and surface morphs are most likely explained by complex interactions traversing these levels of molecular signaling, numerical expansion of taste buds, and higher cortical functions.

The developmental basis for taste system expansion

For years, a persistent belief in the literature was that constructive evolution of the taste system occurred as a "compensatory" mechanism (Schemmel, 1967; Zilles et al., 1983) to counteract loss of vision. Accordingly, in the complete absence of light, there is no visual input. To survive, animals must still obtain nutrition for which the taste system is expected to play a central role. Yamamoto et al. (2009) showed that *sonic hedgehog* may be the molecule that unites vision loss with gustatory (and mandibular) expansion across a critical period of development. Overexpression of *sonic hedgehog* at specific times during embryogenesis linked together eye degeneration with increased numbers of oral taste buds (Yamamoto et al., 2009). Consistent with this, F_3 hybrids generated from surface x cavefish crosses showed an inverse relationship between taste bud numbers and eye sizes (Yamamoto et al., 2009). Collectively, this work suggested that elevated sonic signaling in cavefish increases taste bud numbers at the expense of eye development.

Other studies examining the timing of taste bud appearance have focused largely on oral taste buds, and their appearance up to about 3 weeks post-fertilization. Interestingly, at 5 days post-fertilization (dpf), there are minimal differences in the numbers of taste buds on the lower jaws of cave and surface morphs. By 22 dpf, however, cavefish had roughly three times the number of oral taste buds as surface fish (Varatharasan et al., 2009). Moreover, this numerical increase in taste buds is not uniform for the upper and lower jaws. Rather, by 22 dpf they observed the numerical expansion of taste buds was greater in the upper compared to the lower jaw (Varatharasan et al., 2009). These authors concluded the expansion is attributable to an acceleration in the rate of taste bud development in cavefish. They further suggested that this timing of expansion is coincident with the timing of eye degeneration – suggesting a potential link between these developmental processes. Finally, although the differences were modest, these authors found that cavefish harbored more cells per taste bud at all three stages of development when compared to surface fish (Varatharasan et al., 2009).

To examine the mechanistic link between eye regression and taste bud expansion, Dufton et al. (2012) performed experimental lens ablations (which lead to eye loss) and examined consequences on different sensory systems. Ablating the lens did not impact the number of taste buds on the jaw (Dufton et al., 2012). Further, Protas et al. (2008) found that eye size "was not significantly correlated" with the number of taste buds (as well as two measures of jaw size). Interestingly, this study showed that the lengths of the dentary and maxillary bones, and taste bud numbers, were significantly correlated (Protas et al., 2008). Thus, although there is evidence of a developmental interaction between vision loss and taste system expansion, the underlying genetic bases for these trait differences remain incompletely understood. Further, because the timing of numerical expansion of extraoral taste buds is unknown, the extent to which eye loss is associated with external taste bud expansion is unclear.

Genome-level analyses of gustation in *Astyanax*

A recent analysis of the genes encoding taste receptors in cavefish was performed following the release of the first draft cavefish genome (Shiriagin and Korsching, 2019). The *Astyanax* genome revealed a typical number of *T1R* gustatory genes (i.e., four) – which matched the orthologues in zebrafish. Interestingly, however, this study found that cavefish harbor a dramatic expansion of *T2R* genes which encode bitter receptors (Shiriagin and Korsching, 2019). Specifically, this study reported the presence of 24 *T2R* genes (three are pseudogenes) in the Pachón cavefish genome, which contrasts starkly with the typical numbers of *T2R* genes found in other teleosts, which is generally between 3 and 5 (Dong et al., 2009). This gene family expansion in cavefish was unexpected, as the *TR* gene repertoire is believed to be tuned to the nutritional environment. Since cavefish live amidst nutrient-poor conditions, the relevance (or value) of an expanded *T2R* bitter gene repertoire is unclear. Interestingly, a dN/dS analysis was suggestive of positive selection in two subclades of *T2R* bitter genes, however three other subclades did not show evidence of positive selection (Shiriagin and Korsching, 2019).

The authors estimated the *T2R* gene family expanded tens to hundreds of millions of years ago. This timing estimate would imply the bitter gene repertoire expanded prior to cave colonization (i.e., present in surface fish populations as well), thereby representing a 'pre-adaptive' trait (Shiriagin and Korsching, 2019). Importantly, at the time of publication a draft surface fish genome had not yet been completed. Thus, the ecological relevance of the *T2R* gene expansion is unclear, but the authors suggested it may endow cavefish with a broader bitter sense, which may facilitate avoidance of (bitter-tasting) toxic compounds in the cave.

A more recent publication utilizing an updated genome draft identified only 7 *T2Rs* in the cavefish genome, as opposed to 21 family members (Bhatia et al., 2022). Interestingly, however, a cell-based analysis showed that receptors encoded by four bitter genes (*T2R1*, *T2R3*, *T2R4* and *T2R114*) are functional and responsive to compounds in fish food, as well as a bitter compound (quinine; Bhatia et al., 2022). Future studies examining the surface fish genome are anticipated to provide an important comparison and insight to the timing of the *T2R* gene family expansion, and the extent to which it is associated with the cave habitat.

Gaps in knowledge and areas of future focus

Although the value of an enhanced gustatory system is intuitive, for an organism adapted to total darkness, it must be stressed that the precise function of cavefish extraoral taste buds remains unclear and largely unexplored. Moreover, although expanded taste bud domains at the periphery is tempting to regard as adaptive, it is unknown if this re-positioning is perhaps a consequence of other evolutionary mechanisms (e.g., indirect pleiotropy). To avoid weak inferences regarding the adaptive significance of extraoral taste buds (see Gould and Lewontin, 1979) rigorous analyses of taste system function are necessary. Along these lines, one recent study argued that external taste buds are used for preliminary assessment of food items during random swimming or targeted searches for food (Kasumyan and Marusov, 2015). Extraoral taste buds thus carry an importance for determining whether to pursue (or avoid) a food item. This work evaluated the sensitivity of *Astyanax* cavefish to two different amino acids, L-glutamine and L-phenylalanine. At present, it is unclear the extent to which olfaction was implicated in their study since amino acids can excite olfactory receptors. It is interesting, however, to note that L-glutamine is preferred by cave morphs and may be specifically linked to extraoral taste sensitivity (Kasumyan and Marusov, 2015).

To gain a more comprehensive understanding of the constructive evolution of taste, we identified several areas of opportunity. First, with respect to taste perception, it will be important to design studies that discriminate between gustatory and olfactory sensation. Well-designed olfaction studies have been performed in cavefish (Bibliowicz et al., 2013), and similar studies are needed for gustation. With respect to extraoral taste buds, a broader survey of taste bud numbers and distributions will be useful for determining if this feature is uniformly associated with cave life, and how these distributions compare across different populations. Further, presence (and large numbers of) extraoral taste buds may predictably lower the threshold for tastant sensitivity – such a relationship has never been experimentally established. Therefore, studies examining how numerical diversity of taste buds on the external body facilitate food-finding in cavefish will be essential. Relatedly, a remaining unsolved mystery is why cavefish harbor a different feeding posture, if this derived posture does not relate to the expanded spatial domain of extraoral taste buds?

One potential solution to this mystery is an explicit analysis of the genetic architecture (and genetic bases) for the numerical expansion of taste buds. Classic studies revealed a genetic component for these features (Schemmel, 1980; Wilkens, 1988), therefore contemporary genetics techniques may hold promise for identifying the gene(s) responsible for divergent extraoral taste bud phenotypes. How do these genetic changes differ between different populations, such as Pachón and Tinaja?

It is unclear how the expansion of extraoral taste buds at the periphery is involved in the lower threshold for sensitivity to tastants. More broadly, it is unclear how molecular differences in cavefish taste buds (e.g., Prox1, Substance P), neuroanatomical differences (e.g., higher axon density at the TBs, larger telencephalon) are potentially implicated in the elevated sensitivity to tastants.

From a developmental standpoint, a major unanswered question is what developmental process mediates the expanded numbers of taste buds in cavefish. Some work suggests a heterochronic shift in the development of oral taste buds (Varatharasan et al., 2009), but what explains the dramatically increased number and distribution of extraoral taste buds? What is the life history timing of this expansion? Is the expansion of taste bud numbers linked to vision loss, as suggested in the literature (Zilles et al., 1983)? If so, how does this process relate to extraoral taste bud expansion, which may occur over a much more protracted period of life history?

Finally, from a genomic perspective, what is the bitter taste receptor gene family (*T2R* genes) representation in the recently updated surface fish genome? The absence of this information necessarily limits our ability to infer the evolutionary mechanism(s) mediating this genomic feature. Moreover, taste receptor structure and expression are purportedly tuned to the nutritional environment. Thus, are there numerical and/or structural differences are in taste receptors of cave and surface fish, given that they occupy such different (nutritional) environments? Identifying such differences may be key to discerning how the taste system evolves in natural environments differing markedly in their nutrient composition.

Conclusion

Gustatory expansion is a commonly regarded example of constructive evolution in *Astyanax* cavefish. Owing to a robust comparative paradigm, several differences have been identified between cave and surface morphs over the past ~90 years (Figure 2A). The question of how these differences inter-relate to yield adaptive improvements to taste sensation remain unclear. Certain assumptions, e.g., relating external taste bud expansion to feeding posture differences, are not supported by contemporary results. Future work examining diverse knowledge gaps hold promise for understanding how the differences in taste identified between cave and surface fish ultimately confer an adaptive advantage in the subterranean environment. This work will provide insights to both the evolution of gustation in this fascinating system, but also provide key insights to the broader nature of constructive trait evolution.

Author contributions

DB and JBG conceived the project, wrote and prepared the manuscript, and reviewed and summarized the primary literature. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Troglomorphic adaptations on the northern European frontier: the phylogeny of the cave *Pseudosinella* (Hexapoda, Collembola) in the Western Carpathians

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Introduction: Using an integrative taxonomic approach, we investigated the morphological and molecular characters to identify the species of the genus *Pseudosinella* occurring in caves of the Western Carpathians and to clarify their phylogenetic relationships. Based on morphological characters, we hypothesized that *Pseudosinella aggtelekiensis* (Stach, 1929) and *Pseudosinella paclti* (Rusek, 1961) originated from different phyletic lineages.

Methods: We used the barcoding fragment of the mtDNA COI gene from 87 individuals from 16 caves to reconstruct the phylogenetic history of the genus *Pseudosinella*.

Results: The molecular phylogenetic tree revealed two distinct species groups with allopatric distributions. The first group consisted of *P. aggtelekiensis* populations from the Slovak Karst and three undescribed species from fragmented and isolated karst areas in southeastern Slovakia. The second group, *P. paclti* populations in the Central Western Carpathians, was merged. This group included *Pseudosinellamuranensis*, a new species taxonomically described in this work. It is characterized by highly developed troglomorphic features and is restricted to a small karst area, the Muranska planina Plateau. The phylogeny of the genus *Pseudosinella* from the caves of the Western Carpathians revealed a Miocene diversification. According to molecular calibration, the two distinct *Pseudosinella* lineages were separated in the Middle Miocene, about 14.51 Mya, followed by further diversification in the *P. paclti* lineage 10.89 Mya and in the *P. aggtelekiensis* lineage 11.14 Mya.

Discussion: This phylogeny is consistent with the uplift of Triassic limestones during the early formation of the Western Carpathians in the Paratethys region and the initial development of caves during this period. The study provides further important evidence that the Western Carpathians played a significant role as an independent speciation center of the obligate cave fauna in Europe.

KEYWORDS

biospeleology, short-range endemism, cryptic diversity, divergence, molecular calibration

1. Introduction

Collembolans are "wingless" hexapods, one of the most abundant and diverse groups of soil mesofauna with nearly 9,000 species described to date (Bellinger et al., 1996–2022), probably accounting for only about 20% of the hypothetical diversity of this group (Potapov et al., 2020). Collembolans are also among the most diverse and abundant invertebrates in caves and other subterranean habitats (Juberthie, 2000), with over 400 troglobiotic (obligate cave) species known (Deharveng and Bedos, 2018).

The genus *Pseudosinella* (Schäffer, 1897) belongs to collembolan genera with high species diversity. Members of the genus inhabit a wide range of habitats, from xerophilous environments to wetlands and caves (Winkler et al., 2021). Currently, it includes 374 species (Bellinger et al., 1996–2022; Kováč, pers. database) with a predominantly Holarctic distribution, although recent discoveries in subterranean environments of Brazil (Cipola et al., 2020) and Australia (Guzik et al., 2021) suggest that the diversity of this genus in the Southern Hemisphere is far from well understood. More than 140 species (about 37%) are obligate cave dwellers (Lukić, 2019) with more than 100 species distributed in southern Europe (Fiera et al., 2021).

This genus is apparently a polyphyletic taxon, with phyletic lineages derived from various ancestors of the genus *Lepidocyrtus* (Bourlet, 1839) (Christiansen, 1961; Gama, 1984; Soto-Adames, 2002; Wang et al., 2004). *Pseudosinella* representatives can be distinguished from *Lepidocyrtus* relatives essentially by the reduced number of eyes (Christiansen, 1961), an artificial diagnostic morphological feature that does not account for phylogenetic relationships among these taxa. Species of the genus *Pseudosinella* generally have additional distinguishing characteristics, such as loss of pigmentation and modified unguis structure. These characters are related to their mode of life, which is restricted to deeper soil layers, shallow subterranean habitats, or caves.

Phylogenetic relationships among higher Collembola taxa have traditionally been based on morphology, but more recently, phylogenetic studies have benefited from molecular data. Internal relationships within Entomobryomorpha subfamilies have been investigated in several molecular-based studies (Leo et al., 2019; Sun et al., 2020; Cucini et al., 2021, Guzik et al., 2021). Within the Lepidocyrtinae, Gama (1984) made the first attempt to reconstruct the complex *Pseudosinella* phylogeny which was based on presumably non-adaptive morphological characters, namely the modifications of the basal chaetae on the labial triangle, and the presence/absence of specific chaetae on the abdominal segments II and IV. Morphological characters traditionally used for species diagnosis of Lepidocyrtinae taxa, especially head and tergal chetotaxy, are very conservative indicators of genetic divergence (Soto-Adames, 2002) and represent synapomorphies for the subfamily (Zhang et al., 2015). However, the polyphyly of *Pseudosinella* as indicated by the morphology has not been confirmed at the molecular level (Zhang et al., 2015; Godeiro et al., 2021, 2023).

In terms of geographic distribution, *Pseudosinella* lineages show obvious subterranean radiation in the Pyrenees (Deharveng and Bedos, 2018), the USA, Mexico (Christiansen and Culver, 1987), and Brazil (Cipola et al., 2020). In Southeast Asia, the diversity of troglobiotic *Pseudosinella* is concentrated in Sulawesi and Papua New Guinea (Deharveng, 1988; Deharveng and Bedos, 2012). Recently, Guzik et al. (2021) revealed extreme genetic diversity of this genus in the subterranean calcretes of arid Australia.

Based on a study carried out in caves in the eastern USA, Christiansen and Culver (1987) defined levels of *Pseudosinella* troglomorphy that are confined to specific morphological adaptations to the subterranean domain. Troglomorphic species are essentially recognized by a larger body size, an elongated unguis with reduced internal and lateral teeth, acuminate tenent hair, and an elongated apical mucronal tooth. In contrast to these forms, the highly troglomorphic species represent a more advanced stage of morphological adaptation to caves in which unguis is extremely elongated, the internal and lateral teeth are completely absent, the two basal teeth greatly reduced, tenent hairs acuminate and shortened, and the apical mucronal teeth are markedly elongated. In addition, these "advanced troglomorphs" often have distinctly elongated antennae, *Pseudosinella christianseni* (Salmon, 1965) being an extreme case. Identical characters of *Pseudosinella* in the advanced troglomorphy stage were specified by Deharveng (1988). Worldwide, there are about 70 troglomorphic species described mainly from Europe, of which 19 species show advanced troglomorphy. The distribution of these forms agrees well with the observations of Christiansen and Culver (1987), which showed a clear biogeographic pattern of decreasing geographic range with increasing troglomorphy. Moreover, these authors arrived at two important assumptions (implications) that increasing troglomorphy (1) decreases the ability to disperse and (2) points to increasingly earlier times of initial cave colonization.

The geologically complex mountain unit of the Western Carpathians in Central Europe is part of the Carpathian Mountains, one of the biodiversity hotspots in Europe (Mráz and Ronikier, 2016). The present study builds on systematic surveys of cave biota in the Western Carpathians in Slovakia carried out over the last two decades (Kováč et al., 2014). Slovakia is relatively rich in karst, covering an area of over 2,700 km^2. In total, more than 7,200 karst and pseudokarst caves have been documented in the country to date (Bella et al., 2018).

So far, two troglomorphic *Pseudosinella* species are known from the Western-Carpathian caves, but Kováč and Rusek (2012) indicated the presence of several undescribed species. Furthermore, Kováč et al. (2016) pointed out the important role of the Western Carpathians as glacial refugia for subterranean Collembola. The authors recognized five obligate cave *Pseudosinella* species, three of them undescribed, based on morphological characters. They found that these species are allopatric in distribution and restricted to karst caves in the central and southern parts of the Western-Carpathian region.

The profound differences in morphological characters led us to hypothesize that *Pseudosinella aggtelekiensis* (Stach, 1929) and *Pseudosinella paclti* (Rusek, 1961) represent separate phylogenetic lineages. These lineages probably diverged during the pre-Quaternary period when significant palaeogeographic changes occurred in the former Paratethys area. We also considered species with highly evolved troglomorphic features to be evolutionary old taxa.

The aim of the present study was (1) to reconstruct the basic phylogeny of *Pseudosinella* populations occurring in the Western-Carpathian caves using an approach of integrative taxonomy, i.e., a combination of molecular and morphological characters; and (2) to estimate divergence times of individual *Pseudosinella* cave lineages based on molecular calibration.

2. Materials and methods

2.1. Morphological examination

For morphological examination, specimens were mounted individually on slides in Swann medium (Liquido de Swann), modified from Rusek (1975), and examined with a Carl Zeiss Axio 5 phase-contrast microscope and a Leica DM 2500 microscope equipped with DIC optics (differential interference contrast), a measuring micrometric eyepiece, and a drawing arm. Images were captured using a Carl Zeiss Axiocam 208 color camera and ZEN imaging software. The drawings were processed using Adobe Photoshop CS6.

Abbreviations used in text, tables, and figures are as follows: Abd., abdominal tergum; al, anterolateral sensillum; accp, accessorial p–sensillum; Ant., antennal segment; a.s.l., above sea level; cm, conical microchaeta; IBE FS UPJS, Institute of Biology and Ecology Faculty of Science, P. J. Šafárik University, Košice; mac, macrochaeta; mic, microchaeta; psp, pseudoporus; ms, microsensillum; Ti., tibiotarsus; Th., thoracic tergum.

2.2. Nomenclature of chaetae

The dorsal chaetotaxy of the head follows the two systems of Gisin (1967) and Soto-Adames (2010), with Gisin's original system retained for consistency with older taxonomic literature on the genus. Notation of labial palps is following Fjellberg (1999), labial chaetotaxy following Gisin (1964), and postlabial chaetotaxy following Chen and Christiansen (1993) and Cipola et al. (2020). Dorsal chaetotaxy of thoracic and abdominal segments follows both Gisin's (1967) and Szeptycki's (1979) systems, and the notation of sensilla (specialized S–chaetae) on terga follows Zhang and Deharveng (2015).

2.3. *Pseudosinella* material for the study

The specimens of *Pseudosinella* used in this study were collected from 16 caves in the Western Carpathians, Slovakia (Table 1). We used two main methods to collect specimens: (1) visual searching and hand collection from cave walls, sediment, rotten wood, and the surface of standing water (small sinter ponds and puddles in mud), and (2) pitfall trapping with 4% formaldehyde solution or 95.6% ethanol as a fixative liquid. Traps were exposed to cave sediment for no longer than 5 months to minimize their negative impact on local arthropod communities.

2.4. Molecular data analysis and species delimitation methods

A total of 87 *Pseudosinella* specimens from 16 caves in the Western Carpathians (see Supplementary Table S1) were analyzed in the molecular laboratories of the Institute of Entomology, Biology Center AS CR, Ceské Budějovice, Czech Republic, and the Department of Zoology, IBE FS UPJS, Košice, Slovakia.

To avoid contamination, all DNA laboratory work was performed under sterile conditions and using barrier tips. Total DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's modified protocol (see Parimuchová et al., 2017). A polymerase chain reaction (PCR) (Saiki et al., 1988) was performed with a reaction volume of 12.5 µl consisting of 1 µl template DNA (not quantified), 10 × PCR buffer (TopBio), 12.5 mM dNTP mix, 5 µM of each primer, and 0.125 units of Taq polymerase (TopBio) on a GenePro (Bioer Co., Ltd, China) thermal cycler. A fragment of the COI gene (660 bp) was amplified with the universal primers LCO1490 (5′-ggt caacaaatcataaagatattg g-3′) and HCO2198 (5′-taa act gggtgaccaaaaaat ca-3′; Folmer et al., 1994). Thermal cycling conditions were as follows: 94°C for 1 min followed by 37 cycles (94°C for 20 s, 47°C for 40 s, and 70°C for 50 s), followed by 1 min 30 s at 72°C. After verification on agarose electrophoresis, the reaction products were purified using Exo I/FastAP (Thermo Fisher Scientific). Sequencing of the purified products was performed using LCO1490 according to the Sanger method (SEQme s.r.o. in Dobris, Czech Republic). In cases where the primer did not produce a high-quality chromatogram, sequencing was performed with the reverse primer. Sequences were edited with Geneious Prime 2022.1.1 (Copyright © 2005–2022 Biomatters Ltd.) to remove unreadable short segments (∼30 bp at the 5′ and 3′ ends). Because none of the sequences contained stop codons or indels in ORF, they were assumed to be true mitochondrial copies and not nuclear copies. All sequences were checked for their similarity to relatives of the family Entomobryidae using the GenBank BLASTn search (Mega Blast algorithm with the default setting). Sequences were aligned using Geneious Prime 2022.1.1 software (Copyright © 2005–2022 Biomatters Ltd.) according to the Muscle (Codons) algorithm using the Invertebrate Mitochondrial GeneCode and default parameters.

Species delimitation was performed using both barcoding gap and evolutionary models. A total of 87 COI sequences (length 609 bp) were used for analyses. FaBox (Villesen, 2007) was used to assemble the final set of DNA sequences into unique haplotypes. The Assemble Species by Automatic Partitioning (ASAP) method (Puillandre et al., 2021) used genetic distances to propose hypotheses about the species. The Kimura (K2P) model with default parameters was used to group sequences together.

The Poisson tree processes (PTP) model, used to delineate species based on the number of substitutions, was performed using online software (Zhang et al., 2013). Unique haplotypes were only used for this purpose. A maximum likelihood (ML) tree was derived using the auto-substitution model and a 1,000-ultrafast bootstrap analysis (Hoang et al., 2018) in the software IQ-TREE (Nguyen et al., 2015).

TABLE 1 List of caves with *Pseudosinella* populations used for morphological and molecular studies.

