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A new genus and species of nicoletiid silverfish (Insecta, Zygentoma, Nicoletiidae) from caves of northern Alabama, USA

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Abstract

A new genus and species of troglobiotic nicoletiid (Insecta, Zygentoma, Nicoletiidae) is described from northern Alabama, USA. The type species was collected from three caves in the Highland Rim section of the Interior Low Plateau physiographic province on the northern side of the Tennessee River Valley. Morphological and genetic analysis using the mitochondrial 16S rRNA locus show that *Spinanycta alabamensis* **sp. nov.** is quite distinct from related nicoletiids in North America. The species differs from members of other genera by its urosternum I, which in males is modified with a central pointy extension. The new species significantly extends the distribution of cave-dwelling members of the family into the southeastern United States and suggests that additional nicoletiid diversity remains to be discovered from karst regions of the eastern United States.

Keywords

Insect, Interior Low Plateau, subterranean**,** Thysanura, troglobite, troglobiont

Introduction

Among the insects, Silverfish (order Zygentoma) are some of the most intriguing. This group diverged before the appearance of wings and thus are considered among the earliest, most primitive insects. Their predecessors were among the first arthropods to colonize terrestrial habitats and were present during the Silurian Period more than 400 million years ago (Grimaldi and Engel 2005). Within Zygentoma, members of Nicoletiidae typically live in habitats lacking light, with distinctive characteristics for a subterranean existence, such as lack of pigment and eyes. While intrinsically interesting because of their specialized ecology, our understanding of their biodiversity has been greatly constrained, like many troglobionts (Niemiller et al. 2018; Mammola et al. 2019; Niemiller and Taylor 2019), by the difficulties inherent in collecting samples from subterranean environments. For example, the subfamily Cubacubaninae is a seldom encountered group of silverfish that were considered a minor group of little importance primarily restricted to the Neotropics, where they are found under rocks and logs (Espinasa et al. 2007). However, Cubacubaninae is now known to be a much more diverse group with a range extending into southern North America owing to recent studies by cave biologists (Espinasa et al. 2014; Fig. 1). Members of the genus *Texoreddellia* Wygodzinsky, 1973 have been described and documented from more than 150 caves in Texas, USA, and northern Mexico (Espinasa et al. 2016, 2023). *Speleonycta ozarkensis* Espinasa et al., 2010 is known from eight caves in the Ozarks Highlands region of Oklahoma and Arkansas (Espinasa et al. 2014; M.E. Slay, personal communication). *Speleonycta anachoretes* Espinasa et al., 2012 is known from two caves in Arizona, and three caves from California are inhabited by an undescribed species of *Speleonycta* Espinasa et al., 2010.

Here we describe a new genus and species of cave nicoletiid from caves of the Interior Low Plateau karst region in northern Alabama, recently discovered during cave biosurveys in the region. The new species significantly extends the distribution of cave-dwelling members of the family into the southeastern United States and increases the likelihood that additional diversity remains to be discovered in the Interior Low Plateau and other karst regions of eastern North America.

Material and methods

Specimen collection and approach

Specimens were collected as part of a biological inventory of cave life in northern Alabama. Biosurveys consisted of visual encounter surveys (VESs) by a minimum of two people for at least two-person hours in all caves. VESs of terrestrial habitats included the cave floor, walls, and ceiling, as well as underneath rocks, logs, and other debris when available. Any cover objects were returned to their original positions to minimize microhabitat disturbance. Specimens encountered were collected by hand and preserved in 100% ethanol, then stored at -20 °C for molecular analysis. Specimens were accessioned into the Entomology Collection at the Auburn Museum of Natural History.

Morphological analyses

Specimens were examined and dissected using a Motic stereomicroscope. Photographs of some structures were recorded using an iPhone 12 mini camera attached to the dissecting microscope. Photographs were taken in multiple focal planes and processed so that the whole image was in focus using the Zerene Stacker software package (Zerene Systems LLC, Richland, Washington, USA). Drawings of some pertinent structures were prepared with the aid of camera lucida attached to a Motic dissecting microscope.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using Qiagen's DNEasy® Tissue Kit by digesting a leg in lysis buffer from five specimens from Muddy Cave in Madison County, Alabama (Alabama Cave Survey (ACS) cave no. AMD1095) collected on 7 July 2022, one from Rockhouse Cave in Limestone County, Alabama (ACS no. ALM312) collected on 21 May 2020, and the holotype and three paratypes from Bobcat Cave in Madison County, Alabama (ACS no. AMD1283) collected on 26 November 2019.

PCR amplification and Sanger sequencing of a portion of the mitochondrial 16S rRNA locus followed standard protocols and primers (*16Sar* and *16Sb*) used in the past for nicoletiids (Espinasa and Giribet 2009). PCR products were purified using QIAquick® PCR Purification Kit and sequenced by Elim Biopharmaceuticals Inc (Hayward, California, USA). Resulting sequences were visualized and assembled into contigs using the sequence editing software Sequencher™ 3.0. Sequencing primers were trimmed and not included in subsequent analyses.

Phylogenetic analyses

BLAST analysis was done to determine the most similar sequences in GenBank. Sequences of all other nicoletiid species available were aligned and a preliminary tree analysis was performed in ClustalW2 (ML tree was inferred using PhyML v20160115 ran with model and parameters: -o tlr -f m --pinv e --bootstrap -2 --alpha e --nclasses 4. Branch supports are the Chi2-based parametric values return by the approximate likelihood ratio test). DNA sequences for specimens are in NCBI's GenBank (accession no. PP057689).

Conservation assessment

We conducted both NatureServe and IUCN Red List conservation assessments for the newly described species. For the IUCN Red List assessment, the risk category was calculated using RAMAS Red List 3.0 (Akcakaya et al. 2007) following criteria in IUCN (2010, 2012). For the NatureServe assessment, we employed the NatureServe Rank Calculator v3.186 (Faber-Langendoen et al. 2012) following criteria in Master et al. (2009). We calculated two different measures of geographic range size using the web-based program GeoCAT (Bachman et al. 2011; available at https://geocat.kew.org): extent of occurrence (EOO) as a minimum convex hull and area of occupancy (AOO) using a grid size of 2 km (4 km^2). Finally, we employed the threat classification scheme proposed by Salafsky et al. (2008) to determine the overall threat impact to the species.

Results

Class Insecta Linnaeus, 1758
Order Zygentoma Börner, 1904
Suborder Neozygentoma Engel, 2006
Infraorder Euzygentoma Grimaldi & Engel, 2005
Family Nicoletiidae Escherich, 1905

***Spinanycta* Espinasa, Gutierrez & Niemiller, gen. nov.**
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Diagnosis. An American nicoletiid with Urosterna II–VII subdivided into two coxites and one sternite. Urosterna I, VIII and IX of male entire. Coxites on segments II–IX with styli. Urosternum I of males modified with a central pointy extension. Urosternum VIII of male flat posteriorly, without emarginations or projections in between the styli of this segment. Paramera with a distal semi-eversible vesicle and short chaetae, their length being about 1/4 the width of the paramera. Subgenital plate of females subtriangular. Tergum X with one distinct macrochaetae on posterior angles. Cercus of male with sensory pegs (spines) and appendix dorsalis without sensory pegs.

Description. Pedicellus of adult male with unicellular glands. Mouthparts not specialized. Mandible strongly sclerotized apically with usual teeth. Apex of galea with two conules; one longer than wide and the other wider than long. Lacinia heavily sclerotized distally. First process of lacinia pectinate. Labium without prominent lateral lobes.

Tarsi with four articles. Praetarsi with three simple claws. Median claw glabrous, slender and smaller than lateral claws. Urosterna II–VII subdivided into two coxites and one sternite. Urosterna VIII and IX of male entire. Median portion of sternites with 1+1 sublateral macrochaetae at hind borders, as well as 1+1 macrochoetae near suture at about middle of segment. Coxites on segments II–IX with styli. Eversible vesicles on segments II–VI, pseudovesicles on VII. Urosterna I of males modified with a central pointy extension. Urosterna III and IV of adult males apparently without modifications. Urosternum VIII of male straight posteriorly, without emarginations or projections in between the styli of this segment. Tergum X with 1+1 distinct macrochaetae on posterior angles.

Point of insertion of paramera apparently slightly deep. Paramera with a distal semi-eversible vesicle, but less prominent than in *Texoreddellia* (Wygodzinsky 1973) or *Speleonycta* (Espinasa et al. 2010) and with distal chaetae short, their length being about 1/4 the width of the paramera. Stylus IX apparently without sensory pegs in males as seen in some species *Prosthecina* Silvestri, 1933 or *Anelpistina* Silvestri, 1905 (= *Cubacubana* Wygodzinsky & Hollinger, 1977; syn. = *Neonicoletia* Paclt, 1979) (Espinasa et al. 2007). Opening of penis longitudinal. Cercus of male with sensory pegs. Appendix dorsalis without sensory pegs. Female with a subtriangular subgenital plate.

Molecular diagnosis. Sequences generated for the mitochondrial 16S rRNA locus for all ten specimens from the three different caves were 529 bp long and identical.

BLAST analysis showed this 16S haplotype to be quite different from any sequence available in GenBank (maximum of 77.26 Per. ident.). Within the American Nicoletiidae, *Spinanycta* differs from the genus *Allonicoletia* Mendes, 1992 by 130 bp (24.5%), from *Speleonycta* by 132 bp (24.9%), from *Texoreddellia* by 134 bp (25.3%), from *Squamigera* Espinasa, 1999 by 138 (26.0%), from *Gibboletia* Espinasa & Smith, 2023 by 145 bp (27.4%), from *Prosthecina* by 148 bp (27.9%), from *Anelpistina* by 155 (29.3%), and from *Nicoletia* Gervais, 1843 by 153 bp (28.9%). Using the 16S rRNA fragment sequences of nicoletiid species across the subfamily Cubacubaninae (Espinasa et al. 2007), it can be observed that pairs of specimens from different populations of the same species differ by an average of 3.4 nucleotides (range 0 to 13; n = 22) and by 31.2 nucleotides (range 10 to 64; n = 14) among sister species. The 130+ bp between the Alabama specimens and any previously described species of American nicoletiid suggests these populations represent a new species and genus.

Maximum-likelihood phylogenetic analysis showed that members of genera *Speleonycta*, *Allonicoletia*, *Texoreddellia* and *Nicoletia* form a well-supported clade, with the Alabama specimens as sister to this group, and also in a clade distinct from other nicoletiid genera including *Anelpistina*. These results indicate that the Alabama nicoletiids belong to a group independent from any previously described genera for which 16S rRNA sequences are available. *Acanthonima* Espinasa, 2005 has yet to have its DNA sequenced, but morphologically it is very different from the Alabama specimens (See below). Resolving the placement of all aforementioned genera within subfamilies was beyond the goals of the current study.

Type species. *Spinanycta alabamensis* sp. nov.

Etymology. *Spinanycta*. From *spina* = Greek for spine. It references the diagnostic spine in urosterum I, and *nycta* = Greek for night. It references in Greek mythology the occupation of caves by Nyx, the primordial goddess of night.

Remarks. *Spinanycta alabamensis* belongs to the American Nicoletiidae characterized by subdivided abdominal sterna II–VII and fused coxites of abdominal segments VIII and IX (Cubacubaninae + Nicoletiinae; Mendes 1988), or to a still undescribed subfamily that shares these characteristics. *Spinanycta alabamensis* is distinguished from all genera of these subfamilies by the urosterna I of males modified with a central pointy extension, although male sexual secondary characters in urosterna I–IV are highly variable among species.

The type species of the new genus shares some characteristics with *Speleonycta*, *Allonicoletia*, *Texoreddellia*, and *Nicoletia*, such as the paramera with semi-eversible vesicles, urosternum VIII of male straight posteriorly without emarginations or projections in between the styli of this segment. None of these characteristics are present in any of the other genera of Cubacubaninae. The new genus can easily be distinguished from *Speleonycta* because it lacks the very long and specialized chaetae in the distal semi-eversible vesicle of the paramera. From *Allonicoletia* they can further be differentiated by the presence of styli on urosternite II. From *Texoreddellia* and *Squamigera* by the absence of scales. From *Prosthecina* by the absence of conspicuous lateral lobes bearing numerous glandular pores in the submentum. From *Anelpistina* as defined by

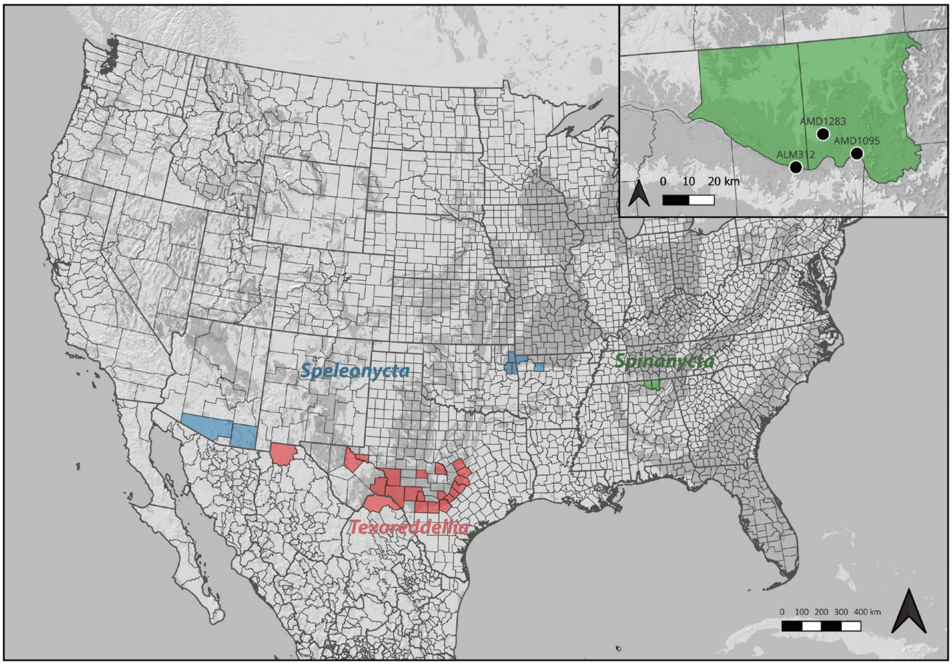

Figure 1. Distribution of cave-dwelling nicoletiid genera in northern Mexico and the southern United States. Shaded areas are municipalities and counties in Mexico and the United States, respectively, with documented occurrences for each genus. Dark gray shaded areas are karst exposures in the United States. Inset is the distribution of *Spinanycta alabamensis* sp. nov. in northern Alabama with Alabama Cave Survey cave codes labeled. Bobcat Cave (AMD1283) is the type locality.

Espinasa et al. (2007), by its urostenum VIII without emarginations or projections in between the styli of this segment and its distinctive paramera. From *Acanthonima* by the absence of sensory pegs in the appendix dorsalis. From *Nicoletia* by its gonapophysis with more than 12 annuli.

Distribution. This genus is known from just three cave systems on the north side of the Tennessee River Valley in southern Madison and southeastern Limestone counties, Alabama, within the Highland Rim section of the Interior Low Plateau physiographic province (Fig. 1).

Spinanycta alabamensis Espinasa, Gutierrez & Niemiller, sp. nov.

https://zoobank.org/A7588D17-B2DA-4B00-839B-6E49C30CF3D6
Figs 2–4

Type material. ***Holotype***: male 8.5 mm, from Bobcat Cave (34.6686°N, 86.7130°W), Alabama Cave Survey (ACS) no. AMD1283. Redstone Arsenal, Madison County, Alabama, USA, deposited into the Entomology Collection at the Auburn Museum of Natural History (AUM 221500), collected 26 November 2019 by Matthew L. Niemiller, Joseph Lamb, Amata Hinkle, and Brian Stoltz (field code MLN 19-050).

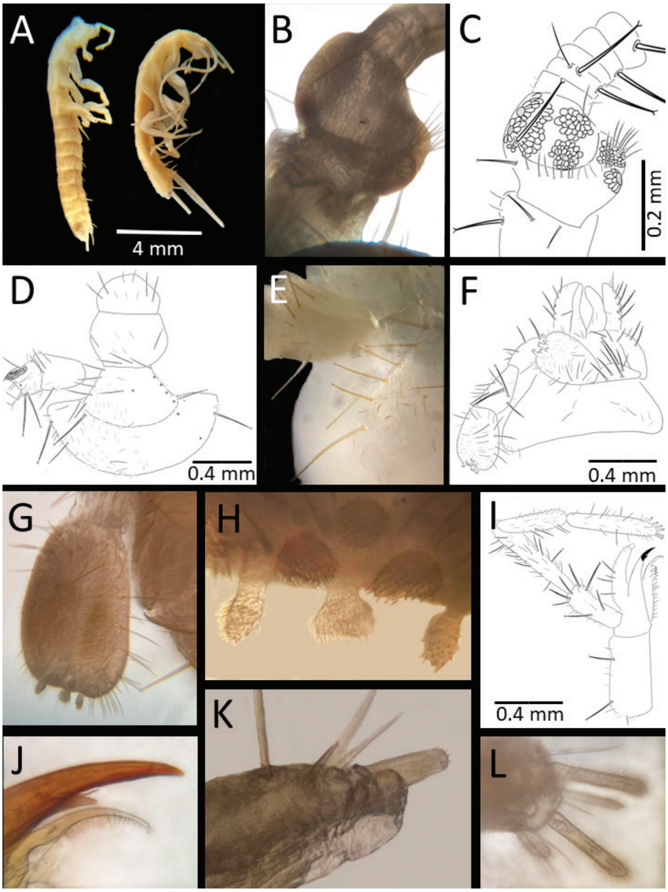

Figure 2. *Spinanycta alabamensis* sp. nov. **A** male holotype (left) and female paratype (right) **B–L** male holotype **B, C** pedicellus **D** head **E** macrochaetae by insertion of antennae **F** labium **G** last article of labial palp **H** sensilla of maxillary palp **I** maxilla **J** apex of lacinia **K** conules of galea **L** sensilla of maxillary palp.

Paratypes: 3♀ paratypes with same collection information as holotype (AUM 221501–221503); 1♂, 3♀ paratypes from Bobcat Cave collected on 6 October 2020 by Matthew L. Niemiller, Joseph Lamb, and Amata Hinkle (field code MLN 20-036.6) (AUM 221504–221507); and 1♂, 1♀ from Bobcat Cave collected 13 September 2023 by Amata Hinkle, Brendan T. Cramphorn, Jared P. Higgs, and Eric C. Maxwell (field code BTC 23-019-1) (AUM 221508–221509).

Additional material: 2♂, 2♀ from Muddy Cave (ACS no. AMD1095), Madison County, Alabama, USA, collected 15 June 2020 by Matthew L. Niemiller, K. Denise Kendall Niemiller, Amata Hinkle, Joseph Lamb, and Katherine E. Dooley (field code MLN 2020-021.1) (AUM 221510–221513); 1♂, 2♀ from Muddy Cave collected 7 July 2022 by Matthew L. Niemiller, Brendan T. Cramphorn, and Eric Cline (field code BTC 22-110-3) (AUM 221514–221516).

1♀ from Rockhouse Cave (ACS no. ALM312), Wheeler National Wildlife Refuge, Limestone County, Alabama, USA collected 21 May 2020 by Matthew L. Niemiller, K. Denise Kendall Niemiller, Amata Hinkle, and Katherine E. Dooley (field code MLN 20-020.6) (AUM 221517).

Etymology. *S. alabamensis*. Derived from the word Alabama, the state where the species was discovered.

Type locality. USA. Alabama, Madison County, Bobcat Cave (ACS no. AMD1283) on Redstone Arsenal. Redstone Arsenal is a 154.8 km^2 United States Army installation in southwestern Madison County adjacent to the city of Huntsville. Redstone Arsenal is host to more than 75 tenant agencies, including the Department of Defense, Department of Justice, Federal Bureau of Investigation, and, most notably, the National Aeronautics and Space Administration's Marshall Space Flight Center. Bobcat Cave is located near the western border of Redstone Arsenal (34.6686°N, 86.7130°W) on a small knoll. Redstone Arsenal and Bobcat Cave are situated within the Tennessee River District of the Highland Rim section of the Interior Low Plateau Province. The cave is developed in the Tuscumbia Limestone with a mapped length of 454.1 m (1,490 ft) and vertical extent of 6.4 m (21 ft). Much of the cave consists of a series of low and broad passages connected by tight crawlways with little exposure of bedrock. Water levels in the cave respond quickly to heavy precipitation and can flood completely. Significant flooding events continually deposit mud on nearly all surfaces in the cave. It is on these mud substrates and under the few mud-encased rocks nearer the entrance that most *Spinanycta alabamensis* have been found. In addition, individuals have been observed on the surface of isolated pools in the Shrimp Room, the largest room in the cave (61 m by 30.5 m).

Description. Male holotype 8.5 mm (Fig. 2A) from Bobcat Cave, collected 26 November 2019. Thorax width 1.5 mm. When complete, antennae about 1.25× the length of the body and tails about 0.75×. General color whitish, yellow (Fig. 2A). When complete, antennae are about 1.25× the length of the body and tails about 0.75× the length of the body. Pedicellus about half the length of the scape, with unicellular glands clustered ventrally in four groups with a row of microchaetae bordering them in form of a "U". On its lateral outside side there are an extra two groups of unicellular glands in a large, bulbous projection (Fig. 2B, C). Female pedicellus simple and slightly less than half the length of the scape. Head with about 6+6 macrochaetae near the insertion of each antenna (Fig. 2D, E).

Mouthparts as shown in Figs 2F–L, 3A. Appendages longer than epigeomorphic species, but shorter than highly troglomorphic nicoletiids. Labial palp's apical article's long. Its width is about 2/3 its length and slightly longer than the penultimate article (Fig. 2F–G). As in other cave-adapted nicoletiids with long appendages, the bulge containing 2 macrochaetae in the penultimate article of the labial palp is not too prominent (Fig. 2F). Labium and first article of labial palp with macrochaetae. The sensilla of the labial palp are longer than other American nicoletiids due to being supported by a longer than common trunk (Fig. 2H). Last article of maxillary palp 1/3 longer than the penultimate article (Fig. 2I). Apex of lacinia as in Fig. 2J. Apex of galea with two conules; one longer than wide and the other wider than long (Fig. 2K). Apex of maxillary palp as in Fig. 2L. Mandible chaetotaxy with 4 macrochaetae (Fig. 3A).

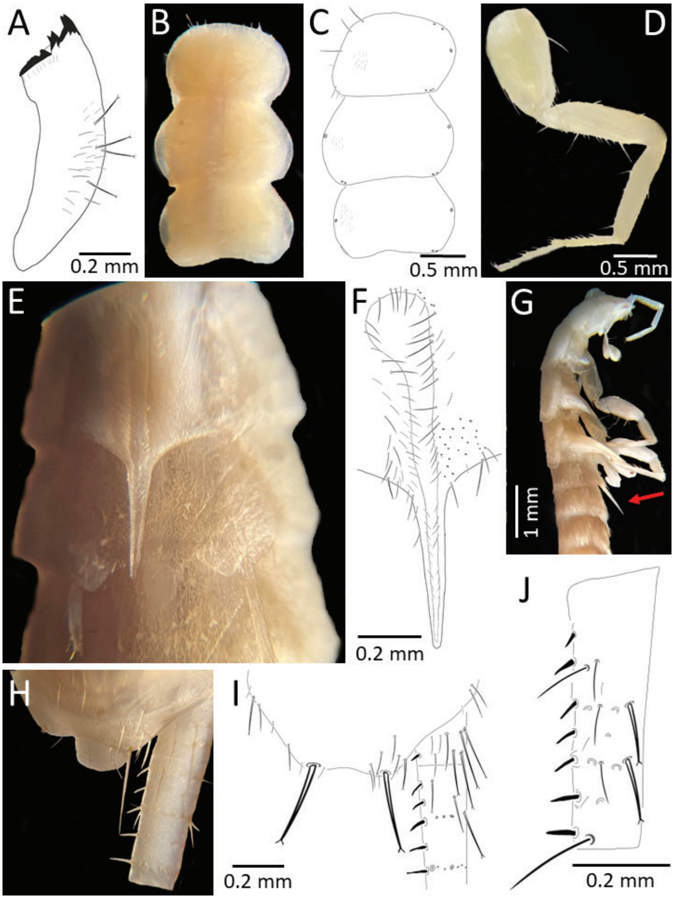

Figure 3. *Spinanycta alabamensis* sp. nov. **A–C, E–J** male holotype **D** female paratype **A** Mandible **B, C** thorax **D** hind leg **E, F** ventral view of spine of urosternum I **G** lateral view of male holotype highlighting (red arrow) the spine of urosternum I **H, I** urosterguite X **J** cercus.

Thorax as in Fig. 3B, C, with 1+1 macrochaetae on their lateral sides plus several posterolateral. Legs not too long when compared to other troglobitic nicoletiids, as shown in Fig. 3D. Hind tibia approximately 5 times longer than wide and approximately 3/4 length of tarsus. Claws of the praetarsus and empodium with a hairy appearance covered basally with small microtrichiae, similar to Molero-Baltanás et al. (2020; fig. 23). Abdominal sterna II–VII subdivided into coxites and sternites (Fig. 3E). Sterna I, VIII and IX of male entire. Styli on sterna II–IX. Sterna I of males modified with a long, narrow point extending to the apex of sterna II (Fig. 3E–G). This point has in its center a groove surrounded by many macrochaetae that extends to the base of sterna I (Fig. 3F). No apparent modifications in male's sterna III and IV.

Posterior end of urotergite X with a 1+1 long macrochaetae (Fig. 3H, I), similar to *Nicoletia phytophila* Gervais, 1844 (Espinasa et al. 2011; Fig. 1C, D). Distance between macrochaetae about the same as the length of the macrochaetae. Area between the distinct macrochaetae with a small concavity (Fig. 3I). Cercus of adult male with a longer than wide basal annulus with one small spine, followed by a longer annulus, with multiple subequal spines (Fig. 3I, J). Female cerci without modifications.

Urosternum VIII of adult male flat between the insertion of the styli (Fig. 4A), without an emargination bordered by projections, as in other Cubacubaninae. Point of insertion of parameres in urosternum IX deep and with coxal processes with several setae slightly more sclerotized (Fig. 4A, B). Parameres as in Fig. 4A, B. Parameres barely surpass the base of styli. Paramera with a distal semi-eversible vesicle and with some short, modified setae (Fig. 4B). Stylus IX with 2 macrochaetae and an extra subapical pair, and larger than other styli (Fig. 4A). Other styli with 1 macrochaetae plus subapical pair. Terminal spine with one small tooth.

In adult females, the ovipositor surpasses apex of stylus IX between 3 and 3+2/3 the length of the stylus IX (Fig. 4G). Gonapophyses with about 21–22 divisions. The subgenital plate has a subtriangular shape (Fig. 4E, F) that ends in a slight point (Fig. 4G), similar to the one in *Nicoletia phytophila* (Espinasa et al. 2011; Fig. 1A, E), although the slight point is less prominent in the new species.

Postembryonic development. Males at 5.5 mm have no glands in pedicellus, no spine in urosternum I, or spines in cerci, but they already have paramers. At 6.5 mm they have glands in the pedicellus, the spine in urosternum I reaches about ¾ of the apex of urosternum II, and spines in cerci are small. At 7 mm the spine reaches the apex of urosternum II and by 8.5 mm it surpasses by 1/10 the length urosternum II.

Females at 5.5 mm have an ovipositor that barely surpasses the apex of stylus IX and gonapophyses with about 15 divisions. By 7 mm ovipositor surpasses apex of stylus IX by about 2+2/3 the length of the stylus and the gonapophyses has the adult number of 21–22 divisions. Between 7.2 mm and 10.5 mm in the largest available specimen the ovipositor surpasses stylus IX by between 3 and 3+2/3 the length of the stylus.

Geographic distribution. The species is known to date only from three caves, all developed in the Tuscumbia Limestone and located along the northside of the Tennessee River in southern Madison and southeastern Limestone counties. The largest population at Bobcat Cave is located on Redstone Arsenal. Muddy Cave is owned and managed by the Land Trust of North Alabama and is located 15.1 km SE of Bobcat Cave adjacent to a large residential area. Rockhouse Cave is located 16.5 km SW of Bobcat Cave and is developed in an exposure of limestone, Rock House Bluffs, on the north shore of the Tennessee River on Wheeler National Wildlife Refuge. The current distribution represents an EOO of 124.1 km^2 and AOO of 12.0 km^2.

Habitat and ecology. At Bobcat Cave, *Spinanycta alabamensis* has been observed actively crawling on mudbanks and underneath mud-covered cobble and rocks adjacent to pools in the dark zone below the slope from the entrance and throughout the Shrimp Room (Figs 5, 6). On two occasions, individuals were observed on the surface of a pool. As many as 20 individuals have been observed during a survey at Bobcat Cave (Table 1). At Muddy Cave, individuals have been observed on mudbanks and underneath mud-covered cobble near pools primarily before the tight crawl to the northern section of the 348-m (1143-ft) cave system. A single individual has been

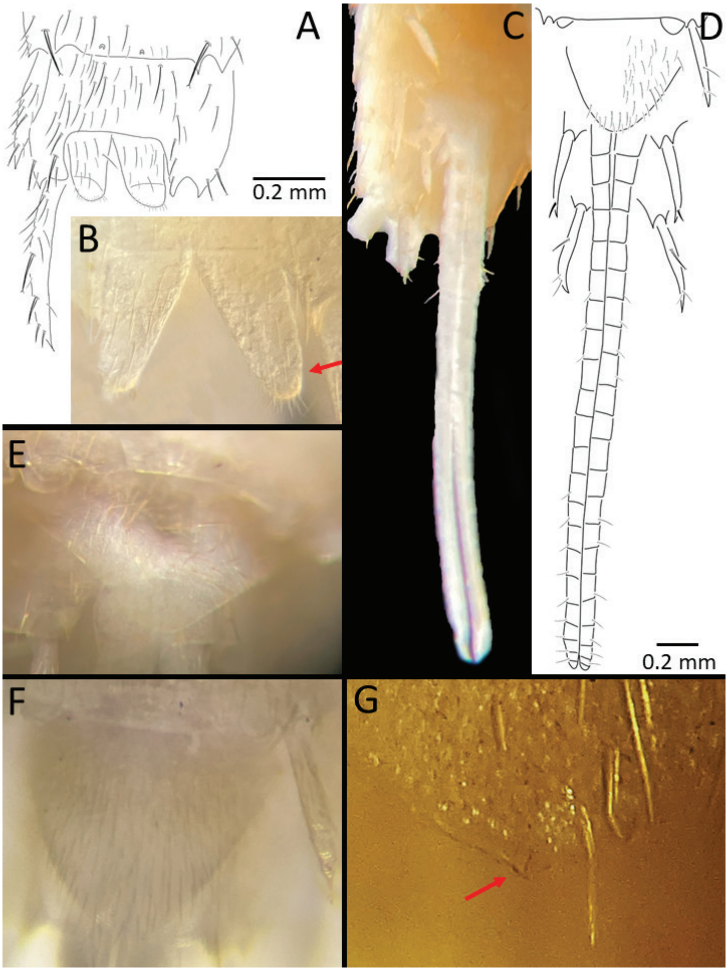

Figure 4. *Spinanycta alabamensis* sp. nov., **A, B** male paratype **C–G** female paratypes **A** male genital area **B** parameres highlighting (red arrow) distal semi-eversible vesicle **C, D** ovipositor **E, F** variability in the subtriangular shape of the subgenital plate **G** apex of subgenital plate highlighting (red arrow) its slight point.

observed and collected at Rockhouse Cave. A female was found under a small (12-cm diameter), loose, but mud-covered rock adjacent to an isolated pool in a fissure in the northern section of the 384-m (1259-ft) long cave system. All observations to date have been made in sections of the three cave systems that completely flood during heavy precipitation events. *Spinanycta alabamensis* co-occurs with several other troglobiotic taxa, including the spiders *Phanetta subterranea* (Emerton, 1875) and *Liocranoides unicolor* Keyserling, 1881, millipede *Scoterpes* sp. Cope, 1872, springtail *Pseudosinella* sp. Schaeffer, 1897, two-pronged bristletail *Litocampa* sp. Silvestri, 1933, and isopod *Miktoniscus* sp. Kesselyak, 1930.

Conservation. *Spinanycta alabamensis* is known currently from three caves in southern Madison and southeastern Limestone counties, Alabama. The species was

Table 1. Observations of *Spinanycta alabamensis* sp. nov. from the three known localities in northern Alabama, USA.

	Date	No. observed
Bobcat Cave (AMD1283), Madison Co., Alabama	26 Nov 2019	7
	6 Oct 2020	5
	13 Sep 2023	20
Muddy Cave (AMD1095), Madison Co., Alabama	15 Jun 2020	13
	7 July 2022	4
	25 Sep 2022	1
	27 Aug 2023	0
Rockhouse Cave (ALM312) Limestone Co., Alabama	21 May 2020	1
	24 Nov 2021	0
	3 April 2022	0

Figure 5. *Spinanycta alabamensis* sp. nov. female in life, observed on 13 September 2023 at the type locality Bobcat Cave, Madison County, Alabama, USA. Photograph by Eric C. Maxwell.

assessed as Imperiled (G2) under NatureServe criteria and as Endangered B1a under IUCN Red List criteria because of few known occurrences, a small and potentially restricted geographic range, high intrinsic vulnerability, and several current and potential threats to populations. All three caves occur on protected lands: Bobcat Cave on Redstone Arsenal, Rockhouse Cave on Wheeler National Wildlife Refuge, and Muddy Cave is owned by the Land Trust of North Alabama. Populations face several existing and potential threats. Pollution from surface runoff associated with continued urbanization in the Huntsville metropolitan area and emerging missions and infrastructure

Figure 6. Searching for *Spinanycta alabamensis* sp. nov. among and under mud-covered rocks at the type locality, Bobcat Cave, Madison County, Alabama, USA. Photograph by Amata Hinkle.

projects on Redstone Arsenal threaten cave life, including *Spinanycta alabamensis*, at all three cave systems (Bearden et al. 2022; USFWS 2022). At present, Bobcat and Muddy caves receives little human visitation; however, Rockhouse Cave receives a high level of unauthorized visitation despite its closed status, which has led to substantial vandalism and accumulation of trash in many sections of the cave.

Remarks. In many species of the subfamily Cubacubaninae, the area between the 3rd legs and the base of the abdomen has very distinct sexual secondary characters. In *Speleonycta ozarkensis*, the tibia of third leg has a large bulge with three distinctly long, sclerotized, and curved macrochaetae (Espinasa et al. 2014). *Anelpistina mexicana* (Espinasa, 1991) has on the lateral sides of urosternum III an extension with the shape of a hook or spine. Many species of *Anelpistina* have an articulated appendage in urosternum IV and *Prosthecina avita* Espinasa, 2000 has both the hook of urosternum III and the appendages in urosternum IV. Interestingly, the presence or absence of these characters follows little phylogenetic predictability, and sister species may lack the character, but the character may be present in distantly related species. With the new species, a new sexual secondary character is described, the spine in urosternum I.

It appears that during reproduction, males in this group may use this part of the body. Evolution has developed a plethora of variable structures. All these structures include some type of chaeta. Sensory input during their interaction with females during courtship may be involved. Unfortunately, their reproductive behavior has yet to be described. Future research may resolve what is the function of all these intriguing structures.

Discussion

Before the description of *S. alabamensis*, 9 troglobiotic nicoletiids have been described from caves of Chihuahua and Coahuila in northern Mexico and the United States, including the states of Arkansas, Arizona, Oklahoma, and Texas. At least two undescribed species are known from caves of California. With the discovery of *S. alabamensis* in Alabama, the distribution of troglobiotic nicoletiids is extended into the Interior Low Plateau karst region in the southeastern United States. Additional diversity may be discovered in the future. Peck (1995) reported eyeless and unpigmented nicoletiids from three additional caves in northern Alabama: Cemetery Cave (ACS no. ADK444) in DeKalb County, Talley Ditch Cave (ACS no. AJK248) in Jackson County, and Shelta Cave (ACS no. AMD4) in Madison County. Unfortunately, the whereabouts of specimens from these caves are currently unknown. Shelta Cave is developed in the same geological formation as caves inhabited by *S. alabamensis* and is 13.4 km northwest of Bobcat Cave. Moreover, both caves, as well as Muddy and Rockhouse caves, share several troglobiotic and stygobiotic species (Cooper 1975; Peck 1989, 1995; Rheams et al. 1992; Niemiller et al. 2019; Niemiller unpubl. data). The nicoletiid from Shelta Cave may be *S. alabamensis* or a closely related species. The identity of nicoletiids reported from Cemetery and Talley Ditch caves is unclear. Cemetery Cave is located on the eastern escarpment of Sand Mountain, while Talley Ditch Cave is in the Crow Creek drainage on the eastern escarpment of the Cumberland Plateau. Both caves are located well east of the range of *S. alabamensis* in a different physiographic province. Thus, *S. alabamensis* is likely the first of several additional species of cave-dwelling nicoletiid to be formally described from karst regions of the southeastern United States.