Abbrev.	Cave	GU	E	L	D	T
ZLA	Zlatnica Cave	M	940	150	34	4.8–6.7
JP	Jelenia priepasť Abyss	M	1,070	323	55	4.2–4.9
LJM	Ľadová jama na Muráni	M	865	35	19	4.0–5.0
MAR	Márnikova jaskyňa Cave	H	770	487	–	No data
DIE	Dielik Cave*	M	570	223	35	6.4–6.9
BOB	Bobačka Cave	M	680	4,653	142	6.6–7.0
HARM	Harmanecká jaskyňa Cave	VF	821	3,216	75	6.3–6.7
PUS	Pustá jaskyňa Cave	LT	943	4,106	202	5.5–9.0
VC	Važecká jaskyňa Cave	KC	784	530	–	7.2–7.4
ZC	Zápoľná jaskyňa Cave	KC	755	1,848	59	6.8–8.2
HRU	Hrušovská jaskyňa Cave	S	295	1,139	–	11.0–11.3
HCA	Hačavská jaskyňa Cave	S	795	202	–	No data
MC	Marciho diera Cave	S	852	150	–	5.3–10.3
AC	Ardovská jaskyňa Cave	S	314	1,510	–	7.9–10.7
DR	Drienovská jaskyňa Cave	S	245	1,588	84	9.4–9.7
SK	Ochtinská aragonitová jaskyňa Cave—Štôlňa Kapusta Tunnel	O	642	585	30	7.6–8.4
BRC	Brekovská jaskyňa Cave	B	260	200	30	6.4

GU, geographical units; S, Slovak karst; O, Ochtinský kryptokarst; B, Brekovský karst; VF, Veľká Fatra Mts.; LT, Low Tatras Mts.; KC, Kozie chrbty Mts.; M, Muránska Planina; E, elevation of cave entrance (m a.s.l.); L, cave length (m); D, cave depth (m); T, internal cave air temperature (°C). GU and cave parameters E, L, and D after Bella et al. (2018). Air temperature measured with digital thermo hygrometer COMET in deep static cave zone (Papáč, 2011; P. Ľuptáčik, pers. comm.).
*Specimens studied only morphologically.

The estimation of divergence time was performed according to the scheme proposed by Katz et al. (2018) and Katz (2020). Input data were processed in BEAUTI v 2.6.7, and further analyses were performed in BEAST 2 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) via the CIPRES Science Gateway (www.phylo.org; Miller et al., 2012). For molecular clock calibration, we used COI = 3.54%/Mya as recommended by Papadopoulou et al. (2010), Katz et al. (2018), and Katz (2020). bModelTest (Bouckaert and Drummond, 2017) was used to estimate the site model. A relaxed log-normal clock model was applied, with the clock rate parameter set to 0.0168 and the Yule process for speciation priorities. Analyses were run twice with an MCMC chain of 100 million generations (sampling every 10,000 generations). The ESS values (>200) and convergence were checked in Tracer 1.7.1 (Rambaut et al., 2018) with 10% burn-in. The tree with maximum clade reliability was created using TreeAnnotator v.1.4.7 (Drummond et al., 2012) and visualized in FigTree v 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

All new sequences are publicly available in GeneBank, and the accession number for each specimen is provided in Supplementary Table S1.

The research complied with the conditions of licenses No. 3102/2009-2.1, No. 7905/2013-2.3, and No. 2661/2017-6.3 of the Ministry of Environment of the Slovak Republic, Certificate of Competence according to Law No. 543/2002.

3. Results

3.1. Molecular species delimitation and divergence time estimation

A 609 bp fragment of COI was aligned for 87 *Pseudosinella* specimens from 16 caves. A total of 28 haplotypes were identified, including nine singletons (haplotypes represented by only one individual). We used two delimitation methods to define molecular operational taxonomic units (MOTUs) and combined them with morphological traits and geographic distribution to evaluate MOTUs at the species level (Figure 1). The ASAP method delimited eight MOTUs, and the best partition had an ASAP score of 1.0 ($p < 0.1$). The distribution of K2P distances showed a clear barcode gap, with haplotypes that diverged at a distance >9% belonging to a different species. The bPTP method estimated nine MOTUs with support ranging from 0.519 to 1.0, overestimating the ASAP delimitation (Figure 1). *Pseudosinella aggtelekiensis*, the species endemic to the Slovak and Aggtelek Karst (identified as AC, HCA, MC, and HRU), was well defined by ASAP, morphology, and geography, while bPTP recognized two distinct MOTUs within these populations. One of them inhabits Ardovská jaskyna Cave (AC), located at the edge of this geomorphological unit. Another known species, *P. paclti*, is well-defined by its morphology and distributed in three karst areas in the central Western Carpathians (VF, LT, and KC). Three different MOTUs were detected within

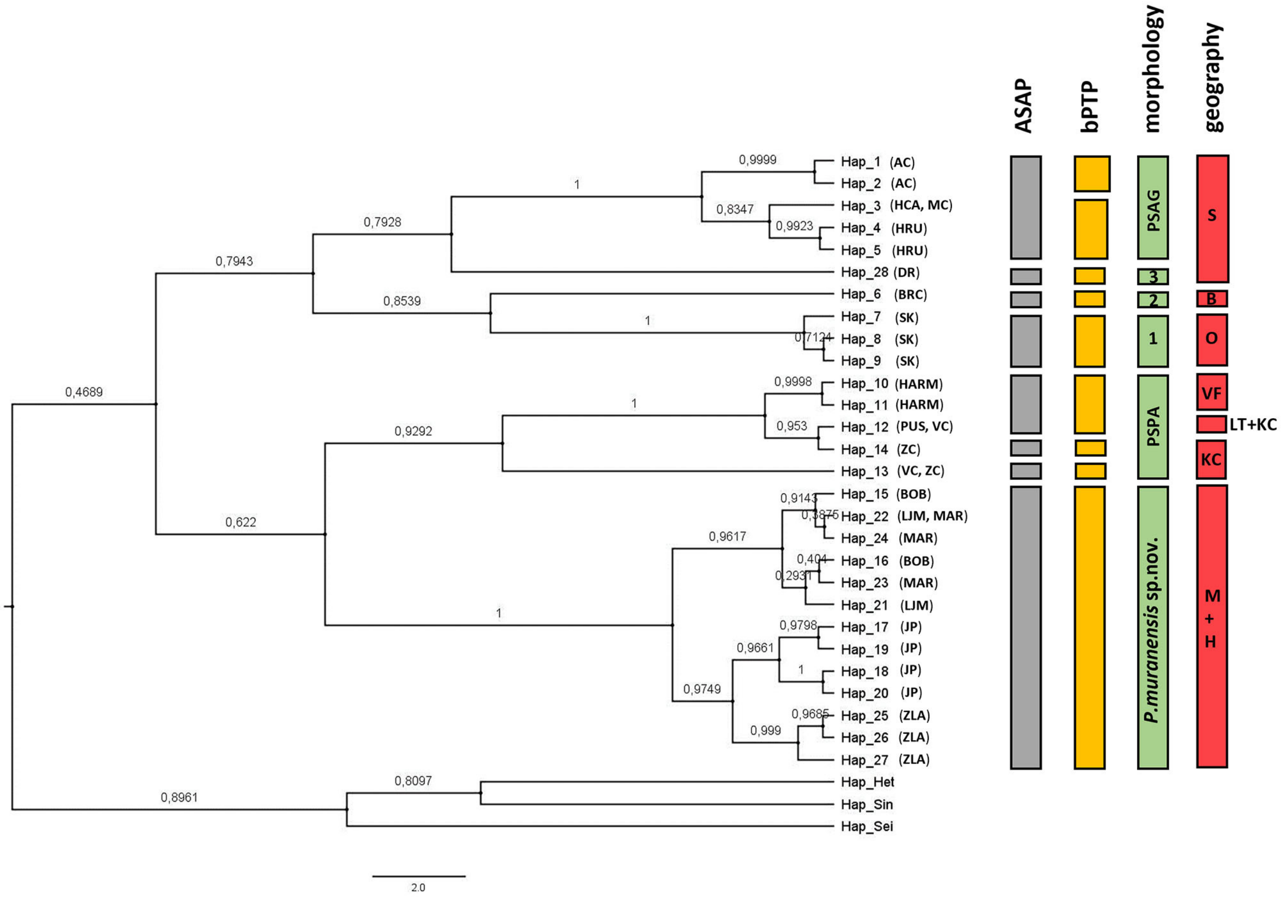

FIGURE 1
Species delimitation of cave *Pseudosinella* with ML tree based on COI marker. The values above the branches are Bayesian posterior probabilities. *Heteromurus nitidus* (MT611220.1), *Sinella curviseta* (NC 042755.1), and *Seira dowlingi* (MW419950.1) were used as outgroups. The graph is supplemented with a species delimitation (ASAP and bPTP) based on morphological characteristics and geographic distance. S, Slovak karst; O, Ochtinský kryptokarst; B, Brekovský karst; VF, Velká Fatra Mts.; LT, Low Tatras Mts.; KC, Kozie chrbty Mts.; M, Muránska Planina; H, Horehronské podolie. For abbreviations of caves in parentheses, see Table 1.

the populations using both molecular methods. The MOTUs designated as JP, LJM, MAR, BOB, and ZLA were confirmed by both molecular methods. It is a highly troglomorphic new species *Pseudosinella muranensis* endemic to the caves of the Muránska planina Plateau and the neighboring Horehronské podolie Valley. The species is described below in the taxonomic part of the Results section under the scientific name *P. muranensis* (Kováč and Parimuchová). The delimitation analyses ASAP and bPTP resulted in three new *Pseudosinella* MOTUs supported by diagnostic morphological characters and geographical distribution. Thus, they represent new species, designated as *Pseudosinella* sp. 1, 2, and 3 (Figure 1, Table 2). *Pseudosinella* sp. 1 inhabits a small cryptokarst area of Devonian origin in the southern Western Carpathians, *Pseudosinella* sp. 2 was detected in a small and well-isolated karst area in the eastern part of the Western Carpathians. And finally, *Pseudosinella* sp. 3 was found in the Drienovská jaskyna Cave (DR) in the Slovak Karst, i.e., within the distribution range of *P. aggtelekiensis*. The geographic range of the studied *Pseudosinella* cave populations and their assignment to species based on integrative taxonomy is shown in Figure 2.

The divergence time of the *Pseudosinella* phylogenetic tree was estimated using representatives of the family Entomobryidae as outgroups. Appropriate sequences of COI genes from mitogenomes deposited in GenBank were used for *Heteromurus nitidus* (MT611220.1), *Sinella curviseta* (NC 042755.1), and *Seira dowlingi* (MW419950.1; Figure 3). The graph (Figure 3) shows that two distinct *Pseudosinella* lineages—"*paclti*" and "*aggtelekiensis*"—diverged from the common ancestor during the Middle Miocene ca. 14.51 Mya, followed by subsequent diversification in the *P. paclti* lineage 10.89 Mya and *P. aggtelekiensis* lineage 11.14 Mya.

3.2. Taxonomy—Description of the new species

Class Collembola (Lubbock, 1873)
Order Entomobryomorpha (Börner, 1913)
Family Entomobryidae (Schäffer, 1896)
Subfamily Lepidocyrtinae (Wahlgren, 1906)
Genus *Pseudosinella* (Schäffer, 1897)
Pseudosinella muranensis (Kováč and Parimuchová), new species
Figures 4–12

TABLE 2 List of eyeless *Pseudosinella* with 32/0201+2 medial tergal macrochaetae on Th.II–Abd.IV segments.

Species	Author and year	Cave/soil/MSS	Troglom.	Country	Dorsal head mac.	Labial triangle	Tenent hair
alfonsii	Dallai and Malatesta, 1982	Cave	TM	I	R001	$m_1m_2rel_1l_2$	a
germanica ssp.*	Gama, 1973	Cave	TM	D	R001	$M_1M_2rEL_1L_2$	a
pongei	Gama, 1979	Soil	No	F	R001	$M_1M_2rEL_1L_2$	a
subvirei	Bonet, 1931 *sensu* Gisin and Gama (1972)	Cave	TM	ES	R001	$m(M)_1m_2rel_1l_2$	a
virei	Absolon, 1901	MSS	TM	F	R001	$m_1m_2rel_1l_2$	a
aelleni	Gama, 1973	Cave	No	D	R011	$M_1M_2rEL_1L_2$	a
lamperti	Schäffer, 1900	Cave	Weakly TM	D	R011	$M_1M_2rEL_1L_2$	a
noseki	Rusek, 1985	Soil	no	CZ	R011	$M_1M_2rEL_1L_2$	a
gineti	Cassagnau, 1955	Cave	TM	F	R011	$m_1m_2rel_1l_2$	a
jacetanica	Jordana and Baquero, 2007	Soil	No	ES	R011	$M_1M_2rEL_1L_2$	a
subefficiens	Gisin and Gama, 1970	Cave	No	F	R011	$M_1m_2(M_2)rE(l_1)L_2$	c
subilliciens	Mateos, 1993	Soil	No	ES	R011	$M_1M_2rEL_1L_2$	c
jesusi	Beruete and Jordana, 2002	Cave	TM	ES	R101	$m_1m_2rel_1l_2$	a
immaculata	Lie-Petersen, 1897	Cave	No	Eur	R101	$m(M)_1m_2rel_1l_2$	a
aueri	Gisin, 1964 *sensu* Stomp et al. (1991)	Cave	HTM	CH, A	R111	$M_1M_2rEL_1L_2$	a
csafordi	Winkler and Mateos, 2018	Soil	No	H	R111	$M_1M_2rEL_1L_2$	c
arrasatensis	Beruete and Jordana, 2002	Cave	No	ES	R111	$m_1m_2rel_1l_2$	c
baztanensis ssp.*	Beruete and Jordana, 2002	Cave	TM	ES	R111	$m(M)_1m_2rel_1l_2$	a
dobati	Gisin, 1965	Cave	Weakly TM	F, MA	R111	$m_1m_2rel_1l_2$	a
duprei	Beruete and Jordana, 2002	Cave	TM	ES	R111	$M_1m_2rel_1l_2$	a
inflata	Bonet, 1931	Cave	TM	ES	R111	$M_1m_2rel_1l_2$	a
*longicornis**	Bonet, 1929	Cave	TM	ES	R111	$m_1m_2rel_1l_2$	a

(Continued)

TABLE 2 (Continued)

Species	Author and year	Cave/soil/MSS	Troglom.	Country	Dorsal head mac.	Labial triangle	Tenent hair
pieltaini	Bonet, 1929 *sensu* Gisin and Gama (1972)	Cave	TM	ES	R111	$m(M)_1m_2rel_1l_2$	a
subinflata	Gisin and Gama, 1969	Cave	TM	ES, F	R111	$m(M)_1m_2rel_1l_2$	a
subterranea	Bonet, 1929 *sensu* Gisin and Gama (1972)	Cave	TM	ES	R111	$m(M)_1m_2rel_1l_2$	a
tarraconensis	Bonet, 1929 *sensu* Gisin and Gama (1972)	Cave	TM	ES	R111	$m_1m_2rel_1l_2$	a
unguiculata	Bonet, 1929 *sensu* Gisin and Gama (1972)	Cave	HTM?	ES	R111	$m_1m_2rel_1l_2$	a
melatensis	Gisin and Gama, 1969	Cave	TM	F	R211	$M_1m_2rel_1l_2$	a
oxybarensis	Gisin and Gama, 1969	Cave	TM	F	R211	$m_1m_2rel_1l_2$	a
jeanpierrei	Beruete and Jordana, 2002	Cave	HTM	ES	R221	$M_1m_2rel_1l_2$	a
paclti	Rusek, 1961 *sensu* Kováč and Rusek (2012)	Cave	TM	SK	R221	$M_1m_2rEL_1L_2$	a
pyrenaea	Bonet, 1931	Cave	TM	ES	R221	$m_1m_2rel_1l_2$	a
muranensis	**n. sp.**	Cave	HTM	SK	R221	$m_1m_2rel_1l_2$	a
sp. 1	(SK)	Cave	TM	SK	R111	$M_1m_2reL_1L_2$	a
sp. 2	(BRC)	Cave	TM	SK	R221	$M_1M_2rEL_1L_2$	a
sp. 3	(DR)	cave	TM	SK	R221	$M_1M_2rEL_1L_2$	a

Dorsal head macrochaetae and dorsal Abd.II chaetotaxy sensu Gisin (1967) and labial triangle chaetae sensu Gisin (1964). All species with this combination have dorsal Abd.II chaetotaxy pABq1q2 and European distribution.
Troglom., troglomorphy; no, without troglomorphic characters; TM, with troglomorphic characters; HTM, with highly troglomorphic characters. HTM character expression was essentially judged by the shape of the foot complex and the shape of the mucro, see also Discussion. Tenent hair: a- acuminate, c - clavate. *P. germanica* ssp.* subspecies of *P. lamperti*; *P. baztanensis* ssp.* subspecies of *P. subterranea*; *P. longicornis** originally described as subspecies of *P. tarraconensis*.
Country: A, Austria; CH, Switzerland; CZ, Czech Republic; D, Germany; ES, Spain; Eur, Europe; F, France; H, Hungary; I, Italy; Ma, Morocco; SK, Slovakia.

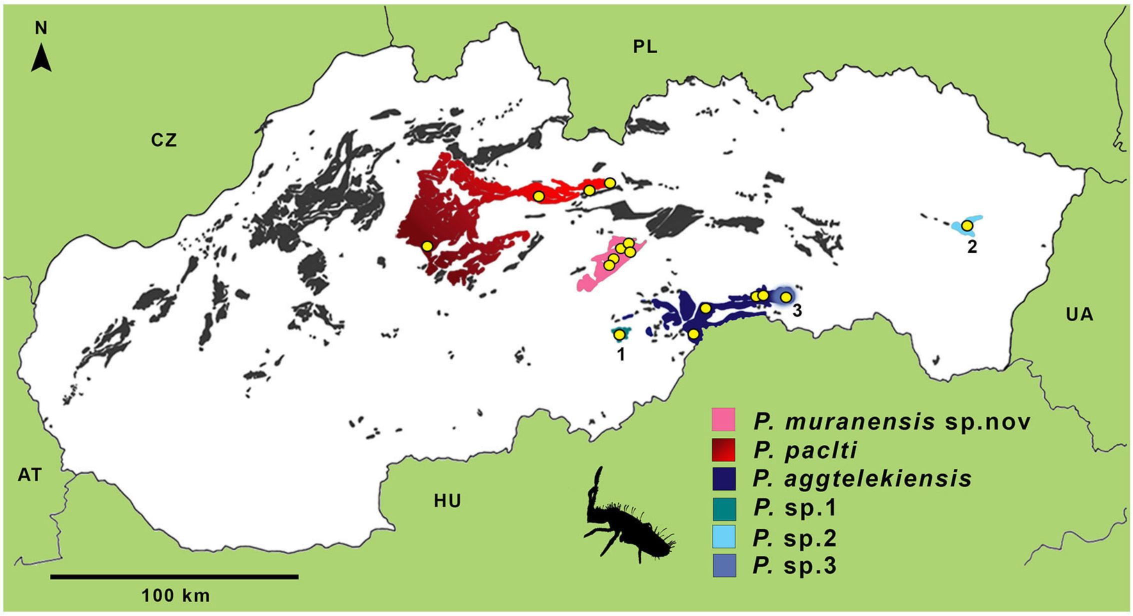

FIGURE 2
Distribution of *Pseudosinella* taxa in the caves of the Western Carpathians as a result of the integrative taxonomy study. The yellow circles stand for the sampling locations, the spots for the karst areas: black-red, Velká Fatra and Low Tatras; purple, Muránska planina Plateau; dark-blue, Slovak karst; 1, Ochtinský kryptokarst with *P.* sp.1; 2, Brekovský karst with *P.* sp. 2; 3, Drienovská Cave in the Slovak Karst with *P.* sp. 3. Karst areas were digitally processed by P. Gažík.

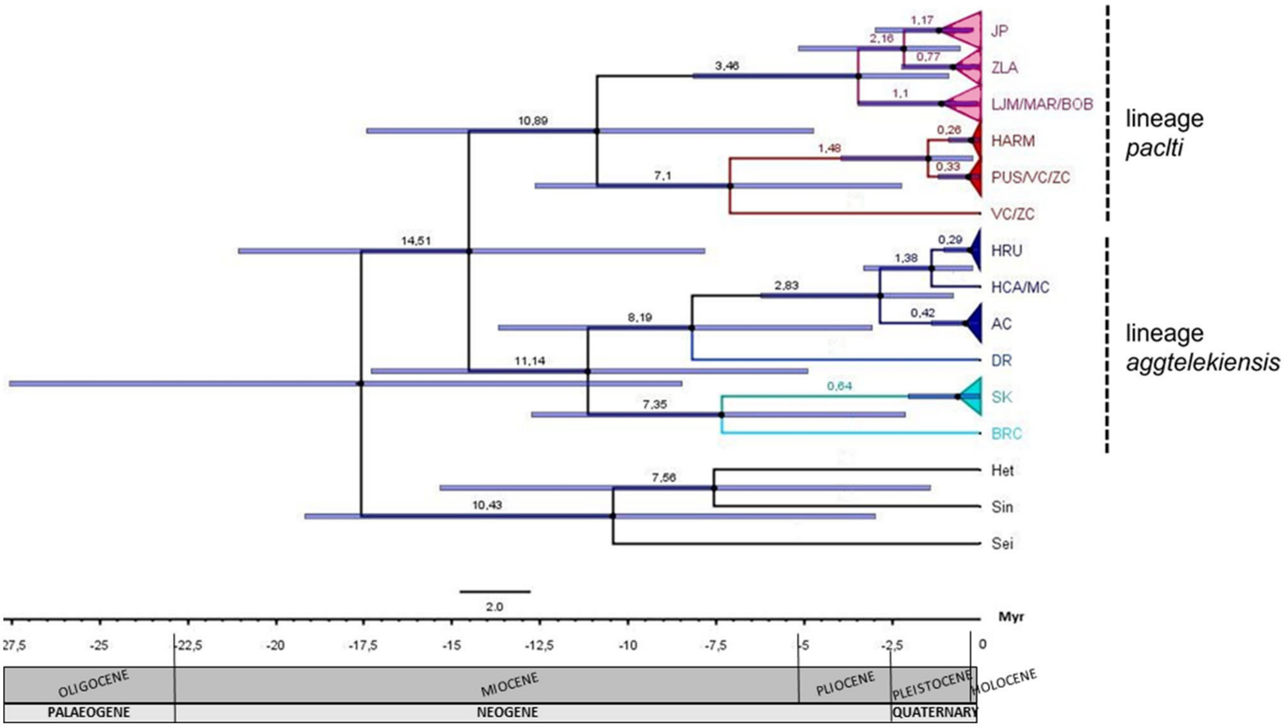

FIGURE 3
Bayesian tree in BEAST 2 for the *Pseudosinella* populations from the Western Carpathians caves based on the COI marker (visualized by FigTree). The purple boxes around each internode correspond to the 95% confidence intervals of the divergence time for each branch of the *Pseudosinella* phylogeny. For illustration, the geologic time scale is shown below the phylogeny. The different karst areas are represented by triangles of different colors: the Muránska planina Plateau in pink, the Low Tatras in red, the Slovak Karst in dark blue, the Ochtinský kryptokarst in green, and the Brekovský karst in turquoise. *Heteromurus nitidus* (Het), *Sinella curviseta* (Sin), and *Seira dowlingi* (Sei) are used as outgroups. The value 3.54%/Myr was used for the calibration of the molecular clock. For abbreviations of caves, see Table 1.

FIGURE 4
Pseudosinella muranensis, general habitus of the new species. Jelenia priepasť, Muránska planina Plateau. Photo: Ľ. Kováč and A. Parimuchová.

Pseudosinella paclti (Rusek, 1961)—Kováč et al. (2002)
Pseudosinella cf. *paclti* (Rusek, 1961)—Kováč et al. (2014)
Pseudosinella sp. 3—Kováč et al. (2016)
Pseudosinella sp.—Parimuchová et al. (2020)
https://zoobank.org/NomenclaturalActs/12BE12B0-154A-4A05-B59F-9A49BB683F8C

3.2.1. Material

Type material. *Holotype*: Slovakia. ♀ on the slide (Nr. 204-00), Muránska planina Plateau, Bobačka Cave (680 m a.s.l.), Riečna chodba Passage, pitfall trap, 9 Nov. 2000, leg. Ľ. Kováč. *Paratypes*: Muránska planina Plateau, Bobačka Cave, 9 Nov. 2000, leg. Ľ. Kováč: eight ♀ and one ♂ on the slide (Nr. 204-00), one ♂ (Nr.: 205-00), three juveniles (Nr. 207-00), same data as holotype; one ♀ (Nr. 200-00) and one juvenile (Nr. 201-00), Koncový sifón Passage, pitfall trap, leg. Ľ. Kováč; one ♀ (Nr. 214-00), Veľryba Passage, pitfall trap, one ♀ (Nr. 200-00); six ♀ and three ♂ (Nr. 175-00), hind part, hand collecting, 5 Oct. 2000, leg. Ľ. Kováč and P. Ľuptáčik; one ♀ and one ♂ (Nr. 422-11), Chodba tušenia Passage, 5 Sep. 2011, leg. Ľ. Kováč.

Other materials.

Muránska planina Plateau, Jelenia priepasť Abyss (1,070 m a.s.l.): Biely dóm Hall, four ♀ and two specimens with undetermined sex (Nr. 561-11), central part, hand collecting on rotten wood, 2 Sep. 2011, leg. Ľ. Kováč; Sien pagody Hall, nine ♀ and two specimens with undetermined sex (Nr. 117-18), hand collecting on the surface of sinter pool, 18 May 2018, leg. A. Parimuchová;
Muránska planina Plateau, Zlatnica Cave (940 m a.s.l.): hind hall, 11 ♀ and two juveniles (Nr. 232-18), hand collecting on rotten wood, 14 June 2018, leg. Ľ. Kováč, V. Papáč, A. Mock, P. Ľuptáčik, A. Parimuchová; second hall, two ♀ (Nr. 233-18), hand collecting of surface of the water pool, 14 June 2018, leg. A. Mock; entrance part, one ♀ and one ♂ (Nr.87-09), hand collecting on stony debris, 1 Oct 2009, leg. V. Papáč; Antifa Hall, four ♀ (Nr. 85-09), pitfall trap, four ♀ (Nr. 69-09), hand collecting on sediment, two ♀ and one ♂ (Nr. 70-09), hand collecting on rotten wood, Oct 2009, leg. V. Papáč; SNP Hall, eight ♀ (Nr. 23-09), hand collecting on rotten wood, 21 May 2009, leg. V. Papáč;
Muránska planina Plateau, Ľadová jama na Muráni (Studna) Cave (1,165 m a.s.l.), three ♀ and one juvenile (Nr. 273-19), hand collecting on rotten wood, 3 June 2019, leg. A. Parimuchová; one ♀ (Nr. 61-08), hand collecting on rotten wood, 7 July 2008, leg. V. Papáč;
Muránska planina Plateau, Dielik Cave (570 m a.s.l.), Hall of fossils, three ♀ (Nr. 4-10), hand collecting on clay, one ♀ (Nr. 3-10), hand collecting on surface of water pool, 5 Jan. 2001, leg. V. Papáč;
Horehronské podolie, Márnikova jaskyňa Cave (770 m a.s.l.), six ♀ and 16 juveniles (Nr. 237-18), hand collecting on speleothems, 14 June 2018, leg. V. Papáč, A. Parimuchová; three ♀ (Nr. 240-18), pitfall trap, 13 March 2018, leg. J. Lakota.

All material is deposited in the IBE FS UPJS.

3.2.2. Description

Diagnosis: Eyes and pigmentation are absent. Diagnostic characters *sensu* Gisin (1967): (1) labium with basal chaetae as **m1m2rel1l2**, chaeta **r** minute, all labial chaetae smooth, (2) basic pattern of dorsal macrochaetae as **$R_1R_2$221/32/0201+2**, with number of cephalic macrochaetae M-S-Pa varying as 221 or 111, (3) chaetal pattern of Abd. II tergum **pABq1q2**, and (4) supplementary chaeta **s** anterior to trichobothrium on Abd. IV present; labral chaetae smooth; postlabial part of head and Abd. II–V terga with numerous additional ciliated mesochaetae; posterior macrochaetae

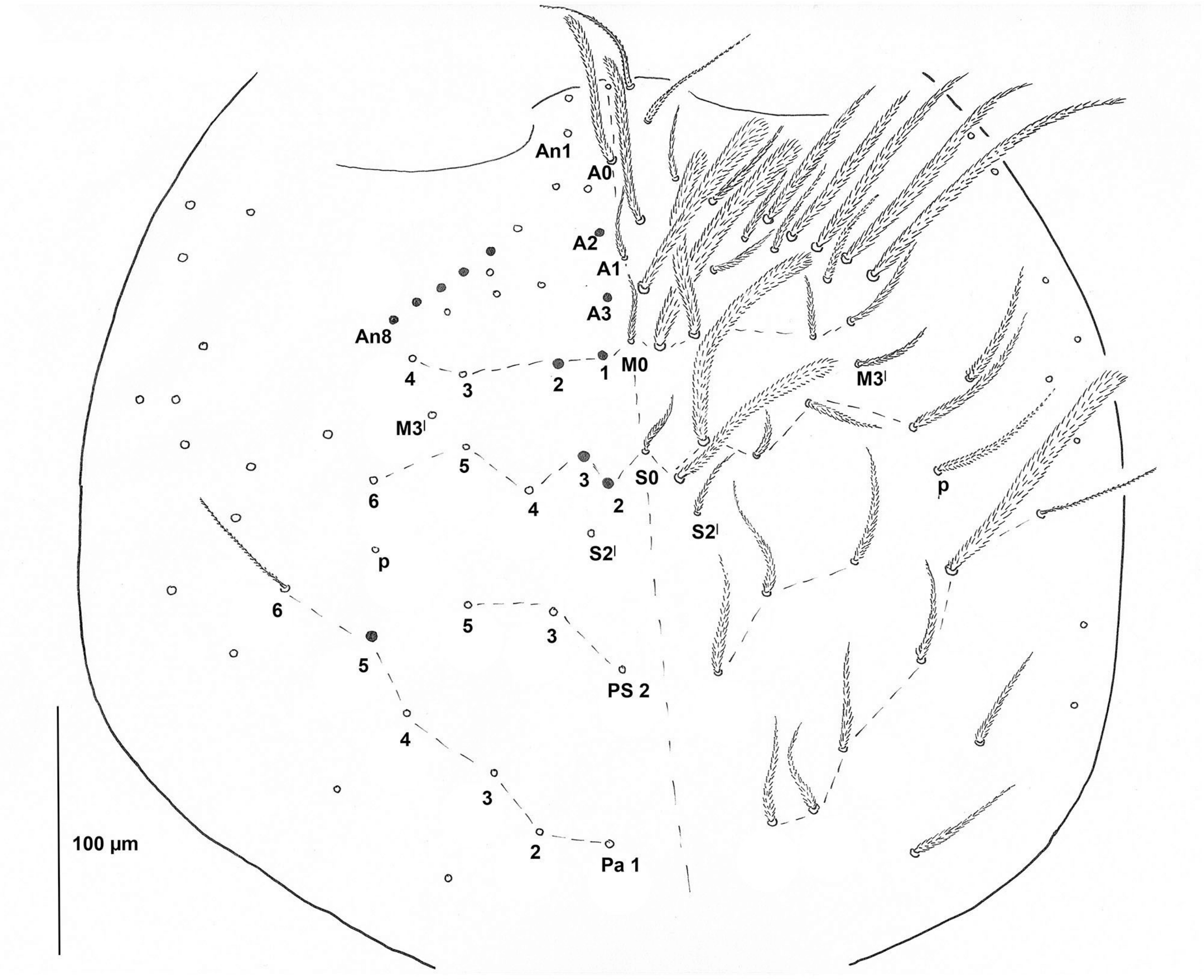

FIGURE 5
Pseudosinella muranensis, chaetotaxy of the head (*sensu* Soto-Adames, 2010). Trichobothria Pa6 drawn in full length.

of Abd. IV–V distinctly elongated; antennal segments I, II, and postlabial part of the head with conical microchaetae c**m**; unguis distinctly elongated, internal and lateral teeth absent, and basal teeth strongly reduced; external lamella of unguiculus is smooth; tibiotarsal tenent hair short and acuminate; trochanteral organ on leg III with 10 smooth microchaetae; and mucro with distinctly elongated apical teeth.

Habitus: General habitus as in Figure 4; Body length 2.1–2.4 mm (head 460 µm, thorax 550 µm, and abdomen 1,340 µm); White, without traces of pigmentation; Scales on antennae and legs absent, ventral side of manubrium with scales.

Head: Eyes absent; basic pattern of dorsal mac: A2, A3, M1, M2, S2, S3, and Pa5 that is R_1R_2221 *sensu* Gisin (1967), or R_1R_2111 (see Variability); An1–3 as mesochaetae and An4–8 as macrochaetae; Ocular area with mic **p**; Trichobothrium (Pa6) 50 µm long (Figure 5); Chaetotaxy of clypeus not seen well. Labrum with 4 prelabral and 5, 5, 4 labral chaetae, all smooth (Figure 6B); papillae on labral edge not differentiated; Pattern of basal chaetae on labium with **m1m2rel1l2** (*sensu* Gisin, 1967; Figure 6A), chaeta r minute, all chaetae of labial triangle smooth; Outer maxillary palp with one basal chaeta and three smooth sublobal hairs; Labial papilla D (*sensu* Fjellberg, 1999) with three guard chaetae, papilla E with finger-shaped lateral process, barely reaching the top of papilla and four guard chaetae; Postlabial part of head plurichaetotic, chaetae mostly ciliated, except for four smooth chaetae in G-row and smooth chaeta J1; two ciliated chaetae in **b.c.** position; and three chaetae **cm** situated apically.

Antennae: Length ratio of antennae and head diagonal (measured from apex of the labrum to posterior edge of head) 20.9; Relation of antennal segments I–IV as 1:2.2:2:3.5 (holotype); Ant. I with a ventral group of up to 20 slightly curved microsensilla and an external group of about 5 conical microchaetae (Figure 7C); External side of Ant.II with numerous curved sensilla, slightly curved microsensilla, and several conical microchaetae; distally with a dorso-external group of 5 leaf-shaped sensilla (Figure 7A); Antennal segments I and II with conical microchaetae **cm**; Ant. III organ with two thick leaf-shaped sensilla partly behind a cuticular fold, two thin guard sensilla, and 1 microsensillum (Figure 7B); Ant. III segment with numerous thin and curved sensilla, numerous straight microsensilla, and several **cm** on its

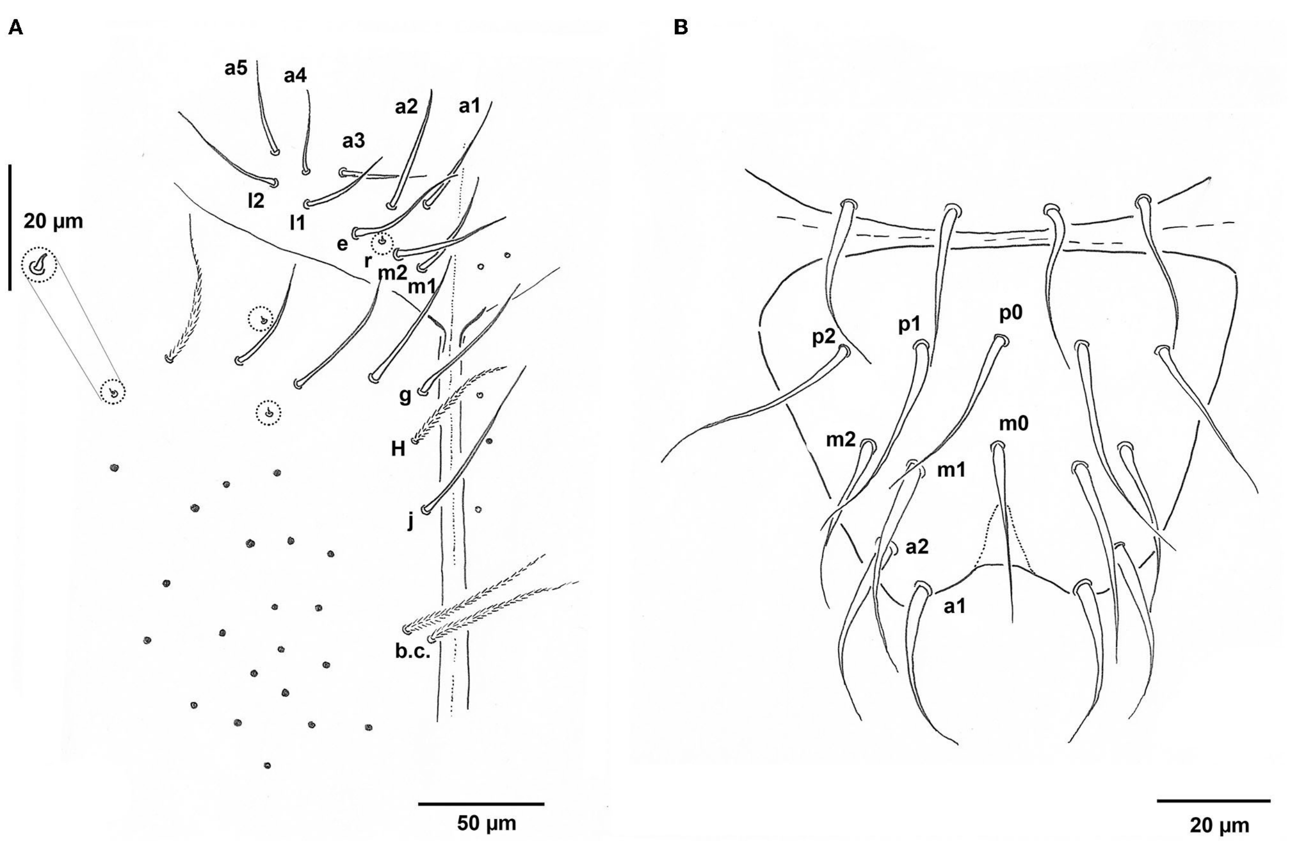

FIGURE 6
Pseudosinella muranensis. **(A)** Labium with postlabial chaetae (enlargement of microchaeta on the left); **(B)** Labrum.

dorsal side (Figure 7D); Ant. IV dorsally with seven types of chaetae: numerous long and thin blunt-tip sensilla, thick and band sensilla, smooth thin acuminate sensilla, six leaf-shaped sensilla at external side of segment, short spine-like chaetae, many ciliated mesochaetae, and apically one dorso-external modified chaeta (type a according to Cipola et al., 2020) apical bulb absent (Figure 7E).

Thorax and abdomen: Dorsal mac formula from Th. II to Abd. IV *sensu* Gisin (1967) 32/0201+2; Th. II with 3 mac, 1 sensillum **al,** and 1 microsensillum **ms** in anterolateral position; Th. III with 2 **mac** and S-chaeta **al,** S-chaeta **ms** absent.

Abd. I with lateral S-chaeta **ms**; Chaetotaxy of Abd II–III as in Figure 9A; Chaetal pattern of Abd. II between 2 dorso-medial trichobothria (m2, a5) **pABq1q2** *sensu* Gisin (1967); sensu Szeptycki (1979) as **p = a2p, A = a2, B = m3, q1 = m3e,** and **q2 = p4**; Chaeta **d3** on Abd III absent; Chaetotaxy and trichobothrial complex of Abd IV in 1st instar, juvenile, and adult developmental stages as in Figures 10, 11. Mac B5, B6, C1, D3, E2, E3, E4, and F1 are thick and long with broad sockets, and B5, B6, and D3 are extremely elongated (350 µm); Numerous thinner **mac** with smaller sockets: A1, A1’, A4, B4, C4, T5, T7, Te7, D2, De1, De3, E1, E4p2, F2, F2p, F3, F3p, Fe4, and 3 chaetae in r row; Abd IV with 4 fan-shaped supplementary chaetae anteriorly to anterior trichobothrium T2: D1, **s**, **a**, **m**; Numerous ciliated mesochaetae associated with longitudinal T-row of chaetae with two trichobothria (T2, T4); Two **as** and 1 **ps** S-chaeta, long dorsal S-chaetae on Abd. IV absent. Dorsal chaetotaxy of Abd V as in Figure 9B. Three S-chaetae present, **as**, **accp4,** and **accp5;** seven thick mac on each side, m2, m3, and m4 extremely elongated (237.5 µm), and 1 unpaired mac p0.

Length of trichobothria: Head: p6–50 µm; Abd. II: m2–100 µm, a5–115 µm; Abd. III: m2–275 µm, a5–300 µm, m5–115 µm; Abd. IV: T2–350 µm, T4–125 µm.

Tubus ventralis with scales, with 5–7 subequal ciliated chaetae on the anterior side and 10–12 subequal ciliated chaetae on the posterior side; lateral side with around 10 ciliated and 2 smooth chaetae apically per half (Figure 12B).

Legs: Legs with scales; Subcoxa I with 3–4 chaetae and 2 psp; subcoxa II with a-row of 5–6 chaetae, p-row with 4 chaetae, and 2 psp; subcoxa III with a row of 6 chaetae and 2 posterior psp; Trochanteral organ on leg III with up to 10 thin and smooth spine-like chaetae distributed in V-shaped pattern; Internal edge of Ti. I–III with numerous thick acuminate ciliated chaetae approximately as long as claw, external edge with 0,1,1 long mac (more than three times the Ti. width) with a blunt apex; Tibiotarsal tenent hair short and acuminate; Supraempodial chaeta on Ti. III smooth and acuminate, ratio of supraempodial chaeta/unguiculus around 0.9; Unguis and unguiculus as in Figure 8; Unguis narrow and distinctly elongated; Internal and lateral teeth absent, basal teeth strongly reduced: external tooth strong and internal minute; Ratio pretarsus width/unguis length from 1:2.3 to 1:3.1; Unguiculus lanceolate, broad, tapering strongly toward apex; lamellae smooth and without teeth.

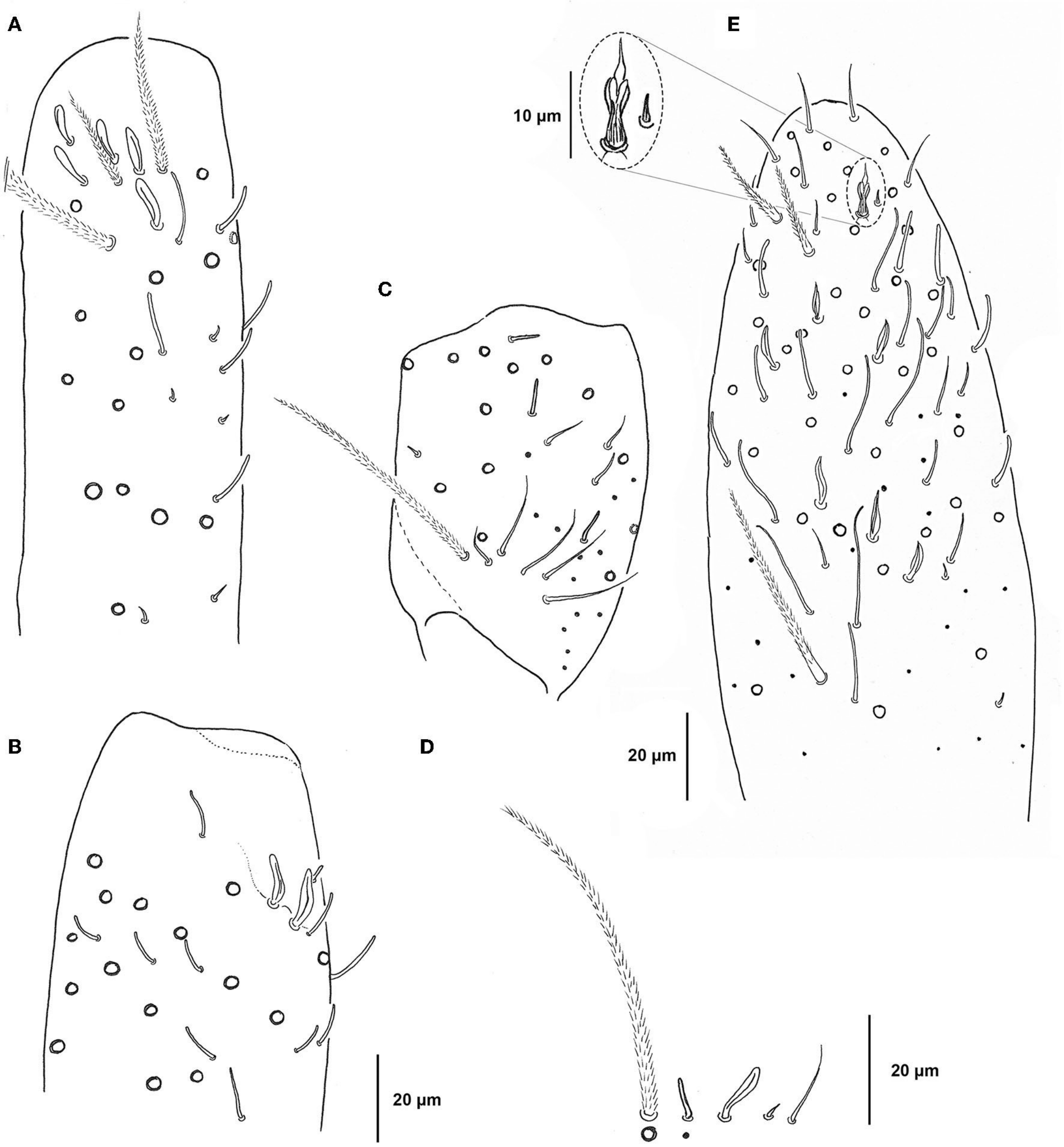

FIGURE 7
Pseudosinella muranensis, antennal segments I–III. **(A)** Apical part of right Ant.II (lateral view, all five sensilla shown); **(B)** Apical part of right Ant.III with AOIII (lateral view); **(C)** Right Ant.I (ventro-lateral view); **(D)** type of chaetae on Ant.I–III; **(E)** apical part of right Ant.IV with enlargement of subapical chaeta (empty circles—ciliated Mac, full dots—long thin sensilla).

Furca: Manubrium ventrally with scales and 2 + 2 terminal ciliated chaetae; Dorsal manubrial plate without scales, with 2 larger inner chaetae and 2 chaetae external to 2 psp, all ciliated (Figure 12A); Length of the not ringed apical part of dens about 1.5 times the length of mucro; Mucro with distinctly elongated anterior tooth; mucronal basal spine short, not reaching the tip of anteapical tooth (Figure 12D); Ratio manubrium/dens/mucro as 7:9:1.