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RESEARCH ARTICLE

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Cavefish dorsoventral axis angle during wall swimming: laterality asymmetry

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Abstract

The *Astyanax* fish exhibits two morphs: an eyed, pigmented surface morph and an eyeless, depigmented cave morph. Previous studies have shown that blind morphs swim nearly parallel to the wall and can sense detailed information about objects by gliding alongside them and sensing changes in the flow field around their body using their lateral line sensory system. Hence, cavefish can build hydrodynamic images of their surroundings. Field observations showed that one of their presumptive prey, mysid shrimp, is predominately found not on the floor, but crawling on the walls. In our study, the angle of the body axis with respect to a vertical wall was measured while fish swam in a tank. Results show that when swimming by a wall, cavefish incline the vertical axis of their body away from the wall. But most significantly, this angle is different when the right side or the left side of their body is oriented towards the wall. Intriguingly, cavefish have a leftward-biased dorso-cranial bend, where the convex side of the head is towards their right side. Other studies have shown behavioral "handedness". When exhibiting Vibration Attraction Behavior (VAB), cavefish in the field show laterality on the preponderant side they circle to explore a vibrating stimulus. Likewise in larval prey capture (LPC) behavior, larvae strike towards prey preferentially located on one side. Our results support that cavefish also express behavioral lateralization during passive swimming by walls and/or when searching for food that is perched on the walls, such as mysid shrimp.

Keywords

Biomechanics, El Abra, handedness, stygobites, troglomorphy

Introduction

The *Astyanax* fish belongs to the Characidae family. It has an eyed morph that lives in surface streams and an eyeless morph that has evolved in subterranean environments, where light is absent, and food resources are often distributed unevenly (Espinasa et al. 2017). The *Astyanax* cavefish has garnered significant attention in the scientific community due to its unique ecological niche and remarkable adaptations. The way it navigates its surroundings in the absence of vision and the feeding posture with which it searches for food while swimming stand out as crucial components of its adaptive strategy (Schemmel 1980). Understanding these dynamics sheds light on the evolutionary processes that have shaped this species and offers valuable insights into the broader field of adaptive behavior in aquatic organisms.

Studies suggest that the *Astyanax* cavefish employs a range of feeding behaviors, which are modified depending on the nature of available food and the environmental local conditions (Espinasa et al. 2021). This adaptive flexibility suggests a sophisticated sensory-motor integration that allows them to efficiently navigate and locate food sources in their subterranean habitat. For example, an increase in the number of their head neuromasts is responsible for the emergence of VAB (Yoshizawa et al. 2010) and an increase in the number of serotonin neurons in the cavefish hypothalamus is responsible for their persistent food-searching behavior (Elipot et al. 2014). Unraveling the basis of these behaviors not only enhances our understanding of the species but also provides valuable comparative insights into the evolution of feeding strategies across different aquatic environments.

To cope with the challenges of foraging in such environments, these fish have developed specialized feeding behaviors that optimize their chances of locating and capturing prey. The posture and the angle at which *Astyanax* cavefish search for and pick up food lying in the bottom of the tank have been identified as a critical aspect of their feeding behavior. As seen in Fig. 1A–C, surface fish fed at a high average angle from the horizontal (average 74°), while two populations of cavefish, the Pachón cavefish (38°) and the Tinaja cavefish (49°), fed at significantly lower angles (Kowalko et al. 2013). This behavioral change in feeding posture is not due to changes in cranial facial morphology, body depth, or to take advantage of the expansion in the number of taste buds (Kowalko et al. 2013). Quantitative genetic analysis showed that two different cave populations have evolved similar feeding postures through a small number of genetic changes, some of which appear to be distinct (Kowalko et al. 2013).

Foraging behavior has also been described when food is floating on the surface. Cavefish respond to the vibrations generated by a potential prey by approaching using the neuromasts of the left side of their face or their right side followed by highly responsive circling (Espinasa et al. 2022). Of notice, different cave populations showed in the field different “handedness”, expressed by preferentially circling to the right side or the left side. Likewise, in LPC behavior, cavefish larvae from different populations strike at prey that is preferentially on their right side or their left side.

While foraging behavior has been described for food on the bottom of the floor and the surface, such behavior has not been described for food on the walls. That blind cavefish exhibit a preference for remaining near the walls of a novel enclosure was noted

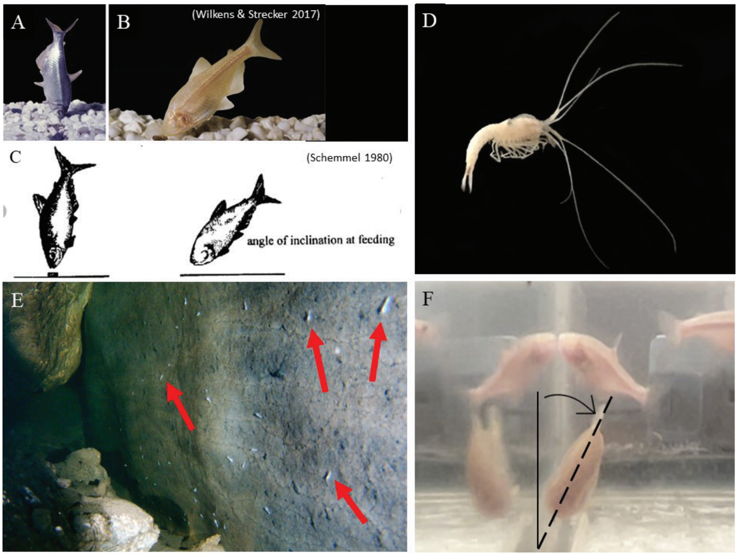


Figure 1. Feeding behavior on the floor and on the walls **A–C** *Astyanax* surface fish search for and pick up food lying on the ground at a high angle from the horizontal. Cavefish fed at lower angles (Modified from Wilkens and Strecker 2017 and Schemmel 1980) **D** in the field, Mysid shrimp are one of the sources of food for *Astyanax* **E** Mysid shrimp at Toro cave. Notice that mysid shrimp are mostly found crawling on the walls (four are highlighted by arrows) and seldom on the ground **F** *Astyanax* cavefish tilt their dorsoventral axis when cruising parallel to a glass wall in a tank they are acclimatized to live in.

by early investigators (Gertychowa 1970; Weissert and von Campenhausen 1981), and subsequently measured in numerous experiments (Teyke 1985, 1988, 1989; Abdel-Latif et al. 1990; Hassan 1992). Patton et al. (2010) then showed that wall-preference was unaffected by wall curvature regardless of being a straight, concave, or convex wall. Sharma et al. (2009) hypothesized that wall following has become more enhanced in blind morphs for exploratory purposes, in large part due to the enhanced, active-flow sensing abilities of the lateral line. In the videos used in Espinasa et al. (2021), it is notable that there is a preference for bottom of the pool or wall following in the native habitat by cavefish, although authors did not provide data as this was not the purpose of the paper.

The purpose of this study is to determine if cavefish and surface fish use a different posture when navigating by a wall. Feeding behavior and the way *Astyanax* cavefish navigates its surroundings in the absence of vision represent a captivating area of research that bridges the fields of ecology, biomechanics, and genetics.

Materials and methods

Field observations of sources of food

Most field studies conducted on *Astyanax* cavefish have been performed at sites accessible to bats and thus, bat guano has been reported as a main source of food (Espinasa et al. 2017). However, many cavefish populations exist beyond sumps in underwater tunnels that are inaccessible to bats. We were interested in studying what are some of the potential food sources in these remote areas. For this, we performed an initial exploration in January 2020 at the deepest portion of Tinaja Cave in the Lake Room Siphon, and in February 2023 at Sabinos Cave in the section beyond Elliott's swim, which is connected to the main cave through a passage with just a few centimeters of air above the water. Both sections have less access to bats. Observations were done with snorkeling equipment and underwater lamps for scuba diving.

A third exploration took place in November 2023 to the Calera system, which has multiple sumps and underwater passages. In this case, remotely operated vehicles (ROV) were used to explore and capture videos. The underwater drone models used were the Fifish V6 and the Fifish V6s with a camera sensor of 1/2.3" SONY CMOS. Effective Pixels 12MP. ISO Range 100–6400 in Auto / Manual. Lens Field of View 166°. Aperture f/2.5. Min Focusing Distance 0.4 m. LED beams with a brightness of 4000 lumens. Two different underwater galleries beyond sumps were explored. One was 71 m long, and the other was 50 m. Attention was given to potential *Astyanax* food sources in these guano void regions of the cave. When found, it was recorded if they were preferentially localized on the floor or the walls. A description of the caves can be found in Elliott (2018) and Espinasa et al. (2020).

Dorsoventral axis angle

A 5-gallon fish tank was subdivided with a vertical glass. A Handycam DCR-SR42 Sony video camera was positioned perpendicular to the glass in such a way that fish swimming parallel to the glass wall would be swimming directly toward or away from the camera. In total, 16 Pachón cavefish (11 derived from the stock originally kept at Dr. Rohner's laboratory at the Stowers Institute and 5 from the stock originally kept at Dr. Borowsky's laboratory at NYU. Both stocks originated from different field collections), 17 Tinaja cavefish (Rohner's stock), 10 Choy River surface fish (Rohner's stock), and 4 eyeless surface fish (Rétaux's stock) were analyzed. Eyeless surface fish were obtained through lensectomy early in their development. Lens removal was conducted bilaterally in surface fish at 1 to 2 days post fertilization following the procedure outlined by Espinasa et al. (2023b). Eyeless fish were the same ones used in Espinasa et al. (2014).

Fish were left to acclimate for at least 30 minutes. Video recordings were made using the night vision function of the Handycam DCR-SR42 camera over a period of ten minutes, where for five minutes the fish were in the left compartment and five in the right compartment. Every time a fish swam parallel to the glass wall, a videoclip image would be extracted, pasted to PowerPoint, and the dorsoventral axis angle would be measured against the vertical angle. It was noted in each videoclip if the specimen was swimming with the right side or left side of its body towards the glass wall. A total of 170 video clips were analyzed for Pachón cavefish, 177 for Tinaja cavefish, 90 for surface fish, and 91 for eyeless surface fish.

A Mann-Whitney U test was used to establish if there was a difference in the average angle used by Pachón fish versus surface fish, Tinaja cavefish versus surface fish, eyeless surface fish versus surface fish, and to establish if the angle used when specimens swam parallel to the wall with their left side, or the right side towards the wall was different for each of the three populations.

Results

Field observations of sources of food

With the use of an ROV, over 120 m of underwater passages were traversed at the Calera system. It was noticed that in galleries where there were no droppings from bats, one of the main potential sources of food was mysid shrimp, *Spelaeomysis quinterensis* (Fig. 1D). It was also noticed that mysid shrimp were predominately crawling on the walls and ceilings but infrequently on the floors (Fig. 1E). A similar pattern of mysid distribution was noticed when snorkeling at Tinaja and Sabinos (Kopp et al. 2018; Espinasa et al. 2020).

When foraging for bat guano, *Astyanax* can encounter this food by seeking on the surface, while droppings are still floating, or on the floor after the particles have sunk to the bottom. Our observations support that in underwater passages beyond the sumps, where bats can no longer reach, cavefish may have to navigate by the walls while seeking alternate sources of food in the way of aquatic crustaceans.

Dorsoventral axis angle

Surface fish and cavefish when navigating parallel to a wall use different dorsoventral axis body angles (Fig. 2A). Surface fish while in the dark swim parallel to the side of walls with a dorsoventral axis angle just slightly off from the vertical (average = 3.2° +/- 11.6° SD). On the contrary, cavefish incline the vertical axis of their body away from the wall (Figs 1F, 2C, D). Instead of their side being oriented parallel and towards the wall, their ventral underside is partially directed towards the wall and the dorsal back of their body is slightly away from the wall. Pachón cavefish incline their body 14.8° +/- 9.9° from the vertical and Tinaja cavefish 10.4° +/- 11.1°. Both Pachón and Tinaja cavefish swim with a significantly steeper angle than surface fish ($P < 0.00001$ for both).

Eyeless surface fish that were enucleated as embryos, and thus are accustomed to navigating without the use of vision throughout their lives, did not behave like cavefish ($P<0.00001$). They continued swimming with an angle similar to eyed surface fish in the dark (Fig. 2A). Their body axis angle (3.1° +/- 6.5°) was almost vertical and not significantly different from the eyed surface in the dark ($P = 0.560$).

In both Pachón and Tinaja cavefish, when their right side of the body is toward a wall, the angle of inclination is significantly higher than when their left side is towards a wall (Fig. 3). Pachón right = 16.6° +/- 10.2° versus Pachón left = 12.9° +/- 9.3° ($P = 0.012$). Tinaja right = 12.6° +/- 9.6° versus Tinaja left = 7.8° +/- 12.2° ($P = 0.012$). Surface fish did not have a significantly different angle of inclination between sides. Surface right = 6.1° +/- 9.7° versus Surface left = 1.0° +/- 12.5° ($P = 0.214$).

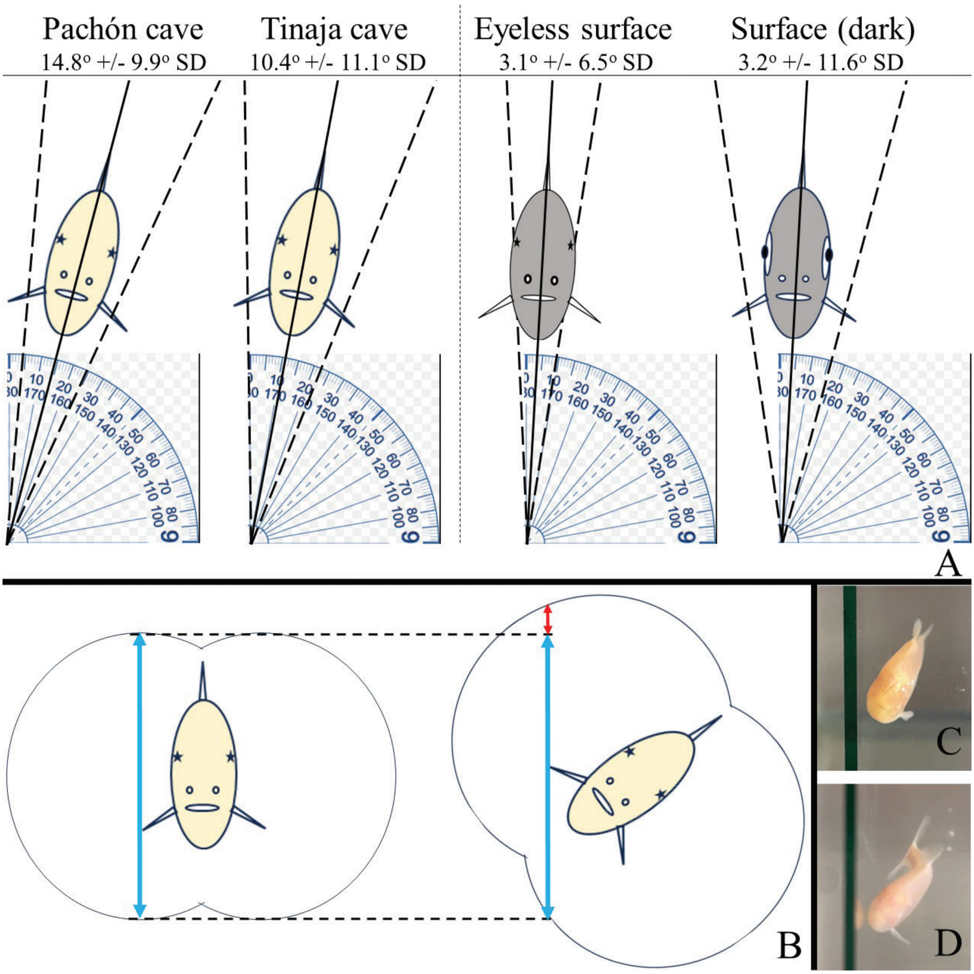
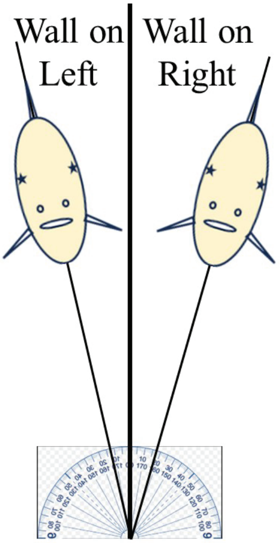


Figure 2. Dorsoventral axis when swimming by a wall **A** when swimming by a wall, cavefish from both Pachón and Tinaja caves incline the vertical axis of their body away from the wall. Eyeless surface fish enucleated as embryos, and surface fish in the dark do not incline as much their vertical axis and swim with a mostly vertical dorsoventral axis. Dotted line = standard deviation from average. Black star = eyeless. **B** When swimming vertically by the side of a wall, the detection field indicated by the semi-sphere on one side of the body can scan an area of the wall (Left; blue arrow). When tilted, a portion of the other side of the body's receptive field or sensory space enlarges the total area of the wall that can be scanned (Blue + red arrow) **C, D** two Pachón cavefish swimming by a glass wall, with the distinct tilt of their dorsoventral axis.

Discussion

Previous studies have shown that bat guano constitutes one of the main sources of nourishment for adult cavefish in passages inhabited by bats (Espinasa et al. 2017). Given that a large portion of the cavefish habitat is in underwater passages beyond sumps, which bats cannot have access to, we used an underwater drone to explore these passages inaccessible to humans without the use of scuba equipment. Our field observations have



	Right	Left	P value
Pachón	16.6° +/- 10.2° N=88	12.9° +/- 9.3° N=82	P=0.012
Tinaja	12.6° +/- 9.6° N=95	7.8° +/- 12.2° N=82	P=0.002
Surface	6.1° +/- 9.7° N=39	1.0° +/- 12.5° N=51	P=.214

Figure 3. When cavefish swim with their right side of the body toward a wall, the angle of inclination is significantly higher than when their left side is towards a wall. Average angles used by Pachón fish are portrayed in the drawing.

shown that in these areas, *Spelaeomysis quinterensis* is very abundant. In the laboratory, *Astyanax* are avid eaters of commercial mysid shrimps and in the field, when a mysid shrimp was deposited in a tank with a cavefish (Espinasa et al. 2020), it was eventually eaten, thus supporting that fish consume mysid shrimp in the field. Of note, it was observed that these mysid shrimps were predominately crawling on the walls and ceilings, but seldom crawling on the floor. As part of their adaptation to the darkness of the underground, cavefish must have evolved a more efficient way to navigate by the walls, and a more efficient foraging behavior when seeking such prey that is not on the floor.

Cavefish can build hydrodynamic images of their surroundings (Windsor et al. 2010). They swim near the walls gliding alongside them, sensing detailed information about objects by the changes in the flow field around their body using their lateral line sensory system (Windsor et al. 2010). Cavefish and surface fish use different angles with respect to the horizontal when searching for food on the ground (Schemmel 1980). Hence, in our study, the angle of the body axis with respect to a vertical wall was measured while fish swam in a tank. Results show that when swimming by a wall, cavefish tilt the vertical axis of their body away from the wall (Fig. 1F). This inclination of the angle of the body axes was significantly higher than surface fish (Fig. 2A).

Why would they incline their body? Espinasa et al. (2023a) described the position and distance from prey when performing a strike during Larval Prey Capture (LPC) behavior. They provided a mathematical model that included a receptive field or sensory detection range as two semi-circles or spheres on each side of the body. At this point, we hypothesize that when swimming vertically by the side of a wall, only the receptive field of one side of the body can scan an area of the wall (Fig. 2B). But when the body is inclined, a portion of the other side of the body's receptive field enlarges the total area of the wall that can be scanned (Fig. 2B). Future studies may confirm this hypothesis.

Results also showed that there was a laterality in the way that cavefish inclined the body axis when swimming by a wall. Both Pachón and Tinaja cavefish used a significantly higher angle when their right side of the body is toward a wall than when their left side is towards a wall (Fig. 3). Previous studies have shown that cavefish have a laterality of "handedness" during foraging behavior. In the field, cave populations preferentially circle to the right side or the left side around a source of vibrations (Espinasa et al. 2022). Likewise, in LPC behavior, cavefish larvae from different populations strike at prey that is preferentially on their right side or their left side (Espinasa et al. 2023a). In the laboratory, individuals from Tinaja Cave showed a moderately consistent preference in VAB for left- or right-side approaches (Fernandes et al. 2022). There may be physiologic or morphologic reasons for this behavioral laterality. For example, there is a positive correlation between the VAB level and the number of left- but not right-side superficial neuromasts in Pachón cavefish (Fernandes et al. 2018). Also, cavefish exhibit directional "bends" in skull shape (Fig. 4A), almost always biased to the left (Powers et al. 2017).

Why would cavefish show a laterality in the way they tilt the body axis when one side of the body or the other is towards a wall? When Espinasa et al. (2023a) described the position and distance from prey when performing a strike during LPC behavior, they proposed in their mathematical model that the receptive field on each side of the body had different radii. The number of strikes shows an enhanced performance for one side, suggesting that each of the receptive field semi-spheres on each side of the body has different volumes (Fig. 4B). As before, we suggest here a hypothesis that will require confirmation in future research. We assume that when cavefish swim with their right side towards a wall, which presumably has a smaller receptive field, if the fish tilts its body to make use of the other side of the body's receptive field, it would enhance its performance (Figs 2B, 4B). But if their left side of the body is towards the wall, which presumably has a larger receptive field, the fish would gain comparatively less enhanced performance by tilting their body axes (Fig. 4C). Future studies may again confirm this hypothesis.

Conclusion

When swimming by a wall, surface fish in the dark and eyeless surface fish swim with a dorsoventral axis angle mostly vertical. On the contrary, cavefish tilt the vertical axis of their body away from the wall. It is hypothesized that when cavefish tilt their body angle, a portion of the other side of the body's receptive field or sensory detection range enlarges the total area of the wall that can be scanned (Fig. 2B). It is also hypothesized that asymmetries in the function of the superficial neuromasts, the bent shape of the cavefish skull, and/or other morphologic of physiologic asymmetries contribute to creating differently sized reception fields on either side of the body. This could explain the different tilt in angle when swimming with their left side or right side of the body towards the wall.

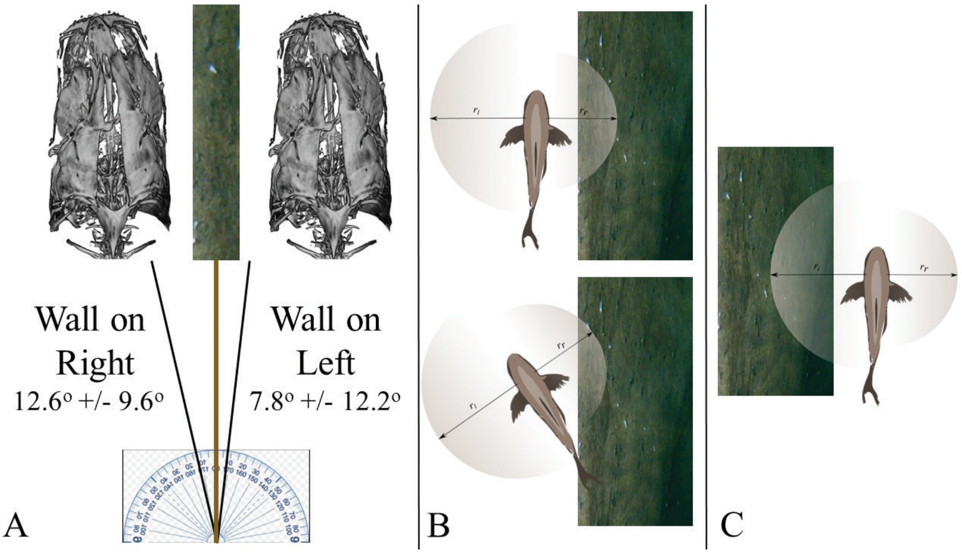


Figure 4. Hypothesis for asymmetrical dorsoventral axis swimming by a wall **A** cavefish have a leftward-biased dorso-cranial bend, where the concave side of the head is towards their left side (Powers et al. 2017). Likewise, Pachón cavefish show a positive correlation of VAB with the number of neuromasts on their left side but not on their right (Fernandes et al. 2018). Results from this study show that when cavefish swim with their left side of the body toward a wall (grey rectangle with white mysid shrimps), the angle of inclination is significantly less than when their right side is towards a wall. Average angles used by Tinaja fish and Tinaja skulls are portrayed in the drawing. By leaning their underside towards the wall (**B**) cavefish receptive fields (grey semicircles with different radii: *Rr=right* and R_l*=left*) from both sides of the body may be combined to increase surface area scanned. Espinasa et al. (2023a) showed that receptive fields on either side of the body in cavefish are asymmetric. It is hypothesized that when cavefish swim with their smaller receptive field (**B**) towards a wall, increasing the dorsoventral axis angle provides a proportionally higher scanned surface area than when cavefish swim with their larger receptive field (**C**) towards the wall. In this case, it is expected that a larger angle will be used when swimming with their right side of the body towards the wall than when their left side is towards the wall.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

Michael Sandone and Michael Girard are the technical specialists and drivers of the remotely operated vehicle (ROV). Without their participation, underwater passages could not have been explored. Thanks to all group members who participated in the January 2020 and February 2023 field trips. In particular Patricia Ornelas and Sylvie Rétaux. This study was supported by the School of Sciences of Marist College.

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RESEARCH ARTICLE

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A new subterranean species of *Oncopodura* Carl & Lebedinsky, 1905 (Collembola, Entomobryomorpha, Oncopoduridae) from a cave in Northeastern Iran

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Abstract

A new species of *Oncopodura* (Collembola, Entomobryomorpha, Oncopoduridae) from the Moghan cave in northeastern Iran, built in a carbonate complex of Kopet Dag mountain range, is described. *Oncopodura moghanensis* sp. nov. can be distinguished from other congeners by (1) 6 long subequal lobes in PAO, each subdivided into 3–6 fingers, (2) dens with 7 dorsal feather-like macrosetae; at basal part with 1 dorsoexternal and 2 dorsointernal hooks, at the distal part with 1 dorsoexternal and 3 dorsointernal hooks, (3) distal part of manubrium with long feather like macrosetae reaching middle part of dens, and (4) mucro with 4 teeth, apical tooth very sharp, and 2 scales at its basal half. A table with diagnostic characters of species related to the *Oncopodura moghanensis* sp. nov. and an updated key to the world species of *Oncopodura* are provided.

Keywords

Cave biology, cave fauna, Kopet Dag, Moghan Cave, springtail, subterranean ecosystem

Introduction

Oncopodura Carl & Lebedinsky, 1905 is an eyeless genus of the family Oncopoduridae (Entomobryomorpha) involving both edaphic and subterranean species. Elongated mucro, the presence of scales on the body, thickened sensilla on Ant IV and the presence of hooks and spines on dens are considered characteristic features of this genus (Yu et al. 2014), which covers 49 species worldwide (Bellinger et al. 2023). Of them, only two species have been recorded from Iran: *Oncopodura ambigua* Christiansen, 1957 and *Oncopodura hamata* Carl & Lebedinsky, 1905 (Mehrafrooz Mayvan et al. 2023).

In terms of biodiversity, Iran is located in the Palearctic biogeographic region and about 54 % of its land area is covered with mountains, including mountain ranges of Zagros, Alborz and Kopet Dag (Azizi Jalilian et al. 2020; Mehrafrooz Mayvan et al. 2015). Karst caves, as the most common type of caves in the world, are the most widespread in the mountainous areas of the country (Juberthie et al. 2001; Ghaderi and Karimi 2014; Malek Hosseini et al. 2015; Malek Hosseini et al. 2016; Moldovan et al. 2018). The present paper is based on the recent speleobiological fieldworks carried out in the Moghan Cave situated in the northeast of Iran in the Kopet Dag mountain range that is formed in massive sedimentary limestones (Manafzadeh et al. 2016; Raziei 2022). Few papers have focused on the subterranean arthropods in Iran, but no studies involving cave Collembola have yet been conducted. This study is aimed at the genus *Oncopodura* Carl & Lebedinsky, 1905, which is highly represented in subterranean habitats with 31 troglobionts altogether (Deharveng and Bedos 2018; Lukić 2019). In this paper, description of a new species of *Oncopodura*, discovered in the Moghan Cave in the Kopet Dag Mountains, northeastern Iran, is provided together with a table summarizing diagnostic characters of world species of *Oncopodura* with PAO with more than 4 lobes and an updated key to the world species of the genus.

Material and methods

Cave descriptions

Moghan Cave takes its name from a village Moghan, it is situated 35 km to southwest of Mashhad, Khorasan-e-Razavi province, on the eastern part of Kopet Dag mountains (36°06'59"N, 59°22'06"E longitude) at altitude of 2193 m above sea level (Fig. 1A, B) The cave has two adjacent entrances located on a relatively steep slope of the mountain with the main entrance 11 m long and 4 m high (Fig. 2A–C). The vegetation in the cave surroundings is very sparse and the entire twilight cave zone (50 m in distance) is completely dry. The cave is about 500 m long, with a maximum depth of 44 m (Fig. 3). It is divided into two floors connected by wells, reaching the height up to 25 m. Moreover, Moghan Cave has five halls, with a water pond located at the end of the fifth hall on the second floor, 5.66 × 6.93 m in area and depth of 30–60 cm (Fig. 2D). The specimens of the new species were collected from the water pool in the deepest part of the cave, which had constant air temperature (12 °C) and relative humidity (RH) of over 73 %, measured with a thermo-hygrometer TFA 30.5015 DTH in December 2023.

Methods

For observation in an optical microscope with phase contrast, initial specimens were mounted in Heinz's medium after clearing in Nesbitt's fluid. To compare effectivity of different clearing methods, additional specimens were boiled softly in evaporation glass dish with 95% ethyl alcohol on electric cooking plate for approximately 1 min to remove fat from the body. For clearing, the specimens were transported to concave glass dish with 10% water solution of KOH for 1 min and then transported to a dish containing chlorophenol until the specimens were transparent. At the end, specimens were mounted on Swann medium. After drying, the cover glasses were ringed with nail polish or Canada balsam to seal the mounting medium. The specimens were observed in Leica DM 2500 light microscope equipped with phase and DIC contrasts, and a drawing arm.

Abbreviations used

Abd – abdominal segment; Ant – antennal segment; PAO – post antennal organ; PS – pseudopore; S – sensillum; Ti – tibiotarsus; VT – ventral tube.

Repositories of investigated material: CoPJSU – Collembola collection of the Department of Zoology, Institute of Biology and Ecology, Faculty of Science, Pavol Jozef Šafárik University, Košice, Slovakia; ZMFUM – Zoology Museum of Ferdowsi University of Mashhad, Mashhad, Iran.

Results

Species description

***Oncopodura moghanensis* Mehrafrooz Mayvan & Kováč, sp. nov.**
https://zoobank.org/8AC9E1C6-F1F1-4F59-8FB6-CB901436009F
Figs 1–11, Table 1

Diagnosis. PAO with 6 long, subequal lobes, each lobe secondarily divided into 3–6 fingers. Unguis normal, not elongated, untoothed; unguiculus acuminate. Dorsal side of manubrium with 11+11 setae. Dens at basal part with 1 dorsoexternal and 2 dorsointernal hooks, at the distal part with 1 dorsoexternal and 3 dorsointernal hooks. Mucro with 4 teeth.

Type locality. Iran, Razavi Khorasan province, Mashhad city, Moghan village, Moghan Cave, Coordinates: 36°06'59"N, 59°22'06"E, 2193 m a.s.l.

Type material. ***Holotype***: female on slide [MoCa101], dark zone, the end part of the cave, 2nd cave floor, collected from water pond surface, 28.ix.2022, temperature = 11.9 °C, RH 73.0%, leg. M. Mehrafrooz. ***Paratypes:*** 4 specimens on slides [MoCa102, MoCa103, MoCa104, MoCa105], *ibidem*, hand collecting on a stalag-

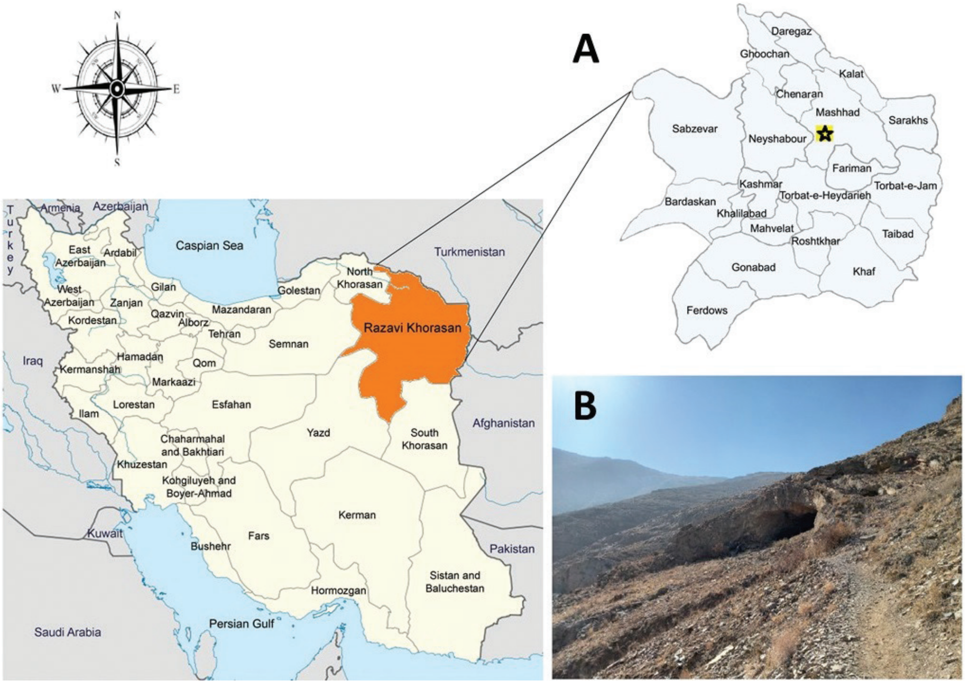

Figure 1. A Razavi Khorasan province in Iran is marked with an orange color; location of the Moghan Cave, where the new *Oncopodura* species was collected, is marked with an asterisk **B** cave entrance and surroundings of Moghan Cave in autumn (Photo: M. Mehrafrooz).

mite, 30.iv.2022, temperature = 12.0 °C, RH 73.4%, leg. M. Mehrafrooz. Holotype and one paratype deposited in UPJŠ; three paratypes deposited in ZMFUM.

Description. ***Body appearance.*** Length 1.05 mm on average (n = 5, max. length 1.2 mm). Colour white without traces of pigment, body covered by hyaline scales.

Antenna. Antenna nearly as long as head, without apical bulb and scales (Fig. 4A–E). length of Ant I: II: III: IV as 0.02: 0.04: 0.06: 0.08 mm respectively. Ant I with 7 thick dorsal setae and 11 normal setae (smaller than those of Ant II–III). Ant II with 1 broad wrinkled apical sensillum similar to those of Ant III, 1 apical blunt sensillum, 11 dorsal and 6 ventral setae and 4 dorsolateral microsetae. Ant III with sensory organ formed by 2 expanded wrinkled sensilla with 1 blunt, basal sensillum, 8 thick setae at their base, and 10 slender, long setae covered by very soft cilia (Fig. 4B). Ant IV not elongated, with a row of 4 thick and pointed sensilla (Fig. 4C–E), equally distant from each other, and 2 subapical modified setae (Fig. 4D). Ventral side of Ant IV with normal setae, dorsal part with normal and elongated setae.

Head. Eyes absent. PAO well developed and located near base of Ant I, very distinct and large, about 24 μm in diameter, with 6 subequal lobes formed around a central circle, each lobe secondarily divided into 3–6 irregular finger-like lobes (Fig. 5A). Labrum with 4 prelabral setae and 4 rows of 2, 3, 5, 4 setae on papillae (Fig. 6A). Left mandible with 4 teeth and right mandible with 3 teeth.

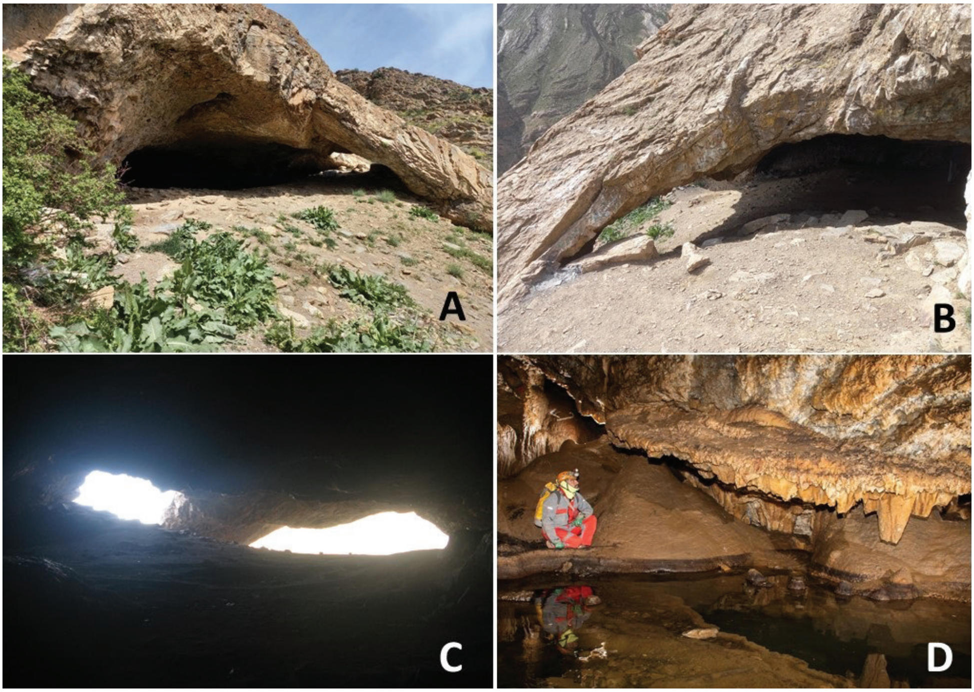

Figure 2. Moghan Cave, northeast Iran **A** main entrance of the cave in spring (Photo: M. Mehrafrooz) **B** second entrance (Photo: M. Mehrafrooz) **C** two cave entrances, view from inside the cave (Photo: M. Mehrafrooz) **D** pool at the end of the cave located on the second floor (Photo: Vahid Ashrafi).

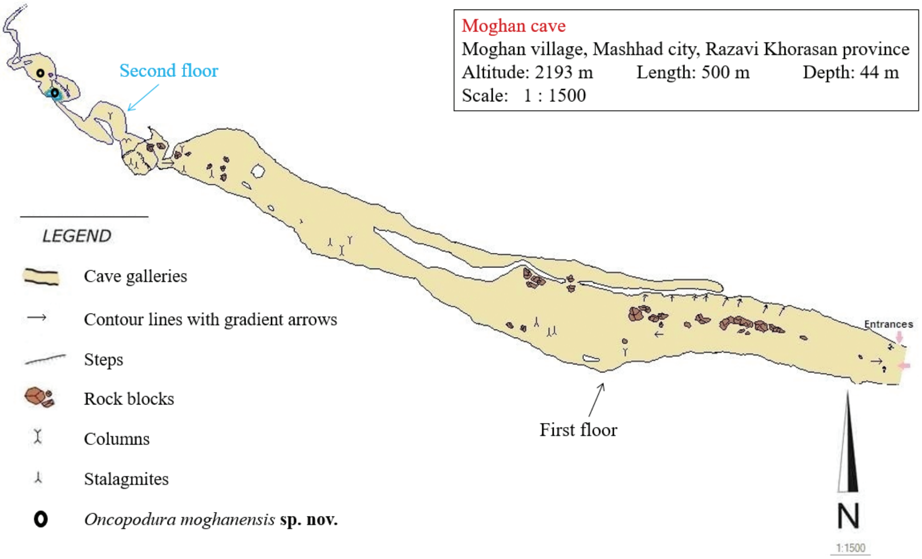

Figure 3. Ground plan of the Moghan Cave (Sketched by V. Ashrafi).

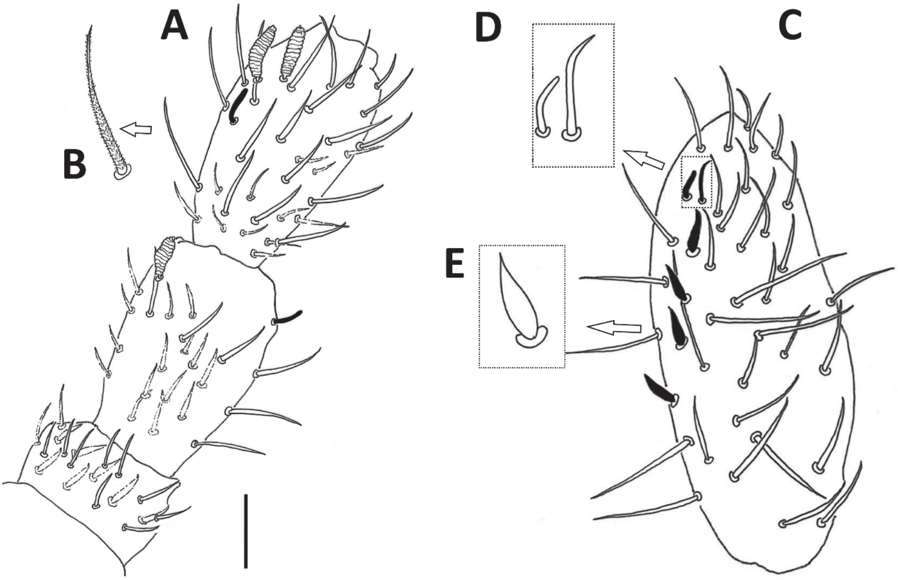

Figure 4. *Oncopodura moghanensis* sp. nov. **A** antennal segments I–III **B** detail of finely ciliated seta **C** antennal segment IV **D** detail of two subapical modified setae near the antennal sensillum **E** detail of thick, pointed sensillum. Scale bars: 0.02 mm.

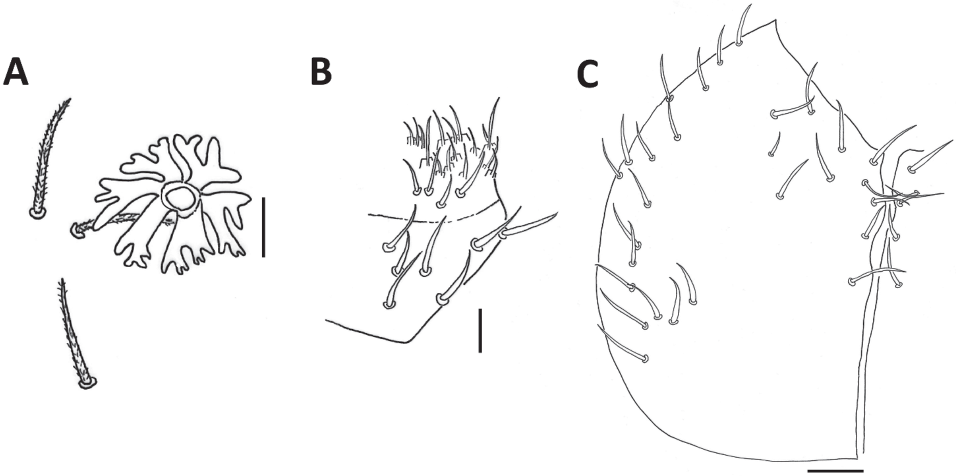

Figure 5. *Oncopodura moghanensis* sp. nov. **A** postantennal organ **B** labium and labial triangle **C** ventral head chaetotaxy. Scale bars: 0.01 mm (**A, B**); 0.02 mm (**C**).

Ventral head without scales, chaetotaxy (setal pattern) as in Fig. 5C, all ventral setae smooth. Head dorsally with 4+4 inter-antennal and 5 lateral macrosetae on each side placed around PAO; 1 short and thick seta posterior to PAO. Two rows of 5 and 6 macrosetae, respectively, in front of prelabral setae. Head with scales, dorsal microsetae as in Fig. 6B. Labial triangle with 7 smooth macrosetae (Fig. 5B).

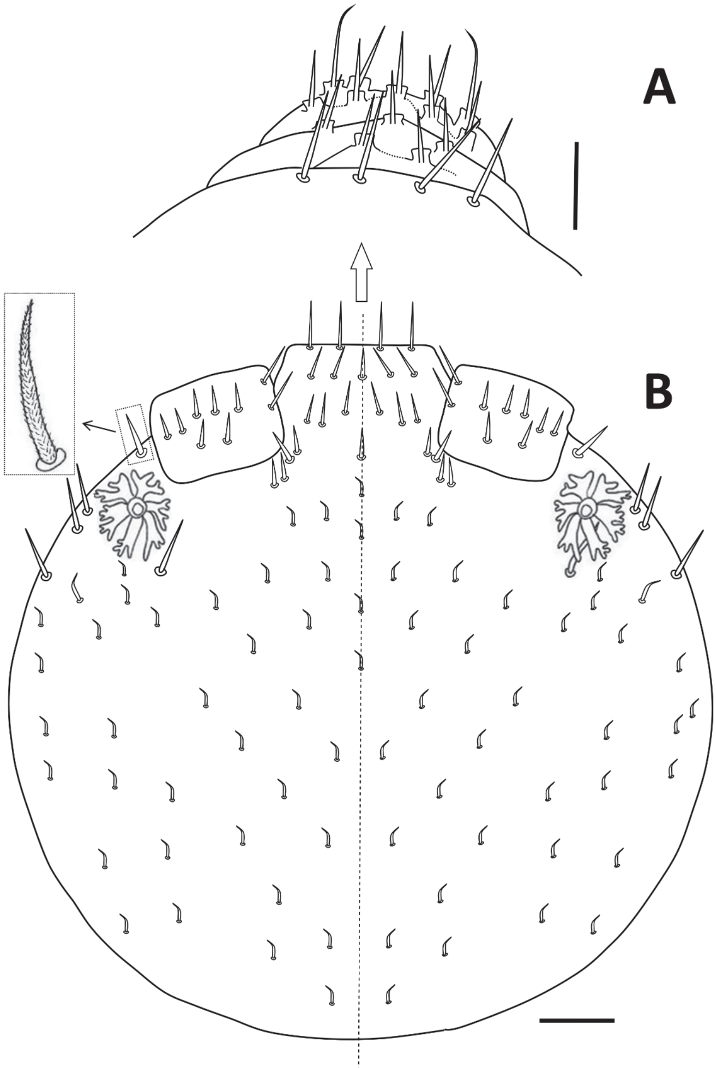

Figure 6. *Oncopodura moghanensis* sp. nov. **A** labrum **B** dorsal head chaetotaxy (dotted circle = interantennal macrosetae). Scale bars: 0.01 mm (**A**); 0.02 mm (**B**).

Body chaetotaxy. As in Fig. 7A, B. The chaetotaxy follows the general pattern described for the genus (Szeptycki 1977; Jordana et al. 2012). Mesothorax extended forward slightly over the head, distally with a row of smooth spine-like setae, laterally with 1 ciliated spine-like seta and 1 trichobothrium and 1+1 medial pseudopores. Metathorax with 2+2 trichobothria, 2+2 lateral spine-like setae and 1+1 medial pseudopores. Abdomen I with 1+1 ciliated spine-like setae and 1+1 medial pseudopores, Abd. II with 1+1 lateral smooth spine-like microsetae, medially with 1+1 ciliated spine-like setae, 1+1 trichobothria and 1+1 medial pseudopores, Abd. III laterally with

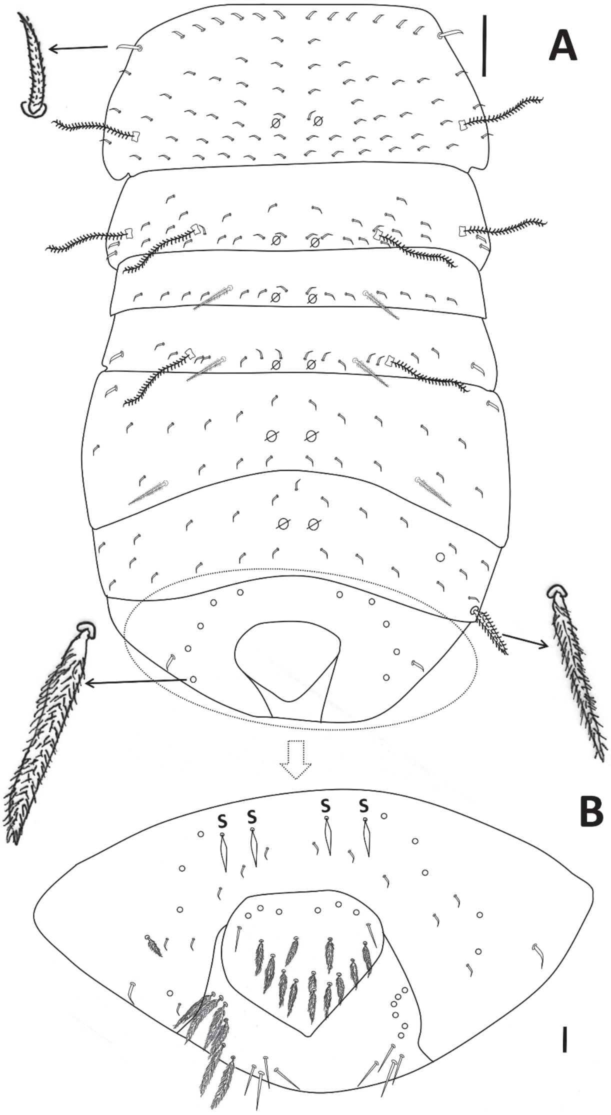

Figure 7. *Oncopodura moghanensis* sp. nov. **A** dorsal chaetotaxy of thorax and abdomen **B** abd V & VI (S = anterior sensilla; circles with a slash represent pseudopores). Scale bars: 0.096 mm (**A**), 0.01 mm (**B**)

1+1 smooth spine-like microsetae, medially with 1+1 ciliated spine-like setae and 1+1 pseudopores. Abd IV with 4+4 ciliated macrosetae and 1+1 pseudopores. Abd V with 2+2 anterior sensilla, 5+5 ciliated macrosetae (shorter than Abd IV macrosetae) and 1+1 smooth spine-like microsetae. Abd VI: epiproct with 9+9 ciliated and 1+1 smooth setae, paraproct with 12 ciliated macrosetae and 8 smooth setae.

Legs. Legs without scales. Chaetotaxy as in Fig. 8A–C. Leg I: trochanter with 6 setae, femur with 3 whorls of 4, 4, 4 setae, respectively; whorl 2 with 1 microseta and whorl 4 with 2 microsetae; Ti with 5 whorls of 4, 4, 5, 4, 7 setae, respectively. Leg II: trochanter with 5 setae, femur with 3 whorls of 4, 3, 5 setae, respectively; whorl 3 with

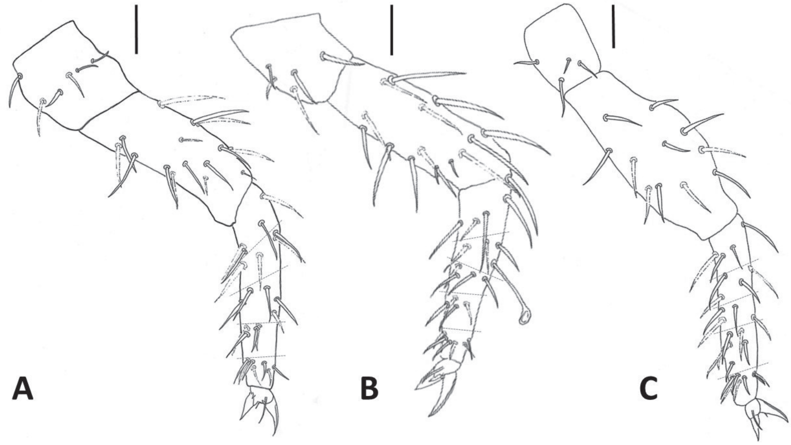

Figure 8. *Oncopodura moghanensis* sp. nov., chaetotaxy of legs **A** leg I **B** leg II **C** leg III. Scale bars: 0.02 mm.

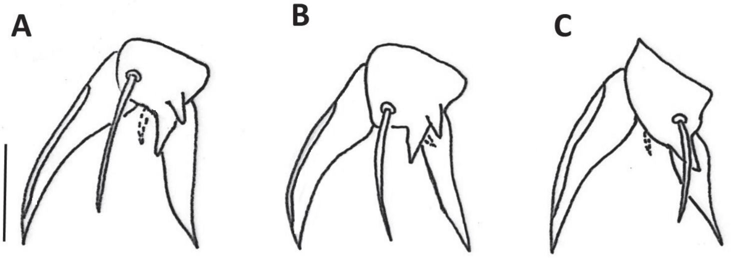

Figure 9. *Oncopodura moghanensis* sp. nov., detail of foot complex **A** leg I **B** leg II **C** leg III. Scale bar: 0.01 mm.

2 microsetae; Ti with 5 whorls of 4, 4, 4, 4 and 6 setae, whorl 2 with 1 stout, clavate (apically expanded) macroseta. Leg III: trochanter with 4 setae, femur with 3 whorls of 3, 3, 5 setae; Ti with 5 whorls of 4, 4, 4, 5, 6 setae respectively, whorl 5 with 1 microseta. Most Ti setae large, acuminate, and extremely finely ciliated; tenent hair slender and acuminate. Unguis (Fig. 9A–C) normal, not elongated, untoothed; inner pretarsal setae long, slightly shorter than the unguiculus, about 1/2 length of internal side of unguis; outer pretarsal setae shorter; unguiculus acuminate.

Ventral tube (collophore) without prominent papillae, with 3+3 setae (2+2 dorsolateral and 1+1 frontal). Tenaculum with 4+4 teeth, setae absent.

Furca. length of manubrium: dens: mucro as 0.046: 0.040: 0.031 mm respectively. Ventral side of manubrium with scales, dorsal side with 3+3 long and 1+1 short smooth setae, 6+6 ciliated axial macrosetae; 1+1 long, feather-like distal macrosetae reaching half of the dens (Fig. 10A). Ventral side of dens with scales; chaetotaxy of dorsal side of dens subdivided into basal and distal part. Basal part with 2 dorsointernal ciliated hooks, 1 dorsointernal smooth conical spine (broad at the base and strongly tapered at the top), 1 dorsoexternal ciliated hook and 5 dorsal ciliated macrosetae (basal tubercle with 2 ciliated macrosetae, of which inner 1 feather-like and thinner than

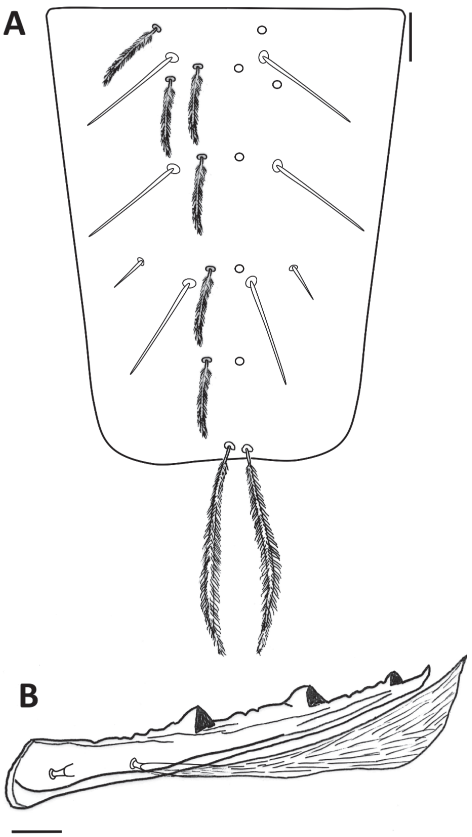

Figure 10. *Oncopodura moghanensis* sp. nov. **A** manubrium dorsal side **B** mucro with basal scale. Scale bars: 0.01 mm.

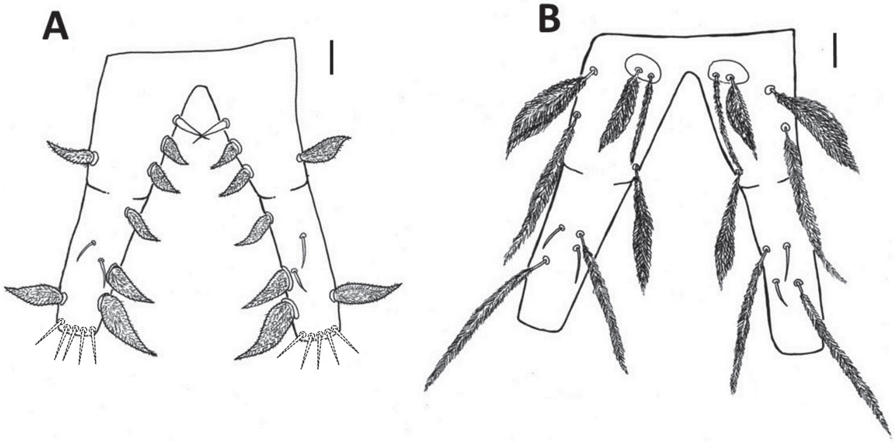

Figure 11. *Oncopodura moghanensis* sp. nov., dorsal side of dens **A** dens with dorsolateral hooks and spines **B** dens with ciliated dorsal macrosetae. Scale bars: 0.01 mm.

outer one). Distal part of dens with 3 dorsointernal ciliated hooks (1 medial, 2 distal), 1 dorsoexternal ciliated hook, 2 dorsal ciliated macrosetae and 2 dorsal short, smooth medial spine-like setae. Dens apically with 5 smooth ventral setae (Fig. 11A, B). Mucro with 4 teeth: 2 apical, 1 medial and 1 basal; 2 scales at its basal half (Fig. 10B).

Table 1. Differential characters of world species of *Oncopodura* with PAO with more than 4 lobes. Characters: **1**, Ant IV medial S number; **2**, Ant IV basal S number; **3**, Ant IV S form, 1 = long, 2 = pointed, 3 = rounded, 4 = short, 5 = trapezoidal; **4**, Ant. III S number; **5**, Ant. II S number; **6**, PAO lobes number; **7**, PAO vesicle fingers number; **8**, Basal dens dorsoexternal hooks number; **9**, Basal dens dorsointernal hooks number; **10**, Basal dens external ciliated macrosetae number; **11**, Basal dens internal ciliated macrosetae number; **12**, Distal dens dorsoexternal hooks number; **13**, Distal dens dorsointernal hooks number; **14**, Distal dens external ciliated macrosetae number; **15**, Distal dens internal ciliated macrosetae number; **16**, Mucro teeth number; **17**, Mucronal basal scales: 0 = absent, 1 = present; **18**, Ungual basal lamella, 0 = absent, 1 = or < ½ unguis, 2 > ½ unguis; **19**, Unguis, 1 = normal, 2 = long. Dif. - number of differential characters in new species *versus* total number of compared characters. * = differences from new species *O. moghanensis* sp. nov. "–"= absence of data; "?" = Insufficient or confusing information in original description; S – sensillum.

Species/Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Dif.
Oncopodura ambigua Christiansen, 1957	4	1*	3*	0*	0*	6	1*	0*	1*	0*	1*	1	1*	0*	0*	4	1	2?*	1	13/19
Oncopodura atoyacensis Bonet, 1943	4	0	4*	0*	0*	6	1*	0*	1*	0*	2*	1	1*	0*	0*	4	0*	0	1	12/19
Oncopodura crassicornis Shoebotham, 1911	4	0	3*	0*	0*	6	1*	1	2	0*	1*	1	3	0*	0*	4	1	0	1	8/19
Oncopodura czmur Szeptycki, 1977	4	0	1*	2*	0*	6	1*	0*	3*	2	3	1	2*	1	1	4	1	0	1	7/19
Oncopodura dethieri Janssens & De Bruyn, 2010	4	0	1*	1	0*	6	23-*	0*	3*	–	–	1	4*	–	–	4	0*	0	1	7/15
Oncopodura egerszogensis Loksa, 1961	0*	0	–	1	1	6	3*	1	1*	2	2*	2*	1*	1	1	4	0*	0	2*	8/18
Oncopodura hubbardi Christiansen & Bellinger, 1996	4	1*	1*	2*	2*	56-*	1*	0*	0*	2	1*	1	1*	1	1	4	1	1*	2*	11/19
Oncopodura itatiaiensis Arlé, 1961	4	0	4*	2*	0*	5*	1*	1	2	1*	1*	1	3	2*	2*	4	1	2?*	1	10/19
Oncopodura kuramaensis Yosii, 1956	4	0	4*	1*	1	6	1*	0*	2	1*	3	1	3	0*	1	4	1	–	–	7/18
Oncopodura moghanensis sp. nov.	4	0	2	1	1	6	36-	1	2	2	3	1	3	1	1	4	1	0	1	
Oncopodura pegyi Gruia, 1994	4	0	4*	3*	1	68-*	1*	0*	2	1*	4*	1	3	1	1	4	1	0	2*	8/19
Oncopodura pelissiei Deharveng, 1988	4	0	3*	3*	2*	6	1*	1	2	2	3	1	3	0*	1	4	1	0	2*	6/19
Oncopodura reyersdorfensis Stach, 1936	3*	0	2	0*	0*	6	12-*	0*	1*	1*	2*	1	1	0*	0*	–	0*	0	2*	12/18
Oncopodura tunica Christiansen & Bellinger, 1980	10*	0	2	0*	2*	5*	1*	0*	1*	1*	2*	1	1*	1	1	4	1	1?*	2*	12/19
Oncopodura yosiiana Szeptycki, 1977	4	0	2	2*	1	6	1*	0*	2	1*	3	1	3	1	1	4	1	0	1	4/19

Etymology. The name of the new species is derived from the Moghan Cave in Kopet Dag Mountains in northeastern Iran, i.e. the locality where the species was discovered.

Taxonomic remarks. So far, 50 species of *Oncopodura* have been described. Based on shape of PAO, *Oncopodura moghanensis* sp. nov. is similar to *O. dethieri* Janssens & De Bruyn, 2010, *O. egerszogensis* Loksa, 1961 and *O. reyersdorfensis* Stach, 1936. The new species is characteristic with 3–6 finger-like lobes in PAO vesicles, while *O. dethieri* has 2–3 fingers, *O. egerszogensis* has 3 fingers and *O. reyersdorfensis* has 1–2 fingers in PAO. Based on dorsal chaetotaxy of dens, *O. moghanensis* sp. nov. is similar to *O. crassicornis* Shoebotham, 1911, *O. pelissiei* Deharveng, 1988 and *O. itatiaiensis* Arlé, 1961. However, Szeptycki (1977) mentioned more complex chaetotaxy of manubrium for *O. crassicornis*, when comparing it with *O. yosiiana* Szeptycki, 1977. The new species has 6 long and subequal lobes in PAO with each lobe secondarily divided into 3–6 fingers, while *O. crassicornis* and *O. pelissiei* has 6 simple lobes and *O. itatiaiensis* 5 simple lobes in PAO. Among other species, *O. yosiiana* differs from the new species by 6 simple lobes in PAO, manubrium with numerous dorsal setae (16+16 feather-like setae, 6+6 smooth mesosetae, 1+1 smooth microsetae and 1+1 ciliated macrosetae), and dens without basal external hook.

Deharveng (1988) recognized two basic groups of *Oncopodura* congeners:

1. *O. crassicornis*–group: Ant IV with 4 short and thick sensilla (S-setae) arranged in a curved line, thickened sensilla at the base of segment absent; PAO large with 6 elongated lobes; unguis without large basal lamella (external tooth); pretarsal setae long; dental hooks finely scaly-ciliated, lacking strong denticles.
2. *O. tricuspidata*–group: Ant IV with 4 thick sensilla (S-setae) arranged in a line, and one thickened sensillum at the base of segment (sometimes absent); PAO small, with 3–4 rounded lobes, or they are absent; unguis with large basal lamella, dental hooks smooth, but with strong denticles on their outer surface.

Recently, numerous species have been described with various combinations of the group-specific characters. In many species of *O. crassicornis*–group, Ant IV sensilla are in a line and in some species Ant IV basal sensilla is present, such as in *O. ambigua* Christiansen, 1957 and *O. hubbardi* Christiansen & Bellinger, 1996. Also, PAO in *O. hubbardi*, *O. itatiaiensis* and *O. tunica* Christiansen & Bellinger, 1980 have 5 lobes and in *O. pegyi* Gruia, 1994 has 6–8 lobes. Unguis basal lamella is present in species including *O. ambigua*, *O. hubbardi*, *O. itatiaiensis* and *O. tunica*.

In some species of *O. tricuspidata*–group, ant IV basal sensilla is absent such as *O. cruciata* Bonet, 1943, *O. equatoriana* Thibaud & Najt, 1987, *O. hoffi* Christiansen & Bellinger, 1980, *O. iowae* Christiansen, 1961, *O. mala* Christiansen & Bellinger, 1996, *O. meridionalis* Cassagnau, 1959, *O. puncteola* Yosii, 1956 and *O. subhoffi* Christiansen & Bellinger, 1998. Also, there are some species with 1 single lobe PAO including *O. cavernarum* Stach, 1934, *O. gledensis* Baquero et al., 2007, *O. mala*, *O. siquierae* Seminario-Cordova et al. 2018 and *O. vioreli* Gruia, 1989. On the other, ungual basal lamella is absent in some species including *O. cruciata*, *O. equatoriana*, *O. hoffi*, *O. iowae*, *O. mala*, *O. meridionalis*, *O. puncteola* and *O. subhoffi*. Thus, practical use of sorting of *Oncopodura* congeners to *tricuspidata-* and *crassicornis*-group has become questionable.

The new species was collected in the deep cave zone but, nevertheless, it does not show morphological adaptations to subterranean environment such as elongation of unguis or increase in the number of sensilla on antennae and body or augmentation of setae over the head, characteristic for some troglomorphic congeners (e.g. Deharveng 1988).

Key to world species of genus *Oncopodura*

The following key was prepared based on the diagnostic characters of worldwide *Oncopodura* species (adapted from Absolon and Kseneman 1932; Bonet 1943; Szeptycki 1977; Mari Mutt 1984; Christiansen and Bellinger 1998). This should be considered a tentative key, since the limited data on several important characters in some species included.

1 PAO absent **2**
– PAO present **18**
2 Ant IV with 4 medial sensilla **3**
– Ant IV with more than 4 medial sensilla **16**
3 Mucro with two teeth .... ***O. bidentata* Delamare Deboutteville, 1948 (Ivory coast, soil)***
– Mucro with more than two teeth **4**
4 Mucro with 4 teeth **5**
– Mucro with more than 4 teeth **11**
5 Dens without basal dorsointernal hooks **6**
– Dens with at laest 1 basal dorsointernal hooks **7**
6 Dens with 1 distal dorsoexternal hook and 1 distal dorsointernal hook. Unguis basal lamella shorter than or equal to half the length of the unguis .... ***O. hamata* Carl & Lebedinsky, 1905 (Crimea, cave)**
– Dens without distal dorsoexternal hook and with 2 distal dorsointernal hooks. Unguis basal lamella longer than half the length of the unguis .... ***O. occidentalis* Bonet, 1931 (Spain & France, cave)**
7 Dens without dorsoexternal hooks .... ***O. tiegsi* Womersley, 1942 (Australia, decaying log)***
– Dens with dorsoexternal hooks **8**
8 Unguis basal lamella shorter than or equal to half the length of the unguis **9**
– Unguis basal lamella longer than half the length of the unguis **10**
9 Ant II with 7 blunt sensilla. Unguis normal .... ***O prietoi* Bonet, 1943 (Mexico, cave)**
– Ant II with 13 blunt sensilla. Unguis elongated .... ***O. jauzioni* Deharveng, 1988 (France, cave)**

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* considered as *species inquirenda*, due to insufficient or doubtful data in an original description.

10 Apex of Ant II and III with many blunt sensilla (more than 15). Dens with 2 distal dorsointernal hooks. Basal scale on mucro present .... ***O. dura*** **Christiansen & Reddell, 1986 (Mexico, cave)**

– Apex of Ant II and III with few blunt sensilla (less than 5). Dens with 1 distal dorsointernal hook. Basal scale on mucro absent .... ***O. delhezi*** **Stomp, 1974 (Algeria, cave)**

11 Mucro with 5 teeth **12**

– Mucro serrated, with numerous teeth (more than 5) **14**

12 Ant II with 16–32 blunt sensilla. Ant III with 6–7 blunt sensilla. Basal scale on mucro present .... ***O. susanae*** **Christiansen & Reddell, 1986 (Mexico, cave)**

– Ant II with 1–2 blunt sensilla. Ant III with 2–8 blunt sensilla. Basal scale on mucro absent **13**

13 Unguis basal lamella shorter than or equal to half the length of the unguis. Ant II with 8 blunt sensilla. Dens with 1 basal dorsointernal hook and without distal dorsoexternal hooks .... ***O. fadriquei*** **Jordana et al., 2012 (Spain, cave)**

– Unguis basal lamella longer than half the length of the unguis. Ant II with 2 blunt sensilla. Dens without basal dorsointernal hooks, with 1 distal dorsoexternal hook .... ***O. alpa*** **Christiansen & Bellinger, 1980 (USA, moss and lichens)**

14 Dens with 1 basal dorsoexternal hook and 2 distal dorsointernal hooks .... ***O. gul*** **Yosii, 1966b (South Korea, cave)**

– Dens without basal dorsoexternal hooks, with 3 distal dorsointernal hooks **15**

15 Dens with 1 basal and 3 distal external coarsely serrated hooks, almost digitated. Basal scale on mucro absent. Unguis basal lamella present, longer than half the length of the unguis .... ***O. kuramotoi*** **Yosii, 1965 (Japan, cave)**

– Dens with 1 basal and 3 distal serrated hooks. Basal scale on mucro present. Unguis basal lamella absent .... ***O. puncteola*** **Yosii, 1956 (Japan, cave)**

16 Apex of Ant III with many blunt setae. Mucro basal scale present. Unguis basal lamella longer than half the length of the unguis **17**

– Apex of Ant III with 2 blunt setae. Mucro basal scale absent. Unguis basal lamella shorter than or equal to half the length of the unguis .... ***O. lebretoni*** **Deharveng, 1988 (France, cave)**

17 Ant IV with 5 medial sensilla and 1 basal blunt sensillum. Dens with 1 basal internal hook. Mucro with strong media lamella .... ***O. fenestra*** **Christiansen & Bellinger, 1996 (USA, cave)**

– Ant IV with 6 medial sensilla and 2 basal blunt sensilla. Dens without basal dorsointernal hooks. Mucro without median lamella .... ***O. frankeae*** **Christiansen & Bellinger, 1998 (USA, cave)**

18 PAO with 1 simple lobe **19**

– PAO with 3 or more lobes **23**

19 Unguis basal lamella absent or if present, its longer than half the length of the unguis **20**

– Unguis basal lamella shorter than or equal to half the length of the unguis **21**

20 Unguis basal lamella absent. Ant III with 2 blunt sensilla, Ant II with 2 blunt sensilla. Distal part of dens with 2 ciliated macrosetae.. .... ***O. mala* Christiansen & Bellinger, 1996 (USA, cave & epigeic)**

– Unguis basal lamella longer than half the length of the unguis. Ant III with 1 blunt sensillum, Ant II without blunt sensilla. Distal part of dens without ciliated macrosetae .... ***O. siquierae* Seminario-Cordova et al., 2018 (Spain, cave)**

21 Mucro with 4 teeth, basal scale present **22**

– Mucro with 5 teeth, basal scale absent... .... ***O. gledensis* Baquero et al., 2007 (Spain, cave)**

22 Unguis basal lamella longer than half the length of the unguis. Ant IV without basal blunt sensilla, Ant II with 2 blunt sensilla... .... ***O. vioreli* Gruia, 1989 (Romania, cave)**

– Unguis basal lamella shorter than or equal to half the length of the unguis. Ant IV with 1 basal blunt sensillum, Ant II with 3 blunt sensilla ....***O. cavernarum* Stach, 1934 (Italy and Slovenia, cave)**

23 PAO with 3 lobes...**24**

– PAO with 4 or more lobes..**26**

24 Mucro with 4 teeth. Ant IV with 4 medial sensilla and 1 basal blunt sensillum, Ant III with 2–3 blunt sensilla **25**

– Mucro with 5 teeth. Ant IV with 5 medial sensilla and 4 blunt sensilla. Ant III with 3 blunt sensilla....***O. equatoriana* Thibaud & Najt, 1987 (Ecuador, cave)**

25 Unguis basal lamella longer than half the length of the unguis. Dens with 1 basal and 2 distal dorsointernal hooks. Ant III with 2 blunt sensilla .... ***O. tricuspidata* Cassagnau, 1964 (France, cave)**

– Unguis basal lamella absent. Dens with 2 basal and 3 distal dorsointernal hooks. Ant III with 3 blunt sensilla ....***O. hoffi* Christiansen & Bellinger, 1980 (USA, cave)**

26 PAO with 4 lobes...**27**

– PAO with 5 or more lobes..**36**

27 Mucro with 4 teeth **28**

– Mucro with more than 4 teeth **35**

28 Dens with 1 basal dorsoexternal hook. Ant IV with 4 sensilla along curved line.. ***O. hyleana* Arlé, 1961 (Brazil, litter in forest)**

– Dens without basal dorsoexternal hooks. Ant IV with 4–7 sensilla in a line **29**

29 Ant IV with 7 sensilla **30**

– Ant IV with 4 sensilla **31**

30 Ant IV with 1 basal sensillum. Ant III with 4 and Ant II with 2 blunt sensilla, respectively. Dens with 1 distal dorsointernal hook. Basal scale on mucro present. Unguis basal lamella present, shorter than or equal to half the length of the unguis .... ***O. nana* Thibaud & Najt, 1987 (Ecuador, cave)**

– Ant IV without basal sensilla. Ant III with 1 blunt sensillum, Ant II without blunt sensilla. Dens with 3 distal dorsointernal hooks. Basal scale on mucro absent. Unguis basal lamella absent.. .... ***O. meridionalis*** **Cassagnau, 1959 (Spain, beech humus)**

31 Unguis basal lamella longer than half the length of the unguis........................**32**

– Unguis basal lamella absent or if present, its shorter than or equal to half the length of the unguis **33**

32 Ant IV with 1 basal sensillum. Ant III with 1 and Ant II with 7 blunt sensilla, respectively. Mucro longer than dens. Dens with 2 distal dorsointernal hooks ....***O. jugoslavica*** **Absolon & Kseneman, 1932 (Bosnia & Herzegovina, Croatia and Chile, cave)**

– Ant IV without basal sensilla. Ant III without blunt sensilla, Ant II with 2 blunt sensilla. Mucro equal or shorter than dens. Dens with 1 distal dorsointernal hook ....***O. arecibena*** **Mari Mutt, 1984 (Puerto Rico, cave)**

33 Dens with 1 distal dorsointernal hook. Ant IV without basal sensilla .... ***O. iowae*** **Christiansen, 1961 (USA, cave)**

– Dens with 2 distal dorsointernal hooks. Ant IV with 1 basal sensillum **34**

34 Ant III with 1 and Ant II with 5 blunt sensilla, respectively. Dens with 2 basal and 1 distal dorsointernal hooks, all are normally serrated. Unguis basal lamella absent .... ***O. cruciata*** **Bonet, 1943 (USA, cave)**

– Ant II and III without blunt sensilla. Dens with 2 basal and 1 distal dorsointernal hooks, all strongly dentated. Unguis basal lamella shorter than or equal to half the length of the unguis ....***O. indica*** **Yosii, 1966a (India, cave)**

35 Mucro with 5 teeth. Ant IV without basal sensilla; Ant III without and Ant. II with 2 blunt sensilla, respectively. Dens with 1 basal and 2 distal dorsointernal hooks. Unguis basal lamella absent..........***O. japonica*** **Yosii, 1956 (Japan, cave)**

– Mucro with numerous teeth, serrated. Ant IV with 1 basal sensillum; Ant III with 2 and Ant II with 4–5 blunt sensilla, respectively. Dens with 2 basal and 3 distal dorsointernal hooks. Unguis basal lamella shorter than or equal to half the length of the unguis............. ***O. subhoffi*** **Christiansen & Bellinger, 1998 (USA, cave)**

36 PAO with 5 lobes...**37**

– PAO with ≥ 6 lobes...**39**

37 Unguis basal lamella longer than half the length of the unguis. Dens with 1 distal dorsointernal hook and without basal dorsoexternal hooks. Ant II with 2 blunt sensilla **38**

– Unguis basal lamella shorter than or equal to half the length of the unguis. Dens with 3 distal internal hooks and 1 basal dorsoexternal hook. Ant II without blunt sensilla ....***O. itatiaiensis*** **Arlé, 1961 (Brazil, litter)**

38 Unguis with well-developed tunica. Ant IV with 10 median sensilla and without basal sensilla. Ant III without blunt sensilla. Dens with 1 basal dorsointernal hook ....***O. tunica*** **Christiansen & Bellinger, 1980 (USA, cave)**

– Unguis without tunica. Ant IV with 4 median sensilla and 1 basal sensillum. Ant. III with 2 blunt sensilla. Dens without basal dorsointernal hooks ....***O. hubbardi*** **Christiansen & Bellinger, 1996 (USA, cave)**

39 PAO with 6 simple lobes...**40**

– PAO with 6 lobes subdivided into 2 or more fingers **47**

40 Mucro with 4 teeth **41**

– Mucro with 5 teeth .... ***O. czmur* Szeptycki, 1977 (North Korea, forest litter)**

41 Unguis basal lamella present.. **42**

– Unguis basal lamella absent.. **44**

42 Unguis basal lamella longer than half the length of the unguis. PAO with 6 lobes **43**

– Unguis basal lamella shorter than or equal to half the length of the unguis. PAO with 6–8 lobes .... ***O. pegyi* Gruia, 1994 (Romania, cave)**

43 Ant IV without basal sensilla. Both Ant III and Ant II with 1 blunt sensillum. Dens with 2 basal and 3 distal dorsointernal hooks .... ***O. kuramaensis* Yosii, 1956 (Japan, edaphic)**

– Ant IV with 1 basal sensillum. Ant III and II without blunt sensilla. Dens with 1 basal and 1 distal dorsointernal hook... ***O. ambigua* Christiansen, 1957 (Lebanon, soil)***

44 Dens with 2 basal and 3 distal dorsointernal hooks. Basal scale on mucro present **45**

– Dens with 1 basal and 1 distal dorsointernal hook. Basal scale on mucro absent .... ***O. atoyacensis* Bonet, 1943 (Mexico, epigeic)**

45 PAO lobes relatively long .... ***O. pelissiei* Deharveng, 1988 (France, cave)**

– PAO lobes short.. **46**

46 Basal tubercle of dens with 2 ciliated macrosetae. Ant III with 2 and Ant II with 1 blunt sensillum, respectively. Posterior setae at male genital plate feathery ..... ***O. yosiiana* Szeptycki, 1977 (Japan, North Korea and USA, epigeic)**

– Basal tubercle of dens with 1 ciliated simple macroseta and 1 ciliated leaf-shaped macroseta. Ant III with 1 blunt sensillum, Ant II without blunt sensilla. Posterior setae at male genital plate smooth, pointed.... ***O. crassicornis* Shoebotham, 1911 (England, France, Poland and Hungry, eipigeic and cave)**

47 Basal scale on mucro present. PAO lobes subdivided into 3–6 fingers. Ant IV with 4 medial sensilla. Dens with 1 basal and 1 distal dorsoexternal hook. Dens with 3 basal and 3 distal dorsointernal hooks....... ***O. moghanensis* sp. nov. (Iran, Cave)**

– Basal scale on mucro absent **48**

48 Some PAO lobes simple and some lobes subdivided into 2 fingers. Ant IV with 4 medial sensilla. Dens without basal dorsoexternal hooks, with 1 distal dorsoexternal hook. Dens with 2 basal and 2 distal dorsointernal hooks .... ***O. reyersdorfensis* Stach, 1936 (Poland, cave)**

– PAO lobes subdivided into 2–3 fingers. Ant IV with 4 medial sensilla. Dens without basal dorsoexternal hooks, with 1 distal dorsoexternal hook. Dens with 3 basal and 4 distal dorsointernal hooks .... ***O. dethieri* Janssens & De Bruyn, 2010 (Belgium, cave)**

– PAO lobes subdivided into 3 fingers. Ant IV without medial sensilla. Dens with 1 basal and 2 distal dorsoexternal hooks. Dens with 1 basal and 1 distal dorsointernal hook.............................. ***O. egerszogensis* Loksa, 1961 (Hungary, cave)***

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* considered as *species inquirenda*, due to insufficient or doubtful data in an original description.