Both sexes are known. Male genital plate, testiculus, and ductus ejaculatorius are shown in Figure 12C.

Etymology: The species is named after the Murán region in central Slovakia, where the karst area of the Muránska planina Plateau is located.

Variability: A total of 16 specimens were examined for the pattern of the dorsal mac on the head. The pattern R_1R_2221 (M1, M2, S2, S3, Pa5) was observed in 13 specimens, R_1R_2111 (M_1, S_2, Pa5) in one specimen, and a left-right asymmetry 111 vs. 011 in mac numbers was observed in two specimens.

Habitat characteristics: The species inhabits a deep zone of caves of the Muránska planina Plateau with stable microclimatic conditions, characterized by absolute humidity and relatively low air temperature of 5.5–6.5°C. It was observed in numerous specimens on rotten wood, scattered bat guano, and on the water surface of small sinter pools or puddles in cave mud.

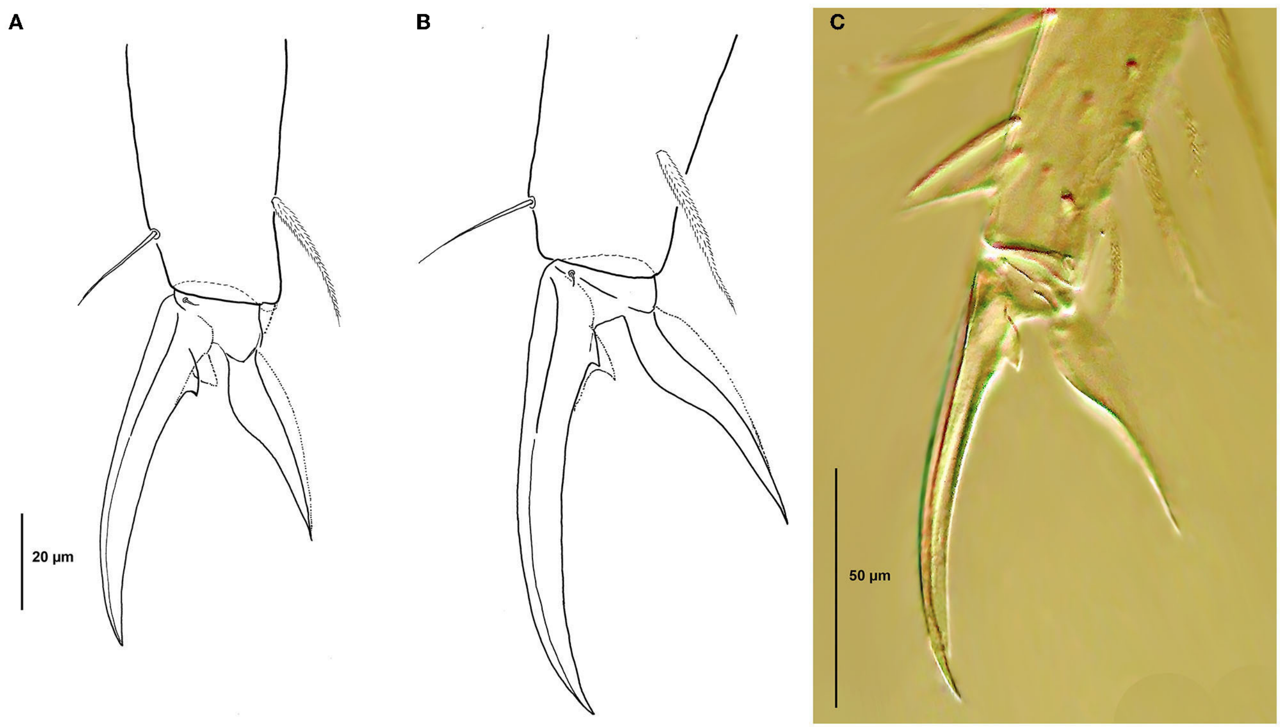

FIGURE 8
Pseudosinella muranensis, foot complex of leg I. **(A)** Shorter type from Bobačka Cave (1:2.3); **(B)** Longer type from Jelenia priepast´ Abyss (1:3); **(C)** DIC contrast image.

It was also frequently sighted on sinter decorations with percolating water.

3.2.3. Taxonomic remarks

Pseudosinella muranensis n. sp. exhibits a combination of morphological characters that represent a high degree of adaptation to deep subterranean habitats:

1. Relatively large body size (up to 2.4 mm),
2. Absence of eyes and pigmentation,
3. Smooth labral and labial chaetae,
4. Number of medial macrochaetae on terga from head to Abd.IV essentially as $R_1R_2221/32/0201+2$ *sensu* Gisin (1967),
5. Plurichaetotic postlabial part of head and Abd. II–V terga, with numerous additional ciliated mesochaetae, associated with longitudinal T-row of chaetae on Abd. IV,
6. Extremely elongated macrochaetae on Abd. IV-V,
7. Extremely elongated trichobothria m2 and a5 on Abd. III and T2 and T4 on Abd. IV,
8. Ant. I–II and postlabial part of the head with conical microchaetae **cm,**
9. Foot complex with distinctly elongated unguis, internal and lateral teeth absent, basal teeth much reduced, unguicular lamellae smooth,
10. Short and acuminate tibiotarsal tenent hair,
11. Distinctly elongated apical teeth on mucro.

The diagnostic characters of the eyeless *Pseudosinella* representatives are similar to *P. muranensis* n. sp. with the common formula of the medial macrochaetae on Th.II–Abd.IV as **32/0201+2** *sensu* Gisin (1967) are listed in Table 2. It is noteworthy that all these congeners share two other common features: (1) European distribution and (2) the chaetotaxy formula of Abd.II *sensu* to Gisin (1967) as **pABq1q2**. Furthermore, most of them are cave dwellers with endemic distributions restricted to caves/cave systems confined to small geographic areas. Combining **R221** macrochaetae on the head and the labial triangle formula as **m1m2rel1l2**, the new species is most similar to *Pseudosinella pyrenaea* (Bonet, 1931) *sensu* Beruete et al. (2002). *Pseudosinella pyrenaea* occurs in five caves in the northern part of the Navarra region, Spain, suggesting the convergent evolution of the subterranean taxa. The new species also resembles *P. paclti* (Rusek, 1961), but this species differs, among other characters, in the formula of the labial triangle **M1m2rEL1L2** and the shape of the foot complex. The differences in important characters between Western-Carpathian relatives are provided in Table 2. Another apparently troglomorphic species, *P. aggtelekiensis*, differs from these congeners mainly in the macrochaetotaxy of the Abd.IV tergum. With respect to Gisin's nomenclatural system, this species is characteristic of the macrochaetae formula **R111/32/0201+3** and the labial triangle setae **M1M2reL1L2**. Among other features, it also has a characteristic shape of the foot complex (Kováč and Rusek, 2012).

FIGURE 9

Pseudosinella muranensis, chaetotaxy of Abd.II–III and Abd V. **(A)** Abd.II–III terga *sensu* Soto-Adames (2010), in parentheses *sensu* Gisin (1967). The additional chaetae are marked with an asterisk; **(B)** Abd.V tergum. Red squares, long Mac (for symbols see legend in Figure 11).

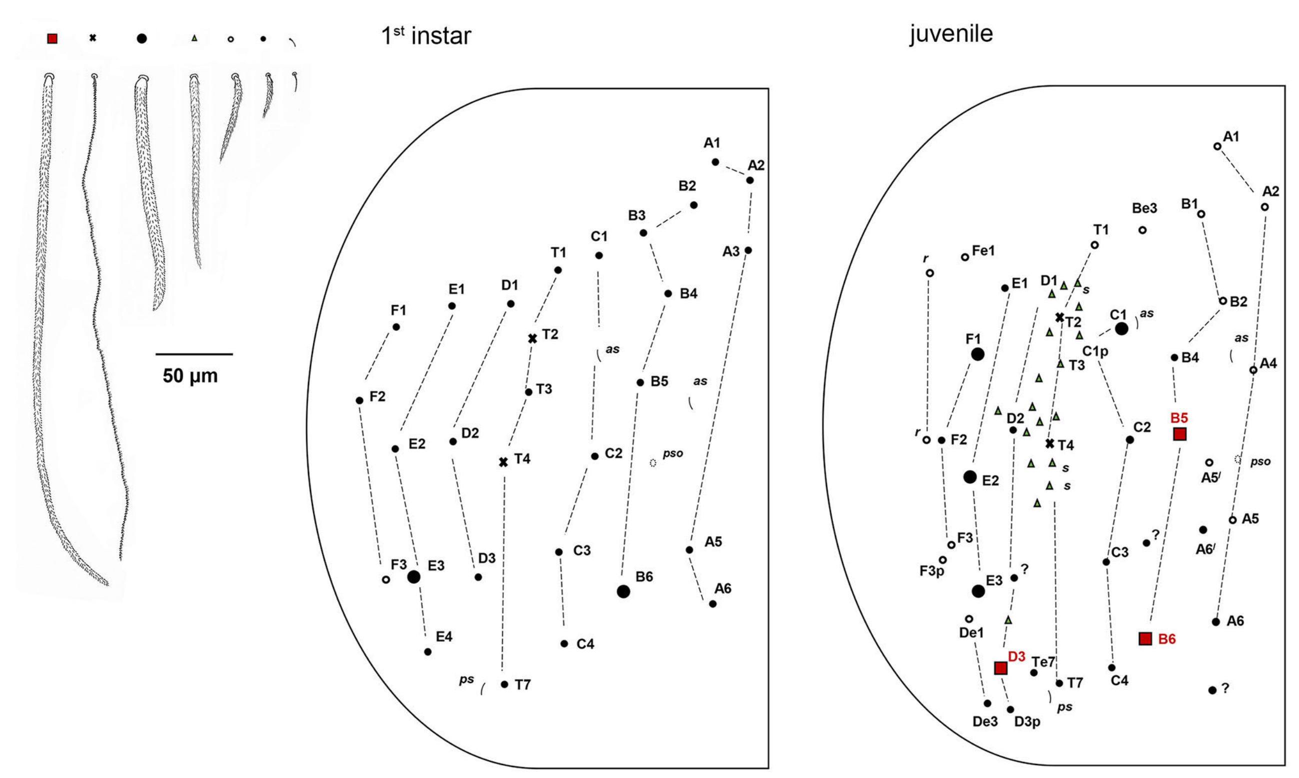

FIGURE 10
Pseudosinella muranensis, chaetotaxy of Abd.IV tergum in 1st instar and juvenile. Upper left—legend to the symbols used in the schemes.

3.2.4. Geographic distribution

Pseudosinella muranensis n. sp. is a short-range endemic species, whose distribution is restricted to the Muránska planina Plateau in the central Western Carpathians. This small karst has an area of 420 km^2 and hosts ∼500 caves (Bella et al., 2018).

Figure 13 shows the distribution of the highly troglomorphic *Pseudosinella* representatives in Europe. Their placement in this category was judged essentially by the shape of the foot complex and the shape of the mucro, and the selection follows Deharveng (1988), Deharveng and Thibaud (1989), and Christian (2002).

(1) Picos de Europa
Pseudosinella chapmani Gama, 1979; Santander, Spain;
(2) Pyrenees
Pseudosinella bessoni Deharveng, 1988; Grotte du Pic d'Orby, Atlantic Pyrenees, France;
Pseudosinella cabidochei Deharveng, 1986; Gouffre de la Pierre Saint-Martin, Atlantic Pyrenees, France;
Pseudosinella unguilonginea Jordana and Beruete, 1983; Navarra, Atlantic Pyrenees, Spain;
(3) Massif Central
Pseudosinella kersmaekersi Stomp, 1974; Grotte du Peintre, Grands Causses, Massif Central, France;
(4) Alps
Pseudosinella carthusiana Gisin, 1963; Grotte du Guiers-vif, Grande-Chartreuse, France;
Pseudosinella salisburgiana Gisin and Gama, 1969; Eisriesenwelt, Totengebirge and Schenkofen cave, Hagengebirge, Austria;
Pseudosinella aueri Gisin, 1964; Almberghöhle, Styria, Austria.

It is evident that the distribution of these forms correlates with the "mid-latitude biodiversity ridge in terrestrial cave fauna" defined by Culver et al. (2006), which includes the main mountain ridges from the Pyrenees to the Alps. The discovery of *P. muranensis* highlights the biogeographic importance of the Western Carpathians for the evolution of invertebrates highly adapted to a subterranean environment.

4. Discussion

4.1. Integrative taxonomy and phylogeny of the cave *Pseudosinella* populations

Cave-dwelling springtails provide excellent material for the study of adaptation (Christiansen and Culver, 1968). Our study of the *Pseudosinella* populations in the caves of the Western Carpathians, in which we took an integrative taxonomy approach, was primarily aimed at answering the question of whether this geologically complex area in Central Europe harbors ancient cave lineages, whose origin may predate the Quaternary. We also wanted to understand the importance of this area for the evolution of the highly adapted subterranean fauna.

As a first step, we examined the morphological characters of local *Pseudosinella* populations. Some characters are obviously unstable, such as the relative length of the antennae, and the relative length and shape of unguis that could be an expression

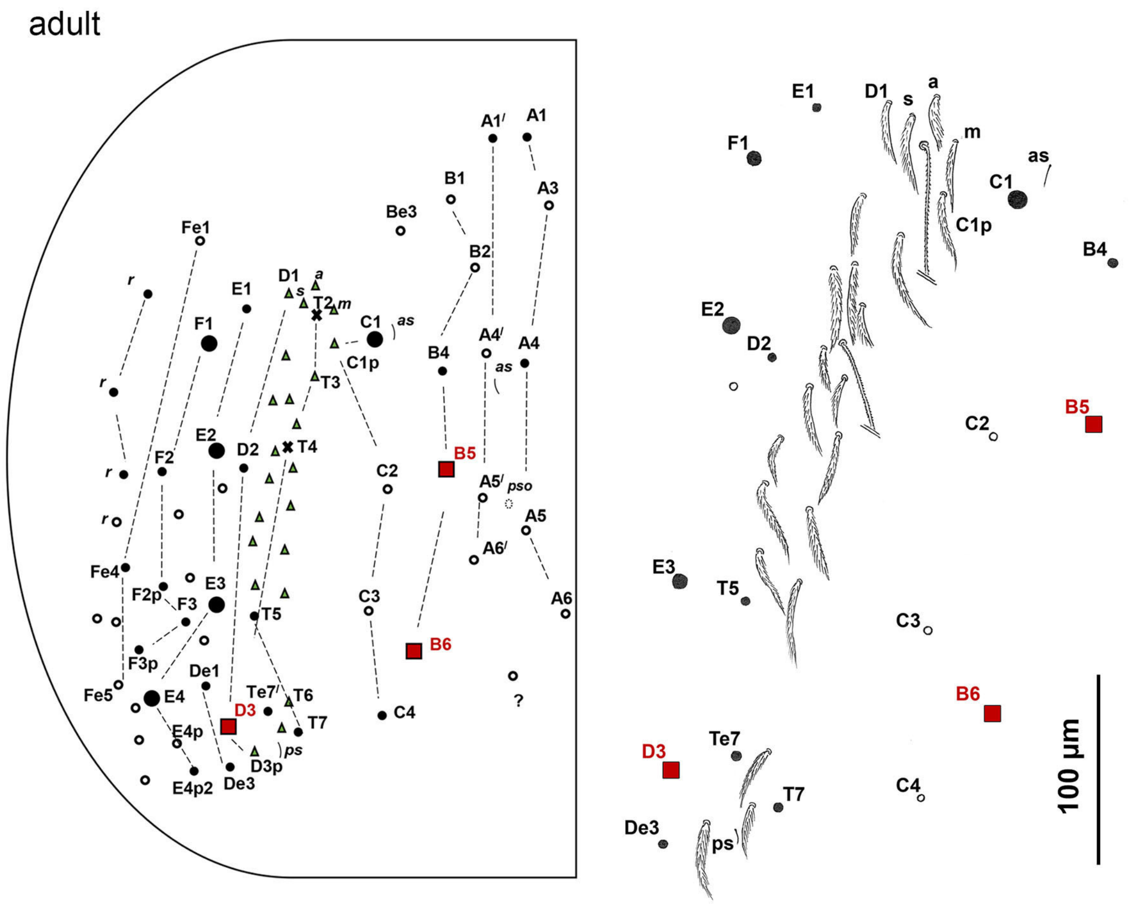

FIGURE 11
Pseudosinella muranensis, chaetotaxy of Abd.IV terga in adults, enlargement of the area with accessory chaetae **(right)**.

of phenotypic plasticity. In contrast, macrochaetotaxy of the terga and labial chaetotaxy are considered complex, stable characters traditionally used for species diagnosis in *Pseudosinella* (Gisin, 1964, 1967; Soto-Adames, 2010). Recently, Zhang and Deharveng (2015) drew attention to the chaetotaxy of tergal sensilla (pattern of S-chaetae) as a powerful tool for revealing the phylogeny and systematics of the family Entomobryidae, which also seems to be very useful for the taxonomy of *Pseudosinella*. In the description of *P. muranensis*, we adopted the approach of Szeptycki (1979) for a detailed study of postembryonic development of tergal chaetotaxy in abdominal segments IV and V from the first instar to the adult stage. This approach helped us characterize the chaetotaxy complexity and assess the degree of adaptation to the cave environment. In this highly troglomorphic species, we observed the multiplication of ordinary ciliated chaetae on the last abdominal terga instead of the simplification of chaetotaxy seen in edaphic *Pseudosinella* counterparts such as *Pseudosinella alba* (Packard, 1873) (Szeptycki, 1979). The enormous elongation of anterior trichobothria on abdominal segments III–IV and posterior macrochaetae on abdominal segments IV–V is very characteristic of this newly described species.

In the molecular section, we identified eight to nine *Pseudosinella* MOTUs using barcoding-gap (ASAP) or evolutionary (bPTP) delimitation analyses, corresponding to observed differences in morphological characters among cave populations and their degree of geographic isolation. Populations of *P. aggtelekiensis* geographically confined to the Slovak-Aggtelek karst unit were also supported by ASAP and morphology. However, the bPTP method suggested two distinct MOTUs within this clade, one of which specifically inhabits Ardovská Cave (AC). This is likely a result of the isolation of the local population from the other four populations due to distance, specifically the 18.4 km (11.4 miles) to the nearest Hrušovská Cave (HRU) population in the same clade. To get a better idea, the five *P. aggtelekiensis* populations were located 7.7–37.7 km (4.8–23.6 miles) from the type locality, the Domica-Baradla Cave System. Based on morphology, the populations from the central Western Carpathians (Velká Fatra, Low Tatras, and Kozie chrbty Mts.) belonged to *P. paclti*, originally described from the Demänová Cave System in the Low Tatras. The four *P. paclti* populations were located 1.7–45.2 km (1.1–28.3 miles) from the type locality. However, both molecular delimitation methods signaled the presence of two other MOTUs in two caves of the Kozie chrbty Mts. At the moment, we are not able to distinguish these taxa morphologically, nor can we explain this sympatric pattern of MOTUs. The populations of *P. muranensis*, morphologically strongly adapted to caves, described in this

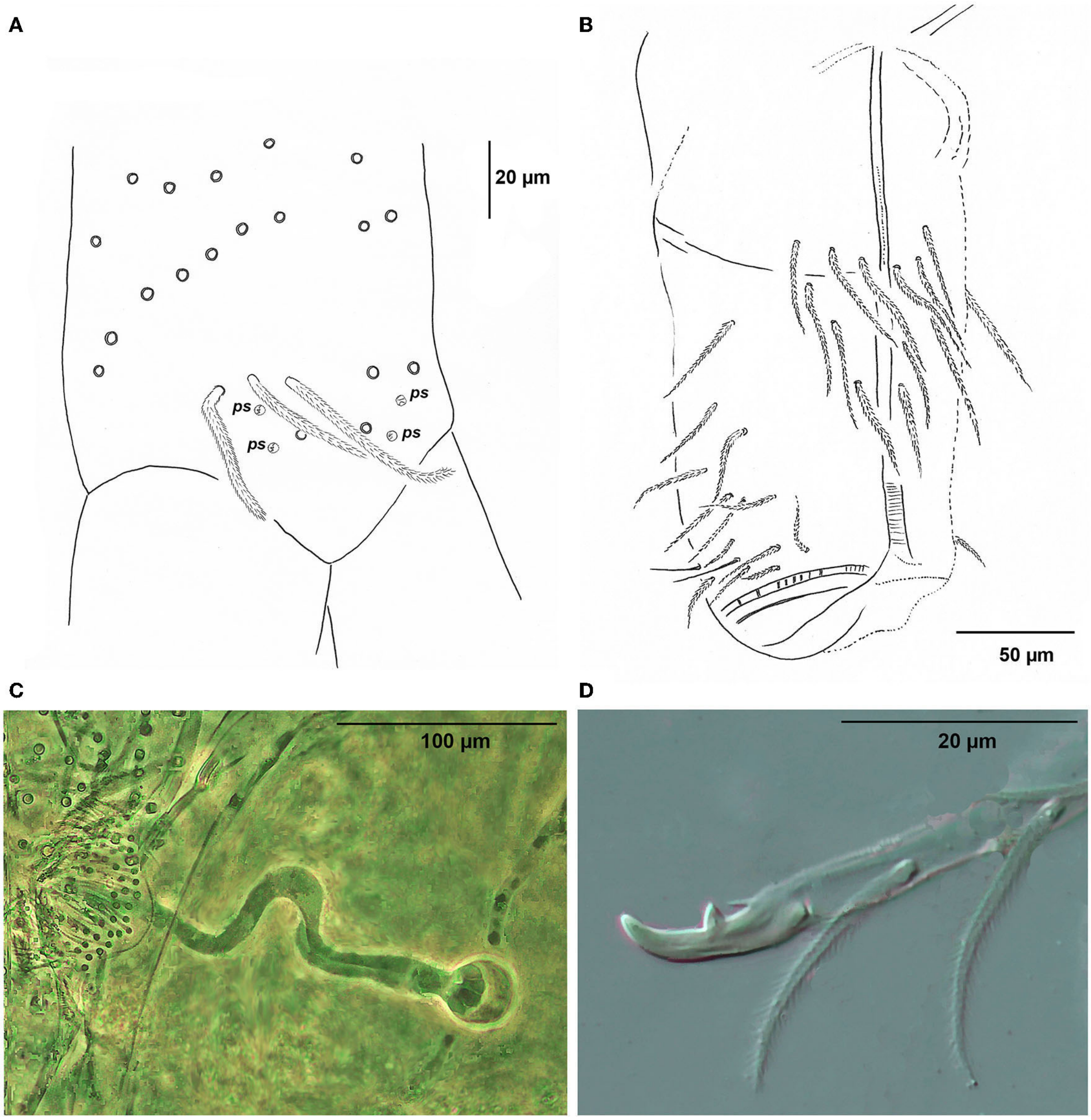

FIGURE 12
Pseudosinella muranensis, **(A)** Manubrium, dorsal chaetotaxy of apical part; ps, pseudopores; **(B)** Chaetotaxy of ventral tube (antero-lateral view); **(C)** Male genital plate with testiculus and ductus ejaculatorius (phase–contrast image); **(D)** Mucro and apical part of dens, lateral view (DIC contrast image).

work formed a compact clade, well-supported by molecular and morphological criteria, as well as by the geographic location of the caves in the Muránska planina Plateau in the central Western Carpathians. We also found three undescribed *Pseudosinella* species. The first is restricted to the Ochtinská aragonite Cave (SK) in a small area of isolated cryptokarst of Devonian origin in southern Slovakia. The populations of the second species, on the other hand, are endemic to the Brekovský kras Karst, a small and well-isolated karst area in eastern Slovakia. The third species was found in the Drienovská jaskyňa Cave (DR) in the eastern part of the Slovak-Aggtelek Karst, but not in sympatry with *P. aggtelekienesis*, which is characteristic of caves in this karst unit. All three species are well supported by both molecular and morphological delimitation criteria. The subterranean *Pseudosinella* from the Western-Carpathian caves were allocated into two main lineages: (1) the "*paclti*" lineage, restricted to the central Western Carpathian, and (2) the "*aggtelekiensis*" lineage, confined to the southern karst areas of the Western Carpathians. Thus, our study supports the main conclusion of Soto-Adames (2002) that several morphological characters traditionally used for species diagnosis in the genera *Pseudosinella* and *Lepidocyrtus* are very conservative indicators of genetic divergence and have a strong phylogenetic signal.