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Metabolic rates of groundwater species as a function of body mass and temperature

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Abstract

Research on the metabolic physiology of groundwater species, particularly regarding oxygen consumption rates (OCR), has made significant advancement, revealing valuable insights into the adaptations of exclusively groundwater-dwelling (stygobitic) species. However, a comprehensive understanding of how these metabolic rates scale with body mass and respond to temperature changes remains elusive. This study aims to bridge this gap by reviewing published data on OCR across a variety of groundwater organisms to elucidate patterns of metabolic rates in relation to body size and temperature. We employed a combination of literature review and quantitative analyses, focusing on the allometric scaling of OCR with body weight and the effect of temperature on metabolic rates. Our findings indicate that OCR scales with body weight in an allometric pattern, with an inter-species slope of 0.80, suggesting non-isometric scaling. Furthermore, our analysis showed that stygobitic species' metabolic rates are less responsive to warming than those of non-stygobitic species at low to moderate temperatures. However, at higher temperatures, metabolic rates in stygobitic species decline faster than in non-stygobitic taxa, highlighting a potential vulnerability to global climate change. This study contributes to our understanding of the metabolic strategies of groundwater species, underscoring the need for further research to fully grasp the eco-evolutionary implications of these findings for groundwater conservation.

Keywords

Allometry, Arrhenius equation, ectotherms, metabolism, oxygen consumption rate, respiration, stygobiont

Introduction

Metabolism encompasses the entirety of biochemical reactions within an organism, essential for sustaining cellular functions and the whole biosphere (Braakman and Smith 2013). This process, which involves deriving energy from fuel molecules, is inherently inefficient: a portion of the energy is invariably converted into heat, a form not practically utilizable (Balaban 2020). Endotherms, such as mammals and birds, harness and regulate metabolic heat to maintain stable internal body temperatures and peak sustained performance (Ruben 1995). In contrast, ectotherms, including invertebrates, reptiles, amphibians, and fish, do not rely on metabolic heat for temperature regulation, leading to internal temperatures that fluctuate with environmental changes (Angilletta 2009). The metabolic rate is the energy an organism consumes over a specific period of time, quantifiable through calorimetry by measuring an organism's heat loss. This measurement can be expressed in joules (J), calories (cal), or kilocalories (kcal) per unit of time (Kaiyala and Ramsay 2011). Alternatively, it can be assessed through respirometry, which calculates the oxygen consumption rate as the volume of oxygen consumed over time (Lampert 1984). Oxygen consumption rates (OCR) vary widely across taxa, and can be influenced by factors such as organism's body mass (Gillooly et al. 2001), environmental conditions (e.g., temperature; Hernández-León and Ikeda 2005) and activity level (Culver and Poulson 1971). Standard OCR are a proxy of standard metabolic rates (SMR), which refer to the metabolic rates measured under conditions of rest, tranquility, absence of stress, and fasting (Angilletta 2009). Typically, SMR scale with body mass in both endothermic and ectothermic species (Gillooly et al. 2001). This relationship is represented by the allometric law (West et al. 1997) in equation 1:

$$SMR = SMR_0 \times M^b \tag{1}$$

Here, SMR_0 is a constant that is characteristic of the kind of organism, M is the organism's body mass, and b is the scaling exponent dictating the relationship's slope. The equation 1 can be conveniently log-transformed as in equation 2:

$$log(SMR) = logSMR_0 + blogM \tag{2}$$

where: $b > 1$ indicates that SMR increase at a faster rate than mass; $b < 1$ indicates that SMR increase at a slower rate than mass and $b = 1$ indicates isometry, i.e. SMR scale proportionally with mass. For organisms to stay in energy balance, metabolism can only vary in proportion to their surface area (Rubner 1883). Kleiber (1932) experimentally found a (close to) 3/4 exponent to describe the relationship between metabolic rate and body mass. Notably, many invertebrate taxa exhibit a mass exponent b in the range of 0.66–0.80, a shared characteristic whose underlying reason remains elusive (Hoppeler and Weibel 2005).

Temperature has profound effects on chemical and biochemical reactions and aerobic metabolism at the cellular level, thereby shaping the metabolic rates of ectotherms

(Schulte et al. 2015). Within permissive thermal ranges, which are temperatures conducive to long-term survival, an increase of +10 °C often leads to a doubling or tripling of metabolic rates (Dell et al. 2011). This effect can be quantified using Q_{10} (the factorial change in metabolic rates resulting from a 10 °C increase) or by Ea (Arrhenius activation energy; Cossins and Bowler 1987). For most ectotherms, Ea typically ranges from 0.5 to 0.8 eV (= 48.26 to 77.22 kJ/mol; Q_{10} = 2 or 3), equating to a 7–12% increase in metabolic rates per degree Celsius (Dell et al. 2011). Initially, in ectotherms, warming positively affects physiological processes like developmental speed, mobility and egg production (MacLean et al. 2019). However, excessive warming accelerates aging and senescence, and beyond a critical temperature threshold, heat-related failure can occur rapidly, with severe consequences (Brown et al. 2004). The physiological causes of heat failure in ectotherms, potentially including protein denaturation, oxygen limitation, cellular excitability loss and membrane dysfunction, are not fully understood (Brown et al. 2004). The severity of temperature stress depends on both the temperature and duration of exposure, with thermal tolerance limits significantly influencing species distributions (Rezende et al. 2014). According to the metabolic theory of ecology, metabolic rate varies with body mass and temperature as a result of internal physical constraints (Glazier et al. 2020). However, various ecological factors may also affect metabolic rate and its scaling with body mass. Glazier et al. (2020), for example, have shown that the effect of temperature on the ontogenetic scaling of resting metabolic rate of the freshwater amphipod *Gammarus minus* depends critically on habitat differences in predation regime.

Groundwater habitats, typically oligotrophic and devoid of light, feature stable temperatures and chemical conditions, which reduce the need for rapid physiological adaptations (Di Lorenzo et al. 2023). Stygobitic species (i.e., species that are unable to complete their life cycle outside of groundwater habitats; Culver et al. 2023), have been shown to generally exhibit lower metabolic rates than their surface water counterparts (Hüppop 1986; Hervant et al. 1997; Di Lorenzo et al. 2015). However, a few studies have demonstrated the opposite pattern. Simčič and Sket (2019) observed that the OCR did not differ significantly between species or subspecies of the same genus from surface and subterranean habitats, but still they responded differently to temperature changes. Undoubtedly, our current understanding of metabolic variations across groundwater metazoans and their surface water relatives is limited. A comprehensive review of the OCR of stygobitic species is still lacking. Additionally, the influence of environmental factors, like temperature, on OCR in stygobitic species and their compliance with allometric scaling principles represent significantly underexplored areas. Gaining a better understanding of the physiological performance of subterranean organisms is of paramount importance, especially in the face of climate change (Vaccarelli et al. 2023). The persistence of species with poor dispersal abilities and high habitat specialization, such as groundwater species, will strongly rely on their physiological capacity to cope with environmental changes (Pallarés et al. 2020).

We aim to address the knowledge gaps in understanding the patterns of metabolic rates in groundwater species by reviewing the published data reporting on OCR of these organisms. The specific objectives are testing, for groundwater organisms, the validity of existing models on metabolic rates' dependencies on a) body size and b) temperature. Since the whole-body metabolic rate is central to the understanding of physiological as well as of ecological function of species, this review endeavors to enhance our understanding of the metabolic physiology of groundwater organisms, offering insights for their effective conservation and management efforts.

Material and methods

Data collection

We systematically searched and collected data from both peer-reviewed and grey literature sources. We conducted the literature search using the Web of Science platform, applying a set of pre-defined criteria. Our selection criteria were specifically designed to target studies quantifying metabolic rates in groundwater metazoans by measuring OCR. Following preliminary investigations in December 2023, various search terms were trialed, leading to the consensus adoption of the search string: TS = ("oxygen consumption rate*" OR "metabolic rate*" OR metabolism) AND TS = (groundwater OR stygob* OR subterranean). The initial search in December 2023 yielded 2,236 papers. We meticulously examined the complete content of each identified article and thoroughly scrutinized the reference lists within these articles to identify any supplementary pertinent sources. Additionally, our research approach extended beyond Web of Science, encompassing an unstandardized exploration of gray literature sources. Half of the studies retrieved from the literature focused on microbiology, engineering, agronomy, and other topics not pertinent to environmental science and ecology. Of the remaining studies, 14% were on terrestrial subterranean species, 10% on marine species, and 24% lacked quantitative OCR measurements (e.g., studies on metabolites). Following the screening phase, we identified 23 papers. In the next step, we meticulously reviewed the full text of each paper and extracted various key data points, including the type of publication, year of study, geographic and taxonomic scope, type of groundwater habitat, life history traits, morphometric data, field observations, acclimation data, and measurements of OCR. Additionally, we extracted data for non-stygobitic species that were examined in some of the papers for comparative purposes. Whenever the results of OCR measurements were not reported in tables or in text, we employed a web-based plot digitizer tool (https://automeris.io/WebPlotDigitizer) to extract OCR data from plots and images when the data were presented only graphically.

We obtained a total 291 OCR measurements from 23 studies (see the Results section for more details). To address our research questions on metabolic scaling and the impact of temperature on metabolic rates, we created two sub-datasets. For detailed information on these datasets, please refer to the relevant paragraphs in the subsequent sections. We converted all obtained data on OCR to a standard unit of μL O_2/mg DW/hour (DW = dry weight). In cases where dry weight was not reported, we estimated it to be 15% of the wet mass, based on the methodology used by Wilhelm et al. (2006)

for two amphipod species, which was in turn based on Taylor et al. (2003). This conversion was applied because approximately 70% of the records with indicated wet weight pertained to amphipod species. Oxygen mass (in µg O_2) and oxygen in moles (µmol O_2) were converted into volume (µL O_2) using a conversion factor of 1.43 (Gnaiger 1983; Peters 1983) and 22.4 (Lampert 1984), respectively. We applied no temperature corrections, as we recorded the temperatures at which each study was conducted.

Metabolic scaling

To investigate the relationship between body weight and OCR, we compiled a data set of 10 stygobitic species and 64 measurements. We collected data on 2 copepod species [*Diacyclops belgicus* Kiefer, 1936 and *Moraria* sp.], 3 isopod species [*Proasellus lusitanicus* (Frade, 1938); *Stenasellus virei* Dollfus, 1897; *Asellus aquaticus cavernicolus* Racovitza, 1925], 4 amphipod species [*Niphargus rhenorhodanensis* Schellenberg, 1937; *Niphargus virei* Chevreux, 1896; *Niphargus krameri* Schellenberg, 1935; *Niphargus stygius* (Schiödte, 1847)], and one amphibian species [*Proteus anguinus* Laurenti, 1768]. We included data only from studies that measured adult specimens at rest that had fasted for no more than 15 days prior to measurements and were not exposed to additional stressors (e.g., temperature stress, anoxia). We excluded studies where the experimental temperatures exceeded the species' permissive ranges, thereby ensuring data at temperatures consistent with their native habitats. Some authors referred to the OCR of resting, fasted animals without additional stressors as “standard metabolic rates” (e.g., Di Lorenzo and Galassi 2017), while others termed them “routine metabolic rates” (e.g., Simčič and Sket 2019). This variation in terminology probably stems from differences among species in their levels of random activity during respirometry trials. However, previous research has shown no discernible effect of author classification on metabolic scaling with body weight for other taxa (Killen et al. 2010; Boyce et al. 2020). We considered the specified OCR values in this study as the most appropriate proxies for standard metabolic rates, defined as metabolic rates measured under conditions of rest, tranquility, absence of stress, and fasting (Angilletta 2009). To prevent bias from multiple measurements per species, we included only one OCR value per species per study, as suggested by Clarke and Johnston (1999). To obtain one OCR value per species, when multiple data points existed for a species within a study, we used the median values to minimize the impact of potential outliers.

We used a linear model to examine the expected allometric relationship between species DW (in mg) and OCR (in µL O_2/h × ind.). We log-transformed OCR and DW values. We assessed the normality of the residuals using both a Shapiro-Wilk test and visual inspection of QQ-plot (Suppl. material 1: fig. S1).

Temperature effects

We compiled data from the literature for 19 stygobitic species and 288 measurements to assess the dependence of metabolic rate estimated from OCR on temperature. Collected data included 2 copepod species [*D. belgicus* and *Moraria* sp.], 6 isopod species [P. *lusitanicus*; *Proasellus valdensis* (Chappuis, 1948); *Proasellus* sp. 1 (gr. *cavaticus*); *Proasellus* sp. 2 (gr. *cavaticus*); *S. virei*; *A. aquaticus cavernicolus*)], 7 amphipod species [*N. rhenorhodanensis*; *N. virei*; *N. krameri*; *N. stygius*; *Stygobromus* sp.; *Stygobromus pecki* (Holsinger, 1967); *Gammarus acherondytes* Hubricht & Mackins, 1940)], 2 decapod species [*Procambarus franzi* Hobbs & Lee, 1976, *Procambarus pallidus* (Hobbs, 1940)], one amphibian species [*P. anguinus*] and a fish species [*Astyanax mexicanus* (De Filippi, 1853)]. Aquatic subterranean organisms must cope with periodic oxygen deficiency in their habitats, which can sometimes involve rapid changes from normoxia (> 3 mg/L O_2) to hypoxia (in the range of 0.3 to 3 mg/L O_2) or even anoxia (< 0.3 mg/L O_2; Malard and Hervant 1999). Previous studies have demonstrated that they are resistant to hypoxia, although they cannot survive severe hypoxia (dissolved oxygen < 0.01 mg/L O_2) for more than two days (Malard and Hervant 1999). Accordingly, the OCR values in our dataset were quite comparable in normoxia and hypoxia for some species. For this reason we included data for OCR measured in both normoxic and hypoxic conditions. This approach allowed us to capture the physiological responses of these organisms to the varying oxygen levels they naturally experience. As in the metabolic scaling analyses, we analysed all data collectively, without differentiation into standard or routine categories. We included in the calculation the OCR (in µL O_2/mg DW × h) and the incubation temperatures at which the animals were acclimatised for at least 5 days prior measurements. For an illustrative presentation of compiled data regarding the dependence of metabolic rate on temperature, we divided all OCR into 11 temperature classes with an interval of 3 degrees, from -2 °C to 30 °C, and calculated mean and standard deviation (SD) for the stygobitic and non-stygobitic groups of species (Suppl. material 1: table S1).

The thermodynamic response of the OCR over the entire temperature range was calculated in terms of the Arrhenius activation energy (Ea), which describes the influence of temperature on the metabolic rate. An estimate of Ea was derived from the slope of the Arrhenius plot of the natural logarithm of the OCR against the reciprocal of the absolute temperature (Robinson and Williams 1993) according to the equation 3:

$$Ea = -R_{gc}S \tag{3}$$

where Ea is expressed in kJ/ mol, R_{gc} is the gas constant (8.314 J/ mol × K), and S is the slope of the Arrhenius plot. We calculated the slope separately for low (from -2 °C to 18 °C) and high (from 19 °C to 30 °C) temperature ranges, as the mean OCR increases up to the 16–18 °C temperature class and after that decreases with temperature (Suppl. material 1: table S1). For comparative analysis, we conducted the same assessments of the relationship between temperature and metabolic rates using comparable data obtained from 12 non-stygobitic species concurrently sourced from the same literature. We compiled data from the literature for: 1 copepod species [*Eucyclops serrulatus* (Fischer, 1851)], 4 amphipod species [*Gammarus fossarum* Koch, 1836; *Gammarus lacustris* G.O. Sars, 1863; *Synurella* sp.; *Niphargus zagrebensis* S.

Karaman, 1950], 2 isopod species [*Asellus aquaticus aquaticus* Linnaeus, 1758; *Asellus aquaticus carniolicus* Sket, 1965], 2 decapod species [*Procambarus pictus* (Hobbs, 1940), *Procambarus clarkii* (Girard, 1852)], 1 cyprinodontiformes [*Poecilia mexicana* Steindachner, 1863], 1 Cypriniformes [*Gobio occitaniae* Kottelat & Persat, 2005] and 1 Characiformes [*Psalidodon fasciatus* (Cuvier, 1819)]. Since most of the data come from studies in which OCR was measured at only one or two temperatures, we pooled the data of all stygobitic or non-stygobitic species into two groups to assess the general trend in the relationship between OCR and temperature for stygobionts and compare it with the relationship for non-stygobionts, as data sets of both groups were obtained using the same approach. We compared the slopes of the Arrhenius plots between stygobitic and non-stygobitic species using ANCOVA to ascertain if there were significant differences in the Ea of the two groups. We used a Shapiro-Wilk test to assess the normal distribution of the data, and a Levene's test to examine the equality of variances. We performed all statistical analyses using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA).

Results

We collected 23 papers originating from Nearctic (Canada, USA, Mexico), and Palearctic Regions (France, Portugal, Spain, Italy, Slovenia) (Fig. 1). Publication years spanned from 1971 to 2023, showing a notable uptick starting in 2000 (Fig. 1). We gathered 291 OCR measurements across five taxonomic classes, encompassing 37 species (Table 1), of which 23 were stygobitic (186 measurements) and 14 non-stygobitic (105 measurements). Within the stygobitic taxa, the order Amphipoda was the most studied with 11 species and 106 OCR measurements, followed by the order Isopoda, with 7 species and 54 measurements (Fig. 1). Studies investigating the impact of temperature on the OCR of stygobitic taxa focused on the orders Harpacticoida, Cyclopoida, Isopoda, and Amphipoda (Fig. 1). However, only amphipods were examined regarding the effects of both warming and cooling (Fig. 1). The stygobitic species examined in this study were primarily collected from caves, followed by karst springs and alluvial aquifers, with no hyporheic species included (Fig. 1). The 291 records of OCR, also including type of groundwater habitat, life history traits, morphometric data, field observations, acclimation data, and measurements of OCR is available in Figshare (https://doi.org/10.6084/m9.figshare.25564377.v4) in Excel (.xlsx) format with read.me text file describing metadata. We reported the average OCR per species in Table 1. Dry weight ranged from 0.001 to 3185 mg (Table 1).

For 25 out of the 37 species in this study, the collection site temperature was below or equal to 18 °C (Table 1). Mean OCR, measured in µL O_2/h × mg DW, varied among species, ranging from 0.043 (*Astyanax mexicanus*) to 6.7 (*D. belgicus*) for stygobitic species, and from 0.13 (*Gobio occitaniae*) to 14.86 (*Gammarus lacustris*) for non-stygobitic species (Table 1). In the analyzed data set, non-stygobitic species demonstrated higher

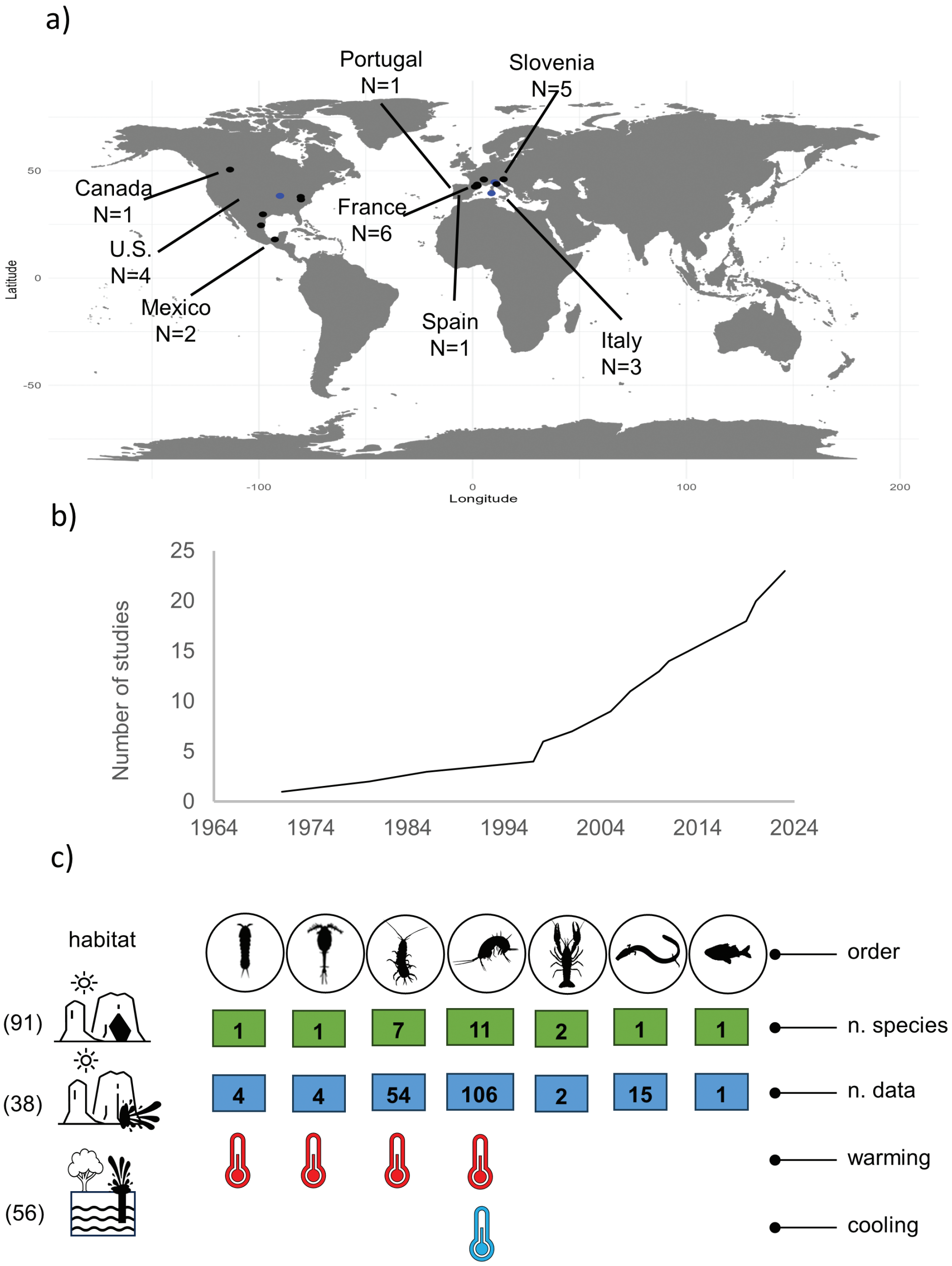

Figure 1. Geographic distribution of the 23 studies included in our dataset (**a**), with the timeline of publication years (**b**). N indicates the number of studies per country. The lower portion of the panel shows the distribution of the 186 OCR (oxygen consumption rate) measurements derived from these studies for stygobitic species (**c**). Icons provide a breakdown of the number of species per taxonomic order, and details on studies that examined the effects of warming and cooling. Information on the number of OCR measurements categorized by habitat type is also depicted (caves, springs and alluvial aquifers).

Table 1. Mean values of oxygen consumption rates per taxon. E = ecology (SB: stygobitic; nSB: non-stygobitic; NA- not well defined); DW = mean dry weight (mg); T (temperature of the collection site; °C); OCR (mean oxygen consumption rates in µL O_2/h × mg DW); reference number as in Suppl. material 1: table S2.

Taxa	E	DW	T	OCR	REF
Copepoda					
Diacyclops belgicus	SB	0.001	15.0	6.722	16,17
Eucyclops serrulatus	nSB	0.003	15.0	8.025	16
Moraria sp.	SB	0.001	8.0	1.122	23
Malacostraca					
Asellus aquaticus	nSB	2.41	11.0	1.159	4,5
Asellus aquaticus carniolicus	nSB	7.26	10.0	0.383	18
Asellus aquaticus cavernicolus	SB	4.37	10.0	0.283	18
Gammarus acherondytes	SB	8.93	12.9	1.311	10
Gammarus fossarum	nSB	4.58	11.0	0.734	4,5,8,9,11,13
Gammarus lacustris	nSB	1.20	12.0	14.867	6
Gammarus minus	nSB			2.347	1
Gammarus troglophilus	nSB	18.46	12.9	2.979	10
Niphargus krameri	SB	2.24	10.0	0.467	9
Niphargus rhenorhodanensis	SB	1.91	11.3	0.420	4,5,8
Niphargus stygius	SB	4.92	10.0	0.931	9,11,13,18,21
Niphargus virei	SB	13.81	11.3	0.247	4,5,12
Niphargus zagrebensis	nSB	4.10	11.0	1.860	18,21
Proasellus lusitanicus	SB	4.70	17.0	0.043	22
Proasellus sp. 1 (gr. *cavaticus*)	SB		9.9	0.516	15
Proasellus sp. 2 (gr. *cavaticus*)	SB		11.0	0.579	15
Proasellus valdensis (PV1)	SB		11.7	0.638	15
Proasellus valdensis (PV2)	SB		6.3	0.525	15
Procambarus clarkii	nSB	3184.50	19.0	2.770	2
Procambarus franzi	SB	714.00	18.0	1.500	2
Procambarus pallidus	SB	1737.00	19.0	0.450	2
Procambarus pictus	nSB	537.00	16.0	3.220	2
Stenasellus virei	SB	1.68	11.0	0.397	4,5
Stygobromus sp.	SB	1.20	7.0	6.089	4
Stygobromus pecki	SB	2.01	23.0	0.493	20
Stygonectes emarginatus	SB			1.793	1
Stygonectes spinatus	SB			2.653	1
Stygonectes tenuis potamacus	SB			2.673	1
Synurella sp.	nSB	1.07	23.0	4.853	20
Amphibia					
Proteus anguinus	SB	2625.00	12.8	0.065	7,19
Teleostei					
Astyanax mexicanus	SB			0.043	19
Gobio occitaniae	nSB			0.137	19
Psalidodon fasciatus	nSB	937.50	25.0	1.818	3
Actinopterygii					
Poecilia mexicana	nSB	38.10	25.0	2.333	14

OCRs compared to their stygobitic congeners in the genera *Asellus*, *Niphargus* and *Procambarus* (Table 1). This trend, however, was not observed in the genus *Gammarus*, where the pattern differed (Table 1). The OCRs of stygobitic cyclopoids appear to be approximately six times higher than those of stygobitic harpacticoids, both belonging to the class Copepoda (Table 1).

Body mass explained most of the linear variation in OCR ($R^2 = 0.908$). In detail, the model (Fig. 2) showed a slope (b) of 0.80 (95% CI: 0.72–0.98, SE: 0.09, $p = 0.00005$) with an intercept of -0.39 (95% CI: -0.57 – -0.21, SE: 0.07, $p < 0.00001$).

The Arrhenius plots of ln OCR against the reciprocal of the absolute temperature are shown for the stygobitic and non-stygobitic species in Fig. 3a and Fig. 3b, respectively. The response of stygobitic species to increasing temperature followed the classic bell-shaped profile (Fig. 3a), while the lack of data for non-stygobitic species made this response less conspicuous (in 5 cases only one data point was provided for a given temperature class; Fig. 3b). The Ea for the low temperature range (from -2 to 18 °C) was 94.7 ± 20.3 kJ/mol for the stygobitic species and 132.2 ± 35.3 kJ/mol for the non-stygobitic ones. In the high temperature range (from 19 to 30 °C), the value was -232.7 ± 63.1 kJ/mol for the stygobitic species and -83.6 ± 48.1 kJ/mol for the non-stygobitic ones. The comparison of the slopes between stygobites and non-stygobites showed no statistically significant difference in the response of the OCR to temperature changes (Table 2).

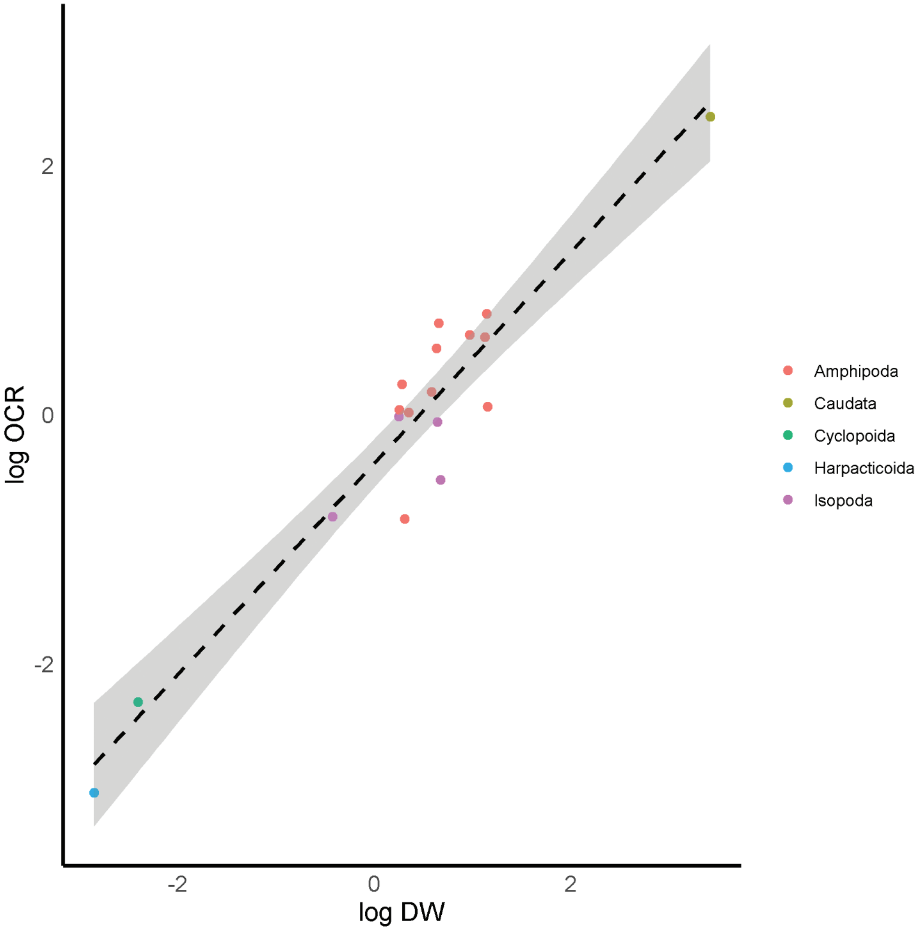

Figure 2. Oxygen consumption rates (in μL O_2 /ind × h) as function of body weight (dry weight in mg) in stygobitic species.

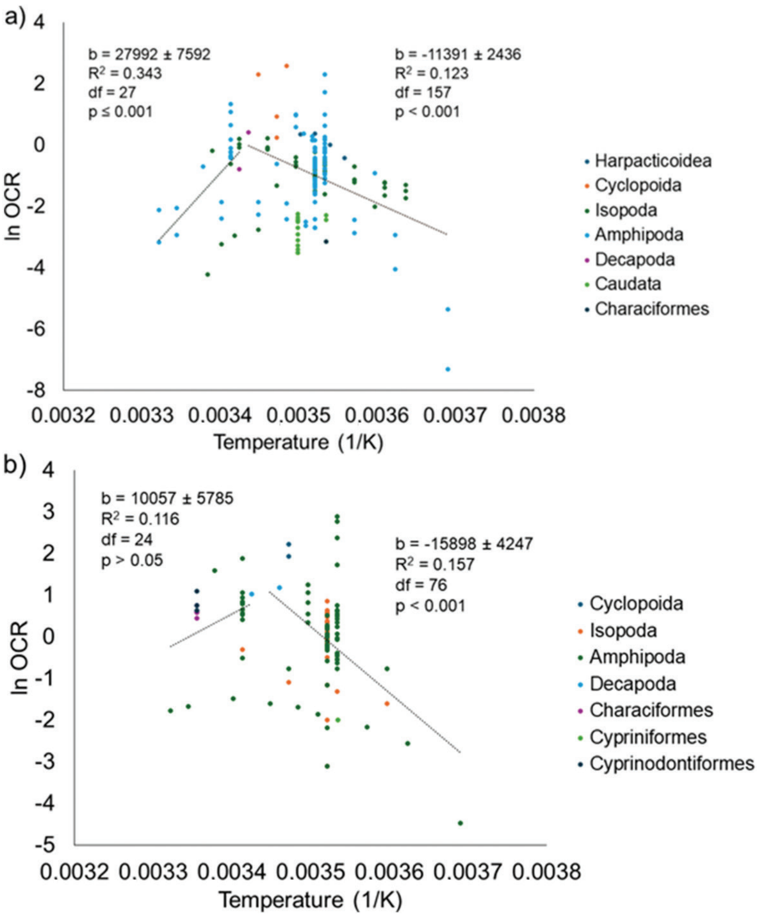

Figure 3. Arrhenius plots of oxygen consumption rates (OCR), in μL O_2/mg DW × h for (a) stygobitic and (b) non-stygobitic species.

Table 2. Results of ANCOVA for the comparison of the slopes between stygobites and non-stygobites (Group) using reciprocal of absolute temperature (Temp) and group as factors, analyzed for two temperature ranges.

	df	MS	F	p
Temp range -2–18 °C				
Group	1	1.296	0.889	0.347
Temp	1	44.052	30.222	<0.001
Group × Temp	1	1.161	0.796	0.373
Error	231	1.458		
Corrected Total	234			
Temp range 19–30 °C				
Group	1	3.918	3.573	0.065
Temp	1	17.200	15.684	<0.001
Group × Temp	1	3.722	3.394	0.071
Error	49	1.097		
Corrected total	52			

Discussion

Our analysis of oxygen consumption rates across groundwater organisms has unveiled significant insights into the metabolic physiology of both stygobitic and non-stygobitic species. This research highlighted a substantial increase in related studies since the year 2000, reflecting the escalating scientific interest in understanding the complex dynamics of groundwater fauna (Koch et al. 2024). The research efforts have been notably focused on specific taxa, such as Amphipoda, underscoring interest in the ecological and physiological adaptations of this order, further evidenced by recent research (e.g., Premate and Fiser 2024). Additionally, the investigation into the effects of temperature on OCR, especially regarding amphipods, emphasizes the significance of temperature—particularly in the context of global warming—as a pivotal environmental factor influencing metabolic rates in groundwater ecosystems (Vaccarelli et al. 2023). The focus on studying species from caves highlights the importance of these habitats in researching subterranean fauna, being caves more directly and easily accessible. Yet, the lack of stygobitic species from the hyporheic zones from this research underscores a significant gap. It would be important to address this omission, particularly given the integral connection between groundwater and surface water ecosystems (Sacco et al. 2024). The range of OCR reflects the metabolic diversity among groundwater communities. These variations are crucial for understanding the ecological roles and adaptations of these species to subterranean life. Notably, non-stygobitic species exhibited higher OCRs compared to their stygobitic counterparts in certain genera, except for *Gammarus*. This pattern indicates the presence of divergent metabolic strategies, possibly mirroring adaptations to different ecological niches and evolutionary responses to the energetically constrained subterranean habitats (Hose et al. 2022). The finding that stygobitic cyclopoids exhibit OCRs roughly six times higher than those of harpacticoids, despite both belonging to the class Copepoda, underscores considerable metabolic variability among closely related groups. This variability indicates that the metabolic rates of groundwater species are determined by a complex array of factors extending beyond temperature and body mass.

Our analyses revealed that OCR of stygobitic species scale with body weight following an allometric pattern, with a slope of 0.80. This indicates that OCR do not scale isometrically; in other words, metabolic rates do not double when the body weight doubles. This scaling pattern is consistent with findings in crustaceans (e.g., Ivlevla 1980; Glazier et al. 2010) and other surface taxa, both aquatic and terrestrial (Peters 1983; Schmidt-Nielsen 1984; Calder 1996). Several reasons underpin non-isometric scaling, encompassing heat exchanges with the environment (larger animals have a lower surface area relative to their volume, which reduces the energy required per unit of weight to maintain body temperature), energy transportation (the circulatory and respiratory systems do not scale linearly with size) and cellular and mitochondrial factors (White and Kearney 2013). If other underlying reasons remain elusive (Hoppeler and Weibel 2005), the principle of allometric scaling of metabolic rates stands as a cornerstone in the realm of biological scaling. It encapsulates the optimization of energy utilization across a spectrum of organism sizes, including those inhabiting groundwater environments, which appear to adhere to this universal pattern without deviation.

A significant portion of the intra-specific metabolic rates' variation can also be explained by differences in the mass. Shokri et al. (2019) demonstrated that individual metabolic rates scale allometrically with body mass in three species of surface amphipods. However, the intra-specific slopes were in the range of 0.32–0.36. This indicates a moderate mass-depended variation of metabolic rates within an individual species, which can allow it to broaden its ecological niche, contributing to greater functional diversity. Noteworthy, OCR appears to be mass-independent in certain stygobitic species (Hose et al. 2022), as observed in studies on *D. belgicus* (Di Lorenzo et al. 2015), *P. lusitanicus* (Di Lorenzo and Reboleira 2022) and *G. acherondytes* (Wilhelm et al. 2006). This pattern (described as ametric in Di Lorenzo et al. 2015) has been regarded as an adaptive trait to energy-limited groundwater habitats, where individuals may increase in size without increasing in their metabolic rates. This phenomenon likely occurs because weight gain is attributed to metabolically inactive reserves (stored fats, water, or other substances that add to the weight but do not participate in respiration) as body mass increases. Stygobitic species possess larger fat reserves compared to their surface-dwelling relatives (Hüppop 1986; Culver et al. 1995). This trait allows stygobitic species to endure prolonged periods without food by relying on stored energy (Hervant et al. 1997), thereby enhancing their resilience to the challenges posed by their habitats (Di Lorenzo et al. 2023 and references therein). On the other hand, the low metabolic variability among individuals of the same species may heighten population vulnerability and diminish the resilience to deviations from the norm, such as those stemming from anthropogenic disturbances (Hose et al. 2022). Further studies are necessary to confirm whether this trait is shared among stygobitic species or not.

The Arrhenius activation plots, which illustrate the effect of temperature on organisms over the range -2 to 30 °C, showed a temperature break in the OCR for both stygobitic and non- stygobitic species that were included in our study according to the procedure described in the Methods. Lower Ea values observed in stygobitic species compared to non-stygobitic ones within the low temperature range (-2–18 °C) indicate that the OCR of stygobitic species increase less with increasing temperature. This implies that the metabolic rates of stygobitic species are less responsive to temperature changes than that of non-stygobitic species. These findings align with previous research examining the response of potential metabolic activity, determined as a proxy for cellular respiration, to temperature changes in *N. stygius* (Simčič and Sket 2019). The reason for the relatively stable metabolic potential could be the reduction in costs for mitochondrial biosynthesis and degradation. The observed pattern could be also related to the low resource availability in subterranean habitats. Furthermore, the cost of maintaining a higher number of mitochondria, e.g. maintaining proton gradients and aerobic enzyme capacities, would contribute to an increase in basal metabolic rate (Rolfe et al. 1999; Pörtner 2002). As a result, this increase would lead to a shift in the energy balance, which would have an unfavorable effect on the accumulation of energy reserves for growth and reproduction (Lannig et al. 2003). Biochemical indicators of metabolic activity are key components of biochemical metabolic pathways that are directly or indirectly linked to processes important for performance, growth and/or reproduction. The basic premise for their use is that adjustments in the rates of physiological processes are necessary to balance metabolic demand with available energy supply (Dahlhoff 2004).

The Ea values for stygobitic species within -2 to 18 °C (94.7 ± 20.3 kJ/mol) are in the range of values reported by Mermillod-Blondin et al. (2013) for three isopod species of the genus *Proasellus* that colonize groundwater habitats with stable temperatures (annual temperature amplitude <1 °C) and by Simčič and Sket (2019) for the hypogean population of *A. aquaticus cavernicolus*. However, the Ea values in this study (0.98 eV and 1.3 eV for -2 to 18 °C and -2.41 and -0.86 eV for 18 to 30 °C, stygobitic and non-stygobitic species), exceed the average 0.48 eV reported for 314 aquatic and terrestrial ectothermic species (fishes, crustaceans, mollusks, amphibians and insects) within their permissive temperature range (0 to 40 °C; Jorgensen et al. 2022). This suggests that the temperature ranges we investigated in this study might not entirely fall within the permissive thermal range for the species analyzed. The high Ea values observed in our study implies that climate change could escalate heat-related energy expenditure for stygobitic and non-stygobitic species, with potential severe outcomes, as observed in many other ectothermic species (Jorgensen et al. 2022). Our findings suggest that even modest global warming scenarios may impose greater metabolic impairments on stygobitic species compared to non-stygobitic ones, in line with results of a recent meta-analysis on the effects of climate change on subterranean ecosystems (Vaccarelli et al. 2023). In fact, in the high temperature range (18–30 °C), the Arrheinius plot showed faster decline in OCR in stygobitic species than in non-stygobitic species. This indicates that hypogean species have a less efficient enzyme system at higher temperatures than epigean species. This might be due to the inactivation of respiratory enzymes that limit enzymatic processes outside their usual temperature range, leading to an irreversible loss of functions (Yurista 1999), as thermal tolerance is subject to phenotypic alteration within genetically defined limits (Cuculescu et al. 1998). Enzyme structure and function are highly sensitive to temperature variations, whether these changes occur rapidly or over evolutionary timescales (Angilletta 2009). The adaptive responses to these stresses have played a significant role in biological evolution (Clarke 2017). Studies have demonstrated that temperature impacts on enzyme properties establish the thermal optima and tolerance limits of metabolic functions, which are crucial for the survival and performance of organisms (Angilletta 2009). It appears that stygobitic species are less capable of maintaining optimal activity at higher temperatures, indicating a greater sensitivity to elevated temperatures compared to non-stygobitic species. The discrepancy indicates that surface species may possess a more effective molecular defense mechanism against thermal stress compared to subterranean counterparts. Most probably due to the fact that non-stygobitic species live in environments with high fluctuations in water temperatures. Previous studies have shown that there are variations in the genes associated with responses to thermal stress between subterranean and surface-dwelling terrestrial species, with a notably larger number of genes showing differential expression in surface taxa, suggesting a more robust heat shock response capability (Beasley-Hall et al. 2022). Although this has not yet been specifically demonstrated for aquatic subterranean species, it carries implications regarding the reduced thermal tolerance of stygobitic organisms at the molecular level.

Our review highlighted that the studies aiming towards understanding of the influence of temperature on metabolic rates of stygobitic species remain disconnected from broader ecological considerations such as energy budgets, food web dynamics and ecosystem functioning. Metabolic heat production and loss, critical in energy transfer efficiency between trophic levels, are yet to be fully integrated into these studies. The reports of such effects on metabolic scaling usually focus on single factors, such as comparison in metabolism between stygobitic and non-stygobitic species, the investigation of different species responses under different temperature acclimation and under different experimental set-ups (hypoxia/normoxia; starvation, exposure to pollutants) or investigation of metabolic rates of species from different localities or habitats, while understanding the possibility of significant interactive effects between multiple factors requires further studies.

This study provides insights in the metabolism of groundwater species, though there are potential limitations and areas that could benefit from further research. Extracting relevant data for our study was hindered by the inconsistent reporting of experiments, which frequently omitted detailed information on body size and weight. We bridged these gaps with calculations and estimations. Nonetheless, the assumption made on computation of the dry weight and the inclusion in the dataset of species that have not yet been described represent a limitation of our study. We also acknowledge potential limitations of our approach aimed at assessing the effect of temperature on the metabolic rates of groundwater species. Pooling data from different species into broad groups (stygobitic and non-stygobitic) might mask species-specific variations, potentially leading to an oversimplified interpretation of the data. Additionally, certain species are overrepresented in specific temperature classes, leading to a potential uneven comparison between the two ecological groups. The potential bias toward frequently studied species and regions signals the direction toward studies should expand in the future. Additionally, while this study explores the influence of environmental factors, such as temperature on OCR, more research is needed to understand the metabolic strategies of stygobitic species fully. Future research should focus on lesser-studied habitats, including hyporheic zones and employ experimental approaches to assess environmental stressors on metabolic rates. Integrating energy budgets, food web dynamics and ecosystem functioning into metabolic studies will provide a deeper understanding of groundwater species' resilience and inform conservation strategies against anthropogenic impacts and climate change.

Conclusion

Our investigation inquired into the metabolic rates of groundwater organisms, focusing on the relationship between oxygen consumption rates (OCR), body weight and temperature. We uncovered an allometric scaling of OCR with body weight and revealed a distinct thermal sensitivity between stygobitic and non-stygobitic species, underscoring a nuanced vulnerability to changing temperatures. However, the reliance on existing literature may introduce biases towards more frequently studied species and regions, potentially skewing the universality of our conclusions. Moreover, the varied methodological approaches across studies present challenges in data standardization, emphasizing the need for uniform reporting standards in future metabolic rate research. The implications of our findings are potentially far-reaching, providing insights for the conservation and management of groundwater ecosystems, particularly in the face of global climate change. For example, understanding the metabolic responses of stygobitic species to temperature changes can inform conservation strategies to protect these species in regions predicted to experience significant warming (Shokri et al. 2022). However, to build upon our work, research should expand to include lesser-studied groundwater habitats and employing experimental approaches to directly assess the impact of environmental stressors on metabolic rates. Such endeavors would enrich our comprehension of subterranean life's resilience and adaptability, informing strategies to safeguard these vital ecosystems against anthropogenic impacts and climatic shifts.

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Supplementary material 1

Supplementary information

Authors: Tiziana Di Lorenzo, Nataša Mori, Tatjana Simčič

Data type: docx

Explanation note: **table S1.** Oxygen consumption rates (μL O2/h × mg DW) for stygobitic and non-stygobitic species divided into 11 temperature classes (TC) with an interval of 3 degrees, from -2 °C to 30 °C. N: number of measurements. **table S2.** List of literature sources with geographic information and aims of the studies. **fig. S1.** Quantile-Quantile (QQ) plot assessing the normality of the residuals from the generalized linear model with data not corrected by temperature (Shapiro test: W = 0.928, p-value = 0.180).

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Arthropod diversity in shallow subterranean habitats of the Appalachian Mountains

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Abstract

Subterranean arthropods are important components of soils and contribute essential food-web functions and other ecosystem services, however, their diversity and community composition has scarcely been assessed. Subterranean pitfall traps are a commonly used method for sampling soil habitats in Europe but have never been widely implemented in the Americas. We used subterranean pitfall traps to sample previously unsurveyed arthropod communities in southwestern Virginia, U.S. Traps were placed in shallow subterranean habitats (SSHs), underground habitats close to the surface where light does not penetrate, and more specifically at the interface between the soil and underlying “milieu souterrain superficiel”—a microhabitat consisting of the air-filled interstitial spaces between rocks (abbreviated MSS). In total, 2,260 arthropod specimens were collected constituting 345 morphospecies from 8 classes, 33 orders, and 94 families. A region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified and sequenced, and objective sequence clustering of 3% was used to establish molecular operational taxonomic units (mOTUs) to infer observed species richness. In all, 272 COI barcodes representing 256 mOTUs were documented for rare soil-dwelling arthropod taxa and are published to build a molecular library for future research in this system. This work is the first taxonomically extensive survey of North American soil-dwelling arthropods greater than 10 cm below the soil surface.

Keywords

Completeness, DNA, endogean, epigean, hypogean, milieu souterrain superficiel, MSS, shallow subterranean habitats, SSH, survey

Introduction

The need to study and describe global biodiversity has never been more urgent. Anthropogenic habitat loss has been implicated as the major driver of the currently ongoing sixth great mass extinction event in geological history (Cowie et al. 2022). Biodiversity is at greatest risk in highly diverse regions known as biodiversity hotspots (Myers et al. 2000; Hamilton et al. 2022). The Appalachian Mountains of eastern North America constitute one such hotspot, and has experienced tremendous habitat loss from exploitative mineral extraction, timber harvesting, and other land conversion practices (Stein 2000).

According to a recent review assessing global declines of arthropod diversity and abundance due to habitat loss and other factors, twice as many species show long-term population declines as those exhibiting population increases (Sánchez-Bayo and Wyckhuys 2020). Subterranean arthropods, and other low-mobility invertebrates, are at elevated risk of extinction due to high rates of endemism and physiological constraints on dispersal (Mammola et al. 2019). Arthropods constitute the most species-rich animal group on Earth, with an estimated 7 million species, of which 5.5 million belong to the class Insecta (Santos et al. 2020). The Catalog of Life (2022) documents 1,128,168 species of arthropods, making up only 16% of the species estimated to exist. As such, arthropods constitute a globally understudied portion of biodiversity at high risk of species loss and anonymous extinction, a process in which a species is lost before it is discovered and described (Lobl et al. 2024). In the face of global decline, assessing and understanding insect and arthropod diversity is of paramount importance.

Research of subterranean organisms in North America has been dominated by taxonomically narrow studies with singular focal species or groups and have yielded important discoveries that hint at a significant but yet hidden diversity (e.g. Derkarabetian et al. 2010; Harden et al. 2024a, 2024b). General sampling and taxonomic assessment of soil-dwelling arthropods in North America has rarely, if ever, been conducted greater than 10 cm below the soil surface. As a result, little to nothing is known about the diversity and taxonomic composition of the arthropod communities that occupy the shallow subterranean habitats (SSH) of North America, and especially those within the Appalachian biodiversity hotspot. Broadly defined, SSH are underground habitats close to the surface where light does not penetrate, and at a basic level include soil and underlying milieu souterrain superficiel (MSS), a microhabitat consisting of the air-filled interstitial spaces between rocks (Mammola et al. 2016). But more broadly SSH can include underground aquatic interstitial habitats (e.g. hyporheic and hypotelminorheic zones), lava tubes, epikarst, and calcrete aquifers, which are defined in Culver and Pipan (2014). These habitats (except for some lava tubes) are typically close to the surface and are variable in depth but on average extend 0.1–5 meters in depth and up to 10 m (Culver and Pipan 2014). The arthropods that live the SSH have morphological and physiological adaptations such as the lack of pigmentation and eyes, shortened legs and elongate flexible bodies (Marek et al. 2021). SSH can be considered ecotones as they serve as transitional zones between adjacent epigean (above ground) and hypogean habitats (Prous 2004; Novak 2014). The term “hypogean” is often used to describe taxa or habitats associated with the rocky substrate that lays below the MSS, including cavernous karst cavities and cave systems distant from the surface (Prous 2004). Hypogean taxa have partially overlapping morphological adaptations to those in the SSH such as pigment and eye reduction, but contrast in having elongated appendages (Deharveng et al. 2024). Establishing tangible distinctions between different subterranean habitats is difficult as they exist more as a gradient than as distinct zones, thereby further contributing to the challenges of characterizing their diversity (Mammola et al. 2017). For the purposes of this study, SSH is used to refer to aphotic soil and MSS habitats 10–67 cm below the soil surface, but not extending into the bedrock, bedrock MSS, or into cave systems.

Sampling subterranean habitats is physically challenging, contributing to our lack of taxonomic and ecological knowledge in these systems. Although the number of studies focusing on subterranean arthropod communities has increased in recent decades, subterranean taxa continue to remain underrepresented in most biodiversity surveys due to difficulties associated with sampling the vertically distinct and ecologically important soil and MSS layers. Most studies focusing on soil invertebrate biota have taken place in the tropical and subtropical areas of the world and have focused on sampling ants (Wong and Guénard 2017) while most MSS studies have taken place in Europe, Japan, and Australia (Mammola et al. 2016; Ledesma et al. 2019; Halse and Pearson 2014). To our knowledge, SSHs have never been generally sampled in North America using ad hoc subterranean sampling.

Due in part to this lack of study, North American subterranean arthropods are vastly understudied. Essential research on them such as species descriptions, identification, and the assessment of biodiversity currently suffer from taxonomic shortcomings in which time, labor, and specialized expertise are direly required (Hebert et al. 2003; Ball and Armstrong 2011; Meierotto et al. 2019). Consequently, they are underrepresented in faunistic and ecological studies, and in major genetic repositories such as the National Center for Biotechnology Information (NCBI) and the Barcode of Life Data System (BOLD). The use of DNA barcodes, when coupled with traditional, morphology-based taxonomy, may help to partially overcome these shortcomings by streamlining identification and biodiversity assessment (Blaxter 2004). However, the taxonomic potential of DNA barcoding cannot be reached for North American soil-dwelling arthropods without the fuller representation of NCBI with expertly identified vouchers representing these taxa.

The aim of this study was to survey the subterranean arthropod communities of previously unexplored SSH in southwestern Virginia, U.S. This work also sought to generate high quality DNA barcodes for the sampled taxa in order to expand a molecular foundation for future research in North American subterranean systems. The COI barcodes generated from this study will contribute to the representation of subterranean arthropod genetic data at NCBI, Ecdysis, Global Biodiversity Information Facility (GBIF) and other biodiversity data repositories, and will serve as a tool for future work in characterizing and understanding subterranean arthropod communities of Appalachia and North America more generally.

Materials and methods

Sampling design and study sites

The subterranean pitfall traps (hereafter subterranean traps, or traps) used in this study follow the design of López and Oromí (2010), which was selected due to its efficiency in sampling subterranean arthropods, and the semi-permanent nature of the installation. The main outer shell of the trap is constructed of a polyvinyl chloride (PVC) pipe perforated with holes for entry of subterranean arthropods. The trap can remain in place, while an internal collecting receptacle itself is removable (Fig. 1). This allows for repetitive sampling without removal of the entire trap and lessens disturbance to the habitat between sampling events, thus reducing the impact of installation on the communities being sampled. The collecting receptacle is composed of a thinner plastic (polypropylene) cup with a smaller plastic (polyethylene) bait chamber secured in the center (Fig 1). Small rubber gaskets, stainless steel washers, and small bolt-nut combinations were used to stabilize the bait chamber within the center of the collecting receptacle. The bait chamber is capped with a perforated lid to allow the bait odors to disperse. Three strings are attached to the collecting receptacle, allowing it to be lowered to the bottom of the trap and then retrieved during collecting events. The perforations in the main outer shell (d = 7.95 mm) allow individuals to enter the trap, but restrict the entrance of vertebrates (e.g., small mammals, reptiles), and begin 10 cm below the soil surface to decrease the likelihood of epigean individuals entering the traps.

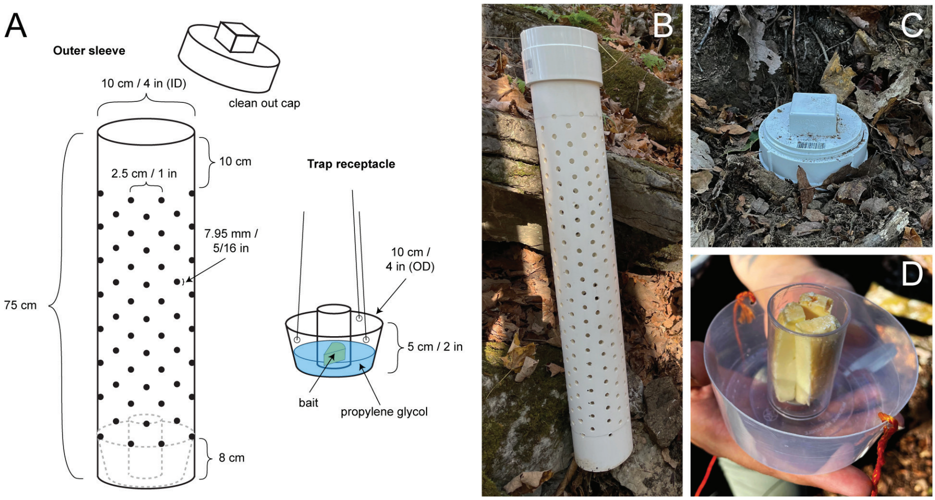

Figure 1. Design of the subterranean pitfall traps used in this study **A** subterranean pitfall trap main outer shell (left) and collecting receptacle (right) with dimensions in centimeters (cm) and inches (in). ID = inside diameter. OD = outside diameter **B** main outer shell **C** buried trap with clean out cap visible above the soil **D** collecting receptacle with bait, Limburger cheese, prior to filling with preservative. (Both metric and imperial measurements provided for some dimensions due to U.S. sourcing of materials, e.g., PVC pipe and 5/16 in. drill bit.).

The trap design allows for sampling within a range of 10–67 cm below the soil surface. The bait chamber receptacle was baited with Limburger cheese and the trap collecting receptacle filled with propylene glycol for specimen preservation. Limburger cheese was selected as bait due to its strong odor and propensity for attracting arthropods (Schneider and Culver 2004). Propylene glycol was selected due to its DNA preservation, thermal, and moisture buffering qualities (López and Oromí 2010).

A total of 20 subterranean pitfall traps were installed across three sites in southwestern Virginia (Table 1). Each site was forested, and traps were installed where evidence of frequent human disturbance was absent. Traps 1–5 were installed near the university's dolomite quarry ("Quarry"; Table 1) and traps 6–10 were installed on university land near Blacksburg ("Fallam"; Table 1). These sites are located within the Ridge and Valley ecoregion of Virginia where the rock substrate is mainly composed of sedimentary rock (Omernik 1995; U.S. Geologic Survey 2021). Dolomite was the rock type most frequently encountered while installing these traps. Traps 11–20 were installed in Floyd County, Virginia ("Starroot"; Table 1). This site is located within the Blue Ridge ecoregion of Virginia with the rock substrate heavily composed of metamorphic rock (U.S. Geologic Survey 2021). Quartzite was the rock type most frequently encountered while installing the Starroot traps. All sites were located within mixed forests dominated by oaks (*Quercus* spp.), with occasional pines (*Pinus* spp.), American beech (*Fagus grandifolia* Ehrh.), and maples (*Acer* spp.) scattered throughout (Virginia Department of Conservation and Recreation 2021). All sites were selected because they offered a topographical variety and evidence of an underlying rocky substrate consistent with the MSS that aligned with the optimal conditions described by López and Oromí (2010).

Table 1. Summary information of the study sites and traps.

Site	Trap #	Latitude, Longitude	Elevation (m)	County	GPS Accuracy (m)
Quarry	1	37.2231, -80.3832	631	Montgomery	3
	2	37.2229, -80.3799	610	Montgomery	6
	3	37.2228, -80.3818	639	Montgomery	5
	4	37.2225, -80.3875	667	Montgomery	9
	5	37.2232, -80.3836	619	Montgomery	2
Fallam	6	37.2124, -80.6093	560	Montgomery	3
	7	37.2127, -80.6085	558	Montgomery	34
	8	37.2132, -80.6054	600	Montgomery	3
	9	37.2133, -80.6049	592	Montgomery	3
	10	37.2119, -80.6090	559	Montgomery	4
Starroot	11	36.9656, -80.4185	751	Floyd	6
	12	36.9663, -80.4177	773	Floyd	3
	13	36.9685, -80.4171	771	Floyd	9
	14	36.9684, -80.4176	778	Floyd	3
	15	36.9673, -80.4170	780	Floyd	3
	16	36.9670, -80.4171	783	Floyd	5
	17	36.9668, -80.4172	781	Floyd	3
	18	36.9657, -80.4189	776	Floyd	3
	19	36.9651, -80.4186	790	Floyd	3
	20	36.9639, -80.4184	772	Floyd	3

There was slight variation in soil and rock composition between sites as well as within sites. Traps were placed in MSS microhabitats with varying sizes and quantities of rocks. Each trap was loaded with bait and preservative and operated for two weeks before the specimens were collected. A handheld Garmin eTrex 10 global positioning system (GPS) was used to record the geographical coordinates of the traps with positional accuracy recorded in meters. Traps were set on 28 December 2021 (winter) and again on 1 June 2022 (spring) for a total of four weeks of baited collection time.

Morphospecies

Specimens were removed from the propylene glycol with a sieve and pooled by order for each trap, stored in 8.0 mL Sarstedt vials with 100% ethanol, and subsequently identified to morphospecies using a Leica M125 stereomicroscope (Leica, Wetzlar, Germany). Morphospecies are operational taxonomic units identified by examination of easily observable morphological characters (Derraik et al. 2010). Because morphospecies are determined solely based on morphology, different life stages of a holometabolous insect species can be designated as separate morphospecies. One to two specimens were selected as representatives of each morphospecies. An individual of each morphospecies was photographed in ethanol in a Z-stack of 7–12 focal planes with a Canon EOS 6D SLR camera equipped with a Canon MP-E 65 mm macro lens and mounted on a Visionary Digital Passport portable imaging system (Canon, Tokyo, Japan; Visionary Digital, Charlottesville, Virginia). Helicon Focus (HeliconSoft, Kharkiv, Ukraine) was used to integrate the focal stacks into a single high resolution composite image. Each morphospecies was imaged from dorsal, ventral, and lateral views. Where appropriate, laterally compressed or coiled specimens were imaged only from lateral perspectives (e.g., some Diplopoda and Chilopoda).

Morphospecies from different sites, even those suspected to be the same species based on morphological similarity, were treated as distinct and unique in order to capture potential cryptic species. Each morphospecies was identified to at least the family level using various resources (Stehr 1991; Goulet and Huber 1993; Arnett and Thomas 2000; Arnett et al. 2002; Government of Canada 2002a, b; Triplehorn et al. 2005; Whitfield et al. 2014). Exceptions included immature mite, dipteran, coleopteran, and hemipteran specimens for which morphological identification resources do not exist, or were not accessible. Identifications of these morphospecies were retained at the order level.

DNA sequencing, barcode generation and analysis

DNA was extracted from each morphospecies using a DNeasy (Qiagen) Blood & Tissue extraction kit. The extraction protocol was modified to be less destructive, keeping specimens largely intact for morphological identification, deposition as vouchers, and potential species description (Gilbert et al. 2007). Rather than homogenizing the specimen by grinding body parts in buffer, a single puncture was made in the cuticle with a flame-sterilized pin and the specimen transferred to the microcentrifuge tube along with the buffer solution. This puncture allows the Qiagen lysis buffer to access the softer tissues within the body without grinding the specimen. The specimen was then recovered from the DNeasy minicolumn following the final buffer wash and stored in an 8.0 mL Sarstedt vials with 100% ethanol. Specimens are deposited in the Virginia Tech Insect Collection (https://collection.ento.vt.edu) under the specimen codes provided in Suppl. material 1: table 1.

A fragment of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene region was amplified utilizing polymerase chain reaction (PCR) employing the primers LCO1490 and HCO2198 (Folmer et al. 1994). These primers were selected as they have been shown to be useful for the amplification of the same COI gene region from a broad diversity of arthropods (Folmer et al. 1994; Elbrecht et al. 2019). In addition, the region corresponds to the often-used barcode region that is ubiquitous in genetic databases such as NCBI and BOLD. The PCR protocol was conducted according to Means et al. (2021a, b). Cleaning, quantification, normalization, and sequencing of amplicons on an Applied Biosystems ABI 3730 capillary sequencer was carried out by Arizona Genetics Core (University of Arizona). The raw forward and reverse chromatograms were assembled into consensus sequences in Mesquite (Version 3.61) by base calling, trimming, and quality assessment using the sequence analysis module Chromaseq (Version 1.52) with the software PHRED and PHRAP (Ewing et al. 1998; Maddison and Maddison 2009; Maddison and Maddison 2021). Sequence quality control and assembly was carried out according to the methods outlined in Vasquez-Valverde and Marek (2022) and a consensus COI sequence approximately 500–600 base-pairs in length was generated for each morphospecies. For the chromatograms that did not contig in Mesquite, additional assembly attempts were made using Geneious and various assembler algorithms (Geneious Version 2022.1.1, Build 2022-03-15). No additional contigs were retrievable. To generate the completed DNA barcodes, the consensus sequences were put in the correct reading frame within Mesquite using the sequence processing tools Reading Frame and Codon Position and aligned within Geneious using MAFFT (Version 7.490; Katoh and Standley 2013). Absence of stop codons were confirmed by visual inspection of chromatograms and examination of any erroneous single nucleotides in Mesquite, followed by designation of the proper reading frame orientation after each nucleotide check.

The barcodes were matched with existing records using GBIF's sequence-id engine querying BOLD (GBIF 2023). An additional local Basic Local Alignment Search Tool (BLAST) analysis was conducted by downloading all arthropod COI sequence data uploaded to NCBI as of 7 March 2023 and utilizing the custom batch BLAST feature in Geneious. BLAST hits with a percent identity match of ≥ 97% were accepted as molecular identifications at the species level. Hits with a percent identity match below 97%, but ≥ 95%, were accepted at the genus level (Srivathsan et al. 2022). Searches with no matches at these thresholds were left with morphological identifications at the family level. Sequences were then clustered at 3% dissimilarity (97% similarity by nucleotide identity) using objective clustering to establish molecular operational taxonomic units (mOTUs) for species binning (Meier et al. 2006; Srivathsan 2022). The divergence threshold of 3% was employed in accordance with the original methods for objective clustering as well as similar arthropod diversity studies employing objective clustering (Hebert et al. 2003; Smith et al. 2005; Meier et al. 2006; Srivathsan et al. 2022).

Sampling completeness

Sample completeness curves with 95% confidence intervals were generated for each of the three sites in iNEXT Online (Hsieh et al. 2016) employing 1,000 bootstrap replicates to analyze the relationship between sample coverage and sample size. iNEXT Online employs Hill numbers calculated from species richness and evenness. Hill numbers estimate sample coverage through rarefaction and to extrapolate the effect of additional sampling on sample coverage (Chao et al. 2014). They represent a standardized method to equitably compare diversity across data sets even when samples originate from highly disparate natural assemblages or various techniques that they were sampled (Hsieh et al. 2016). The sample completeness curves were estimated from Hill number $q = 1$, or the exponential of the Shannon diversity index for each site (Shannon 1948; Hill 1973; Ellison 2010; Hsieh et al. 2016). This metric is also referred to as effective number of species (ENS). Additional sample completeness curves were generated for other Hill numbers, but are not reported here as there were no significant differences from the ENS-based curves. The two collection periods (i.e., winter and spring) were combined for the analysis.

The Starroot site was sampled with ten subterranean traps for four weeks of total collection time, while the Fallam and Quarry sites were sampled with five traps each for four weeks of total collection time, constituting equal sampling times but half the sampling effort employed at the Starroot site. To account for the unequal sampling effort across sites, extrapolated coverage estimates for double the sample size were estimated for each of the sites.

Taxonomic composition and abundance

Observed species richness and abundance were used to characterize the taxonomic composition of the samples and sites. Relative proportions of species richness by class across the three sites were analyzed as hierarchical data and visualized by treemaps in JMP Pro 16 (SAS Institute Inc., Cary, NC). Observed abundance is reported by order and includes all orders.

Results

Sampling, morphospecies, and barcode analysis

Our sampling resulted in 2,260 arthropod specimens representing 345 morphospecies (Suppl. material 1: table 1). Select morphospecies images are included in Suppl. material 1: figs 1–39, and all 717 high resolution composite images taken are deposited at Ecdysis (https://ecdysis.org/, URLs to records in Suppl. material 1: table 2) and in the Virginia Tech Data Repository. Of the 345 morphospecies, 320 successfully amplified with COI primers, and from those amplicons, 272 successfully assembled into barcode sequences. Objective clustering revealed 256 total molecular clusters, or molecularly distinct species bins (mOTUs). Sequence dissimilarity was greater than 3% for 16 morphospecies clusters, indicating that each constituted multiple species. Each of these 16 clusters represented 2–4 species, thereby collapsing the 272 sequenced morphospecies to 256 molecularly distinct species bins. The 71 specimens that did not successfully sequence were left as morphospecies with morphological identifications only. COI barcodes were generated for the 272 sequenced specimens and deposited in NCBI (Suppl. material 1: table 1). Sequence matching via GBIF's sequence-id engine yielded 142 acceptable molecular identifications (percent identity match ≥ 95%) with 59 at the species, 40 at the genus, and 36 at the family level. One specimen first identified as an arthropod was later determined to be a pot worm (Annelida, Enchytraeidae) based on COI barcode (SPT-00078). Annelids were very abundant in the samples but only arthropods were analyzed for this study.

Sampling completeness

The sample completeness curve was highest for the Starroot site where sampling effort was double that of the other sites (Fig. 2). The asymptotic shape of the rarefied curve is indicative of the high sample coverage observed at this site (Table 2). Similarly high sample coverages were also observed for the remaining sites. The effective number of species was highest for the Quarry site and lowest for the Fallam site (Table 2). ENS was comparable between the Quarry and Starroot sites, but the observed sample completeness was higher for the Starroot site.

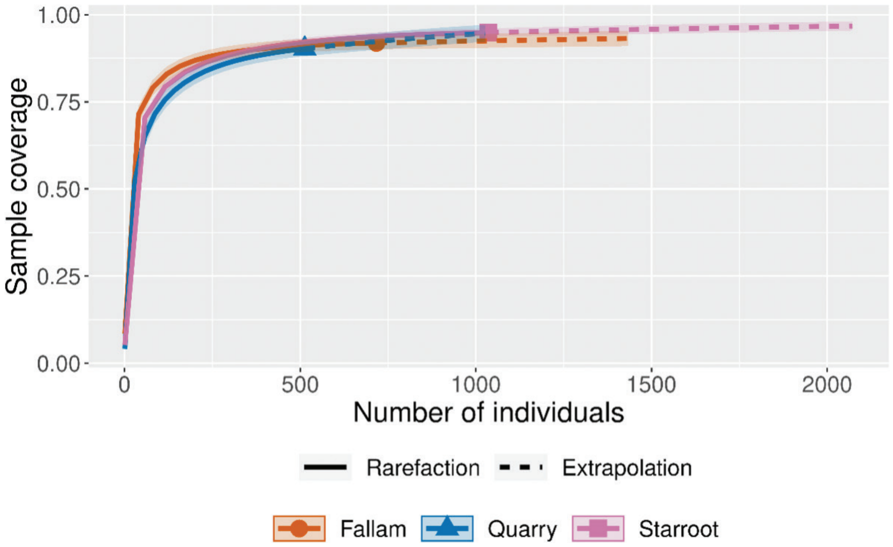

Figure 2. Sample completeness curves, with associated 95% confidence intervals, for the study sites.

Table 2. Effective number of species (ENS), Hill number q = 1, and observed and extrapolated sample coverage for each site.