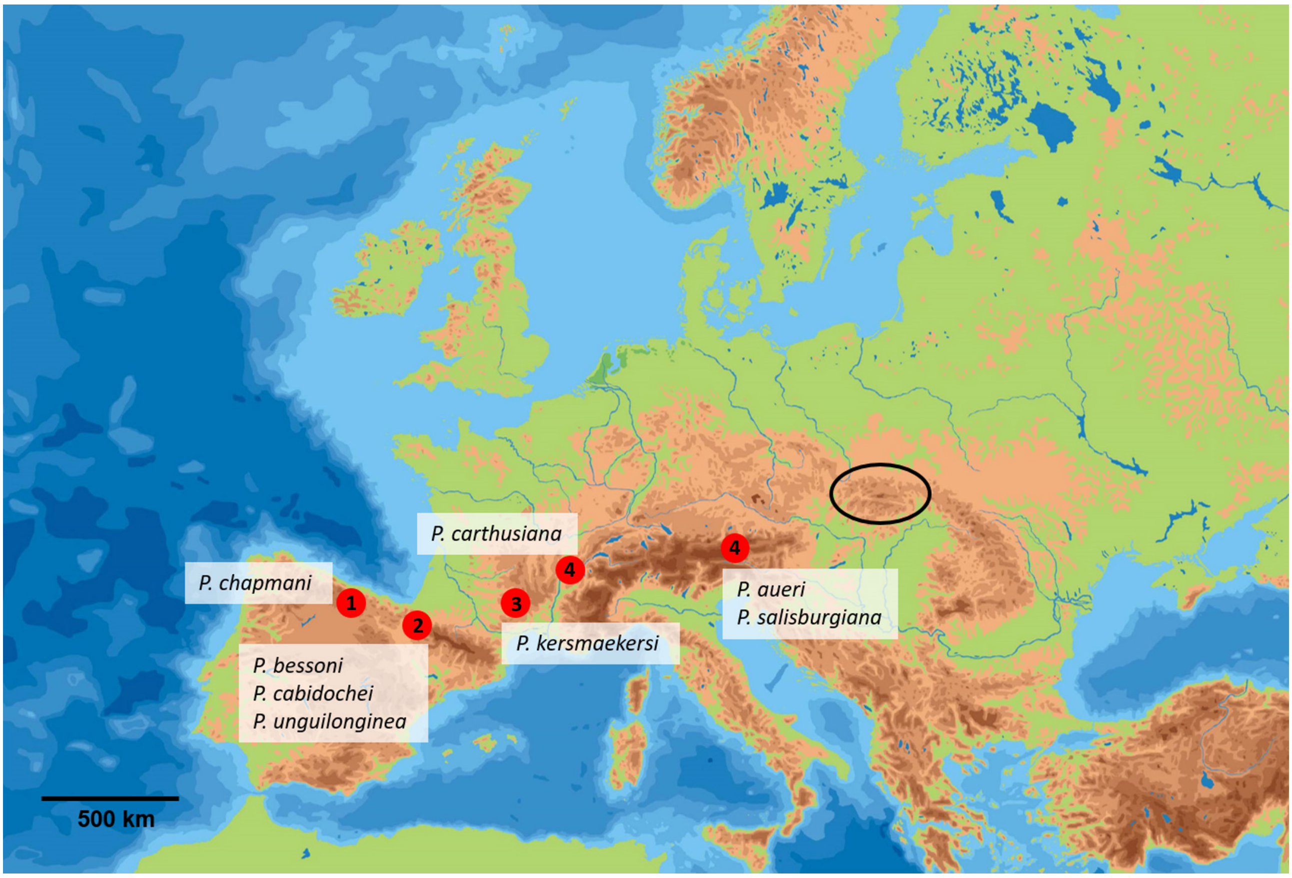

FIGURE 13
Distribution of the highly troglomorphic *Pseudosinella* representatives in Europe. Red dots represent localities, the black ellipse represents the Western Carpathians. (1) Picos de Europa; (2) Pyrenees; (3) Massif Central; (4) Alps (background map from gifex.com).

4.2. Troglomorphy in the Western-Carpathian *Pseudosinella*

Troglomorphisms represent a set of characteristic morphological modifications that are among the most peculiar adaptations of animals to a deep subterranean environment and occur in many phylogenetically unrelated groups of organisms (Christiansen, 1961, 1992; Deharveng and Bedos, 2018; Ribera et al., 2018). Therefore, morphological convergences may have evolved independently in different *Pseudosinella* lineages (Christiansen, 1992).

In terrestrial cave invertebrates, these modifications are divided into (1) regressive traits, such as reduction of eyes and body pigment, and (2) constructive traits, such as enlargement of the body, elongation of appendages, and claws on the legs. Collembola exhibits a great diversity of regressive troglomorphic traits, which results in high taxonomic diversity of troglobiotic forms in this group (Deharveng and Bedos, 2018). In addition, Collembola shows a number of specific constructive troglomorphic modifications such as hypertrophy and multiplication of antennal sensilla, and enlargement of paired sensilla in the Ant. III organ, elongation of unguis, reduction and basal shift of the two internal unguicular teeth, regression of the tenent hairs on the tibiotarsi, and elongation of the distal mucronal tooth (Christiansen and Culver, 1987; Deharveng, 1988; Christiansen, 2012).

It is important to note that each troglomorphic trait evolves independently within the cave lineages (Deharveng and Bedos, 2018). For example, anophthalmy without elongated appendages is common in the cave and edaphic species, whereas some cave species are oculate and weakly pigmented but have elongated appendages, e.g., *Pseudosinella theodoridesi* (Gisin and Gama, 1969), which inhabits caves in Haute-Garonne and Ariége in France. As Deharveng and Bedos (2018) pointed out, extreme appendage elongation is not necessarily accompanied by extreme elongation of unguis, and these differences in evolutionary changes result in complex patterns of troglomorphy. Indeed, patterns of troglomorphy are determined by the characteristics of the microhabitat that species inhabit (Deharveng, 1988; Lukić et al., 2018). The elongation of the unguis is clearly an advantage for locomotion over moist substrates or open water; this trait is also typical of aboveground Collembola inhabiting aquatic biotopes (Christiansen, 1961). However, the shape of the foot complex can vary within populations of the same *Pseudosinella* species (Soto-Adames, 2010). Christiansen and Culver (1987) defined three stages of troglomorphy seen in cave Entomobryinae. The newly described

P. muranensis apparently shows a high level of troglomorphy with distinct morphological adaptations to a cave environment.

4.3. Distribution pattern of cave *Pseudosinella*

The distribution pattern of cave *Pseudosinella* in the Western Carpathians follows one of the basic scenarios whereby aboveground species that colonize subterranean habitats can undergo adaptive radiation and become endemic to a cave system (Christiansen and Culver, 1968). Christiansen and Culver (1987) demonstrated a clear biogeographic pattern of decreasing geographic range with increasing troglomorphy in *Pseudosinella* species in caves of eastern North America, which can be explained by the assumption that increasing troglomorphy decreases dispersal ability. Apparently, parallel speciation occurred in the Western Carpathians, with morphologically similar species evolving independently in different caves, in response to a common selective pressure according to the evolutionary scenario described by Christiansen (1961, 1992). Patterns of genetic differentiation in troglobionts are likely driven primarily by isolation due to physical barriers and reflect vicariant speciation (Katz et al., 2018). This is because obligate cave invertebrates have very limited dispersal abilities and thus have limited ability to migrate between caves; as a result, they may display extreme genetic divergence even across small areas (e.g., Balogh et al., 2020). We observed sympatry of two troglobiotic *Pseudosinella* in only two caves in the Kozie Chrbty Mts., which are among the northernmost sites with troglomorphic invertebrates in Europe (Kováč et al., 2014). This essentially allopatric distribution pattern of *Pseudosinella* in the Western Carpathians suggests that isolation was the main driving force for the evolution of local lineages. Such a distribution indicates the absence of recent and historical gene flow and supports the idea of speciation by vicariance at the time of habitat colonization (Guzik et al., 2021). On the other hand, the co-ocurrence of multiple troglobiotic species as a result of subterranean adaptive radiation would support the "cave as island" hypothesis (Culver and Pipan, 2009).

In our study, a highly troglomorphic species *P. muranensis* belonging to the "*paclti*" phyletic lineage was discovered. It inhabits caves in the Muránska planina Plateau, a small karst area in the central Western Carpathians. *Pseudosinella aggtelekiensis* (Stach, 1929), a species with advanced troglomorphy, is also a short-range endemic, confined to most caves of the Slovak and Aggtelek Karst in the southern part of the Western Carpathians. In contrast, *P. paclti* and three undescribed species of the *P. aggtelekiensis*–group (sp. 1, 2, and 3) occur in more or less "peripheral" karst areas. Thus, this pattern is basically consistent with the concept of adaptive novelty, according to which more troglomorphic *Pseudosinella* forms in caves in the eastern USA tend to be distributed in the center of the area and less troglomorphic ones in the periphery (Christiansen and Culver, 1968, 1987). This pattern is also confirmed by the distribution of the highly troglomorphic collembolan *Deuteraphorura muranensis* (Parimuchová and Kováč, 2020) (fam. Onychiuridae), which is also endemic to the small karst area of the Muránska planina Plateau in the central part of the Western Carpathians Mts. It has the most pronounced troglomorphic traits of all known subterranean *Deuteraphorura* congeners (Parimuchová et al., 2020). The local caves studied are situated at elevations above 700 m and have relatively low internal air temperatures (Table 1), conditions comparable to other mountain caves in the surrounding regions. The long-term historical isolation of the local karst may be a clue to this unusual distribution pattern of highly troglomorphic taxa, despite its partial connection with the neighboring karst areas of Tisovský kras in the southwest and Slovak Paradise in the northeast, both of which are composed of the same Triassic limestone formations.

The distribution of three undescribed *Pseudosinella* species in southern karst areas contradicts the observation of Christman et al. (2005) that single-cave endemism is not high in isolated cave areas on the periphery. To obtain an overall picture, it is important to note that troglomorphic *Pseudosinella* representatives do not occur in the karst of the Tatras Mts. that form the northern ridges of the Western Carpathians (Kováč et al., 2016). The general pattern of a few terrestrial troglobionts in the northern karst areas of Western Carpathians is usually explained by the proximity to continental glaciers during the Pleistocene glacial events.

The distribution of troglomorphic *Pseudosinella* in Europe, their low diversity, and the absence of highly troglomorphic forms in the rest of the Carpathians (Ukraine, Romania) suggest a possible colonization of the Western Carpathians by an ancestral lineage from the Eastern Alps. However, a thorough integrative study of *Pseudosinella* populations from these areas is needed to clarify this assumption. The diversity of cave representatives of this genus in the Balkan Peninsula is still unclear, and so far no highly troglomorphic *Pseudosinella* species is known from the Dinaric caves (Lukić and Deharveng, 2008).

4.4. Divergence time of the subterranean *Pseudosinella* lineages

In general, many subterranean taxa apparently represent old phylogenetic lineages that have persisted for long periods in subterranean refugia (Gibert and Deharveng, 2002; Holsinger, 2012). The distribution range size of a subterranean animal is a good indicator of the age of its lineage, and increasing troglomorphy suggests increasingly earlier times of initial cave colonization (Christiansen and Culver, 1987; Christian, 2002). However, as Trajano (2007) noted, there are limitations of using troglomorphy to estimate phylogenetic age because of the complexity of the mechanisms underlying morphological differentiation, i.e., a complex balance among various genetic, ecological, and biological factors that produce the actual divergence rates. The evolution rate of a troglomorphism may depend on the environment or way of life of a species and its ancestors. For example, old species may be less troglomorphic than younger ones if they have a slower rate of phenotypic evolution (Pipan and Culver, 2012; Ribera et al., 2018).

It is also considered that a putative ancestral species is no longer found near the distributional ranges of troglomorphic species (Christiansen, 2012). However, this assumption still needs to be supported by a molecular study of the cave and edaphic populations of *Pseudosinella* (and edaphic populations of *Lepidocyrtus*) in terms of their phyletic relationships to reveal the evolutionary scenario of these lineages in the Western Carpathian, which is indeed a challenging task.

Estimating the divergence time of the phyletic lineages of *Pseudosinella* populations was one of the main objectives of the present study. Soto-Adames (2002) applied 2.3% sequence divergence per million years as the most commonly used calibration for the COI molecular clock in the *Lepidocyrtus*/*Pseudosinella* complex. In this study, we calibrated the divergence time according to Katz (2020), who estimated relative substitution rates in the COI unit inferred by Bayesian analysis over a topologically constrained phylogeny of Hexapoda using a relaxed molecular clock model. Rates for Collembola were not significantly different from the average rate or from rates estimated for most other groups compared. This suggests that clocks calibrated using "universal" insect rates may be appropriate for estimating evolutionary time scales in this group.

The troglomorphic *Pseudosinella* populations of the Western Carpathians occur exclusively in karst caves developed in Triassic limestone formations of marine origin, suggesting that the geological history of the area had a crucial role in the formation of their present distribution pattern. The phylogeny study of the genus *Pseudosinella* (Hexapoda, Collembola) in the Western Carpathian caves revealed an initial split of an ancient lineage in the Middle Miocene into lineages "*paclti*" and "*aggtelekiensis,*" with the common ancestor colonizing the area about 14.5 Mya. This finding thus supports our assumption that the local troglomorphic *Pseudosinella* populations are descendants of ancient lineages originating before the Quaternary (Kováč et al., 2016). Indeed, the history of a site has a major influence on the history of a lineage, as the characteristics of past environments (e.g., geological setting, contemporary global climate, and biotic composition) have significantly influenced the survival, dispersal, and diversification of phylogenetic lineages that occurred at these sites (Lomolino et al., 2010).

Undoubtedly, the initial diversification within the collembolan genus *Pseudosinella* in the Miocene was associated with complex geological (tectonic) events in the Paratethys region. This is consistent with the uplift of Triassic limestones during the early formation of the Western Carpathians (Popov et al., 2004) and most likely also with the initial development of caves during this time. Continentalization of Europe during the Neogene, triggered by the Alpidic orogeny and the simultaneous retreat of the Paratethys Sea, led to isolated evolution and an increase in the endemicity of the entire local biota (Neubauer et al., 2015). The central Western Carpathians formed an island in the Paratethys during the Burdigalian and Langhian stages of the Miocene, which ranged from 20.4 to 13.6 Mya (Kováč et al., 2017). This long-term isolation undoubtedly led to the independent evolution of the local biota after previous colonization of the territory by lineages from southern European orogenic units (e.g., Kováč, et al. 2014; Haase et al., 2021). The onset of cooling of the contemporary global climate, known as the Middle Miocene Climatic Transition—MMCT (Methner et al., 2020), could promote the colonization of deep subterranean spaces by edaphic invertebrates. Similarly, the large number of *Pseudosinella* subterranean species recorded in the subterranean calcretes of Western Australia is also explained by more rapid climatic changes in history (Guzik et al., 2021). The overall phylogenetic history of the genus *Pseudosinella* is likely longer than shown in our study and extends back to the Oligocene, indicating independent diversification of certain lineages from more than one ancestor and presumed polyphyly of the entire genus, similar to that observed by Guzik et al. (2021). Indeed, the phylogeny of *Pseudosinella* reflects the paleogeographic context. For example, all eyeless relatives with an identical number of dorsal macrochaetae from thorax to abdomen, 32/0201+2 *sensu* Gisin (1967), are European representatives (Table 2).

In their synthesis of the richness of European troglobionts, Culver et al. (2006) assessed the part of Central Europe north of the Dinaric Mountains as an area with very few or no troglobionts, and the reduction in the number of these forms was largely considered to be the result of Pleistocene glaciation events (e.g., Deharveng et al., 2012). However, our study highlights the biogeographic importance of the Western Carpathians as a major speciation center of troglomorphic troglobionts, located at their northern distribution limit in Europe. Not only do highly troglomorphic taxa occur here but the diversity of troglomorphic obligate cave invertebrates includes more than 20 terrestrial species with endemic distribution (Kováč et al., 2014). The palpigrade *Eukoenenia spelaea* (Peyerimhoff, 1902), the pseudoscorpion *Neobisium slovacum* (Gulička, 1977), the rhagidiid mite *Foveacheles troglodyta* (Zacharda, 1988), the millipede *Hylaebainosoma gulickai* (Tajovský et al., 2014), and the collembolans *Megalothorax tatrensis* (Papáč and Kováč, 2013), *D. muranensis* (Parimuchová and Kováč, 2020), *P. aggtelekiensis* (Stach, 1929), *Neelus koseli* (Kováč and Papáč, 2010), and a newly described *Pseudosinella* species are among the local taxa that are very well adapted to the cave environment.

We are aware of certain limitations of the present study. Further studies with larger population datasets and incorporating of additional molecular markers may shed more light on the questions of subterranean biodiversity and phylogeny of the genus *Pseudosinella* and the complexity of troglomorphic adaptations in relation to cave microhabitat structure. Subterranean collembolans of the genus *Pseudosinella* are indeed suitable organisms for studies of evolutionary processes and population genetics, and the same approach can be extended to other subterranean taxa distributed in the Western Carpathians, on the northern European frontier of troglomorphy.

5. Conclusion

The present integrative taxonomic study focused on the subterranean populations of the genus *Pseudosinella* (Hexapoda, Collembola) inhabiting caves in the Western Carpathians in Central Europe. Eight to nine molecular operational taxonomic units (MOTUs) were identified, three of which are undescribed species.

In addition to the typical troglomorphic characters, the newly described subterranean species *P. muranensis* is characterized in the adult stage by a multiplication of the common chaetae on the last abdominal terga, which is probably a common feature of the highly troglomorphic conspecifics. The species is endemic to a small karst area in the central Western Carpathians documenting the short-range distribution pattern of highly evolved troglomorphisms.

Age calibration of the *Pseudosinella* phylogeny in the Western Carpathian caves indicates diversification of the ancient *Lepidocyrtus*/*Pseudosinella* lineage in the Middle Miocene, supporting our hypothesis that local troglomorphic *Pseudosinella* populations are of ancient, pre-Quaternary origin.

Moreover, our study supports the assumption that morphological characters traditionally used in species diagnosis in the genus *Pseudosinella* are conservative indicators of genetic divergence.

Finally, this study highlights the role of the Western Carpathians as an important speciation center of the troglomorphic subterranean fauna at the northern distributional limit in Europe.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on animals in accordance with the local legislation and institutional requirements.

Author contributions

ĽK and AP designed the study. ĽK, AP, and VP collected the data and analyzed morphological traits. AP, NR, and NJ extracted mtDNA and prepared the molecular data. MŽ and AP analyzed the molecular data. ĽK wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1169911/full#supplementary-material

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Adaptive shift of active electroreception in weakly electric fish for troglobitic life

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The adaptive-shift hypothesis for the evolution of cave-dwelling species posits that ancestor species in surface habitats had exaptations for subterranean life that were exploited when individuals invaded caves. Weakly electric Gymnotiform fishes, nocturnal South American teleost fishes, have features that appear to be likely exaptations for troglobitic life. These fishes have active electrosensory systems in which fish generate weak electric fields that are detected by specialized electroreceptors. Gymnotiform fishes use their electric fields for navigation, prey capture (scene analysis), and social communication. Although active electrosensory systems appear to be exaptations for troglobitic life, as fish use these systems to "see in the dark", producing electric fields is energetically costly. Cave habitats, which often are low in resources, may not be able to support such high energetic demands. *Eigenmannia vicentespelaea*, a species of weakly electric fish that is endemic to the São Vicente II cave in central Brazil, surprisingly generates stronger electric fields than their surface relatives. The increase in strength of electric fields may result simply from differences in size between cave and surface populations, but may also be due to lack of predation pressure in the cave or increases in "sensory volumes" and acuity that improve prey localization and capture. *Eigenmannia vicentespelaea* exhibits the classical phenotypes of any troglobitic fish: these fish have small to nonexistent eyes and loss of pigmentation. The closest living surface relative, *Eigenmannia trilineata*, inhabits streams nearby and has eyes and pigmentation. The electrosensory and locomotor behavior of both species of fish were measured in their natural habitats using a grid recording system. Surface *Eigenmannia* exhibited dramatic circadian changes in social behavior, such as hiding under rocks during the day and foraging in groups at night, while cave *Eigenmannia* displayed territorial behavior with no apparent circadian modulations. The territorial behavior involved electrical and movement-based interactions that may be a form of boundary patrolling. Electrosocial behavior and scene analysis are mechanistically interlinked because both stem from active sensing tactics.

KEYWORDS

cave, weakly electric fish, evolution, EOD, territory, troglomorphism

Introduction

Darwin was once befuddled by the unusual phenotypes of cave organisms. He labeled them "*wrecks of ancient life*" and remarked "*As it is difficult to imagine that eyes, though useless, could in any way be injurious to animals living in darkness, I attribute their loss solely to disuse*" (Darwin, 1859). *Absit omen*, we disagree with Darwin – we would like to contend that cave animals are not a wreck, but simply elegant phenotypes that evolved to retain the most important aspects of life. Troglobites feature unusual and idiosyncratic morphologies that arise from a life history adapted to perpetually dark habitats. They brandish regressive features, such as the loss of eyes and pigmentation, as well as compensatory traits, such as a lower threshold for mechanosensation (Culver et al., 1995; Culver and Pipan, 2009; Haspel et al., 2012; Soares and Niemiller, 2013; Niemiller and Soares, 2015; Soares and Niemiller, 2020).

Cave organisms are recognized as unique model systems for testing evolutionary, ecological, and biogeographic hypotheses (Poulson and White, 1969; Jeffery, 2009; Juan and Emerson, 2010; Juan et al., 2010) and are promising systems to increase our understanding of evolutionary and historical processes that promote or constrain biodiversity (Juan et al., 2010). Despite increased interest and study of troglobites in recent years, subterranean biodiversity remains one of the most poorly understood faunas on the planet. Fishes are routinely prominent members of aquatic cave communities, and currently, over 300 species in 10 orders and 22 families (Proudlove, 2010: Soares and Niemiller, 2013) have been identified. Here we review intriguing electrosocial features of a species of weakly electric fish, *Eigenmannia vicentespelaea*, which evolved in a single cave system in central Brazil (Figure 1) and that is yet to be thoroughly studied (Bichuette and Trajano, 2006; Bichuette and Trajano, 2017; Fortune et al., 2020).

Speciation in cavefishes

There are two contrasting hypotheses for the speciation of subterranean fishes: the *climate-relict* hypothesis (Holsinger, 1988; Peck and Finston, 1993; Holsinger, 2000) and the *adaptive-shift* hypothesis (Howarth, 1973; Howarth, 1981; Holsinger, 2000; Niemiller and Soares, 2015). The climate-relict hypothesis suggests that surface ancestors retreated into subterranean habitats in response to climate fluctuations. As surface conditions became more inhospitable, extirpation of surface populations promoted allopatric speciation. In contrast, the adaptive-shift hypothesis suggests that surface ancestors with exaptations to subterranean life (e.g., a nocturnal lifestyle) exploited new subterranean niches that offer reduced competition and quickly evolved in parapatry or sympatry with related surface populations (Juan et al., 2010; Niemiller and Soares, 2015). A key distinction between the climate relict and the adaptive shift hypotheses is whether divergence occurs in the absence (allopatric) or presence (parapatric or sympatric) of gene flow (Niemiller et al., 2008). In both hypotheses, cavefish species are derived from ancestral populations from the surface, whether the original population is extinct or not.

Weakly electric fishes and the adaptive shift hypothesis

Weakly electric fishes have a suite of features that appear to be obvious exaptations for troglobitic life. Most species of weakly electric fishes are nocturnal, and many live in turbid waters. While in the absence of visual cues, weakly electric fishes use electrosensory feedback from self-generated electric fields in social communication, navigation, predator avoidance, and prey capture (Heiligenberg, 1991; Caputi and Budelli, 2006; Skeels et al., 2023). Such active electrosensory systems are considered to be adaptations for nocturnal lifestyles, and thus may be an exaptation for troglobitic habitats.

Specifically, weakly electric fishes have evolved specialized electric organs for the generation of electric fields in the water and have evolved corresponding electrosensory receptors that detect these fields (Hopkins, 1988; Heiligenberg, 1991; Caputi and

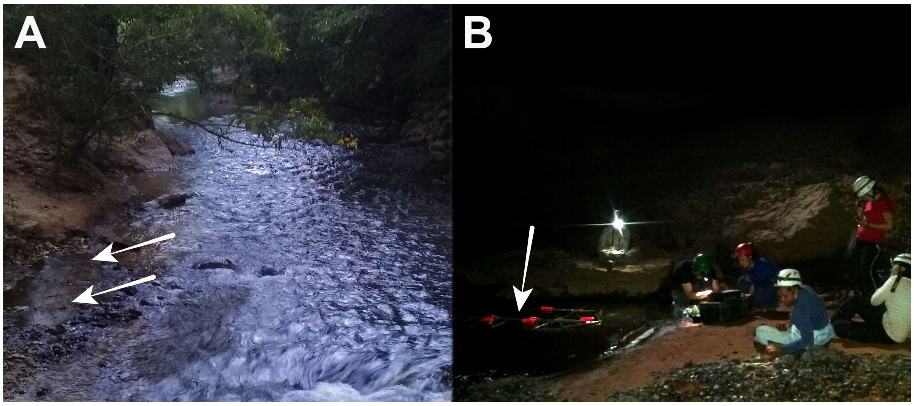

FIGURE 1
Habitats are composed of faster-moving rivers with quiet eddies and pools. **(A)** Surface habitat outside cave Rio de Lapa. Arrows point at the location where *Eigenmannia triliniata* were found and recorded in Fortune et al. (2020). **(B)** Cave Sao Vicente II where *Eigenmannia vicentespelaea* are endemic. Arrows show the recording rig: red floating inflatables maintained the position of the grid in the pool.

Budelli, 2006; Crampton, 2019). In most species, the electric organ is composed of modified epaxial muscle cells, but some species rely on modified spinal axons for the generation of electric fields (Caputi and Budelli, 2006; Shifman et al., 2015; Crampton, 2019; LaPotin et al., 2022). These organs produce transient potential differences between the outside and inside of the fish (Babineau et al., 2006). These potential differences are detected by specialized electroreceptors that have lower resistances than the surrounding skin (Zakon, 1988; Gallant, 2019). Most of the electric current flows across these receptors, which are densely packed in the head of the fish (Heiligenberg and Bastian, 1984; Hopkins, 1988; Zakon, 1988; Nelson and Maciver, 1999). Two categories of signals modulate the flow of current across an individual's electroreceptors: differences in the local resistance near the fish and the electric fields produced by nearby conspecifics and other organisms (Yu et al., 2012).

The electric fields generated by these fishes are affected by local differences in resistance in the surrounding water (Von der Emde, 1999). vzsavcwbcvSuch local differences can be caused by, for example, the presence of rocks (higher resistance) or other organisms (generally lower resistance) (Fotowat et al., 2013). These differences in resistance affect the flow of current through and voltages occurring at an individual's electroreceptors, which are encoded by changes in neurophysiological activity (Assad, 1997; Rose, 2004; Fukutomi and Carlson, 2020). In this way, this active electric sense provides a sensory channel for the control of a wide range of behaviors in which the organism interacts with its surroundings (von der Emde, 2006). Indeed, an elegant set of experiments demonstrated how a species of weakly electric fish, *Apteronotus albifrons*, uses its electrosensory system to capture prey in the absence of visual cues (Bastian, 1981; Nelson and Maciver, 1999; MacIver et al., 2001).

Although these fishes use electric fields to detect nearby objects, evidence from catfish are nevertheless consistent with the hypothesis that the generation of weak electric fields evolved in the context of social signaling (Baron et al, 1994; Orlov et al, 2021). Weakly electric fishes detect the electric fields of nearby conspecifics (Heiligenberg, 1991) and the active electric sense is an important channel for social communication between conspecifics (Henninger, 2015; Caputi, 2017; Crampton, 2019). The ancestors of weakly electric fishes had a category of electroreceptors known as ampullary receptors, which detect low-frequency electric signals in the water. Ampullary receptors can detect, for example, the currents released by muscles that move the operculum (Bodznick and Montgomery, 2005; Grewe et al., 2011), or by the movements of prey items (MacIver et al., 2001).

Variations in muscle cell size (Zakon, 2012), morphology (Mills et al., 1992; Zakon, 1995; Gallant, 2011), and ion channels (Zakon et al., 2006; Gallant et al., 2014; Swapna et al., 2018) in the ancestors of weakly electric fishes may have resulted in increased leakage of current into the water. This increase in current leakage may have made those individuals easier to detect by electroreceptive conspecifics in dark or muddy water, therefore potentially leading to increased reproductive success. Findings from *Clarias gariepinus* catfish, which generate rudimentary electric fields during social encounters (Baron et al, 1994; Orlov et al, 2021), are consistent with this hypothesis.

The costs of producing electric fields

This ability to "see in the dark" using self-generated electric fields, however, comes at a cost. The generation of electric fields in water is energetically expensive, consuming up to 30% of an individual's energy budget (Salazar et al., 2013; Markham et al., 2016). This budget includes the cost of producing the electric field, which involves the cycling of sodium and potassium across cell membranes of electrolytes that comprise the electric organ and the activity of brain areas that both detect the electric fields and control behavioral responses to them.

Another potential cost of generating electric fields is the increased risk of predation by electroreceptive predators. Ampullary electroreceptors are found on many species of fishes that may be predators of weakly electric fishes, including catfish and electric eels. When weakly electric fish produce signals with power at low frequencies, e.g., below about 100 Hz, they may reveal themselves to fish with ampullary electroreceptors. Indeed, electric signals containing low-frequency components that are preferentially detected by ampullary receptors were shown to be more salient to an electric eel than electric signals without these low-frequency components (*Electrophorus electricus*; Stoddard, 1999; Stoddard, 2002).

Electroreceptive predators of weakly electric fishes appear to be overrepresented in the catfish group (Stoddard, 1999). Pimelodidae, a family of piscivorous catfishes, regularly prey upon weakly electric gymnotiform fishes (Reid, 1983; Duque and Winemiller, 2003; Petrere et al., 2004) in South America and the African sharptooth catfish, *Clarias gariepinus* has been described as a predator of mormyrid weakly electric fishes (Merron, 1993).

Weakly electric cavefish

Eigenmannia vicentespelaea from central Brazil is the only known species of gymnotiform weakly electric fish that show complete troglomorphy. The closest epigean species, *Eigenmannia trilineata*, is found in the same watershed (Bichuette and Trajano, 2006). The cavefish show regression of eyes and loss of pigmentation (Bichuette and Trajano, 2006), while surface fish are pigmented and have functioning eyes (Figure 2). Nevertheless, the surface fish *Eigenmannia trilineata* and other species of *Eigenmannia* have been observed to be nocturnal (Tan et al., 2005). *Eigenmannia* hide in roots, rocks, and leaf litter during the day and actively forage at night (Hopkins, 1974; Hopkins, 1981; Deng and Tseng, 2000; Tan et al., 2005; Stamper et al., 2010; Fortune et al., 2020; Gouveia et al., 2022)

We traveled to the São Vicente II cave and measured the electrosensory and locomotor behavior of both surface *Eigenmannia trilineata* and cave *Eigenmannia vicentespelaea* (Fortune et al., 2020). We used a custom array of amplified electrodes, 50cm spacing, that both captured the electric behavior of nearby fish, and allowed us to calculate the positions of these fish (Madhav et al., 2018). These grid recording systems have allowed unprecedented insights into the behaviors of weakly electric fishes in their natural habitats (Raab et al., 2019; Henninger et al., 2020).

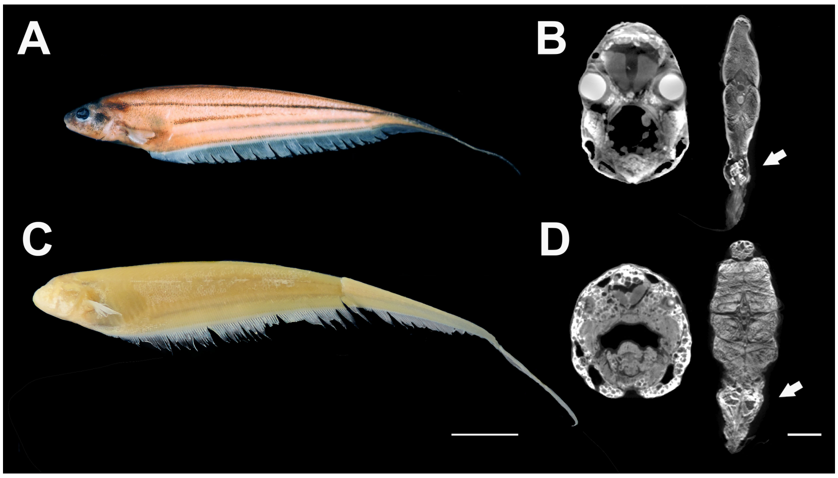

FIGURE 2
Anatomical differences between ancestral surface and derived cave *Eigenmannia*. **(A)** *Eigenmannia trilineata* collected in Rio da Lapa. **(B)** DICEuCT scan through the head and mid-body of *Eigenmannia trilineata*. Note the larger eyes of the surface fish. The arrow points to the electric organ. **(C)** *Eigenmannia vicentespelaea* specimen collected in the cave of Sao Vicente II. **(D)** DICEuCT scan through the head and mid-body of *Eigenmannia vicentespelaea*. Note small and vestigial eyes and larger electric organs (arrows). Scale bars 10 mm and 2 mm.

Effects of troglobitic conditions on the amplitudes of cavefish electric fields

Generating electric fields is energetically costly, consuming up to 30% of an individual weakly electric fish's energy budget (Salazar et al., 2013; Markham et al., 2016). Therefore, if a cave environment is poor in resources, one might expect that weakly electric cavefish might produce lower-amplitude electric fields. Indeed, non-electric species of cavefish have adaptations related to the scarcity of resources (Fišer, 2019). On the other hand, active electrosensation permits weakly electric fishes to image their environment (von der Emde, 2004) and capture prey in darkness (MacIver et al., 2001) and therefore might serve as an exaptation for increasing access to food in cave habitats.

We measured the amplitudes of the electric fields (technical details for these measurements are described in Madhav et al., 2018) produced by the weakly electric cavefish in the São Vicente II cave and of surface fish in the nearby Terra Ronca stream (Figure 1). We were surprised to find that the cavefish produced significantly higher amplitude electric fields (Fortune et al., 2020) compared to their closest living surface ancestors (Figure 3). These more powerful electric fields are likely to be energetically costly, but likely provide benefits to the fish. Stronger electric fields may increase the volume of water around the fish in which it can detect objects including prey – known as the "sensing volume" (MacIver et al., 2001; Snyder et al., 2007). A larger sensing volume increases the likelihood of detecting and capturing nearby prey items. Further, stronger electric fields could potentially result in greater spatial acuity and sensitivity.

We currently do not know the mechanism for the increase in amplitudes seen in the cavefish. The increase in amplitudes may simply result from size differences between the cave and surface populations of *Eigenmannia*: larger fish have larger electric organs, resulting in stronger electric fields. Our impression is that the population of adult cavefish we observed was generally larger than the population of adult surface fish. Other mechanisms that might contribute to increased electric field amplitudes of cave *Eigenmannia* include adaptations of the electric organ, including increases in the relative size of the electric organ in relation to body size, changes in the density or structure of electrocyte ion channels, and/or changes in other structural or resistive features in the skin.

Interestingly, pressure from potentially electroreceptive predators may limit the sizes of surface fish and may also lead to a reduction in the strength of their electric fields. We observed evidence of predation in the surface *Eigenmannia* population, with several adults having obvious morphological signs of regrowth of their tails (Figure 4). Loss of the tail and its regrowth has been considered evidence of predatory attacks (Dunlap et al., 2016). Our conclusion is that surface

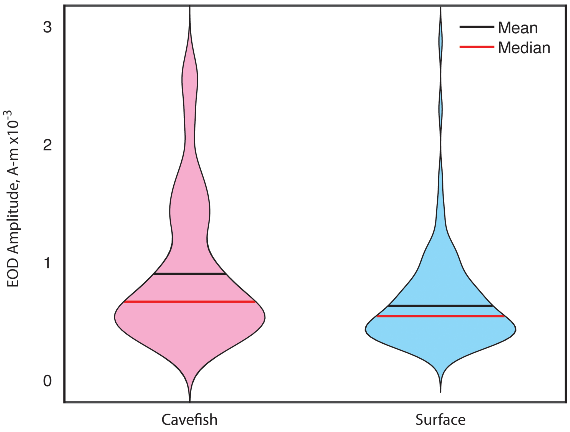

FIGURE 3
A comparison of *Eigenmannia* EOD amplitudes from both habitats shows an increase in the median and mean amplitude in the cavefish (RED line). Also note greater numbers of the strongest EODs in cavefish.

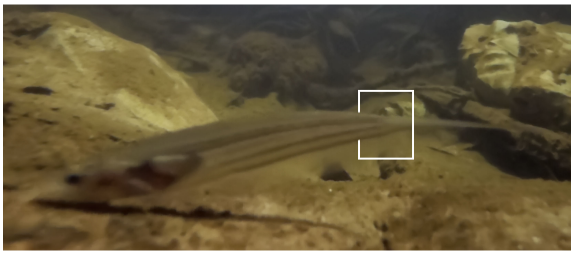

FIGURE 4
Surface *Eigenmannia* showing predation and regrowth of tail. Notice the lack of black stripe and thickness change starting at the outlining box. Image was taken from a video recording in the field (Fortune et al., 2020).

Eigenmannia may face significant predation pressure, supporting the adaptive-shift hypothesis for the emergence of cave populations. If so, the relative increase in the electric field amplitudes of the cavefish might result from a release from predation pressure.

Finally, the area in which Sao Vicente II cave is located has been classified as rupestrian grassland (Barbosa and Fernandes, 2016). This region was not frozen during the last glacial maximum (~21,000 years ago) and in the middle Holocene (~6,000 years ago), making the climate-relict hypothesis an unlikely explanation of the emergence of cave *Eigenmannia*.

Effects of troglobitic conditions on circadian patterns of social behavior

Surface *Eigenmannia* exhibit overt diurnal behavioral cycles. During daylight hours, fish exhibit refuge-seeking behavior, hiding in crevices between rocks and root systems. While hiding during the day, surface *Eigenmannia* are most commonly found in aggregates of several fish (Tan et al., 2005; Fortune et al., 2020). During night hours, surface *Eigenmannia* emerge from their refugia to forage and perform other behaviors. Videos recorded at night of surface *Eigenmannia* in the streams outside the Terra Ronca cave show the fish foraging in groups (Supplemental material, Fortune et al., 2020). These video data corroborate the grid tracking data, which shows groups of several fish swimming near each other at night (Fortune et al., 2020). Most surface *Eigenmannia* were found in groups, and do not appear to maintain territories at any time during 24-hour diurnal cycles (Tan et al., 2005; Fortune et al., 2020).

Unlike surface *Eigenmannia*, the cave *Eigenmannia* appeared to occupy territories 24 hours a day. Cave *Eigenmannia* appeared to perform boundary patrolling behavior on the perimeter of an area of about a square meter (mean = 1.16 sq m, std = 0.76, n = 274). Videos of cave *Eigenmannia* in the São Vicente II karst show the fish engaging in mouth-to-mount conflicts in which both fish swim toward each other (Supplemental material, Fortune et al., 2020), which may be aggressive interactions between individuals defending their territories. We also observed chases between cavefish in the grid recordings in which the retreating fish produced a long frequency rise of its electric frequency (a rise of typically more than 5 Hz over a period on the order of 10s of seconds). In these interactions, the chasing fish did not change its electric frequency, but the retreating fish initiated a long frequency rise seconds before the fish were at their closest during the interaction (Figure 5). The chasing fish followed the retreating fish for about 10 seconds, at which time the retreating fish began to return its electric field to its normal frequency.

Discussion

Eye sizes in *Eigenmannia vicentespelaea*

Eye loss is a trait that is associated with troglomorphy. In all animals studied so far, eyes develop normally in embryonic stages, but eventually the lens undergoes cell death which signals the eyes

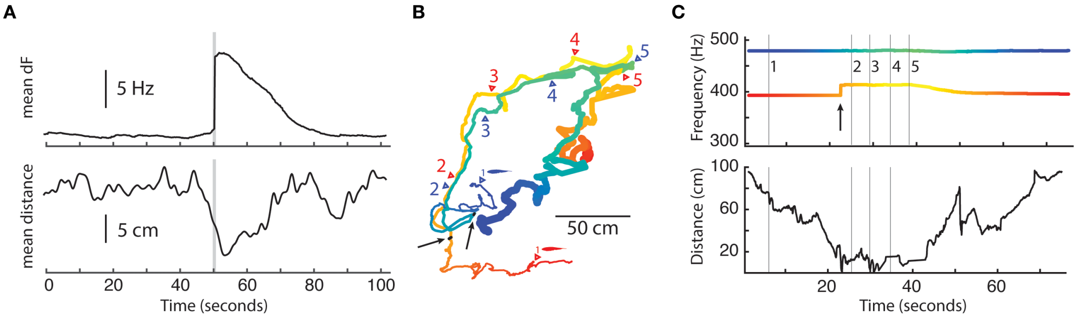

FIGURE 5
Potential boundary patrolling in *Eigenmannia vicentespelaea* (data from Fortune et al., 2020). **(A)** Top panel shows the mean difference (n = 208 events) in electric field frequencies (Hz) between pairs of fish, centered in time on the start of a long-frequency rise produced by one fish in the pair (grey vertical line). Bottom panel shows the average distance between the pairs of fish. Frequency rises were produced as fish approached each other, about 5 seconds before their closest distance. **(B)** Spatial map of the XY positions of two fish during a possible boundary patrolling event (blue fish). Colors of the lines correspond to **(C)**, and the line is thinnest at the start of the epoch and thickest at the end. Numbers correspond to time points shown in **(C)** Black dots with arrows indicate the locations of both fish at the initiation of the frequency rise produced by the red fish. **(C)** Top panel shows the frequencies of both fish over time and bottom panel shows the distance between the fish. In this event, the red fish moved closer to the blue fish. At the moment of the frequency rise produced by the red fish, the blue fish changed direction and rapidly followed the red fish over a distance of well over 2 meters in a period of about 20 seconds. After the event, the blue fish returned to near its original position.

to arrest growth, leading to small eyes sunken into the orbits (Rétaux and Casane, 2013). Interestingly, this mechanism leads to the regression of both eyes. *Eigenmannia vicentespelaea* exhibits a diversity of eye sizes (Bichuette and Trajano, 2006). These include extreme individuals with a full eye on one side and a completely degenerated eye on the other. This suggests that there might be other evolutionary mechanisms at play in eye degeneration in *Eigenmannia vicentespelaea*.

Amplitudes of electric fields

The amplitudes of electric fields increase with the size of fish (Hopkins et al., 1990; Curtis and Stoddard, 2003; Gavassa et al., 2012) and can be used as a nonvisual proxy to estimate body size. However, Gavassa et al. (2013) have shown that social interactions can drive significant changes in the amplitude of electric fields. Further, hormones can increase the amplitudes of electric fields (Markham et al., 2009a; Migliaro and Silva, 2016). We already know that on average, the size of the EOD is larger in the cavefish, the next steps will include correlating them to the size of the fish. For example, we predict that the slope of the relationship between size and electric field amplitude will be steeper in the cavefish in comparison to the surface fish. Such differences would indicate active adaptations rather than simply size effects.

Territoriality in caves

Cave *Eigenmannia vicentespelaea* show boundary patrolling, a form of territorial behavior absent in surface *Eigenmannia*. Territoriality can provide insights into how fish integrate social and environmental cues to produce behavioral responses. There is a cost-benefit in territorial defense, whether from breeding or resource defense (Kaufmann, 1983; Adams, 2001; Christensen and Radford, 2018). Territorial defense is usually associated with breeding males seasonally, but year-round territoriality can establish access to foraging areas or protection from predators (Silva et al., 2020).

A good example of weakly electric fish territorial behavior is shown by the *Gymnotus omarorum*. These fish have territory ranges that are independent of sex but are directly correlated to body size (Silva et al., 2020). During the breeding season, however, Silva et al., 2020 showed that *Gymnotus omarorum* establishes territories, which sizes are correlated to with gonadosomatic index in females and 11-ketotestosterone levels in males. We are interested in the roles of hormones in both regions of the body of the *Eigenmannia vicentespelaea* and *trilineata* populations. It will be interesting if expression differences are correlated with a cave habitat and what are the evolutionary forces driving it.

Social behavior and circadian rhythms

Vertebrate circadian behaviors are based on networks of endogenous clocks across tissues throughout the body of the fish that are synchronized to one another by a master circadian clock in the brain (Ripperger and Schibler, 2001). Master clocks guide physiology and behavior via neural and hormonal cues (Ripperger and Schibler, 2001; Patke et al., 2020).