Site	Quarry	Fallam	Starroot
ENS	39.485	26.882	37.691
Observed Coverage	0.903	0.919	0.949
Extrapolated Coverage	0.947	0.932	0.967

Taxonomic composition

In total, eight classes (Malacostraca, Entognatha, Insecta, Chilopoda, Diplopoda, Pauropoda, Symphyla, and Arachnida) from four arthropod subphyla (Crustacea, Hexapoda, Myriapoda, Chelicerata) were observed across all samples. Observed species richness by class was similar across the three sites (Fig. 3). The three most species rich classes across all sites were Insecta, Arachnida, and Entognatha, followed by Diplopoda, Chilopoda, and Malacostraca. Images of representative individuals from each arthropod subphylum are presented in Fig. 4 and those of each molecularly distinct species and morphospecies observed are included in Suppl. material 1: figs 1–39 [717 high resolution composite images taken are deposited at Ecdysis (https://ecdysis.org/, URLs to records in Suppl. material 1: table 2) and in the Virginia Tech Data Repository, DOI: 10.7294/26397688]. The number of morphospecies by trap, site, and collecting event are shown in Fig. 5.

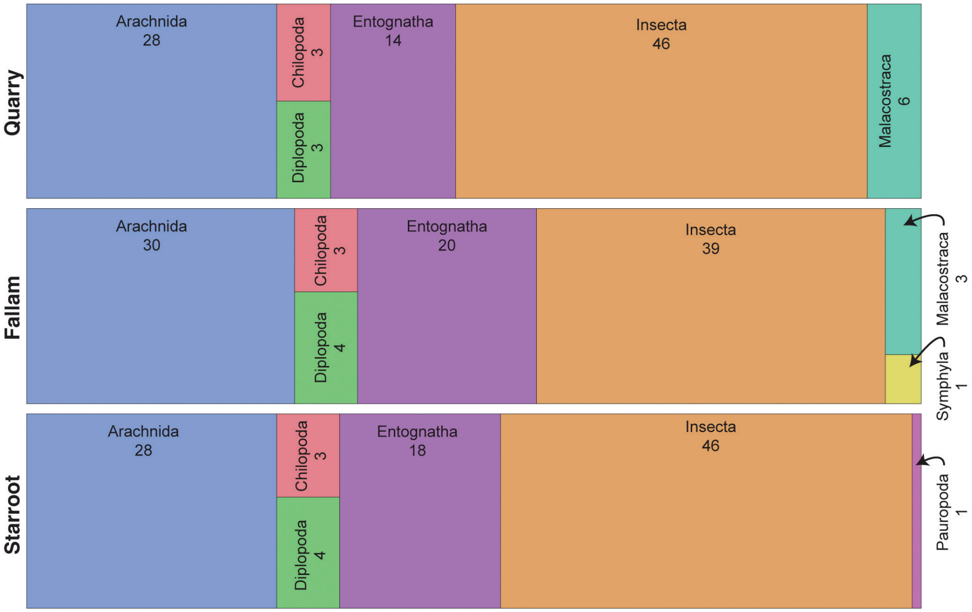

Figure 3. Observed arthropod species richness by class (reported as a percentage) across sites.

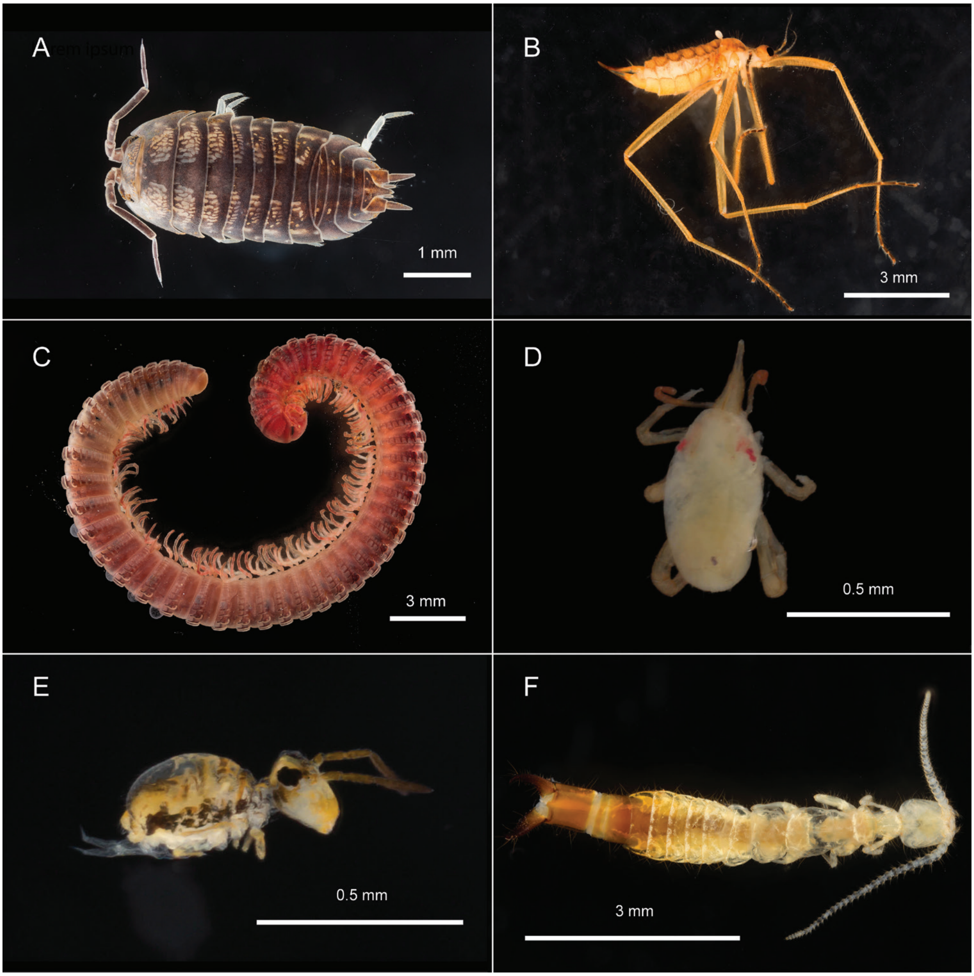

Figure 4. Representative arthropod specimens observed from each subphylum, including two from class Entognatha **A** Crustacea; dorsal view of *Cylisticus convexus*, individual SPT-0001 **B** Hexapoda; lateral view of *Chionea scita*, individual SPT-0020 **C** Myriapoda; lateral view of *Abacion* sp., individual SPT-00119 **D** Chelicerata; dorsal view of Bdellidae, individual SPT-00191 **E** Entognatha; lateral view of Sminthuridae, individual SPT-0067 **F** Entognatha; dorsal view of Japygidae, individual SPT-0061.

Abundance

Overall, the samples were dominated by hexapods and chelicerates with the orders Entomobryomorpha, Sarcoptiformes, Diptera, Hymenoptera, and Coleoptera being among the most abundant across all sites (Fig. 6). Insecta was predominantly represented by members of Diptera (40.13%), Hymenoptera (15.58%) and Coleoptera (33.12%); Arachnida by the subclass Acari or mites (66.67%); and Entognatha by the subclass Collembola (86.15%). The order Hymenoptera was predominantly represented by ants

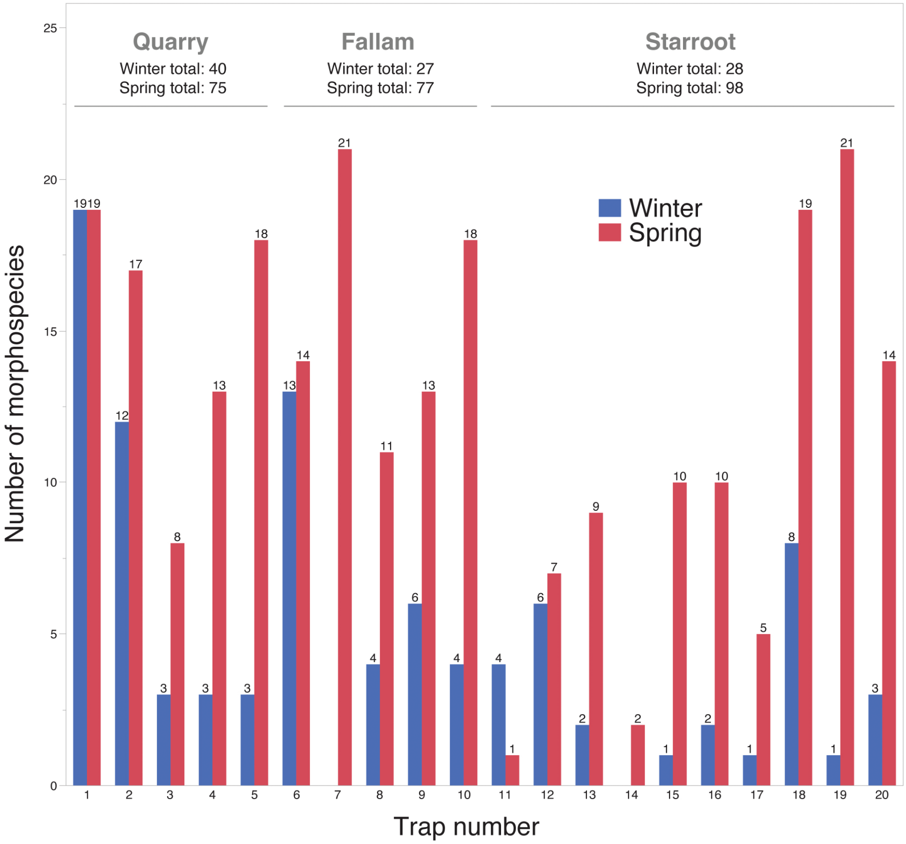

Figure 5. Number of morphospecies by trap, site, and collecting event. Data underlying this figure are in Suppl. material 1: table 3.

(Formicidae; 84.91%) across all sites. While the high abundance of springtails, mites, ants, and beetles in the collected samples is not surprising, the relatively high abundance of Diptera at the study sites can be explained by the high number of early instar larvae of the family Phoridae collected in single traps at the Quarry and Fallam sites.

Discussion

This study is the first taxonomically extensive survey of soil-dwelling arthropods in North America, and the first in the Appalachian Mountains, to employ subterranean pitfall traps capable of sampling to a depth of up to 67 cm below the soil surface. In all, 2,260 individual arthropod specimens were collected constituting 345 morphospecies and 257 molecularly distinct species (mOTUs) representing 8 classes, 33 orders, and 94 families. In total, 272 COI barcodes were sequenced and are published at NCBI. Of these, 102 constitute mOTUs that are new to the NCBI and BOLD databases. Many of the taxa recovered during the study represent new records and have not, or

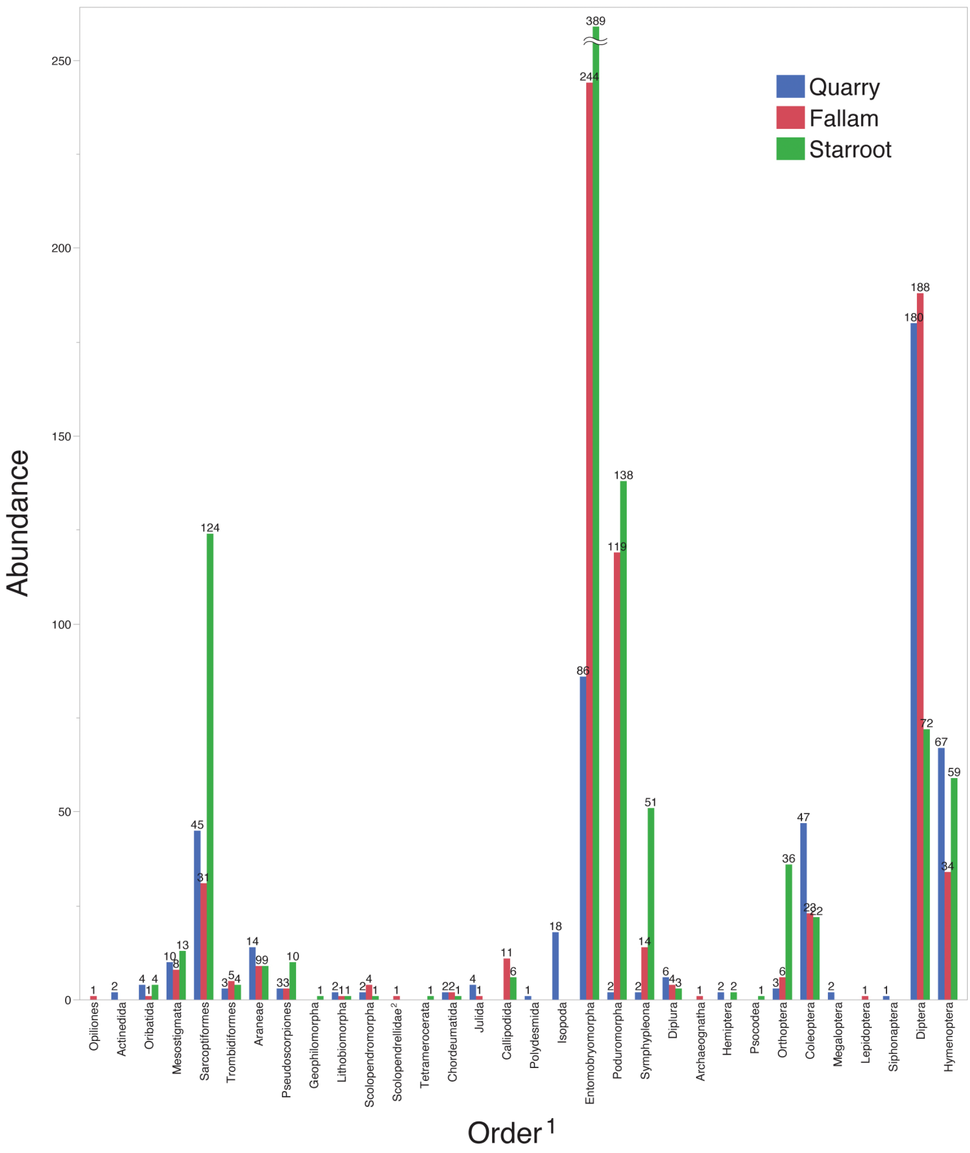

Figure 6. Arthropod orders and their abundance by site. Entomobryomorpha abundance bar for Starroot site extends off the chart and is abbreviated by double squiggly lines. ([1]Phylogenetic arrangement of orders. [2]The class Symphyla does not possess an order rank so family rank used.) Data underlying this figure are in Suppl. material 1: table 4.

have only rarely, been documented in the region. We suspect that a number of the morphospecies recovered are undescribed.

Several morphospecies exhibited hypogean/SSH adaptations: i.e., depigmentation, reduction of eyes and appendage lengthening (hypogean taxa) or shortening (SSH taxa) suggesting that they may be obligate subterranean inhabitants (Deharveng and Bedos 2018; Deharveng et al. 2024). These morphospecies included several species of ants

(Hymenoptera: Formicidae—6 out of 13 morphospecies), beetles (Coleoptera—15 of 53), flies (Diptera—18 of 62), springtails (Collembola—18 of 56), two-pronged bristletails (Diplura: Japygidae—6 of 7), spiders (Arachnida: Araneae—7 of 19), and mites (Arachnida: Mesostigmata, Sarcoptiformes, Trombidiformes—21 of 62). However, most individuals displayed morphology associated with epigean origin, which is consistent with the findings of similar studies outside North America that have shown that SSH are commonly dominated by epigean taxa (Coiffait 1958; Mammola et al. 2017). This dominance of epigean taxa might have contributed to the lower observed richness in winter as the richness and abundance of epigean arthropods, especially insects, in subterranean systems are significantly impacted by seasonality due to surface temperature influencing SSH temperature to some extent. This is not the case for hypogean taxa as climatic stability tends to increase as depth from the surface increases (Mammola et al. 2017). Alternatively, the lower observed richness in the winter may be associated with the decreased mobility of arthropods during cold and dry periods when many are in a winter diapause, or in immobile pupal stages.

The taxonomic composition of our samples is comparable to that of SSH studies in the Canary Islands (Pipan et al. 2011), Italy (Mammola et al. 2017), mainland Spain (Gilgado et al. 2014), Bulgaria (Langourov et al. 2014), and France (Juberthie 2000) with arthropods and annelids being the most abundant groups. Annelids were very abundant in our samples and were mainly represented by potworms (Enchytraeidae). Our findings differ in the detection of mollusks and crustaceans (Isopoda: Oniscidea). We only collected a single mollusk and only a few terrestrial isopods, while both groups were commonly encountered in the studies mentioned above. Our results regarding insect abundance by order align with those of Mammola et al. (2017) in that Diptera, Hymenoptera, and Coleoptera were among the most abundant groups across sites. Our findings are consistent with those of Moldenke and Lattin (1990) in that the families Phoridae, Cecidomyiidae, and Sciaridae were among the most common dipterans encountered. Diptera abundance was highly variable across sites due to, in part, the relatively large numbers of early instar Phoridae larvae observed in single traps at the Quarry and Fallam sites. Ants dominated specific traps at all sites which is consistent with the findings of Mammola et al. (2017). Where ants dominated a trap, relatively few other insects were found, and rarely were two or more ant species found in the same trap. Regarding Coleoptera richness by family, our findings are consistent with those of Moldovan (2005) who reported ground beetles (Carabidae; 14.3% of total abundance in our samples) to be less common than round fungus beetles (Leiodidae; 18.4%). This is in contrast with the study of Mammola et al. (2017), in which carabids were largely observed as larvae with only a single mature specimen collected. In contrast to the studies mentioned above, rove beetles (Staphylinidae; 24.5%) were the most species rich beetle group present in our samples. Hemipterans were rare, consistent with the findings of Langourov et al. (2014). This may be due to bait-bias as phytophagous hemipterans are likely less attracted to protein-based bait than their coleopteran equivalents. Bait-bias has been shown to influence the abundance and richness of taxa recovered in previous surveys of arthropods (Checa et al. 2019). Orthoptera was among the most abundant insect orders at the Starroot site, largely due to the relatively high abundance of the locally common *Ceuthophilus guttulosus*, F. Walker, 1869 (Orthoptera, Raphidophoridae).

Little overlap in molecularly distinct species was observed between the different sites with the Quarry and Fallam sites sharing two species (Hymenoptera: *Stenamma schmittii* Wheeler, 1903; Diptera: *Triphleba aequalis* Schmitz, 1919), and the Quarry and Starroot sites sharing two species (Hymenoptera: *Stenamma schmittii* Wheeler, 1903; Araneae: *Cicurina pallida* Keyserling, 1887). The ant species *Stenamma schmittii* Wheeler, 1903 was observed across all sites. This lack of pronounced species overlap is consistent with Lamoncha (1994) who recorded minimal overlap of oribatid mites between nearby subterranean sites in North and South Carolina, U.S. This trend is consistent with the notion that subterranean arthropod communities are often highly specific to the individual habitats they occupy (Menta and Remelli 2020). That said, these patterns may be a byproduct of insufficient sampling and further work is needed to support these trends, especially for sites with lower sampling effort.

The high sample coverage at the Starroot site suggests that future surveys of similar experimental design should employ ≥ 10 subterranean traps per site and ≥ 4 weeks total of baited-collection time. Further studies are needed to optimize the application of subterranean pitfall traps in North America. Subsequent investigations may also address other factors including presence or choice of baits, duration of sampling, and digging-in effects. Previous work has shown taxon bias associated with baits (Checa et al. 2019) and recency of placement (Mammola et al. 2017). Prior work has shown that baited subterranean traps preponderantly select for epigean taxa such as ants and phorid flies (Mammola et al. 2017). Similarly, epigean taxa may be more abundant in freshly excavated traps and these digging-in effects have been shown in aboveground pitfall traps but not demonstrated in subterranean traps. Future studies without baits and/or including a post excavation and installation settling period may reduce epigean bias and narrow sampling to hypogean (broadly defined) taxa.

Conclusions

Our findings suggest that the understudied SSH arthropod communities of the Appalachian Mountains, and North America, are highly diverse and warrant further study. This is consistent with the findings of several studies showing high arthropod diversity within subterranean assemblages both more broadly and specifically within particular groups such as beetles, spiders, and mites (Lamoncha 1994; Christman et al. 2005; Mammola et al. 2017; Ledesma et al. 2019). The close, but not quite-asymptotic nature of the sample completeness curves for each of our sites indicate that more species remain undocumented, and additional sampling is needed to better characterize subterranean arthropod diversity of southwestern Virginia and the Appalachian region. We still lack solid taxonomic, ecological, and molecular foundations for our understanding of the arthropod communities that inhabit the North American SSH. It is our hope that the primary taxonomic and barcode data generated through this work will serve as a foundation upon which future studies of North American subterranean systems may build upon.

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Supplementary material 1

A combined file

Authors: G. T. Harrison, Howard P. Dunleavy, Luisa F. Vasquez-Valverde, Alejandro I. Del Pozo-Valdivia, Kaloyan Ivanov, Paul E. Marek

Data type: pdf

Explanation note: Morphospecies occurrences, NCBI accession numbers; morphospecies figures 1–39; morphospecies records, including occurrence information and image URLs at Ecdysis.org; data underlying Fig. 5; data underlying Fig. 6.

Link: https://doi.org/10.3897/subtbiol.49.128521.suppl1

Supplementary material 2

Darwin core occurrence file

Authors: G. T. Harrison, Howard P. Dunleavy, Luisa F. Vasquez-Valverde, Alejandro I. Del Pozo-Valdivia, Kaloyan Ivanov, Paul E. Marek

Data type: csv

Explanation note: CSV file of occurrence data in Darwin Core data format.

Link: https://doi.org/10.3897/subtbiol.49.128521.suppl2

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Lost & Found - Rediscovery of H. Hauffen's *Carychium* material in the Dominik Bilimek Collection, BOKU University, including a contemporary assessment within the genus *Zospeum* (Gastropoda, Ellobioidea, Carychiinae)

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Abstract

The syntypes of *Carychium reticulatum* Hauffen, 1856 and *Carychium bidentatum* Hauffen, 1856, thought to be lost to science, have been rediscovered in a recent inventory of the shell collection of Domink Bilimek (1813–1884) at the University of Natural Resources and Life Sciences in Vienna, Austria (BOKU). In this work, we present (1) the historical context of the Dominik Bilimek and Heinrich (also: Henrik) Hauffen collections and (2) provide a contemporary image dataset of Hauffen's original material while considering today's taxonomic understanding of the genus *Zospeum*. We clarify the taxonomic status of the syntypes by using light microscopy, Scanning Electron Microcopy (SEM) and 3D X-ray Micro-CT data in conjunction with Hauffen's original illustrations and compare them to contemporary investigations of their closest congeners. Analysis of *Zospeum reticulatum* (Hauffen, 1856) is supported by historical documentation from Heinrich Hauffen's original annotations and Bilimek's diary entries, as well as from individual collection labels in Hauffen's own script (verified by archive material from the Archive of the National Museum of Slovenia, NMS). Hauffen's descriptions are based on apertural morphology and shell microstructure. Our re-investigation of *Zospeum reticulatum* provides corroborating evidence supporting its assignment to *Zospeum spelaeum* (Rossmaessler, 1839) while that of *Carychium bidentatum* corroborates its status as junior synonym of *Zospeum costatum* (Freyer, 1855).

Keywords
Cave snails, history of sciences, natural history collections, type specimens

Introduction

The importance of original type specimens is unquestionable for taxonomic evaluations and for upholding the international standards of reference that provide objectivity in zoological nomenclature (ICZN 1999). There are many incidences where original specimens were not preserved, became lost (Inäbnit et al. 2019), or got destroyed in the ravages of war (Janssen 2006). However, sometimes valuable material reappears serendipitously during inventories of other collections (Albano and De Mattia 2010; Salvador et al. 2023). We present a case in point, whereby the syntypes of *Carychium reticulatum* Hauffen, 1856 and *Carychium bidentatum* Hauffen, 1856, of the troglobitic genus, *Zospeum* Bourguignat, 1856, from Heinrich Hauffen's original collection, considered lost, reappeared in the natural science collection of Dominik Bilimek, housed at the University of Natural Resources and Life Sciences in Vienna, Austria (BOKU). In this paper, we present the historical context of the Bilimek and Hauffen collections (for biographic information see also Lukeneder et al. 2023) and provide a contemporary image dataset of Hauffen's original material considering today's taxonomic understanding of the genus *Zospeum*. We assess the species using light microscopy, SEM and X-ray Microcomputer-Tomography (X-ray Micro-CT) data. Current geographical information regarding older cave names is provided.

Zospeum reticulatum is considered a junior synonym of *Zospeum spelaeum* (Rossmaessler, 1838) in the most recent work of Inäbnit et al. (2019). Historically, *Zospeum spelaeum* is a much-debated species showing a wide spectrum in representation of shell characters, from smooth shells to ribbed shells to those that are highly reticulated and bidentate or tridentate (Frauenfeld 1856; Hauffen 1856a). Although Hauffen (1856b) found his shells very similar to those of *C. schmidtii* (Frauenfeld, 1854), he and Von Frauenfeld (1856) debated over the taxonomic significance of the number and position of denticles and the density of reticulate microstructure on the teleoconch as unique, diagnostic characters. Meanwhile, contemporary methods of analysis have enabled major access into the shells of these microscopic snails, not possible in 1856. These have revealed new image data providing evidence of the taxonomic significance of signatory structures such as the shape and form of the columellar apparatus, the form of the lamella and the alignment and configuration of the umbilical zone (Inäbnit et al. 2019; Jochum et al. 2024).

In this present work, and in the absence of supporting DNA sequence data from these populations, we delve into Hauffen's and Frauenfeld's morphological quandary by re-investigating this recovered material via new image data while cross checking the potential taxonomic significance of the initial species designation (i.e., *C. reticulatum* versus Frauenfeld's *C. schmidtii* and Freyer's *Z. costatum*). Consequences ultimately would affect species status and taxonomic assignment within *Zospeum*.

Materials and methods

Study material

All shells studied herein stem from the collection of the Institute of Applied Geology at the University of Natural Resources and Life Sciences (BOKU). All specimens include the original collection labels, including information on the determination and the respective author, the site locality and sometimes also the collector. Details about Bilimek's natural science collection are documented in Hopfensperger et al. (2021) and Lukeneder et al. (2023). Heinrich Hauffen catalogued most specimens/groups of specimens with a distinct combination of numbers, which were written on the corresponding label, whereas each combination is unique in his collection (top number corresponds to the species number, base number most probably to the locality number (Fig. 5N). Some labels also include markings with a prominent cross. All inventoried specimens were examined in detail and compared to the material described and illustrated by Hauffen (1856a, b). Localities correspond to Hauffen's documentation. One specimen of *Carychium reticulatum* var. *bidentatum* Hauffen is preserved from Glaven verh. Bibliographic and biographic accounts provide historical context.

Institutional abbreviations

BOKU	Institute of Applied Geology, BOKU University (Vienna, Austria);
IPUW	Department of Palaeontology, University of Vienna (Vienna, Austria)
MCSNTS	Museum of Natural History of Trieste (Trieste, Italy);
NHMW	Natural History Museum Vienna (Vienna, Austria);
PMS	Slovenian Museum of Natural History (Ljubljana, Slovenia).

Image acquisition

Digital imaging and image processing

The individual shells were imaged using a digital Keyence 3D Digital VHX-600 microscope at the IPUW (Micro photography). Additionally, special features were photographed with a Discovery.V20 Stereo Zeiss microscope and processed with the software AxioVision SE64 Rel. 4.9 imaging system at the NHMW (Microphotography). Collection labels were imaged with a Nikon D 5200 SLR, lens Micro SX SWM MICRO 1:1 Ø52 Nikon AF-S, Digital Camera, combined with the graphic tool (freeware) digiCamControl Version V.2.1.2.0 (Macro photography).

Measurements

Shell measurements, including shell height (sh), shell width (sw), aperture height (ah), aperture width (aw), height of last whorl (hlw) and spire angle (SA), were directly made on the Micro-CT images using dimension tools of the software Corel Draw 2017 (Corel Corporation, Ottawa, Ontario, Canada).

X-Ray Micro-Computer tomography (Micro-CT) and image processing

Three selected specimens were imaged intact in the Theoretical Biology Unit of the University of Vienna Evolutionary Biology Department, University of Vienna, Austria, with a Bruker SkyScan 1272 micro-CT system. The tungsten X-ray source was set at 60 kV and 10 W nominal output, with a 0.25 mm Al beam filter. Projection images were acquired at 0.10° steps and 2.0 µm pixel size. Tomographic images were reconstructed using the Bruker NRecon software, resulting in reconstructed voxel sizes of 2.0 or 3.0 µm (isotropic).

The same samples were also imaged using Micro-CT imaging (YXLON FF35 CT, FXE Transmission Beam, Perkin Elmer Y.Panel 4343 CT) at the NHMW. The scanning parameters were set to 110 kV and 400 µA, while using a 0.2 mm copper filter. A total of 3600 projections images were taken with an exposure time of 2 s each, which volume data sets with isometric voxel sizes of 1.8 µm. Further 3D graphical procedures were performed using Amira version 6.4 software (Thermo Fischer Scientific, Electron Microscopy Solutions, Hillsboro, Oregon, USA) with manual segmentation to separate external and internal shell structures. Final visualization was conducted using the Volume Rendering module.

Scanning electron microscopy (SEM)

The microstructure of three selected gastropod shells was imaged using the FEI Inspect S50 at low-vacuum mode at the laboratories of the IPUW. Images were generated using a backscattered electron detector (BSED). Additional photos were taken using the Large Field-Detector (LFD).

Historical context

The collector, Dominik Bilimek (1813–1884), was a Cistercian monk, naturalist and member of the Austrian Volunteer Corps in Mexico (“Freiwilligenkorps”) (1865–1867) (Roth 1965, 2016, 2017) (Fig. 1A), who cultured multiple interests, focusing mostly on entomology and botany. He pioneered biospeleology (in 1846) along with

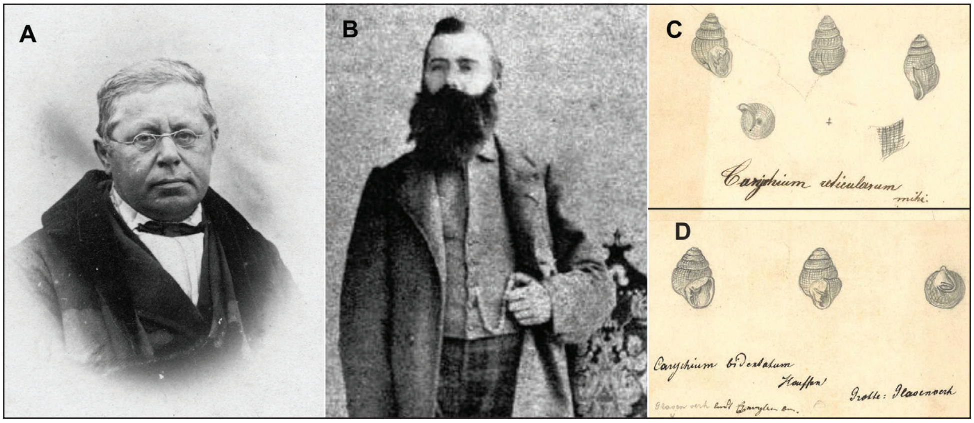

Figure 1. Two collaborating naturalists of the 19th century **A** naturalist and collector Dominik Bilimek (portrait 1864; by courtesy of Fototeca dei Civici Musei di Storia ed Arte Trieste, Italy; inventory no. 4738R) **B** the only preserved portrait of Heinrich Hauffen (Digitalna knjižnica Slovenije, also: Digital Library of Slovenia, reference number 5S74D58Z, Ilustrirani Slovenec) **C** drawing of *Carychium reticulatum* from the H. Hauffen (1856b) manuscript (Archiv NMS, leto 1856, št. 47) **D** drawing of *Carychium bidentatum* from locality Glaven verh; H. Hauffen (1856b) manuscript (Archiv NMS, leto 1856, št. 47).

his contemporaries, including the known 19th century researchers, Adolph Schmidt (1806–1889), Heinrich Freyer (1802–1866) and Heinrich Hauffen. Many of these explorations and contacts are documented in Bilimek's three travel diaries, kept in the archives of the Monastery of Heiligenkreuz (Lower Austria) (Inventory number Rub. 71, fasc. VI, Nr. 10). Bilimek's extensive social networking can additionally be traced throughout his collections, especially on the original collection labels with detailed metadata about collectors and collection dates (Lukeneder et al. 2023). The Bilimek collection also contains material of certain species described by Hauffen - mainly recent snails - including the syntype material of *C. bidentatum* (Hauffen, 1856) and *C. reticulatum* Hauffen, 1856. The corresponding collection labels differ from those of the other labels not only by the handwriting style, but also by the different mode of documentation and the designated boxes containing the associated specimens (see also Lukeneder et al. 2023). Bilimek's collection harbors additional specimens of *C. bidentatum* Hauffen, 1856, now *Zospeum costatum* (Freyer, 1855), and congeners which Hauffen recorded from other Slovenian caves: Douga jama; Kosja jama (better known as: Pri ilavi luzi jark; "Höhle an der Lehmlacke, "Räuberhöhle" in German; see also Wenzel 1992) and Gypsosa jama (near Bischoflack; Hauffen 1858b) (Table 1).

The most remarkable fraction in D. Bilimek's collection of land and freshwater gastropod shells can be assigned to the naturalist, Heinrich Hauffen (1836–1866) (Fig. 1B), an assistant tradesman inspired by the Austro-Hungarian businessman, speleological explorer and naturalist, Ferdinand Joseph Schmidt (1791–1878). Hauffen was especially interested in speleology and zoology and was scientifically tightly affiliated

Table 1. List of specimens including inventory number, cave locality, original Hauffen designated name and number of specimens per locality in the BOKU collection.

Inventory number	locality	taxon	number of specimens
BOKU F3429	Douga jama, Slovenia	*Carychium bidentatum*	5
BOKU F3434	Bidou sturm, Slovenia	*Carychium reticulatum*	12
BOKU F3444	(Grotte hinter) Laak, Slovenia	*Carychium reticulatum*	10
BOKU F3473	Glaven vrh, Slovenia	*Carychium bidentatum*	1
BOKU F3642	Kosja Jama (Kozja Jama), Slovenia	*Carychium bidentatum*	6
BOKU F3643	Gypsosa jama, Slovenia	*Carychium reticulatum*	4

with Heinrich Freyer, zoologist Georg von Frauenfeld (1807–1873) and the naturalist, Dominik Bilimek. Together with Heinrich Freyer, Hauffen pioneered biospeleology, engaging in extensive speleological exploration, investigation, and taxonomic description of cave snails in the region of the former "Krain" (including parts of today's Slovenia and Carinthia, Austria). In an extensive treatise on the genus *Carychium*, Frauenfeld re-investigated zospeid snails (Frauenfeld 1856), including submitted specimens from Heinrich Hauffen from other caves.

Heinrich Hauffen's name occurs in the Bilimek diaries several times. A visit by Bilimek in "Krain" (historical name for the Austro-Hungarian part of Slovenia and Styria (Austria)) (organised by the naturalist Heinrich Freyer) was documented in notes of the first Bilimek travelling diary. He explored the Seeler Grotte (also Željnske jame or Sele'er Grotte) in Unterkrain (today part of Slovenia). This cave is mentioned as the type locality of the coleopterid *Anophthalmus bilimekii* ("Bilimek's Blindlaufkäfer" which Bilimek (Schiner 1854) investigated in 1846 and described in 1847, see also Sturm 1847). On 7 September 1846, the explorers, Bilimek, botanist Stephan L. Endlicher (1804–1849) and another unidentified person visited the nearby cave Križna jama (also Cross Cave, Kreuzberghöhle near Lož/Laas; formerly: Grotte Podlaase) (see also Lukeneder et al. 2023).

From 1865 to 1867, Bilimek and Hauffen both followed Archduke Ferdinand Maximilian Joseph (Archduke Ferdinand Maximilian Joseph Maria from Austria; 1832–1867) and his wife Empress Charlotte (Marie Charlotte Amélie Augustine Victoire Clémentine Léopoldine from Belgium; 1840–1927), as members of the Austrian Volunteer Army Corps to Mexico in May 1865 (Roth 1965). Dominik Bilimek was the 7th museum director and collection custodian at the Natural History Museum in Mexico. Bilimek's and Hauffen's time in Mexico is documented only by few diary entries including a field trip recorded by Hauffen and Bilimek on September 25, 1865, stating, "*on the second mountain behind Quadaloupe*" (Mexico) that they collected eight new plants and two insects. Other entries record November 29, 1865, Bilimek, Hauffen and the Mexican captain, Gonzalez, from the ship Guardia Estable aus Tetela del Oro (see also Roth 1965) collecting insects and cicadas in St. Angel and Petregal (both Mexico) as well as a field trip by Bilimek and Hauffen on December 2, 1865, in Petregal (Mexico) (see also Dominik Bilimek diary no. 2, entry no. 454). A corresponding collection label exists in the Bilimek collection (such as andesitic lava, collected in Mexico, Piedregalle, on 2 December 1865) (Lukeneder et al. 2023).

However, it is not documented, how the Hauffen collection became part of the Bilimek collection. Heinrich Hauffen intended to sell his collection of land and freshwater gastropods, consisting of 10.000 specimens and more than 800 species already in the year 1859 (Frauenfeld 1860). Unfortunately, it is also not documented whether the sale actually took place. Moreover, there are two letters from Dominik Bilimek sent to Emperor Maximilian preserved at the Austrian State Archives (Austrian State Archives signature no. AT-OeStA/HHStA FA MvM 46-2-5), dated 6 April 1866 and 1 May 1866. In one of them, Bilimek requested that the zoologist, Heinrich Hauffen, be dismissed from his position as assistant at the 'future National Museum' in Mexico because he was no longer able to perform his duties due to severe illness (Letter from 1 May 1866. Austrian State Archives signature no. AT-OeStA/HHStA FA MvM 46-2-5). Heinrich Hauffen died in Mexico on 28 June 1866, aged 30. Since they were collaborating, close friends in Mexico, it can be assumed that Bilimek inherited Hauffen's scientific collection after his death.