We know that strongly defined cycles caused by inputs such as light, temperature, and other biological influences such as predation, can synchronize the intrinsic internal clock generated by cryptochrome gene expression. Ripperger and Schibler (2001) demonstrated that the endogenous timekeeping system created by the interaction of these genes can be synchronized by diverse types of zeitgebers (time-givers in German) (Takahashi, 1991; Wilsbacher and Takahashi, 1998).

Surface *Eigenmannia* modulates the amplitude of their wave-type electric organ discharges (EOD) in relation to the light/dark cycle, with greater EOD amplitude at night when these nocturnal animals are more active and a lower EOD amplitude during the day when fish are hidden within refugia (Deng and Tseng, 2000; Markham et al., 2009b; Sinnett and Markham, 2015). In *Brachyhypopomus pinnicaudatus*, a pulse-type weakly electric fish, pulse rate, pulse amplitude, and tau$_{P2}$ – the time constant of recovery for the second phase of the pulse waveform, all increased during the subjective night. These circadian rhythms in electric field waveform were even more pronounced under breeding conditions, suggesting a social context (Silva et al., 2007). Similar to the results we report for surface *Eigenmannia*, *B. pinnicaudatus* EOD rhythms were stronger under photo entrained (12 hours of dark:12 hours of light) conditions than under free-running, or constant photic conditions (Stoddard et al., 2007).

Gymnotus omarorum is another pulse-type weakly electric fish with a circadian pattern in electric field pulse rate. These fish live in such dense vegetation in their natural habitat, that they experience no diurnal variation in photic cues, similar to the cave conditions. Because circadian changes in EOD pulse rate persist in *G. omarorum* when measured in the field along with daily fluctuations in temperature it is postulated that daily temperature fluctuations driven by the sun heating the water during the day, act as the primary zeitgeber in these animals (Migliaro et al., 2018). Indeed, temperature variation has been shown to induce changes in the EOD pulse rate in *B. pinnicaudatus* (Silva et al., 2007).

Study details

The data shown in this review were collected for a previous publication (Fortune et al., 2020). A detailed description of the materials and methods can be found there. In short, the procedures used for these observational studies of Gymnotiform fishes were reviewed and approved by the animal care and use committee of Rutgers University/New Jersey Institute of Technology (Protocol #999900774) and follow guidelines for the use of animals in field research established by the National Research Council. Field research permits in Brazil were granted by the ICMBio and SEMARH/SECIMA.

Study sites

Both species of fish are endemic to the upper Tocantis river basin in the state of Goias, central Brazil (46° 10′–46° 30′ S, 13° 30′–13° 50′ W). The cavefish *Eigenmannia vicentespelaea* population is located in the cave of São Vicente II (13° 58′37″ S, 46° 40′04″ W), while *Eigenmannia trilineata* population is found in the Rio da Lapa (13° 38′44″ S, 46° 38′ 08″ W) approximately 8 km away. Both habitats have moderate water currents, clear water with a conductivity below 20 µS, and substrate composed of sand, rocks, and boulders (Fortune et al., 2020).

Anatomy

We chose to use Diffusible Iodine-based Contrast-enhanced Computed micro-Tomography (DICEuCT) because it permits visualization of soft tissue details without damage to the specimens. We imaged four previously collected specimens from Dr. Bichuette's laboratory collection at the Universidade Federal de São Carlos, Brazil. Briefly, we submerged 70% ethanol fixed specimens in 11.25 Lugol's iodine (I2KI) solution for up to 36 h before scanning. Stained specimens were washed in water to remove excess stain and sealed in rubber containers to prevent dehydration. Stained and unstained specimens were scanned at the Core Imaging Facility of the American Museum of Natural History (New York, NY), using a 2010 GE Phoenix v|tome|x s240CT high-resolution microfocus computed tomography system (General Electric, Fairfield, CT, USA) in 50 mL polypropylene centrifuge tubes. DScans were made at 125 kV, with an exposure time of 60 seconds. Voxel sizes were 20.0–25.9 µm and volume reconstruction of raw X-ray images was achieved using a GE Phoenix datos|x.

Recordings of electric behavior and movement at field sites

We used a hand-held single-electrode probe attached to a custom amplifier and speaker to find the fish. When the probe was within about 1 meter of an *Eigenmannia*, its electric field was audible to the user. Also, we were also able to see the animals directly due to the clarity of the water. To record the electric behavior and movement of *Eigenmannia*, we deployed a grid (50 cm spacing) of active electrodes (Madhav et al., 2018; Fortune et al., 2020) at sites where we had previously observed fish. The surface fish were recorded along the edges of the Rio da Lapa outside of the Terra Ronca cave after sundown. The cavefish were recorded in eddies and side pools of the primary river within the São Vicente II cave.

We used an algorithm developed by Madhav et al. (2018; code available at doi:10.7281/T1/XTSKOW) to identify each fish via the time-varying fundamental frequency of its electric field. The locations and orientations of each individual were calculated in relation to the distribution of power at each electric field frequency across the electrodes. These recordings were made in shallow water of no more than 40 cm depth: position estimates were restricted to the XY plane. Continuous recording sessions using the grid were made both in the cave ($N = 14$) and surface ($N = 5$). Intervals between recording sessions ranged from 5 min to several hours and each recording was from 600 seconds to over 1,200 seconds in duration. The position and electric field frequency data were analyzed in 300 s duration non-overlapping epochs. Other details of the recording procedures and analysis can be found in Fortune et al. (2020).

Perspectives

Our future studies include the identification of the causal evolutionary interactions between categories of behavior: scene analysis and social behavior.

Ethics statement

Written informed consent was obtained from the individuals for the publication of any identifiable images or data included in this article.

Author contributions

MB, DS, and EF contributed to the conception and design of the study. DS, KG, and EF wrote the first draft of the manuscript. All authors contributed to the writing and revision of the manuscript and have read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The biomimetic potential of novel adaptations in subterranean animals

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Subterranean habitats can be considered harsh conditions with lack of light, low nutrients levels and constant high humidity. To thrive under such conditions, cave-adapted species have evolved a range of novel morphological, physiological and behavioural adaptations. These adaptations might have significant biomimetic potential. Biomimetics or biologically inspired design is a relatively new interdisciplinary field that aims to harness the processes and mechanisms in nature that have been optimised over millions of years' evolution to improve our own technology. There are two main approaches to biologically inspired design—the problem-driven approach starting with an engineering problem and searching through biological equivalents and the solution-driven approach, which starts with a biological example or solution followed by the identification of a suitable engineering application. While the former approach is the most popular and is favoured by engineers, the latter remains the most successful and is typically driven by fundamental biological research. However, few biomimetic solutions or concepts have so far been described from subterranean habitats despite the rich potential. In this review paper, I first outline the theory behind biologically inspired design before I review the few biomimetic related studies of cave adapted organisms mainly based on the exceptional lateral line systems in blind cave fish. However, the main body of the review focuses on identifying and discussing subterranean adaptations with a particular strong potential including biomimetics sensors, adhesion in high humidity and wet conditions and biomaterials

KEYWORDS

cave-dwelling animals, cave biomimetics, biologically inspired design, troglomorphs, cave adaptations

Introduction

The dark subterranean environment has long been recognised as a natural laboratory for studies on evolutionary and ecological adaptations (Mammola, 2019). Caves and other subterranean habitats are characterised by constant darkness, constant temperatures and high humidity often resulting in standing pools, dripping water and water film on the cave walls. The lack of light means that no primary production takes place in the majority of caves with only a few sulphide enriched and oxygen deprived caves hosting significant amounts of chemoautotrophs (Culver and Pipan, 2019). This again generally results in nutrient limited conditions where organic material only enters the subterranean system through wind blowing material into the cave, underground streams and percolated water bringing in material from the outside, surface animals accidentally entering the cave, or occasional cave dwellers, such as bats and crickets, leaving and entering the cave on a regular basis (Reichle et al., 1965).

The environmental and ecological constraints in subterranean habitats, especially the constant darkness and lack of nutrients mentioned above, combined with stable temperatures and, usually, very high humidity give rise to a very specialised fauna (Mammola, 2019). Traditionally, although this is not universally agreed on (see Trajano and de Carvalho, 2017), cave inhabiting organisms have been classified into three groups (Culver and Pipan, 2019). Trogloxenes are animals that are occasional or accidental visitors to the cave, but do not rely on subterranean habitats to complete their life cycle and include animals such as moths, flies and small mammals. Trogloxenes are most often found in the entrance zone, the area near the cave mouth, which is characterised by some light and climatic variables fluctuating with the outside climate (Howarth, 1983). They do not show any adaptations to subterranean habitats and will therefore not be discussed further in this paper. Troglophiles are animals that spend at least part of their life inside subterranean habitats and include bats, crickets and spiders. They usually reside in the twilight zone, which is characterised by very reduced light and limited daily and seasonal fluctuations in climatic variables compared to the outside (Mammola and Isaia, 2018). Troglophiles usually do not show any morphological adaptations, but they can show physiological and behavioural adaptations (Howarth, 1983). The troglophile cave spider *Meta menardi*, for example, builds highly modified orb webs lacking a frame structure (Simonsen and Hesselberg, 2021), while another troglophile spider *Metellina merianae*, which also occurs outside of caves, do not show any modifications to their webs (Hesselberg and Simonsen, 2019). Finally, troglobites (or troglobionts) are animals that spend their entire life inside caves and includes most of the well-known cave animals such as the olm (*Proteus anguinus*), the Mexican cave fish (*Astyanax mexicanus*) and many arthropod species (Howarth and Moldovan, 2018). Almost all troglobites inhabit the dark (or deep) zone of caves, which is characterised by a complete lack of light and a stable temperature and a high humidity. Many, but far from all, troglobites show examples of convergent evolution with morphological adaptations (in which case they are often referred to as troglomorphs, although it is worth noting here that for most species, function and fitness benefits of these putative adaptations have not been explicitly studied) including eye loss, depigmentation and elongation of limbs (Howarth, 1983; Pipan and Culver, 2012). Physiological adaptations include enhanced non-visual sensory systems, erratic or a complete lack of circadian rhythms and lower metabolic rates (Hervant, 2012), while behavioural adaptations, which are the least studied of all, include a broadening of diet, reduced social or aggregation behaviours and a shift of reproductive behaviour to producing fewer, but larger offspring (Howarth and Moldovan, 2018; McGaugh et al., 2020).

The insular nature of caves has also resulted in a high proportion of endemic species, sometimes with species known from just one specific cave (Mammola, 2019). Examples include 23% of amphipods and 31% of beetles in known from single subterranean sites in the Western Balkans (Bregović et al., 2019), the guano moth (*Kangerosithyris kotumarensis*) found only in Kotumsar Cave in India (Biswas, 2010), the centipede (*Cryptops speleorex*) found only in Movile Cave in Romania (Vahtera et al., 2020) and the West Virginia Spring Salamander (*Gyrinophilus subterraneus*) known only from General Davis Cave in the US (Grant et al., 2022). However, it is worth noting that caves may be less insular than we think, as many individual caves are linked together through a vast network of small fissures in the karst (Culver and Pipan, 2019; Mammola et al., 2021). Consequently, while most of our knowledge on the hypogean fauna is based on studies from caves, many subterranean organisms may actually be adapted to live in the narrow fissures rather than the open caves, which we are more familiar with (Mammola, 2019).

These convergent adaptations and the large number of endemic species make the subterranean habitat a potential goldmine for developing novel biomimetic technologies. Biomimetics (also referred to as biologically inspired design) is a relatively new interdisciplinary field that aims to extract inspiration for technological breakthroughs from biologically processes and organism traits, refined over millions of years of trial and error through natural selection (Vincent et al., 2006; Lenau et al., 2018). While the examples of commercially successful biomimetic products are still far and few between, the focus on harsh ecosystems is already proving successful. Deserts organisms, for example have generated a number of biomimetics ideas, including algorithms for effective visual vector based navigation in the desert ant *Cataglyphis* (Dupeyroux et al., 2019), anti-erosion properties from the exoskeleton of the sand scorpion *Leiurus quinquestriatus* (Han et al., 2010) and passive water collection technology inspired by desert organisms (Gurere and Bhushan, 2020) such as the tenebrionid beetle *Stenocara gracilipes* from the Namib desert (Parker and Lawrence, 2001) and the Argentinian cactus *Gymnocalycium baldianum* (Liu et al., 2015). However, despite the unique subterranean fauna, very few biomimetic related studies have focused on cave-adapted animals.

The aim of this paper is to review the very few existing studies on cave biomimetics and to suggest adaptations in cave-dwelling species with significant biomimetic potential. I focus on putative adaptations in three areas relating to the environmental characteristics of the habitat; morphological adaptations for adhesion and locomotion on smooth cave rocks, the evolution of alternative sensory systems in response to the lack of vision in the constant darkness, and finally on adaptive changes to biomaterials, especially spider silk, in response to the high humidity and unique environmental and ecological conditions. My hope is to encourage cave biologists to look again at the species they encounter in light of potentially novel functions, so that they, together with engineers, can explore their biomimetics potential.

What is biomimetics and biologically inspired design?

Biomimetics can be defined as the study of biological functions, processes or morphological traits with the aim of applying them for generating inspiration to develop novel technological solutions. The term biomimetics was coined in 1969 by Otto Schmitt, but it first became recognised as an independent discipline (typically nested within mechanical or materials engineering) in the 1990s (Vincent et al., 2006). However, long before the term was coined, humans looked to nature for inspiration with classical examples ranging from early human tools mimicking the function of sharp canines in mammal predators to the unsuccessful attempts by Leonardi da Vinci to develop flying machines based on the wing design of birds and bats (Hesselberg, 2007). The first successful example of biomimetics dates from before the term was coined and is that of Velcro, which was discovered in 1955 by a Swiss engineer when walking his dog. He noticed that burdock seed attached strongly to the fur of his dog, and decided to investigate the reason for that, which led him to discover the many tiny hooks on the burdock seed. He designed Velcro in a similar way with one side consisting of tiny hooks and the other side of furlike hairs (Hesselberg, 2007). Since the 1990s, the field of biomimetics has attracted a lot of attention, and both the number of papers and the number of patents grew exponentially in the early parts of this millennium (Bonser, 2006; Hesselberg, 2007; Lenau et al., 2018). The proportion of papers published in the general area of biomimetics continues to rise to the present day (Figure 1).

Recently, there has been a large push to try to develop a more systematic approach to biomimetics often referred to as the biologically inspired design process, which consists of a number of steps ranging from the initial idea to the finalised commercial product (Lenau et al., 2018; Speck and Speck, 2019). One of the earliest systematic methods is the Biomimicry Innovation Method, developed by the Biomimicry Guild in the early 2000s, which identified 4 steps: Identify function, biologize (translate into biology) the question, find nature's best practice and generate product ideas (Gebeshuber et al., 2009). However, the most well-known and most adopted method is the Georgia Tech model of biologically inspired design, which consists of the following 6 steps: problem definition, reframing the problem, biological solution search, defining the biological solution, principle extraction and principle application (Helms et al., 2009). In general, we can divide the biologically inspired methodology into two; the problem-driven and the solution-driven approach (Figure 2). The problem-driven approach (also known as top down or technology-pull) starts with a technical problem, which is followed by a search for potential biological solutions, before principles are extracted and applied (Speck and Harder, 2006). The solution-driven approach, on the other hand, is usually initiated by biologists, who have discovered a novel function in a biological organism, which is followed by a search for potential technological problems or gaps which the biologically function could solve, before principles are extracted and applied (Speck and Harder, 2006). The later steps in the solution-driven approach require heavy involvement of designers and engineers.

Much effort has gone into developing the problem-driven approach in recent years, especially trying to overcome the obstacles of translating biological concepts into a language that designers and engineers can understand (Vincent et al., 2006). A number of different tools have been developed including BioTRIZ, a biomimetics ontology and AskNature (Vincent et al., 2006; Deldin and Schuknecht, 2014; Vincent and Cavallucci, 2018). The latter is the most immediately accessible tool, allowing anybody to search

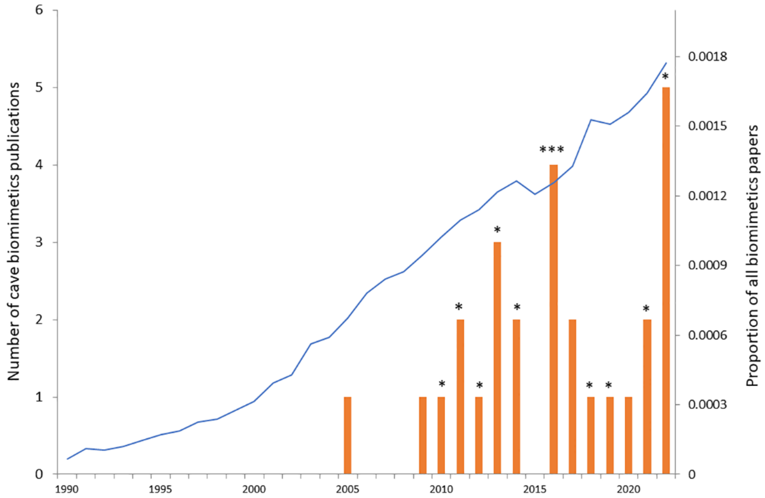

FIGURE 1
Cave biomimetics papers in relation to all biomimetics papers over the past 33 years (from 1990 to 2022). Red bars show the number of published papers in the Web of Science catalogue on cave biomimetics on the 7th of August 2023 {search phrase: *TS=[cave AND (biomim* OR "biologically inspir*" OR bioinsp*)]*}. Each star (*) indicates one of the 12 papers that were of direct relevance to cave biomimetics (the search phrase returned a number of papers not directly related to cave biomimetics). The proportion of all published papers that include biomimetics is shown by the blue curve. For each year the number of published papers on biomimetics [search phrase: *TS = (biomim* OR "biologically inspir*" OR bioinsp*)*] was divided by the total number of published papers in that year.

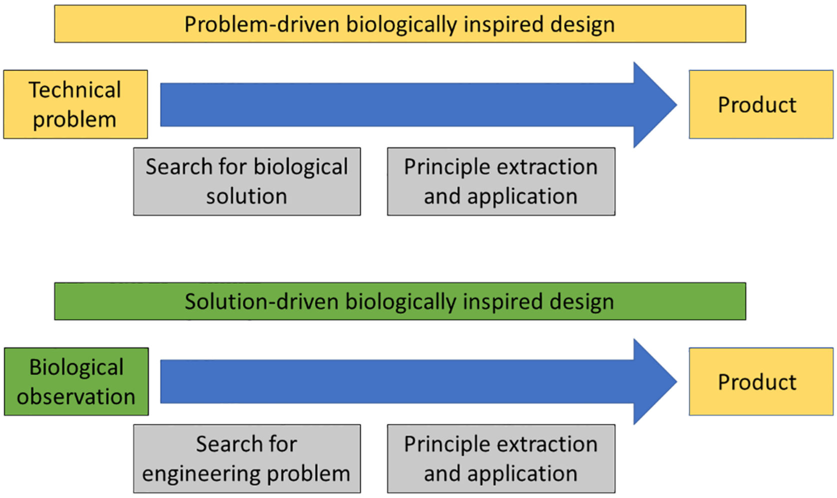

FIGURE 2
Problem-driven biologically inspired design (above) starts with an engineering problem followed by a search for potential biological solutions before principle extraction and application. Solution-driven biologically inspired design starts with a biological observation (or solution) followed by a search for an engineering (or technical) problem before principle extraction and application. Inspiration for this figure comes from Lenau et al. (2018) and Speck and Speck (2019).

for potential biological solutions to technological problems on the internet (asknature.org), although it still struggles, in some cases, with limited explanations and questionable references. Still, the most effective route for the problem-driven approach to lead to a successful outcome is to include biologists into the project team (Snell-Rood, 2016; Graeff et al., 2019). The problem-driven approach has to date delivered few success stories with the most promising ones probably being in the area of medical technology including using biomaterials, such as silk, as scaffolds for tissue regeneration and in surgery (Holland et al., 2019; Song et al., 2020), and developing painless microneedles based on the mouth parts and biting behaviour of mosquitoes (Lenau et al., 2017; Ma et al., 2022). Nonetheless, the most successful methodology remains the solution-driven one to which the above mentioned Velcro belongs. Further promising research with large potential commercial applications include the self-cleaning Lotusan paint based on the Lotus effect in water lily leaves (Neinhuis and Barthlott, 1997; Antony et al., 2016) and strong adhesives based on the intricate morphology of gecko feet, which allows them to cling on to, and move on, smooth vertical surfaces (Autumn et al., 2000; Geim et al., 2003). However, very few out of the estimated 8 million species of living organisms on Earth have been the focus of biomimetics studies suggesting that there remains a large scope for further success stories arising from for the solution-driven approach.

It is here that cave biologists can potentially make a large contribution as this field of biology has until now largely been overlooked in biologically inspired design. Despite the sustained high output of biomimetics papers during the past decades, only 27 hits came up on Web of Science relating to cave biomimetics (Figure 1). A closer scrutiny of the titles and abstracts of these 27 hits, revealed that only 12 of them were specifically about biomimetic inspiration gained from cave adapted organisms (* in Figure 1; Table 1). The remaining hits mainly relate to using bioinspired robotics to explore cave systems. Nine out of the 12 papers focus on developing biomimetic hydrodynamic flow sensors based on the lateral line system of various species of cave fishes (predominantly, but not exclusively the blind Mexican cavefish *Astyanax mexicanus*). Thus there is a large untapped potential for using the solution-driven biologically inspired design approach to develop novel biomimetic technologies based on a broader range of cave-dwelling species. I discuss a few promising cave adapted animals where the solution-driven approach might be particularly fruitful in the section "Promising cave adaptation examples" below.

Cave biomimetics

We saw in the section above, how few biomimetics related studies have so far been carried out on the subterranean fauna (Figure 1; Table 1). In this section, we cover three of the most interesting and promising examples; two relating to the field of biomimetics sensors and one relating to the field of novel biomaterials.

The best studied example is that of the enhanced lateral line system in blind cave fish compared to epigean fish (Figure 3). The lateral line system (also referred to as the lateral line organ) consists of a series of flow sensors, called neuromasts that are found either on the surface (superficial neuromasts) or embedded in fluid filled canals just below the skin (canal neuromasts) (Figure 3). The two types of neuromasts have complementary functions with the superficial neuromasts being directly exposed to the flow and able to directly respond to different flow regimes, while the canal neuromasts, which are exposed to the water via pores are able to detect differences in pressure and inertial forces (McHenry and van Netten, 2007). The neuromasts are made up of sensory hairs enclosed in a gelatinous dome-like cupula and innervated by afferent nerves (Bleckmann and Zelick, 2009). While all fish

TABLE 1 Biomimetics cave papers directly relating to gaining inspiration from cave-adapted organisms.

Year	Title	Journal	Cave organism	Technology	Source
2010	Biomimetic Lateral-Line System for Underwater Vehicles	IEEE Sensors	Fish: Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Franosch et al. (2010)
2011	Air vortex ring communication between mobile robot	Robotics and Autonomous Systems	Insect: African cave cricket (*Phaeophilacris spectrum*)	Alternative biologically inspired robotic communication (vortex signals)	Russell (2011)
2012	Coping with flow: behavior, neurophysiology and modeling of the fish lateral line system (REVIEW)	Biological Cybernetics	Fish: Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Mogdans and Bleckmann (2012)
2013	Electrospun nanofibrils encapsulated in hydrogel cupula for biomimetic MEMS flow sensor development	IEEE 26th International Conference on Micro Electro Mechanical Systems (MEMS)	Fish: Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Kottapalli et al. (2013)
2014	Bio-Inspired Electromagnetic Orientation for UAVs in a GPS-Denied Environment Using MIMO Channel Sounding	IEEE Transactions on antennae and Propagation	Various species of cave swiftlets	Biologically inspired acoustic navigation method.	DeFranco et al. (2014)
2016	Biomimetic Survival Hydrodynamics and Flow Sensing (REVIEW)	Annual Review of Fluid Mechanics	Fish: Multiple including Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Triantafyllou et al. (2016)
2016	Biomimetic Hydrogel Cupula for Canal Neuromasts Inspired Sensors	IEEE Sensors	Fish: Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Bora et al. (2016)
2016	Nanofibril scaffold assisted MEMS artificial hydrogel neuromasts for enhanced sensitivity flow sensing	Scientific Reports	Fish: Mexican blind cavefish (*Astyanax mexicanus*)	Biologically inspired hydrodynamic flow sensors	Kottapalli et al. (2016)
2018	Insights into the Social Behavior of Surface and Cave-Dwelling Fish (*Poecilia mexicana*) in Light and Darkness through the Use of a Biomimetic Robot	Frontiers in Robotics and AI	Fish: The cave molly (*Poecilia mexicana*)	Biologically inspired robotics, social behaviour	Bierbach et al. (2018)
2019	Flow field perception based on the fish lateral line system	Bioinspiration & Biomimetics	Fish: Multiple, including Chinese cavefish in the genus *Sinocyclocheilus*	Biologically inspired hydrodynamic flow sensors	Jiang et al. (2019)
2021	Adhesive Droplets of Glowworm Snares (Keroplatidae: Arachnocampa spp.) are a Complex Mix of Organic Compounds	Frontiers in Mechanical Engineering	Insect: Fungus gnats/ cave glowworms in the genus *Arachnocampa*	Biologically inspired adhesion, biomaterials	Wolff et al. (2021)
2022	Maximized Hydrodynamic Stimulation Strategy for Placement of Differential Pressure and Velocity Sensors in Artificial Lateral Line Systems	IEEE Robotics and Automation Letters	Fish: The Chinese blind cavefish *Sinocyclocheilus tianlinensis*	Biologically inspired hydrodynamic flow sensors	Yang et al. (2022)

Based on a Web of Science search on the 22nd of June 2022 with the search phrase: *TS=[cave AND (biomim* OR "biologically inspir*" OR bioinsp*)]*.

possess a lateral line system, it is particularly well developed in blind cave fish (Yoshizawa et al., 2010) suggesting that there will be particular technological benefits by focusing on cave fish over their surface counterparts. The Mexican blind cave fish (*Astyanax mexicanus*) is, for example, able to detect minute changes down to 0.4 cm/s in oscillatory fluid flow (Montgomery et al., 1997). It is therefore unsurprising that cave fish in general, and the Mexican cave fish in particular, have been used as a model for developing biomimetic flow sensors in a relatively large number of studies (Table 1). Examples range from the development of individual flow sensors to whole artificial lateral line systems to be used on swimming robots (Liu et al., 2016). In the former category, we find a range of inexpensive and low-powered micro-electromechanical systems (MEMS) sensors inspired by the superficial neuromasts of the Mexican blind cave fish (Triantafyllou et al., 2016). Interestingly, it has been shown that embedding these MEMS sensors in a hydrogel structure inspired by the cupula increases the sensitivity of the flow sensors 3.5–5 times compared to a naked sensor (Kottapalli et al., 2016). A similar functional MEMS based sensor with a hydrogel based capula inspired by canal neuromasts was developed with material and mechanical properties similar to the canal neuromasts in *A. fasciatus* (Bora et al., 2016). Complete artificial lateral line systems consisting of a series of flow sensors have also been developed and implemented on robotic fish (Liu et al., 2016). However, one of the main challenges with implementing effective artificial lateral line systems is finding the optimal location for placing them on the robotic fish (Yang et al., 2022). To address this issue, Yang et al. (2022) developed a hydrodynamic model based on the geometry of the Chinese blind cavefish (*Sinocyclocheilus*

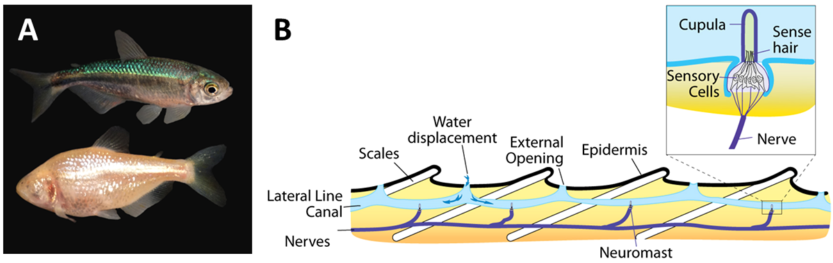

FIGURE 3
The lateral line system in blind cave fish. **(A)** A surface (above) and a cave (below) morph of the Mexican blind cave fish *Astyanax mexicanus*. Photo by Daniel Castranova, National Institute of Health, courtesy of FlickR (in public domain). **(B)** A schematic overview of the lateral line system showing the canal neuromasts consisting of nerves, sense hairs and the cupula. Created by Thomas Haslwanter and made available courtesy of Wikimedia Commons (CC BY-SA 3.0).

tianlinensis), which has a characteristic dorsal hump and horn. They compared results from their hydrodynamic model with flow sensors on the robot and found that the model was able to correctly predict where the superficial and canal neuromasts were placed on the real fish. This suggests that their biomimetic hydrodynamic simulation model can be used to predict optimal placement of flow sensors on robots and other small underwater vehicles (Yang et al., 2022).

Another interesting, albeit far less studied, example of a potential biomimetics sensor inspired by cave-dwelling species is that of the use of air vortex rings as a form of short distance communication in the African cave cricket, *Phaeophilacris spectrum* (Heinzel and Dambach, 1987). *P. spectrum* is found in East Africa and shows an affinity for caves for at least part of their life cycle (a likely troglophile), but it can be easily maintained in the laboratory (Heidelbach and Dambach, 1997). It is not troglomorphic, as it does not have any apparent reduction in pigmentation or eyes, although it does have relatively long antennae (Saussure, 1878). Unlike epigean crickets, *P. spectrum* males do not rely on stridulation (the sound produced when crickets rub their wings against each other) or other auditory signals to signal to females, possibly due to the lack of control of direction of acoustic signals due to echo in caves (Heinzel and Dambach, 1987). Instead they communicate over short distances of up to 15 cm by rapidly flicking their wings to generate travelling air vortices (Heinzel and Dambach, 1987). It looks likely that two different messages can be communicated; a threat display of a single vortex to nearby conspecifics and a courting display to nearby wingless females consisting of a rapid sequence of vortices (Heinzel and Dambach, 1987; Russell, 2011). This novel method of communication was implemented on a mobile robotic system carrying 8 air disturbance sensors that were found to be able to successfully respond to directional cues from a stationary vortex generator (Russell, 2011). Robotic communication by air vortices thus could be an additional form of inter-robot communication in close quarters, where traditional forms of communication such as wireless, acoustic or optical signals may not work or be undesired including underground, in high turbulence and turbidity water, or if part of secret military operations (Russell, 2011).

The final example relates to biologically inspired material properties in the form of adhesion under the high humidity conditions seen in caves based on the silk threads of cave glowworms. There are 9 described species of cave glowworms in the genus *Arachnocampa*, all of which are endemic to New Zealand and Australia (Baker, 2010). The short lived adult fungus gnats are capable of flight, but typically stay close to where they emerged, while the bioluminescent larvae can live up to 12 months with most populations inhabiting caves or cave-like habitats, although a few can also be found in other dark habitats such as sheltered tropical forests where they, interestingly, are more pigmented than the populations found in caves (Meyer-Rowchow, 2007; Baker, 2010). The larvae spin hanging silk snares that contain mucous adhesive droplets that are regularly arranged liked beads on a string (Meyer-Rowchow, 2007). The bioluminescence produced by the larvae lures flying insects such as moths, gnats and mosquitoes into the snares (von Byern et al., 2019). Both larval survival and the adhesiveness of the snares require the high humidity found in caves and rain forests (von Byern et al., 2019). The ability of the snares to function effectively at high humidity, was recently investigated by Wolff et al. (2021), who found that polar substances and hygroscopic compounds in the glue droplets, especially urea and a range of amino acids, were responsible for their function, although interestingly, variation was found both between species and between forest and cave populations (Wolff et al., 2021). Thus, glowworm glue droplets may serve as inspiration for developing moisture resistant and moisture activated adhesives that are required to function in environments with a constant high humidity, which would, for example, be useful as tissue adhesives in surgery (Wolff et al., 2021).

Promising cave adaptation examples

We have seen in the previous sections that while biomimetics studies on cave-adapted species should in theory provide a wealth of inspiration for novel technology, very few studies have in practice been carried out. In this section, I provide a flavour of the vast potential by highlighting a number of unusual adaptations in cave species, that I believe have significant biomimetics potential. Although again we should note that function, fitness benefit and heritability of the putative adaptation have not, in most cases, been tested explicitly. The examples I provide here roughly belong to one of three categories: adhesion and locomotion to smooth and wet surfaces, non-visual biomimetic sensors and wet resistant biomaterials and structures.

Adhesion and locomotion

Caves are characterised by often having very high humidity often with dripping water and water films covering the cave walls (Mammola, 2019). This makes it potentially challenging for cave-dwelling species to adhere to, and effectively move on, the smooth rock surface. One group of cave-adapted species that has been particularly well studied in this regard are the cave springtails, which, due to their size, in effect need to attach to, and move on, vertical water surfaces on the cave wall (Christiansen, 1961; Christiansen, 1965). They achieve this primarily by having claws with a longer, and to some extent more serrated, tooth (the unguis), which allows for a much better grip by anchoring the claw into the water film (Christiansen, 1961; Christiansen, 1965). Springtails are very common in caves with more than 300 described troglobiotic species from European caves alone (Fiera et al., 2021). Undoubtedly, more species awaits discovery, some of which may show additional variation and novel adaptations of their claws (Fiera et al., 2021). A case in point is the recent description of the troglomorphic springtail *Troglaphorura gladiator* from a cave in the Caucasus (Vargovitsh, 2019). This species has extremely long and thin claws, which are either completely submerged in or entirely rising above the water. This alternating sequence makes it in effect walk on water, while simultaneously ensuring a firm grip on the surface preventing the springtails from being blown away by air currents (Vargovitsh, 2019). These adaptations in springtail claws are reminiscent of the intricate setal morphology present in marine polychaete worms required to crawl on muddy and slippery substrate (Hesselberg and Vincent, 2006). The number, morphology and placement of these setae were studied from a biomimetic point of view and implemented on a simple crawling robot (La Spina et al., 2005) with the aim of using them for inspiration for developing novel self-moving colonoscopes (Hesselberg, 2007). The cave springtails may provide a similar inspiration for medical robots or devices required to move on watery and slippery surfaces.

Another potential and larger-sized group of organisms to study in this respect are the millipedes. Detritivorous millipedes are often found in caves, primarily in Europe, but also in relatively large numbers in tropical countries (Liu et al., 2017). Very little is known about the locomotion of cave millipedes, but they do show a number of convergent evolutionary traits including larger size and longer limb length and frequently elongated primary tarsal claws, although surprisingly not elongated antennae (Liu et al., 2017). Three recently described cave-adapted species from continental Portugal and Madeira show an interesting unique adaptation of their tarsal pads with very short accessory claws thought to be an adaptation to climb on the hard, smooth cave walls (Reboleira and Enghoff, 2014; Reboleira and Enghoff, 2018). We need more biomechanical studies on locomotion and foot morphology in troglomorph millipedes, since they might be useful for the ongoing development of biorobots inspired by the morphology and locomotion of aboveground millipedes (Lu et al., 2018; Garcia et al., 2021). A final intriguing example comes from the cave angel fish, *Cryptotora thamicola*, which is endemic to caves in Northern Thailand (Figure 4A), where it is found living in fast flowing streams (Brooke et al., 2016). It has a unique ability to climb hard, smooth rock surfaces in waterfalls using a tetrapod-like walking gait enabled by a robust pelvic girdle and other morphological adaptations otherwise only seen in terrestrial vertebrates (Brooke et al., 2016). We do not yet know how this ability might be facilitated by specialised adaptations of their fins to allow better grip with the smooth surface, but the combination of tetrapod-like crawling with normal fish swimming may in itself have potential for the development of amphibious biorobots (Ijspeert, 2020).

Biomimetic sensors

In the previous section, we discussed the relatively large effort that has gone into developing biomimetic flow sensors based on the lateral line system of blind cave fish. Interestingly, one cave-adapted fish, the catfish *Astroblepus pholeter*, has relatively few neuromasts and instead seems to rely on innervated dorsal skin teeth (denticles) to detect hydrodynamic forces (Haspel et al., 2012). The catfish is endemic to Jumandi Cave in Ecuador, where it lives in a very fast

FIGURE 4
Some cave adapted species with biomimetic potential. **(A)** The cave angel fish (*Cryptotora thamicola*) is adapted to climb slippery rocks in fast flowing streams. Photo: Chulabush Khatancharoen via FlickR (CC BY 2.5). **(B)** The springtail (*Troglobentosminthurus luridus*) has extremely long antennae, which likely function as effective direct mechanosensors. Photo modified from de Souza et al., 2022 (CC BY 4.0). **(C)** The cave adapted midge (*Troglocladius hajdi*) is capable of flying in total darkness, likely using the its long forelegs as feelers. Photo taken by J. Bedek and modified from Anderson et al., 2016 (CC BY 4.0).

flowing and turbulent stream suggesting that these denticles could provide inspiration for a different type of biomimetic flow sensor. In addition to fish, many other cave adapted species are likely to have evolved heightened non-visual sensory systems to compensate for the lack of light suggesting a rich source for inspiration in developing novel mechanosensory systems. Many invertebrates, especially arthropods, have convergently evolved elongated limbs and sensory structures, presumably in order to increase the range of their contact mechano-sensory system (Howarth, 1983; Christiansen, 2012), although more function based studies are needed to confirm that these are in fact adaptations. The recently described cave springtail, *Troglobentosminthurus luridus*, from the Água Clara cave system in Brazil (Figure 4B), shows, among other troglomorphic traits, extremely elongated antennae (de Souza et al., 2022). The antennae are more than twice the length of the body and consist of 44 subsegments. While the behaviour of this new species is virtually unknown, gut content analyses suggest that they, unusually for a springtail, may act as predators (de Souza et al., 2022). Future studies on this, and other troglobites with very long antennae, should provide interesting data on how they move efficiently with such long antennae, and how they manage to sweep them effectively to detect obstacles, prey and predators, which might have significant biomimetic potential for developing subterranean search and rescue robots (Rouček et al., 2020).

Most cave-dwelling insects, except for the species found in the entrance and twilight zones, have lost or reduced wings and hence do not fly (Wagner and Liebherr, 1992), which make sense given that insect flight is heavily dependent on vision for optomotor flight control (Fry et al., 2009; Lecoeur et al., 2019). Nonetheless, a few troglomorph species of flying insects have been discovered including the pale midge, *Troglocladius hajdi*, with has reduced eyes, long legs and normal wings (Andersen et al., 2016) (Figure 4C). It is known only from one chamber 800–1000 meters down the vertical Lukina jama–Trojama cave system in Croatia. It has so far never been observed in flight, but its morphology suggests that it could engage in slow flight with its long legs stretched ahead of it acting as feelers (Andersen et al., 2016), which could provide alternative inspiration for non-visual sensory structures in micro-air vehicles required to operate in dark and spatially complex environments.

Biomaterials and structures

In the previous section, we saw how the silk used in glowworm capture threads may have biomimetic potential due to their ability to operate effectively at a very high relative humidity. However, glowworms are not the only animals that rely on silk in subterranean habitats. Spiders are relatively common in caves with more than 1000 troglobiotic species worldwide (Mammola and Isaia, 2017). No studies on the mechanical properties of the silk of spiders inhabiting the deep zone have yet been conducted, but one study on the Tasmanian cave spider, *Hickmania troglodytes*, which build large horizontal sheet webs within the twilight zone, found that the structural silk of these webs are as tough as that in vertical orb webs and that they show a unique ontogenetic shift with larger spiders constructing webs using tougher silk (Piorkowski et al., 2017). More than 80% of spiders found in caves build webs (Mammola and Isaia, 2017; Mammola et al., 2022). This combined with the unique environment, especially in relation to the occasionally strong and turbulent airflows near the mouth of many caves (Tuttle and Stevenson, 1977; Gomell and Pflitsch, 2022), suggests that it might be worth paying closer attention to these silk structures. *Meta* cave spiders, for example, construct their orb webs in the twilight zones of many temperate caves (Hesselberg et al., 2019). Their webs differ from epigean orb webs in that they are smaller, less dense, lack frame threads and have many more attachment points to the cave walls (Simonsen and Hesselberg, 2021). While these modifications are primarily thought to be adaptations to capture non-flying prey on the cave walls, they could also reduce web damage caused by wind, as reduced size and less dense spiral turns are generally seen in orb webs built in windy conditions (Vollrath et al., 1997; Wu et al., 2013). Thus, more biomechanical studies on the silk and geometry of these webs might yield insights that could be beneficial in the development of strong lightweight biomimetic structures (Leach et al., 2014; Vollrath and Krink, 2020). This potential was highlighted in a study of the tensile strength of the silk stalks of the eggsac of *Meta menardi*, which are often built close to the cave mouth (Lepore et al., 2012). The study found that these eggsacs have the stretchiest stalks ever measured allowing them to regularly extend up to 300% before breaking (Lepore et al., 2012).

Conclusion

In this review, I have shown that the unusual environmental and ecological factors that drives evolution in subterranean habitats have resulted in highly unique adaptations with significant biomimetic potential, especially within the areas of biomimetics sensors, biomaterials, adhesion and biologically inspired robotic locomotion. Our discussion has mainly focussed on cave-adapted species found in terrestrial or aquatic habitats in the deep zones of large caves, where convergent evolution has often resulted in depigmentation and eye loss enabling the development of effective non-visual sensory systems, and in limb elongation increasing the effective mechanosensory range (Culver and Pipan, 2019; Mammola, 2019). Undoubtedly, many more discoveries on adaptations with biomimetics potential awaits discovery in these zones, but it is worth noting that troglobiotic species are also found in other subterranean habitats such as lava tubes, mines and under stones and boulders in scree habitats (Růžička et al., 2013; Culver and Pipan, 2019). It has also been argued that many cave-adapted species actually spend most of their time in the small fissures and cracks adjacent to the large chambers in which we observe them (Howarth, 1983; Mammola et al., 2021). Adaptations to live in such small spaces may offer inspiration for search-and-rescue robotics, similar to how the amazing compressibility of aboveground cockroaches have been used to develop robust and versatile biorobots (Jayaram and Full, 2016). Additionally, species found in other types of caves may offer significant biomimetic potential. Remipedes, for example, are eyeless crustaceans found in marine caves, where they swim in an unusual upside-down way, while continuously beating their trunk limbs in a metachronal manner (Koenemann et al., 2007). Their flexible and elongated trunks give them excellent steerability, but they are also capable of sudden jumps through the water while beating their limbs simultaneously suggesting overall high biomimetic potential for the development of versatile autonomous underwater robots (Kwak and Bae, 2018).

The present study highlights that more research effort needs to go into discovering and describing new cave-adapted species, which allows us to identify suitable candidates species for further biomimetic studies. However, equally importantly is more in depth functional studies on physiological and behavioural processes of already discovered species given that the most promising biomimetic potential relate to the biomechanical function of morphological structures in terms of locomotion, adhesion and mechanosensory sensitivity. Traditionally, such studies have been difficult to carry out in caves due the large number of impediments facing cave researchers including difficult working conditions, low abundance of target species and difficulties in studying cave-dwelling species in ex-situ laboratories (Mammola et al., 2021). However, recent technological advances in computer simulation and remote sensing/bioinspired robotics as well as more appreciation of the value of having laboratories in caves should allow us to overcome many of these impediments (Mammola et al., 2021). Studies on adaptations have also recently been identifies as one of the most important areas of research for subterranean biologists (Mammola et al., 2020), so it is likely that we will see a huge growth of biomimetics studies on cave-dwelling species in the near future.

It is also my hope that this paper will provide an additional case for the importance of conserving subterranean habitats. While caves are to some extent buffered from many of the main threats facing aboveground ecosystems including climate change, pollution, habitat fragmentation and invasive species (Brook et al., 2008), troglobites, evolved to survive in stable conditions nonetheless face serious threats from even minor disruptions to environmental conditions caused by climate change or human visitors and tourisms, as well as large scale destruction of habitats caused by mining or excessive groundwater extraction (Mammola et al., 2019). The present study gives an overview of some of the many unique adaptations found in cave-adapted species, but also suggests that there is a vast reservoir of species and adaptations that we have not yet discovered. We must therefore preserve as much of the cave biodiversity as possible since it is very likely that future technologies including biorobotics, internal surgery and novel adhesive structures depend on the inspiration we can get from studying subterranean species.

Finally, the present study also highlights the importance of convergent evolution in identifying biomimetics potential. Arguably, convergent adaptations, i.e. the independent evolution of similar traits in response to similar environmental drivers, suggest a more optimal solution to a given problem than non-convergent traits that may be more evolutionarily constrained (Graeff et al., 2020). However, to make full biomimetics use of convergence, we need many more studies on the adaptive value of traits (i.e. function, fitness benefits and heritability) from not only subterranean environments, but also from other harsh ecosystems such as deserts, polar regions and hydrothermal vents.

Author contributions

TH developed the idea and wrote up the manuscript.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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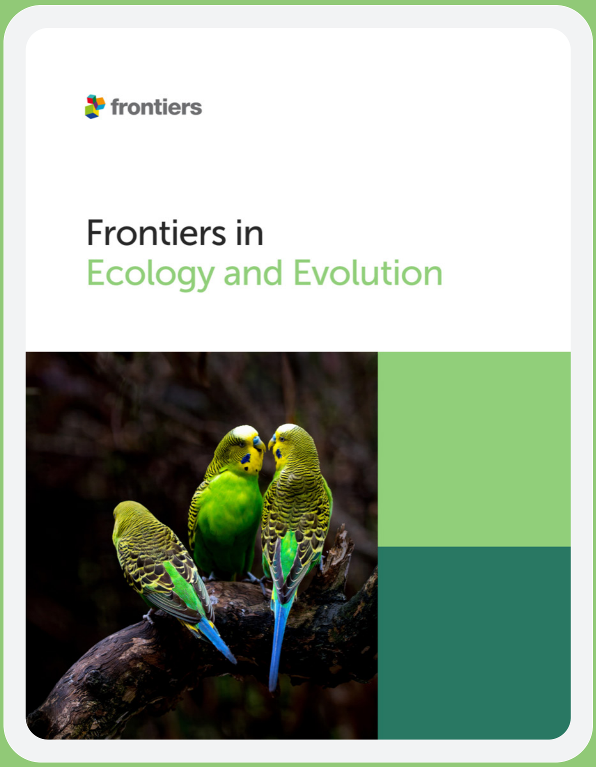

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