Data availability

All used data are included within the manuscript and are available upon request from the authors.

Results

The six syntype lots from the Bilimek Collection - catalogued at the BOKU - are listed in chronological order by their collection registration number in Table 1. The specimens are imaged and discussed in more detail below. Information about their synonymy and chresonymy is provided.

Systematic section

Genus *Zospeum* Bourguignat, 1856

***Zospeum spelaeum* (Rossmaessler, 1839)**
Figs 2, 3, 4

Carychium spelaeum Rossmaessler, 1839, Icon., (1) 2 (1/2): 36–37, pl. 49 fig. 661 (shell) [in der Adelsberger Höhle in Krain an Stalaktiten (Postojnska jama, near Postojna)].

Carychium reticulatum Hauffen, 1856, Verh. zool.-bot. Ges. Wien, Abh., 6: 623–624, pl. 7 fig. 4 (shell) [In der Grotte Bidou sturm (Hauffen leg.; Dobruška jama, near Vodice)].

Zospeum spelaeum: twelve junior synonyms are attributed to *Z. spelaeum* (see Inäbnit et al. 2019).

Figure 2. Light microscopic images of Hauffen's specimens of *Carychium reticulatum* var. *bidentatum* Hauffen, 1856 (syn. *Z. spelaeum* Rossmaessler, 1839) BOKU F3434 from the type locality cave, Bidou sturm in Slovenia **A–D** Shell specimen BOKU F3434b in apertural view (**A**), dorsal view (**B**), apical view (**C**), umbilical view (**D**) **E–H** shell specimen BOKU F3434a in apertural view (**E**), dorsal view (**F**), apical view (**G**), umbilical view (**H**) **I–L** Shell specimen BOKU F3434j in apertural view (**I**), dorsal view (**J**), apical view (**K**), umbilical view (**L**). Scale bars: 1 mm.

Material examined. *Carychium reticulatum* Hauffen: imaged syntype BOKU F3434b, other syntypes BOKU F3434a, BOKU F3434j, Cave Bidou sturm [= Dobruška jama], coll. Bilimek ex Hauffen 1856.

Diagnosis (Hauffen original from German). "Distinct vertical and horizontal lines form an entirely complete network pattern unique to this species".

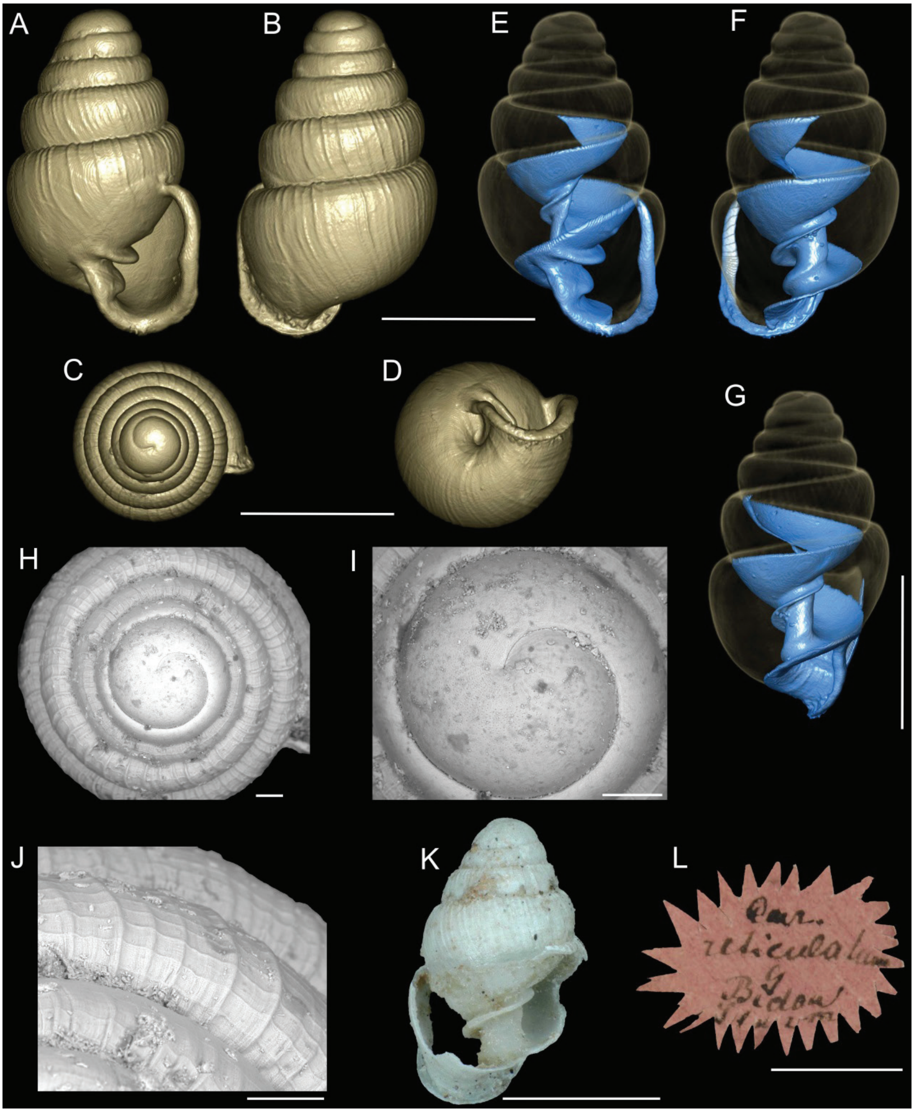

Figure 3. Hauffen's specimens of *Carychium reticulatum* Hauffen, 1856 from the type locality, Bidou sturm in Slovenia. Inventory number BOKU F3434 **A–G** 3D visualizations of Micro-CT data of specimen BOKU F3434b showing the columellar apparatus; apertural view (**A, E**), dorsal view (**B, F**), apical view (**C**), umbilical view (**D**), lateral view (**G**) **H–J** scanning Electron Microscopy images of shell F 3434j (apical view) **I** detail of **H** showing protoconch **J** reticulated sculpture of *Zospeum reticulatum* Hauffen, 1856 **K** broken shell of specimen BOKU F3434d in dorsal view exhibiting collumellar apparatus **L** collection label of BOKU F3434. Scale bars: 1 mm (**A, B, C, D, E, F, G**); 100 μm (**H, I, J**); 1 cm (**L**).

Description (Hauffen original from German). "Shell with 6 whorls, the last is relatively large; suture moderate; shell glassy and transparent; the sculpture is ribbed with the ribs largest by the suture, between these are often smaller ribs which extend to the middle of the whorl, the long ribs begin in most cases at the third whorl and continue over the cross ribs; near the suture they are closer to each other, in the middle of the whorl [zone of greatest convexity] they are however further apart, these crossed over ribs form a network, hence the name; umbilicus somewhat open at the beginning, however closing rapidly, aperture elongate, peristome rim bent backwards, lipped, dented in on the right side; columella somewhat swollen, on which a small denticle is in the middle, which lies deeper inside the aperture, on the inner parietal side are two denticles, the one nearest the columella is the larger and begins closest to the opening, it is next to the smaller one, which lies deeper in the aperture, bending to the right, this smaller interior denticle is visible in some shells and barely visible in others, the parietal shield shows tiny pitting. Height 2.0–2.2 mm. Width 1.5 mm."

Measurements. (BOKU F3434b from Bidou sturm): sh = 2.14 mm; sw = 1.26 mm; ah = 1.01 mm; aw = 0.84 mm; hlw = 1.43 mm; SA = 58.55 deg.

BOKU F3429 from Douga jama: sh = 1.96 mm, sw = 1.18 mm; ah = 0.94 mm; aw = 0.78; hlw = 1.29; SA = 59.90 deg.

Distribution (Hauffen *Z. spelaeum*). Caves Bidou sturm (= Dobruška jama) and Douga jama.

Remarks. Hauffen also recorded this species from a cave behind Laak. Re-investigation of his specimens from that cave herein (Fig. 5) revealed that they are not conspecific with *Z. spelaeum* from Bidou sturm (Figs 2, 3) but rather, with the species *Z. costatum* (Freyer, 1855) (see Inäbnit et al. 2019, fig. 5, C1–3).

Enhanced description of *Z. spelaeum* based on integrative taxonomic analyses of Inäbnit et al. (2019) and Micro-CT and SEM Data in this work.

Diagnosis. Shell ca. 1.96 mm, transparent, elongate, or elongate-conical with an auriform and more or less thickened peristome, bearing two to three apertural barriers; columella with a single lamella.

Description. Shell conical, often more slender than other species, translucent when fresh; whorl never well rounded [as, for example, in *Zospeum frauenfeldii* (Freyer, 1855)], usually ovate-conic in form; shell surface usually ribbed, but variable, which has been used to differentiate subspecies in the past (present on the whole whorl as in "*Z. s. costatum*", present on the upper part of the whorl as in (*Z. s. schmidtii* (fig. 4f–n) or completely absent as in (*Z. s. spelaeum*) (fig. 4a–e) (Inäbnit et al. 2019)), but the spectrum of variation within populations is too broad to establish congruence here; aperture taller than wide, the palatal side usually shouldered; the parietal shield well-differentiated from the lip, its shape either convex or straight; parietalis well-developed in the aperture, extending one whorl into the shell; columellaris visible in the aperture, in some cases disappearing within half a whorl into the shell or continuously weakening; a more or less oblique lamella is present above the middle of the short columella; a basal bulge is present just above the umbilicus; angularis in most cases present as a tooth of variable prominence; palatalis usually present. Protoconch covered with pits; interconnected pits on top of the protoconch densely aggregated into rows; pits

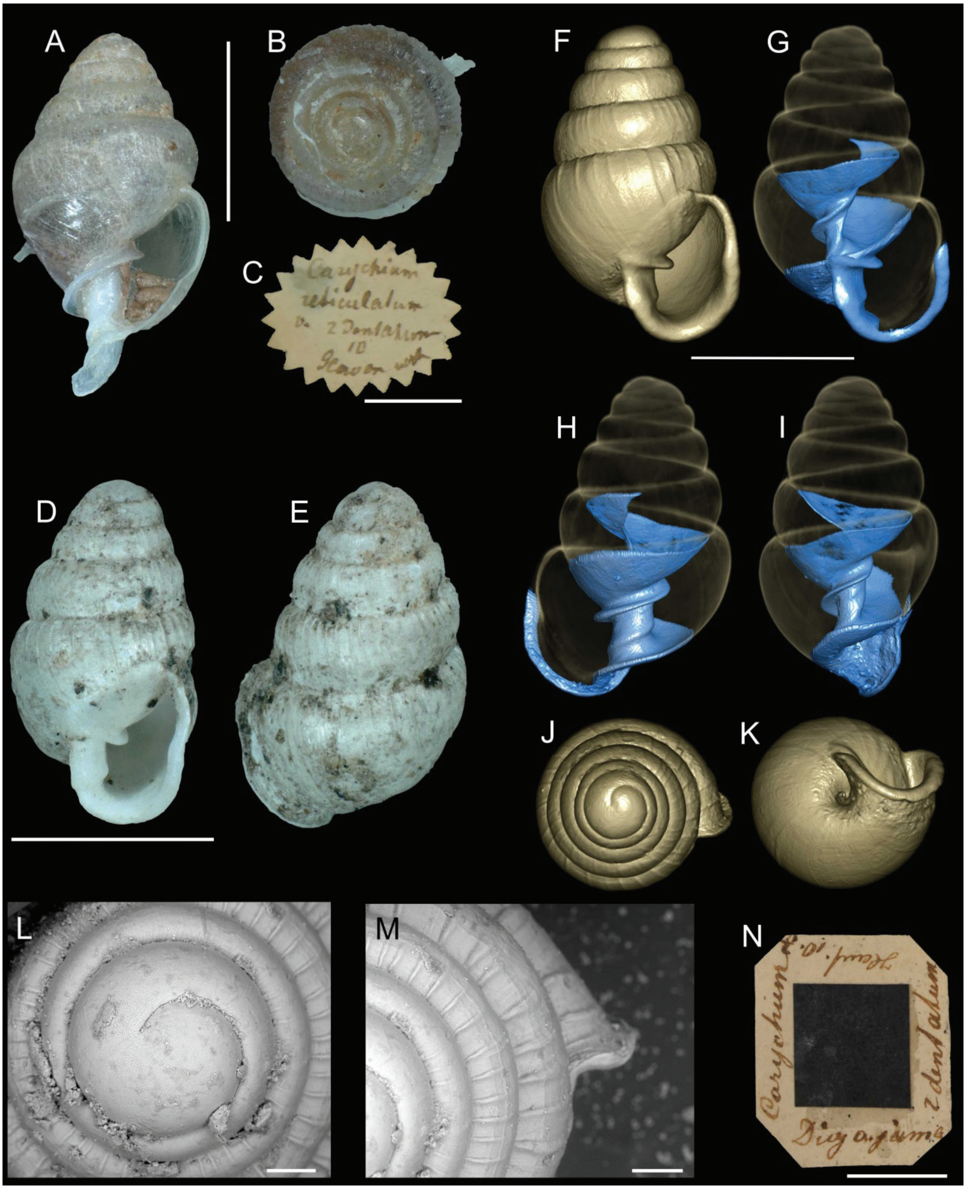

Figure 4. Hauffen's specimens of *Carychium*" *reticulatum* var. *bidentatum* Hauffen, 1856 (syn. *Z. spelaeum* Rossmaessler, 1839) from Glaven verh (Slovenia) and from Douga jama cave (Slovenia) **A, B** light microscopic images of broken shell BOKU F3473a from Glaven verh showing apertural view (**A**), apical view (**B**) **C** collection label of broken shell BOKU F3473a **D–K** specimens of BOKU F3429 from Douga jama (Slovenia) **D, E** light microscopic images of specimen BOKU F3429a showing apertural view (**D**), dorsal view (**E**) **F–K** 3D visualizations of Micro-CT data of specimen BOKU F3429b; apertural view (**F**), apertural view showing columellar apparatus (**G**), dorsal view showing columellar apparatus (**H**), lateral view (**I**), apical view (**J**), umbilical view (**K**) **L, M** scanning Electron Microscopy images of shell of BOKU F3473a from Glaven verh (**L** apical view **M** detail of reticulate microstructure on teleoconch) **N** collection label of BOKU F3429 with notation referring to Douga jama locality. Scale bars: 1 mm (**A, B, D, E, F, G, H, I, J, K**); 100 µm (**L, M**); 1 cm (**C, N**).

on the side of the whorl distributed more randomly. Details of the radula and the *Z. spelaeum* clade within the most recent phylogeny of *Zospeum* are available in Inäbnit et al. (2019).

The *Z. spelaeum* shells herein (BOKU F3434b and BOKU F3429) show a well-formed oblique lamella on a centrally aligned columella, the base of the columella shows a basal, lamella-like bulge in dorsal and aperture facing right views (Figs 3F–G, 4H), forming the more or less pronounced columellaris, which ends deep in the shell and is visible behind the peristome on the columellar side (Figs 3A, E, 4D, F); the robust and well-developed parietalis extends one whorl into the shell, tightly exiting the columellar side of the peristome far up on the parietal shield, ending either before or up to its outermost edge (Fig. 3A, D); umbilical depression deep; columellar side of peristome arches over and oblique to umbilical depression on ventral side (Figs 3D, 4K), alignment of last ¼ whorl not compact. Central part of protoconch partially covered with interconnecting pits and singular pits aggregated in rows (Figs 3I, 4L); onset of ribbing begins at suture of second or third whorl and with increased reticulation of the third whorl some distance from the suture. More or less enhanced reticulation begins at the fourth whorl (Fig. 3H).

Considering the 148 specimens investigated in Inäbnit et al. (2019) and those of this study, *Zospeum spelaeum* differs from *Z. costatum* by a less expanded upper section of the palatal side of the aperture; from *Z. lamellatum* Bole, 1974 by its greatly reduced external ribbing and number and size of lamellae and apertural barriers; from *Z. lautum* (Frauenfeld, 1854) by its usually more slender shell, the presence of ribs, and its bearing a simple inclined lamella versus the more elaborate 3-tiered lamellar configuration in *Z. lautum*; from *Z. trebicianum* Stossich, 1899 by its larger size.

Distribution. *Z. spelaeum* is known from the region of Ljubljana, Slovenia westwards to the Brenta River in Italy, the region of Trieste, Italy, the island of Cres, and eastwards where the Sava River enters the Julian Alps and from the Vrlovka cave in the northeastern part of Croatia (see also De Mattia 2003).

Zospeum costatum **(Freyer, 1855)**
Fig. 5

Carychium costatum Freyer, 1855, Sitzungsber. mathem.- naturw. Cl. kais. Akad. Wiss. Wien, 15 (1): 20, pl. [1] fig. 5a–c (shell) [Von Herrn Ferd. Schmidt in der Grotte bei Goričane unweit des fürstbischöflichen Schlosses Görtschach entdeckt (Babja Luknia, near Goričane)].

Carychium bidentatum Hauffen, 1856, Verh. zool.-bot. Ges. Wien, Abh., 6: 701, pl. 7 fig. 5 (shell) [„in der Grotte am Glaven vrh“ (Hauffen leg., 17 exx.)] (Hauffen 1856b).

Material examined. *Carychium bidentatum* Hauffen: imaged syntypes BOKU F3444a (Grotte hinter Laak), BOKU 3473a (Glaven vrh); other syntypes BOKU F3444b, BOKU F3444d, Cave behind Laak, coll. Bilimek ex Hauffen 1856.

Diagnosis (Freyer, original from German). Vertically ribbed, bidentate carychid.

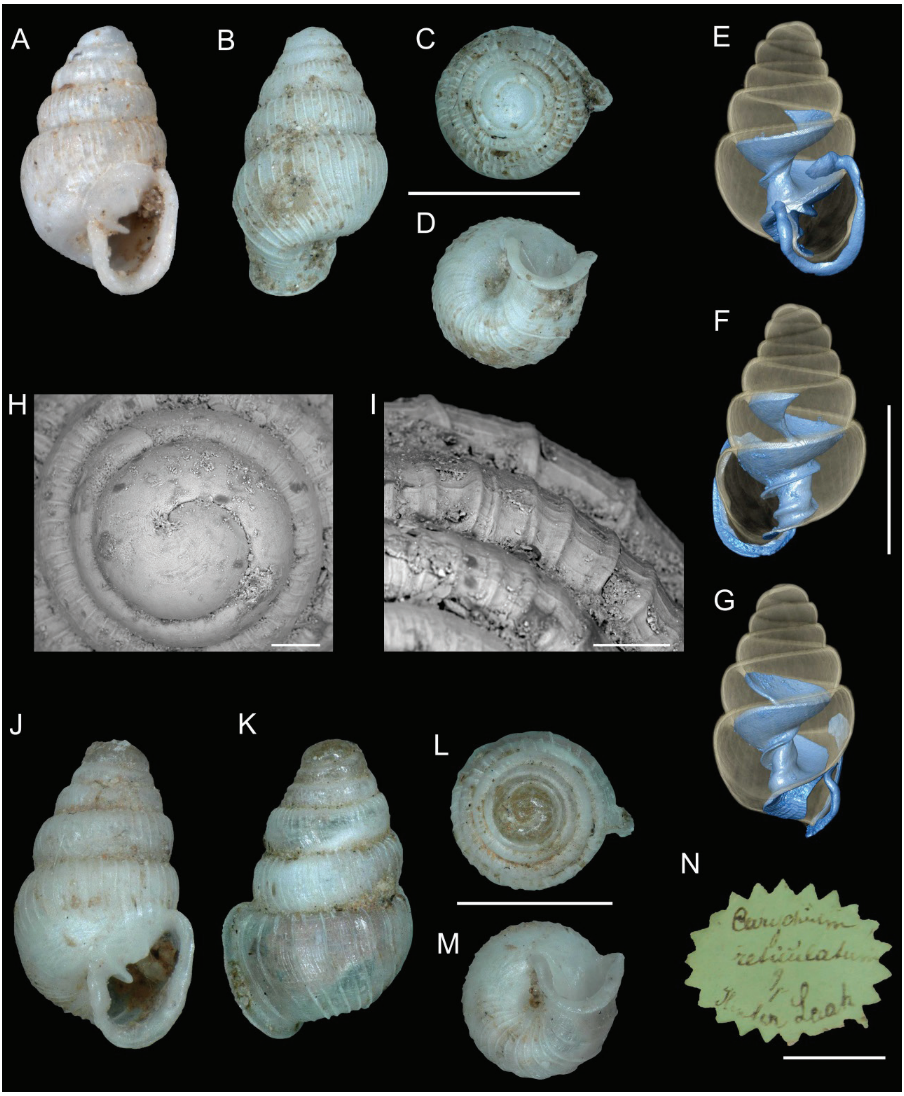

Figure 5. Hauffen's specimens of *Zospeum costatum* (Freyer, 1855) from Laak in Slovenia. Inventory number BOKU F3444 **A–D** shell specimen F3444b in apertural view (**A**), dorsal view (**B**), apical view (**C**), umbilical view (**D**) **E–G** 3D visualizations of Micro-CT data of shell specimen BOKU F3444a showing the columellar apparatus. Apertural view (**E**), dorsal view (**F**), lateral view (**G**) **H, I** scanning Electron Microscopy images of shell specimen BOKU F3444b (apical view) **J–M** shell specimen F3444d in apertural view (**J**), dorsal view (**K**), apical view (**L**), umbilical view (**M**) **N** collection label of BOKU F3444 with characteristic handwriting of Heinrich Hauffen. Scale bars: 1 mm (**A, B, C, D, E, F, G, J, K, L, M**); 100 µm (**H, I**); 1 cm (**N**).

Description (Freyer, original from German). Shell white, spired, overlayed with strongly pronounced vertical ribs. Those of the first four graded whorls are weaker, those of the sixth whorl do not reach the ventral side, hence the ventral side [umbilical zone] (fig. c) is smooth before the aperture; about 1/3 the distance beyond the peristome, five ribs extend from the umbilicus nearly fusing together with some obliquely oncoming ribs from the upper part of the whorl as shown in Fig. 5C. The peristome is flatly bent back, the palatal lip is dented, the left columellar side sinks straight down, flattening at the rounded basal part of the aperture. Aperture shaped like a gourd. The ridge-like denticle nearest the columella [columellaris] reaches to the leftmost side of the parietal shield; the second, shorter ridge-like denticle [parietalis] extends from the parietal side and lies directly across from the shouldered edge of the right apertural lip. Height 2 1/8 mm, Width 1 1/3 mm.

Measurements. (BOKU F3444 from "Grotte hinter Laak", Slovenia): sh = 2.14 mm; sw = 1.09 mm; ah = 0.86 mm; aw = 0.67 mm; hlw = 1.17 mm; SA = 59.08 deg.

Distribution. (Hauffen *Z. costatum*) Cave behind Laak, Slovenia.

Enhanced description of *Z. costatum* based on integrative taxonomic analyses of Inäbnit et al. (2019) and Micro-CT and SEM Data in this work.

Diagnosis. Shell ca. 2 mm, transparent, ribbed, with weak spiral lines, elongate-conical with an auriform and more or less thickened peristome, bearing four apertural barriers, two of which continue on the columella as lamellae.

Description. Shell conical, translucent when fresh; whorls somewhat shouldered, usually stronger than in *Z. spelaeum*; pronounced, equidistantly-spaced axial ribs present on each whorl, though not always covering an entire whorl; weak spiral striae are usually visible; aperture taller than wide, the upper half of the palatal rim shouldered and expanded; the parietal shield well differentiated from lip, its margin more curved than straight; parietalis well developed in the aperture, extending one whorl into the shell; columellaris visible in aperture, extending 3⁄4 of a whorl into the shell; palatalis always present; angularis sometimes present. Internally, the base of the columella continually widens from below the point of attachment of the short, oblique lamella, forming a robust base above the umbilicus (Fig. 5E). The low columellaris increases structural form as it swells up towards the base of the columella (aperture facing right) (Fig. 5G), reaching its fullest size at half-way its distance on the columella (dorsal perspective) (Fig. 5F). Visible also in NHMW-MOL-71847 from Babja luknja, Goričane (fig. 5c1–3) (Inäbnit et al. 2019).

The *Z. costatum* shells (BOKU F3444a and BOKU 3473a) show the same lamella to columellar configuration as that seen in NHMW-MOL-71847 (Inäbnit et al. 2019) as well as the mode and degree of smoothness versus interconnected ribbing described by Freyer (1855) on the body whorl and the ventral side (Fig. 5J–K, M).

Considering the 10 specimens investigated in Inäbnit et al. (2019) and the two individuals in this study, *Z. costatum* differs from *Z. spelaeum* by the generally more expanded shoulder of the upper palatal rim (not visible in the broken shell of BOKU 3473a from Glaven vrh) and the swollen, wider basal configuration of the columella; differs from *Z. lamellatum* by its smaller, single lamella and reduced dentition; differs from *Z. lautum* by the marked presence of ribs and presence of a single lamella; differs from *Z. trebicianum* by its enhanced degree of ribbing and larger size.

Discussion

Conchological differences of considered morphological characters including fine or course ribbing, development or absence of spiral lines or the presence of a second parietalis, have long confounded taxonomists of the radially ribbed, zospeid morphs found in Dinaride caves and which were later lumped together under *Zospeum spelaeum schmidtii* (Frauenfeld, 1854) (Bole 1974). Later studies, based on comparative anatomical investigations (De Mattia 2005) and DNA sequence data (Inäbnit et al. 2019), rejected the subspecific dichotomy for *Zospeum spelaeum* (Rossmaessler 1839) in which the two species, *Carychium bidentatum* Hauffen 1856 and *Carychium reticulatum* var. *bidentatum* Hauffen 1856 became shuffled. All the while, type material of the two species could not be studied because it was considered lost until now. Though topotypic specimens of both species (Kuščer coll. Nrs. 437 and 10137 respectively) are known in the Ljudevit Kuščer collection in Trieste (MCSNTS) (De Mattia 2005), no other known *Zospeum* syntypes described by Heinrich Hauffen are preserved at the PMS, nor at the NHMW nor in the Freyer Collection housed at the MCSNTS (Inäbnit et al. 2019).

We remark that geographical names of towns and caves have changed through time via the historical reshuffling of geographical borders and languages such that there is no congruency, with today's names and those before WWII. Complicating the situation further, Hauffen tended to interchange annotation of locality names. For example, the type locality of *Z. reticulatum*, Bidou sturm, was recorded by Hauffen in 1856. In 1858, he refers to this locality as Bidov šturm at the Dobrus Mountain. Later, in 1932, the same cave locality was recorded by Kuščer as Bidov šturm (De Mattia, 2005). According to the Cave registry of the Speleological Association of Slovenia (Kataster JZS), the original name is considered Dobruška jama (sin. Jama nad Dobrušo) 46,175061, 14,476235; altitude 399 m a.s.l. (pers. com. Rajko Slapnik, June 2023). On the other hand, the type locality of *Z. bidentatum* was considered by Hauffen to be from a cave in a gorge at the Glavni verh near Habach castle (also: Schloss Habbach; Grad Jablje; Slovenia) in one publication (Hauffen 1858b). This locality is also recorded by Hauffen as Glavni verh in his earlier documentation (Hauffen, 1858a) and spelled differently (also: Glaven vrh, Glaven verh, Glavni vrh; meaning "main peak"; "Hauptgipfel" in German) on his labels accompanying the Bilimek Collection material here. According to the Cave registry of the Speleological Society of Slovenia (pers. com. Rajko Slapnik, Sept. 2023) and Bole (1974), the cave, Jama pod Sp. Dobenim is synonymous with Glaven verh, where the speleologists Kuščer, Bole, and Velkovrh are recorded to have collected *Zospeum* shells (Bole 1974). Kuščer (1932) mentions a small cave, with limited access, just below the village Dobeno near Mengs. Jo*ž*e (1977) identifies Glaven verh as kind of a flooded cave below Ručigaj (in Spodnji Dobene near Mengš) (Fig. 6).

Hauffen's manuscript (Hauffen 1858b; Archiv NMS, leto 1856, št. 47) provides some further information about the characteristics of the small-scaled Glaven verh cave (Fig. 6), which should be located within a small canyon. The cave appears to be difficult to enter for ancient cavers (personal comm. Matija Křižnar, 2024). The walls devoid of stalactites and the floor is covered with sand. A small stream should flow through the middle of the cave. Ručigaj corresponds to Ručigajev izvir, while “izvir” can be translated as “spring”. LiDAR (Light Detection and Ranging) photos confirm its position within a canyon.

Hauffen (1856a) loosely includes mention of the “Grottenschnecken” (= “cave snails”), *Carychium lautum* in “Glaven verh” and *Z. alpestre* (Freyer, 1855) in "Douga jama", which he also noted for harbouring *C. reticulatum* var. *bidentatum* in the same

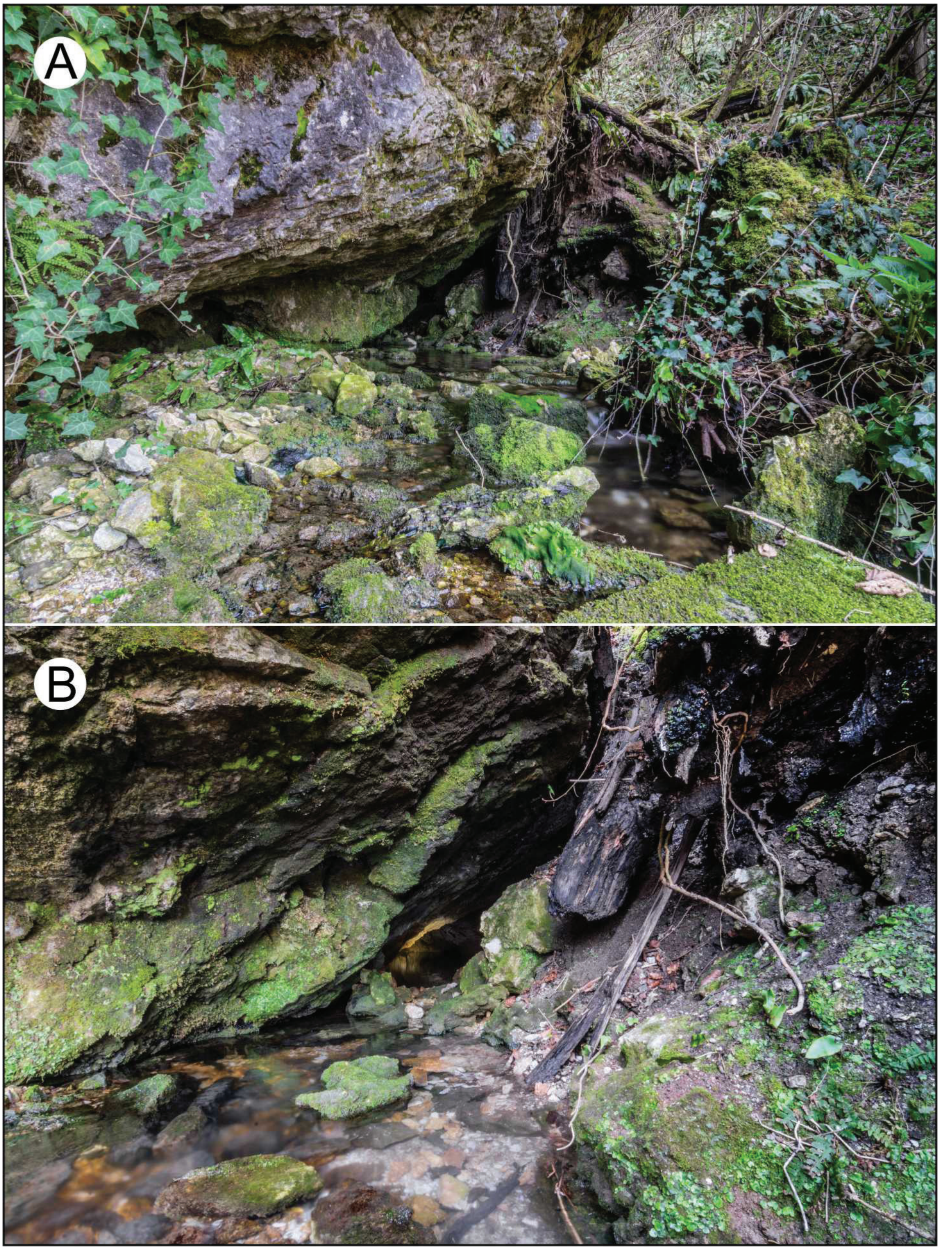

Figure 6. The narrow entrance of Glaven verh identified as Ručigaj = Ručigajev izvir, is located in a small canyon as described by Hauffen (1858b). Photos: Matija Križnar, 2024.

publication. Nevertheless, it is not clear if he actually sent this specific material, as stated therein to Frauenfeld, nor, if he later designated this material differently in his or another collection. We assume his notation referring to Glaven vrh all means the same cave by the Habach Castle (also known by 4 variable names, including Jablje Castle in Slovenian). Notable here, is that Inäbnit et al (2019) documents 3 shells (fig. 5d, NMBE 553383) from the nearby cave Jama 2 pri Jabljah (GPS: 46°08'30.9"N, 14°33'12.6"E) near Loka pri Mengšu (Laak bei Mannsburg). According to the Cave Registry of the Speleological Association of Slovenia, there is a very high density of caves within a very small radius in the region of Loka pri Mengšu of which Glaven verh (Ručigaj = Ručigajev izvir) is one.

By comparing our data to that of congeners in recent works (Inäbnit et al. 2019; Jochum et al. 2024), we reveal that the columellar apparatus of Hauffen's broken syntype of *C. reticulatum* from Glaven verh and the syntypes from "Grotte hinter Laak" (Hauffen 1856b) match well with that of *Zospeum costatum* (Freyer, 1855) in Inäbnit et al. (2019) (fig. 5c1–3). On the other hand, the columellar apparatus, aperture shape, whorl and spire formation and the umbilical configuration of Hauffen's *C. reticulatum* shells from Douga jama unequivocally match those within the spectrum of the species, *Z. spelaeum* (Rossmaessler 1839) in Inäbnit et al. (2019) (fig. 4b1–6). We emphasize that contemporary tools such as 3D X-ray Micro-CT and SEM imaging profoundly aid in the re-interpretation of former species designations while underscoring the significance of past evaluations in taxonomic investigations today.

The taxonomic examinations in this study therefore show that Hauffen's syntypes correspond morphologically to those of known species namely, *Zospeum spelaeum* (BOKU F3434b and BOKU F3429) and *Zospeum costatum* (BOKU F3444 and 3473a). By comparing key zospeid diagnostic characters beyond size, surface structure and dentition, additional ones, such as internal coiling configuration of the columellar apparatus, lamella formation in relation to the columella, the degree and size of the umbilical depression in conjunction with the formation of the final coiling in the umbilical zone (ventral perspective) (Jochum et al. 2024), this work underscores the considerations of previous taxonomists, Frauenfeld (1856), Freyer (1855) and Bole (1974) (i.e. *Z. spelaeum schmidtii*) in its current taxonomic placement within the *Z. spelaeum* clade in Inäbnit et al. (2019).

Conclusions

This work documents *Zospeum* syntypes considered lost to science from the original collection of Heinrich Hauffen and which later landed in the Dominik Bilimek collection at the BOKU in Vienna, Austria. The absence of reliable type material precluded their fullest consideration in the documentation and interpretation of *Z. costatum* (Freyer, 1855) and *Z. spelaeum* (Rossmaessler, 1839) in the most recent integrative taxonomic examinations of the genus *Zospeum* (Inäbnit et al. 2019). Our contemporary morphological analysis of *C. reticulatum* using light microscopy, SEM and X-ray Micro-CT data in conjunction with Hauffen's original documents and figures, illustrates the manifestation and degree of phenotypic variation on the shells (BOKU F3434b and BOKU F3429) of two populations of *Zospeum spelaeum*. Though we know little about the ecology of the respective caves, cave systems in the region of Loka pri Mengšu are densely represented, with many being contiguous and thus, sharing subterranean drainage systems. *Zospeum* snails get washed through these systems during seasonal water flows and displacement of fauna within a concentrated system of caves is not unusual (Culver and Pipan 2009). Considering that *Z. s. schmidtii* was reported from many caves in the region and in several cases sympatrically with *Z. costatum* (Bole, 1974), the potentiality of mixing of populations is high. Reticulated microstructure of the teleoconch is also known in other species such as *Z. frauenfeldii* (Freyer, 1855) and *Z. costatum* (Freyer, 1855). We confirm that although the reticulated microstructure of Hauffen's specimens of *C. reticulatum* is more pronounced than in most other forms of *Z. spelaeum* of which it is considered a junior synonym, the shells (BOKU F3434b and BOKU F3429), and those of other former congeners such as the more or less reticulated variant, *Z. s. schmidtii* (see Bole 1974; Inäbnit et al. 2019), are otherwise, not differentiated enough to assign species status based on this character alone. We confirm that *C. reticulatum* Hauffen 1856 lies within the spectrum of character states taxonomically recognized for the species *Z. spelaeum* (Inäbnit et al. 2019). However, we remark that although this study considers the reticulated shells from the caves Bidou šturm and Douga jama to represent a phenotypic variation within the morphological spectrum known for *Z. spelaeum*, DNA sequence data from new material collected in these caves (and those of geographically proximal caves), would ultimately further our understanding of the *Z. spelaeum* clade. Since we do not rule out the potentiality that Hauffen‘s *C. reticulatum* populations might represent genetically separated evolutionary lineages of *Z. spelaeum* (i.e. having distinct DNA barcodes and nuclear DNA) (Weigand et al. 2014), this work especially opens new perspectives for future exploration and molecular investigations. Hence, we strongly encourage further speleological exploration and specimen collection in Bidou šturm (= Dobruška jama) as well as in Hauffen's listed cave localities (Table 1). Lastly, we emphasize that this study demonstrates the long-term scientific value and significance of the preservation and maintenance of historical, natural science collections.

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Collecting empires: Dominik Bilimek and early subterranean zoology between politics and field research

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Abstract

The Moravian Cistercian monk and collector Dominik Bilimek (1813–1884) is considered one of the earliest European naturalists to conduct zoological research on subterranean fauna in Latin America. During the second French invasion of Mexico, from 1861 to 1867, he accompanied Maximilian of Habsburg, the newly enthroned emperor of Mexico, to the region. There, he explored the Grutas de Cacahuamilpa near Taxco de Alarcón, comparing its fauna to his earlier discoveries in Postojnska jama in Carniola (Slovenia). After the victory of the Mexican republican forces and Maximilian's execution, Bilimek retained his role as curator of the emperor's collections, which he took back with him to Europe and exhibited at Miramare Castle near Trieste. Prior to his Mexican venture, Bilimek had embraced the imperial goals and intellectual agendas of the Viennese central administration while teaching at the monarchy's military institutes. Following the revolutions of 1848/49, extensive research undertakings—including geoscientific, biological, and archaeological surveys—were initiated to preserve the Habsburg monarchy as a supranational entity amidst internal and external crises, and to legitimize its territorial framework as both a natural and cultural unit. These developments coincided with the initial surge of zoological interest in Carniolan caves, which spurred efforts to gather similar findings in other karst regions of the monarchy. Drawing on historical sources, this article explores the intersection of political concepts and fieldwork practices in the early study of cave biology. Special attention is given to the sites, networks, and modes of collecting during this transformative period.

Keywords

Bilimek, cave fauna, collecting, entomology, Habsburg monarchy, history of zoology, Mexico

Introduction

> My companion, endowed with an entomological instinct, meticulously explored every crevice [of Koblarska jama in Slovenia] where insects might hide. His perseverance was richly rewarded when he discovered, beneath scattered rocks, the eyeless cave beetle *Anophthalmus schmidti*. It was first identified by the eminent Carniolan entomologist, Ferdinand Schmidt, initially in Predjama and later in another cave on Mount Krim in Carniola, always in very limited numbers. Thus, [my companion] found the third known location of this beetle and, to his indescribable delight, captured 14 specimens. Two of them are now preserved in the Imperial Collections in Vienna (Skofitz 1847: 218, translated from German by me).

The lucky finder was none other than the Cistercian monk Dominik Bilimek, a notable naturalist and avid collector who made significant contributions to cave entomology in the 1850s and 1860s. Beginning with the earliest discovery of a cave-dwelling insect in Postojna jama in 1832, the search for these specimens over the next two decades would focus mainly on the karst area between Ljubljana and Trieste. These efforts were driven primarily not only by learned communities within the Habsburg monarchy but also by individual foreign scholars. Through comparative observations, these scientists and collectors formulated initial hypotheses about the interdependence of cave specimens and their unique ecosystems.

This article explores the political and social landscapes that fostered the emergence of subterranean zoology within and beyond the Habsburg monarchy, examining how these frameworks shaped research agendas and practices. Approaching the topic from a history of science perspective, I draw on the valuable works of Bellés (1992), Juberthie (2005), Negrea (2007), and Romero (2009), who explored the history of cave biology through the lens of specialty formation and scientific ideas, as well as specific regions like France and the Balkan Peninsula. Considerable attention has been paid previously to the Romanian zoologist and explorer Emil Racoviță (1868–1947), whose influential manifesto "Essai sur les problèmes biospéologiques" (Essay on biospeological problems, 1907) synthesized extant knowledge on the origin and evolution of subterranean fauna and coined the term "biospéologie" (now biospeleology) (Vandel 1964; Tabacaru and Danielopol 2020; Culver and Pipan 2023). Notably, Christian (2003) and Polak (2005) provided comprehensive accounts of nascent cave entomology in both Austria and Slovenia, thereby situating these endeavors within the burgeoning international context.

Bilimek, who has recently gained attention in museological and postcolonial scholarship (Bueno Bravo 2013; Lukeneder et al. 2023) because of his controversial activities as a collector of pre-Columbian artifacts during his stay in Mexico, serves as a pertinent case study for two reasons. First, Bilimek's approach mirrors the established traits of many of his mid-nineteenth-century European naturalist contemporaries studying (cave) fauna: transdisciplinary curiosity, meticulous fieldwork rooted in a penchant for collecting and the notion of orderliness, and extensive networks for exchanging specimens (Kohler 2006). Second, Bilimek's exceptional global scale of operations, facilitated by imperial power relations, and his integration into Vienna's local scientific communities provide avenues for exploring both the methodological and sociopolitical dimensions of early studies in cave biology.

This article argues for a deeper consideration of geopolitical agendas and scholarly exchange in the development of knowledge about subterranean organisms and its emergence as a distinct field of study in the nineteenth century, later defined and systematized by Racoviță and his French collaborator René Jeannel (1879–1965). My essay seeks to shift the understanding of these early steps by viewing them not only as the achievement of a few "professional" scientists but also as a collaborative effort of diverse local, regional, and (trans)national practitioners motivated both by the prospect of public benefit and the hope for personal gain. Based on a (re-)evaluation of published and archival historical sources on cave fauna in the Habsburg monarchy, I will examine the changing conditions, goals, and profit expectations that motivated various stakeholders to engage in cave entomology; the resources they tapped; and the (collaborative) practices they developed. A key focus will be on Bilimek's role, the methods he employed, and the impact of his international ventures. I will assess how these factors shaped the emergence of subterranean zoology, considering the influence of the increasing specialization and imperial-colonial dynamics extant at that juncture. To this end, I will first introduce Bilimek as a scientific collector, highlighting his lesser-known interest in cave fauna. I will then analyze how the study of cave specimens developed under the Habsburg monarchy and conclude with Bilimek's exploration of Mexican caves and his comparative approach.

Dominik Bilimek: Collector and early cave biologist

The second son of a German-speaking butcher in the small Moravian town of Nový Jičín (Neu Titschein, today in the Czech Republic), Bilimek entered the Cistercian monastery of Neukloster in Wiener Neustadt after completing his schooling (Fig. 1). While serving in the monastery's scientific cabinet, he cultivated a deep interest in natural history and antiquarianism. He was ordained a priest in 1837 (Roth 2004). In the course of his subsequent pastoral duties, Bilimek undertook numerous excursions into the nearby mountains of Lower Austria, particularly the Schneeberg and the Raxalpe, which he is reported to have climbed more than a hundred times (Moser 2008). His passion gradually extended beyond the local flora to encompass the systematic study of phanerogams and insects. From 1844 onward, Bilimek's explorations took him to the Adriatic coast and Hungary, where he collected the fauna and flora of Lake Balaton and the karst of the Bakony Mountains.

In the later stages of his career, Bilimek was employed as a professor of natural history and theology at the Convent School in Wiener Neustadt and at various military educational institutions, including the Cadet Institutes in Hainburg, Łobzów (Krakow), and Eisenstadt and the Military Academy in Wiener Neustadt (Roth 1965). While in post, he curated extensive teaching collections there and maintained close

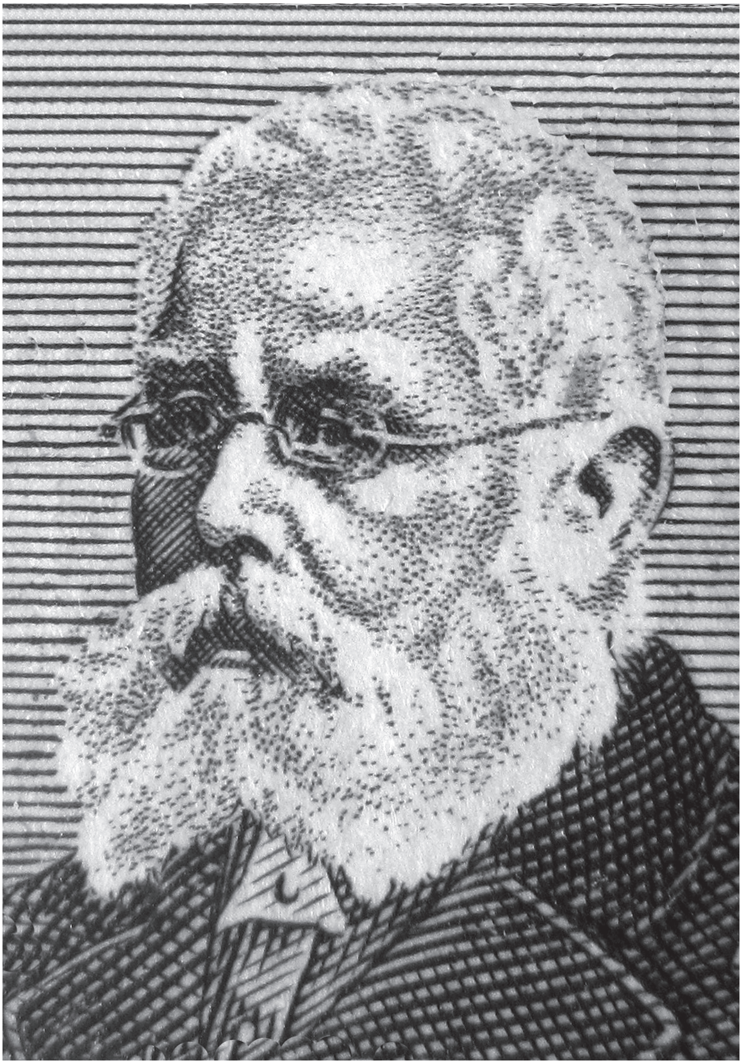

Figure 1. Lithograph of the elderly Dominik Bilimek (1813–1884), curator of the Imperial Mexican Collection at Miramare Castle near Trieste. Source: Monastery Archive Heiligenkreuz (Lower Austria).

contact with Vienna's emergent learned societies and prominent scientists, such as the geologists Dionýs Štúr (1827–1893) and Eduard Suess (1831–1914). Bilimek also expanded his interests to study cryptogam flora, paleontology, and classical antiquity, engaging in excavations and collecting artifacts in the former Roman town of Carnuntum in Lower Austria (Mader 2002).

In 1865, Bilimek entered the service of the Habsburg Archduke Ferdinand Maximilian (1832–1867), who had taken the Mexican crown as Emperor Maximilian I amid the second French invasion of Mexico. On his arrival in the Americas, Bilimek was appointed curator of the Imperial Mexican Museum at Chapultepec Castle in

Mexico City (Riedl-Dorn 2001). Modeled on the Vienna Imperial Collections, this museum and other learned institutions founded by Maximilian, such as the Mexican Academy of Sciences and Literature, emulated their Austrian equivalents (Azuela et al. 2009). During his two-year tenure in Chapultepec, Bilimek traveled to the nearby Querétaro region and the central highlands, buying, collecting, or simply taking numerous natural history specimens and unique ethnographic objects. Bilimek also took some pre-Columbian artifacts from culturally significant caves, for example, those near the town of Orizaba in Veracruz (Bilimek 1878). Following the execution of Emperor Maximilian in 1867, Bilimek managed to save himself and arranged the transport of the extensive Mexican collections to Europe. Today, in light of postcolonial reconciliation measures, his actions are viewed under a critical light (Bueno Bravo 2013; Schönberger 2021). Bilimek continued his stewardship as curator of the imperial collections exhibited at Miramare Castle near Trieste. In subsequent years, he traveled extensively to North Africa and Palestine to enhance the collections further. Once the Mexican specimens and antiques were finally transferred to Viennese museums, particularly to the anthropological–ethnographic collections of the Natural History Museum (1877–78), Bilimek successfully negotiated a pension for himself. This arrangement allowed him to settle in the capital with his housekeeper, thereby avoiding a return to the monastery of Heiligenkreuz (Roth 1965; Lukeneder et al. 2023).

A lesser-known aspect of Bilimek's multifaceted biography, shaped by the imperial politics of the time, was his profound interest in caves and karst. Raised during a period when research in subterranean zoology was just gaining momentum with the first descriptions of cave fauna, Bilimek and his fellow collectors placed special emphasis on underground habitats (Christian 2003). This trend was especially pronounced in the Habsburg crown lands, notably in Carniola, where in the first half of the nineteenth century, a lively commerce in the aquatic salamander *Proteus anguinus* had originated (Mattes 2018; Aljančič 2019) and cave-dwelling insects were first discovered. These included the cave beetle *Leptodirus hochenwartii* Schmidt, 1832, the cave cricket *Troglophilus cavicola* Kollar, 1833, and the blind cave ground beetle *Anophthalmus schmidtii* Sturm, 1844. As a result, starting from the 1840s, caves around Postojna became frequent destinations for naturalists, not only for study but also because cave insects had significant exchange value, leading to a robust trade among collectors (Polak 2005; Zagmajster et al. 2021).

At the time of his trip to Lower Austria and his journey to the Bakony Mountains in Hungary, Bilimek probably explored caves, although published records of these expeditions are scarce. There are references to his 1846 trip to Carniola and the Austrian Littoral, jointly undertaken with the botanist and pharmacist Alexander Skofitz (1847), founder of the Vienna Botanical Exchange Society (*Botanischer Tauschverein*) and the Austrian Botanical Weekly (*Österreichisches Botanisches Wochenblatt*). It was during this expedition that Bilimek focused on collecting cave insects, including the first specimen of the cave beetle *Typhlotrechus bilimeki* Sturm, 1847, which he found in Željnske jame. Additional collecting trips took Bilimek to the Drachenhöhle near Mixnitz (Styria) and into the Moravian Karst (Lukeneder et al. 2023). Despite his contributions, Bilimek did not enjoy widespread recognition as a scientist because of his lack of formal academic training and limited publication record, as well as the transdisciplinary approach he adopted. While he was probably one of the first Europeans to investigate cave-dwelling organisms in Latin America, Bilimek (1867) published only a single, concise article during his lifetime, describing his study of the Grutas de Cacahuamilpa in Mexico. From the 1860s onward, the trend toward specialization and discipline-specific research set boundaries that gradually pushed naturalists, collectors, and patrons to the margins of scientific knowledge production.

To understand the collecting practices of Bilimek and his fellow naturalists, they must be located within a larger societal context. Collecting was not just a passion; it was a crucial method of appropriating nature and, arguably, the most important way to study the natural world at that time (Heesen and Spary 2001; Kohler 2002). The compilation, completion, and systematic ordering of specimens not only made knowledge about the world more accessible but also transformed natural history cabinets into microcosms of the world itself and the unity of knowledge about it (Findlen 1996). In the Habsburg monarchy, natural research was closely associated with aristocratic demands for prestige and representation. Many naturalists, like Bilimek, pursued their scientific interests alongside civil service duties, thereby endowing collecting with significant political implications (Raffler 2007; Rampley et al. 2021). By the latter half of the nineteenth century, the exploitation of the empire's natural resources, the centralization of specimens (both domestic and overseas) in collections in capital cities, and their public display became integral to imperial discourse (Gahtan and Troelenberg 2006; Habermas and Przyrembel 2013). This aspect will be explored further in the following section.

Imperial politics, troglobites, and the k. k. Zoological-Botanical Society of Vienna

The multinational Habsburg monarchy, Europe's second largest state after Russia at the time, was a complex mosaic of diverse territories and cultures stretching from the Alps to the Balkans and the Adriatic to the Carpathians. In contemporary travel reports, learned journals, and descriptions of the empire's natural wonders published in the early nineteenth century, mountains, caves, and other underground sites were often highlighted (Sartori 1807–1809; Pichler 1809; Salm-Reifferscheidt 1814; Costa 1820). The newly established provincial museums (*Landesmuseen*) in the crown lands, beginning with the Joanneum in Graz in 1811, specially focused on these previously unexplored sites and their natural wealth when building their collections (Južnič 2006; Pakesch and Muchitsch 2011). Caves, in particular, came to symbolize regional identity in the Habsburg territories, most notably in Carniola, Hungary, and Moravia. As a result, the earliest descriptions of karst areas and their caves often featured detailed descriptions of the history and culture of the respective crown land (Hohenwart 1830–1832; Vass 1831).

Habsburg policymakers viewed state unity and regional identity not as contradictory but rather as complementary. They aimed to foster "unity in diversity," a slogan marking the deliberate recognition of the empire's natural and cultural diversity (Ash and Surman 2012; Feichtinger and Uhl 2016; Coen 2018). This approach included the state authorities' expressed interest in the exploration, development, and use of caves and other underground sites for touristic, economic and illustrative purposes. When members of the imperial family or the high aristocracy paid an official visit to a crown land, caves often featured prominently on their itinerary. For instance, Emperor Franz (1768–1835) visited the Moravian Karst in 1804 and the Carniolan Karst in 1818, and Crown Prince Ferdinand (1793–1875) toured the Aggtelek Karst (Hungary) in 1811. Infrastructure improvements, such as new paths and lighting, were often made to facilitate these visits and, occasionally, led to the discovery of previously unknown passages (Shaw 2008). As a result, several caves or sections of them explored in the nineteenth century were named after members of the imperial family. In the case of Postojnska jama, certain sections were dedicated to significant landmarks along the road between Vienna and Carniola (Shaw 2006).

In 1824, Postojnska jama came to be officially supervised with the establishment of the k. k. (imperial–royal) Cave Management (*Grottenverwaltung*) and the introduction of regular guided tours (Vekar 1989). This marked an important point in the management of the monarchy's natural heritage, especially its underground treasures, and it had a profound effect on public perceptions of Postojnska jama. Its fame spread far and wide through its inclusion in guidebooks, on postcards, on school wall maps, and the inclusion of specimens in natural history collections, contributing to its reputation as the "foremost among its sister [caves] in the empire" (Schaffenrath 1834; Schmidl 1854: 39; Radics 1861). Over the following years, Postojnska jama became the archetype of an "imperial" grotto par excellence (Fig. 2). Because of its status, other well-known caves throughout the monarchy, such as Baradla-barlang in Aggtelek (Hungary), Jama Vilenica in Lokev (Austrian Littoral), or Sloupsko-šošůvské jeskyně near Blansko (Moravia), were often compared to Postojnska jama (Mattes 2015; Shaw and Čuk 2015). Adolf Schmidl (1802–1863), an official of the Imperial Academy of Sciences in Vienna, toured the principal karst regions of the Habsburg monarchy, documenting their unique characteristics and similarities in scientific publications, guidebooks, and newspaper articles. Schmidl's (1863: 270) comprehensive studies led him to argue that all "Austrian caves," around 200 were known at the time, should be officially "recognized as the property of the crown lands" and administered by the salient provincial assembly (*Landtag*). He passionately campaigned for the preservation, maintenance and equal recognition of these caves, similar to Postojnska jama.

After the revolutions of 1848/49, the Habsburg central administration promoted the formation of scientific societies in Vienna, particularly in fields, such as biology and geography, not addressed by the few state-run research facilities. These private societies, comprising up to 2,000 members from across the empire and abroad, including aristocrats, scholars, bureaucrats, teachers, and military officers, saw themselves as extensions of existing state institutions (Mattes 2024). They played crucial roles as symbols of the

Figure 2. View of “Calvary Mountain” in Postojnska jama, 1864. Artist: Franz von Kurz zum Thurn und Goldenstein. Source: National Museum of Slovenia.

monarchy’s unity and sought to thereby mitigate national tensions. To this end, the societies undertook large-scale cooperative projects that defined the state territory as a natural and cultural unit. One notable institution was the k. k. Zoological-Botanical Society, founded in 1851 to catalog the fauna and flora of the monarchy (Anonymous 1853; Punz 2001). As part of this initiative, the Society enriched the collections of the empire’s schools by providing them with duplicates of specimens submitted free of charge by its members (Frauenfeld 1867; Lack 2006). In return, the Society received financial compensation from the Ministry of Education, highlighting the imperial endorsement of scientific collecting. These learned societies became vital embodiments of statehood and territoriality, amassing natural specimens from every corner of the empire, as later exemplified by the Vienna Natural History Museum.

The Zoological-Botanical Society, of which Bilimek was among the first board members, emerged as a significant forum for the study of cave biology (Christian 2003). Following the identification of a distinct subterranean fauna by Copenhagen zoologist Jørgen Schiødte (1815–1884) in 1849, based on specimens collected in Carniola, the Society’s members expanded their investigations into the empire’s underground animal world. The Society not only published information on newly discovered species in its journal but also served as a hub for the exchange of knowledge and specimens through its monthly meetings. Expertise could compensate to some extent for a modest social background. For example, Prince Richard Khevenhüller-Metsch (1813–1877), the Society's first president, was officially represented by the ichthyologist Johann Heckel (1790–1857), the son of a music teacher and a warden in the imperial collections. Early on, large numbers of troglobites (species that live exclusively in caves) were collected by members of the Society, and numerous specimens were donated to the Vienna Imperial Collections and various provincial museums (Schiner 1854; Schmidt 1860; Wankel 1861).

Often, collectors such as Bilimek did not identify the new species themselves but entrusted this task to the curators of the Imperial Zoological Cabinet. Notable among these curators were the entomologists Vincenz Kollar (1797–1860) and Ludwig Redtenbacher (1814–1876) (Christian 2003). Such collaborations within the Society advanced the understanding and documentation of cave fauna significantly. This was important given the increasing nationalization of the monarchy's research landscape and the attendant professionalization of science, characterized by its emergence as a distinct occupation. In this context, the "unity" of the biological sciences, often emphasized at the Society's meetings, served as a significant symbolic gesture of cohesion, despite the increasing relevance of specialization in terms of day-to-day collaboration (Klemun 2001). Further, the meticulous lists of specimen donors published in the Society's journal aimed to highlight the mutual respect and recognition of both "professional" researchers with formal academic training and institutional affiliations, as well as "amateur" contributors such as dedicated naturalists and collectors.

The significance of subterranean zoology during the Society's two formative decades is illustrated by the president's regular reports of his excursions to Carniola. Prince Khevenhüller-Metsch, a dedicated ornithologist and collector, visited the caves near Postojna annually in the early 1850s, and documented his findings meticulously:

> On August 9th, I arrived in Adelsberg [Postojna] with all the equipment I deemed necessary for this expedition and promptly secured reliable guides. … We searched diligently until around five o'clock in the afternoon and discovered three exquisite specimens of *Leptodirus* in the canyons of Mount Calvary [in Postojnska jama]. … The *Leptodirus* appears to be nocturnal, as we predominantly found the beetles in the evening. Its habitat seems confined to Mount Calvary, where it moves slowly in the deepest gorges, undisturbed by visitors' torches, on very clean, damp stalactite walls that are not excessively wet. It likely also takes shelter in these cavities. Its natural adversaries include the *Obisium* [*Neobisium spelaeus*] and undoubtedly the eyeless cave spider *Stalita taenaria* [Schiødte, 1847]. … From this account, you will appreciate that I have diligently endeavored to explore every facet of organic life in this cave, and I may perhaps boast that few can match my wealth of results and experiences (Khevenhüller-Metsch 1851–1852: 106–109, 43, translated from German by me).

Khevenhüller-Metsch's hours spent crawling through crevices in search of troglobites were somewhat unconventional, given his high aristocratic status. Nonetheless, he shared his passion for entomology with members of the Habsburg family, notably the abdicated Emperor Ferdinand (1793–1875), who commissioned artists to illustrate insects, including numerous cave specimens, in detail (Fig. 3). In addition to Khevenhüller-Metsch's deliberate "hunt" for *Leptodirus*, which was to be rediscovered in 1847 after a partially damaged specimen was found in 1832, he successfully collected the first specimen of *Bathysciotes khevenhuelleri* in Postojnska jama (Polak 2005). This cave beetle was to be later described by the coleopterist and ministerial official Ludwig Miller (1852), and an illustration appeared in the inaugural volume of the Society's journal (Fig. 4).

Ferdinand Josef Schmidt (1791–1878), a German-speaking merchant and collector based in Ljubljana, held a longstanding monopoly on the lucrative trade in cave insects (Polak 2005). Despite his lack of formal academic training, Schmidt acquired great expertise in coleopterans, capitalizing on his fluency in Slovenian and extensive knowledge of prime collecting sites. For years, German entomologists eagerly paid three Friedrichs d'or (the equivalent of 20 grams of gold) for each specimen of *Leptodirus hochenwartii* (Kraatz 1878). In the 1860s, Schmidt was succeeded by Nicolaus Hoffmann, a skilled cutler from Ljubljana, who collected and sold subterranean fauna on a large scale and at more affordable prices. Prompted by the improved transport links provided by the southern railway line from Vienna to Trieste, eminent members of the Zoological-Botanical Society, including President Khevenhüller-Metsch, Secretary General Georg Frauenfeld (1807–1873), and board members Alois Pokorny (1826–1886) and Ignaz Rudolf Schiner (1813–1873), would undertake extensive collecting trips to the karst areas around Postojna. They documented their new findings meticulously in the society's journal, thus contributing to understanding these still largely unknown ecosystems (Pokorny 1853; Schiner 1853; Frauenfeld 1854). Adolf Schmidl's participation in these joint trips culminated in Schiner's (1854) seminal article, wherein he classified cave fauna according to ecological criteria into troglobites, troglophiles, and occasional cavernicolous, classifications still used today, albeit with some modifications.

Until the early 1850s, apart from the blind fish *Amblyopsis spelaea* De Kay, 1842 and the trechine beetle *Neaphaenops tellkampfii* Erichson, 1844 from Mammoth Cave, Kentucky (Tellkampf 1844; Flack 2022), almost all known troglobites were discovered in the Habsburg monarchy, particularly in Carniola. This spurred growing efforts to locate cave specimens in other crown lands, such as Hungary, the Alpine region, and Moravia. Key collectors, for example, Janos Frivaldszky (1822–1895) from the Hungarian National Museum, the Viennese coleopterist Clemens Hampe (1802–1884), the Bohemian-born zoologist Camill(o) Heller (1823–1917), and the Blansko physician Heinrich Wankel (1821–1897), played major roles in documenting and describing new species from these regions. Their findings include the ground beetle *Duvalius* (*Biharotrechus*) *redtenbacheri* I. Frivaldszky von Frivald & J. Frivaldszky, 1857; the bathysciine *Pholeuon angusticolle* Hampe, 1856; the blind isopod *Mesoniscus alpicola* Heller, 1858; and the springtail *Arrhopalites pygmaeus* Wankel, 1860.

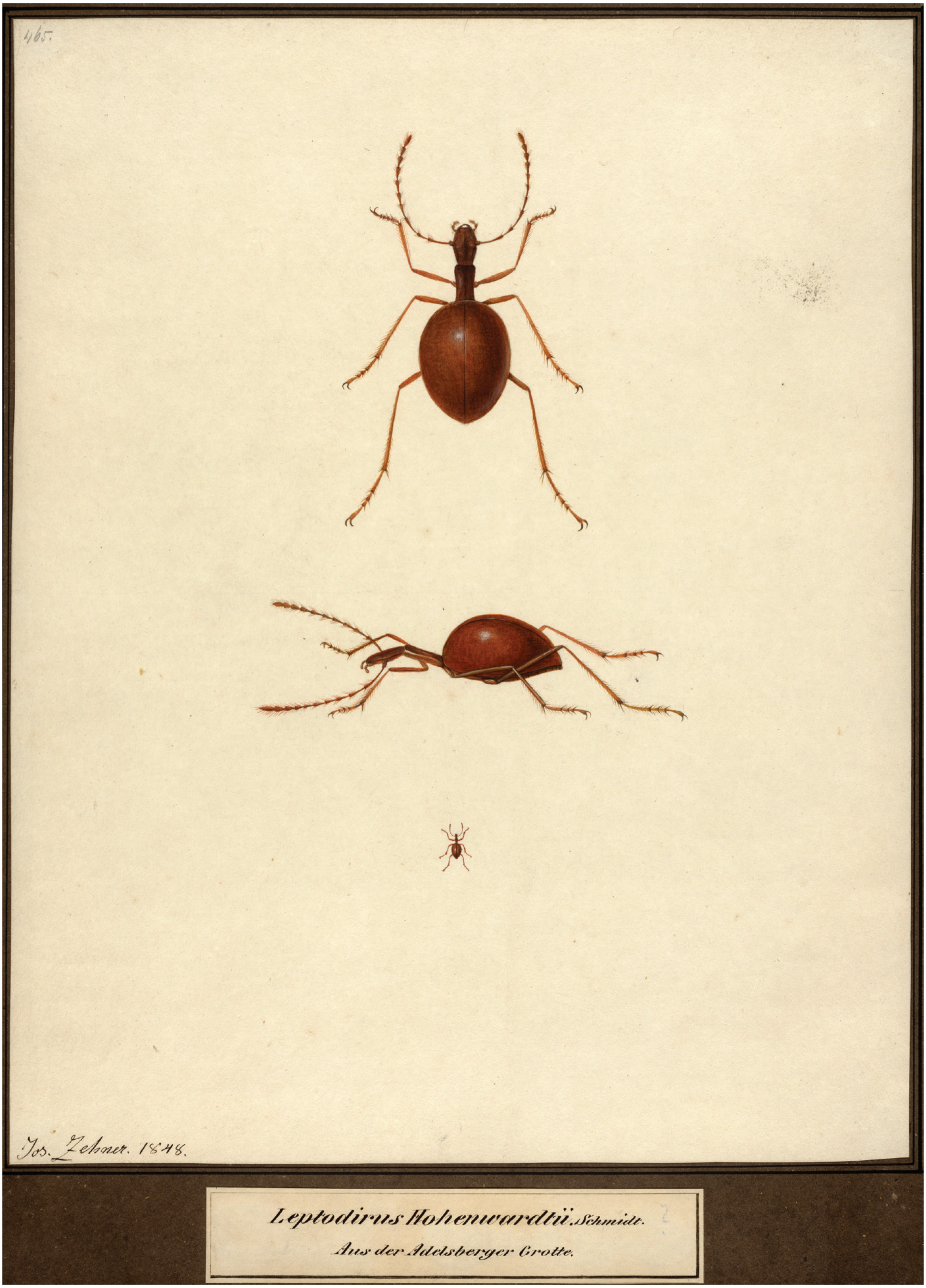

Figure 3. Drawing of the cave beetle *Leptodirus hochenwartii* in the art collection of Emperor Ferdinand of Habsburg, 1848. Artist: Joseph Zehner. Source: Austrian National Library, Bildarchiv (Pk 509, ZOOL32, 465).

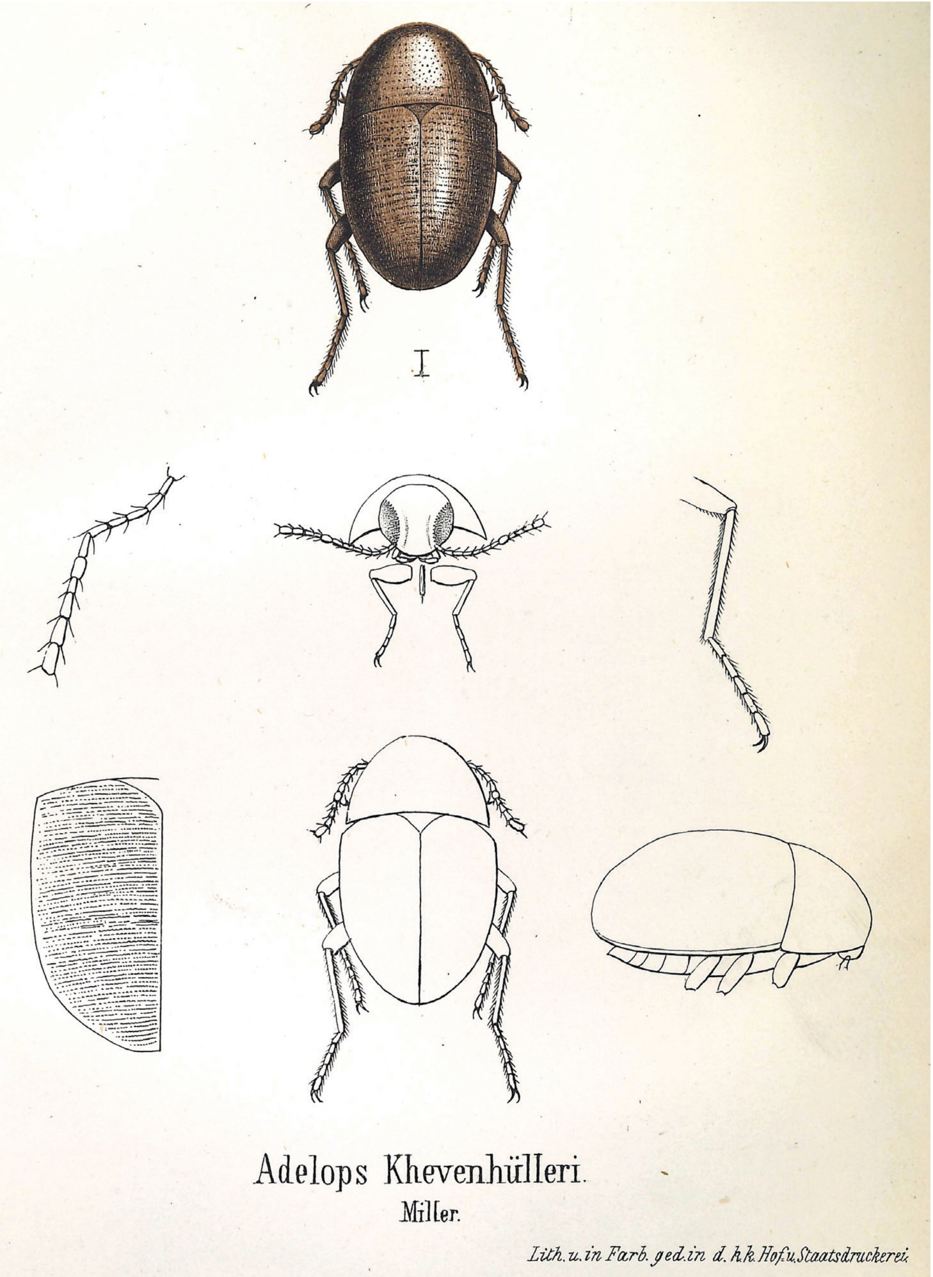

Figure 4. Drawing of the cave beetle *Bathysciotes khevenhuelleri* L. Miller, 1852. Source: Miller (1852, Tab. I).

The discovery and naming of new species held significant prestige and symbolic importance within the zoological–botanical community. Following nomenclatural conventions, the author who described and named the species is identified after the species name, while a specific epithet (the second part of the binomen) is often selected to honor the discoverer or a prominent scholar. By immortalizing the names of researchers and their collaborators, this practice has been and continues to be essential for sharing scientific credit, thereby ensuring that contributors receive due recognition for their work. A lithograph by entomologist Ernst Heeger (1783–1866) (Fig. 5), published alongside Schiner's (1854) article in Schmidl's "Zur Höhlenkunde des Karstes" (On the Study of Caves in the Karst), illustrates various cave insects known to be from Carniola and the Habsburg monarchy at that time. This artificial arrangement of individual species that do not occur together naturally is only found in natural history collections. The symmetrical arrangement of the specimens on the lithograph can be interpreted as a visualization of the unity in diversity of the subterranean fauna. Additionally, four of the ten species are named after their finders or patrons of speleology. These individuals, along with the authors to whom both the name and the validating description were ascribed, symbolize the unity of biological research. In the Habsburg monarchy of the 1850s and 1860s, systematic zoology, particularly the study of cave fauna, was characterized by collaboration and mutual recognition between finders, patrons, and scientists.

Bilimek's visit to the Grutas de Cacahuamilpa and his comparative approach

In January 1866, Bilimek, accompanied by Wilhelm Knechtel (1837–1924), a Bohemian botanist and gardener at Chapultepec Castle, set out on a collecting trip from the Mexican emperor's country house in Cuernavaca. After a 70-kilometer journey, they finally arrived at the impressive entrance to the Grutas de Cacahuamilpa (Fig. 6). This cavern, long known to indigenous people, had become a popular destination since the 1830s among an array of travelers, artists, and politicians. Its distinguished visitors included Mexican President Ignacio Comonfort (1812–1863), who served from 1855 to 1858 (Uribe Salas and Valdivia Moreno 2015).

Although Bilimek (1867: 901) had been "assured from all sides that nothing was living in the cave," he succeeded in collecting eleven different species in a seven-hour exploration, which included the first known discovery of the cave silverfish *Anelpistina anophthalma*. Moreover, Bilimek observed that the Grutas de Cacahuamilpa were comparable in size, stalactite richness, and diversity of fauna to the famous Postojnska jama, establishing the former as a second "imperial" cave. This insightful comparison caught the attention of the Habsburg–Mexican imperial couple. Although Maximilian could not carry out his planned visit because of urgent political commitments, his wife Carlota of Mexico (1866: 3) visited the Grutas de Cacahuamilpa the same year. There, she inscribed her name, proudly recording that she had "written her name in a place more distant than the [inscription] of [former president] Comonfort," emphasizing her desire "not to let the empire be forgotten in this remote place."

Bilimek's rather sober account of his zoological expedition to the Grutas de Cacahuamilpa, presented to the Vienna Zoological-Botanical Society six months after Maximilian's death, was a significant milestone as it was possibly the first article on cave-dwelling

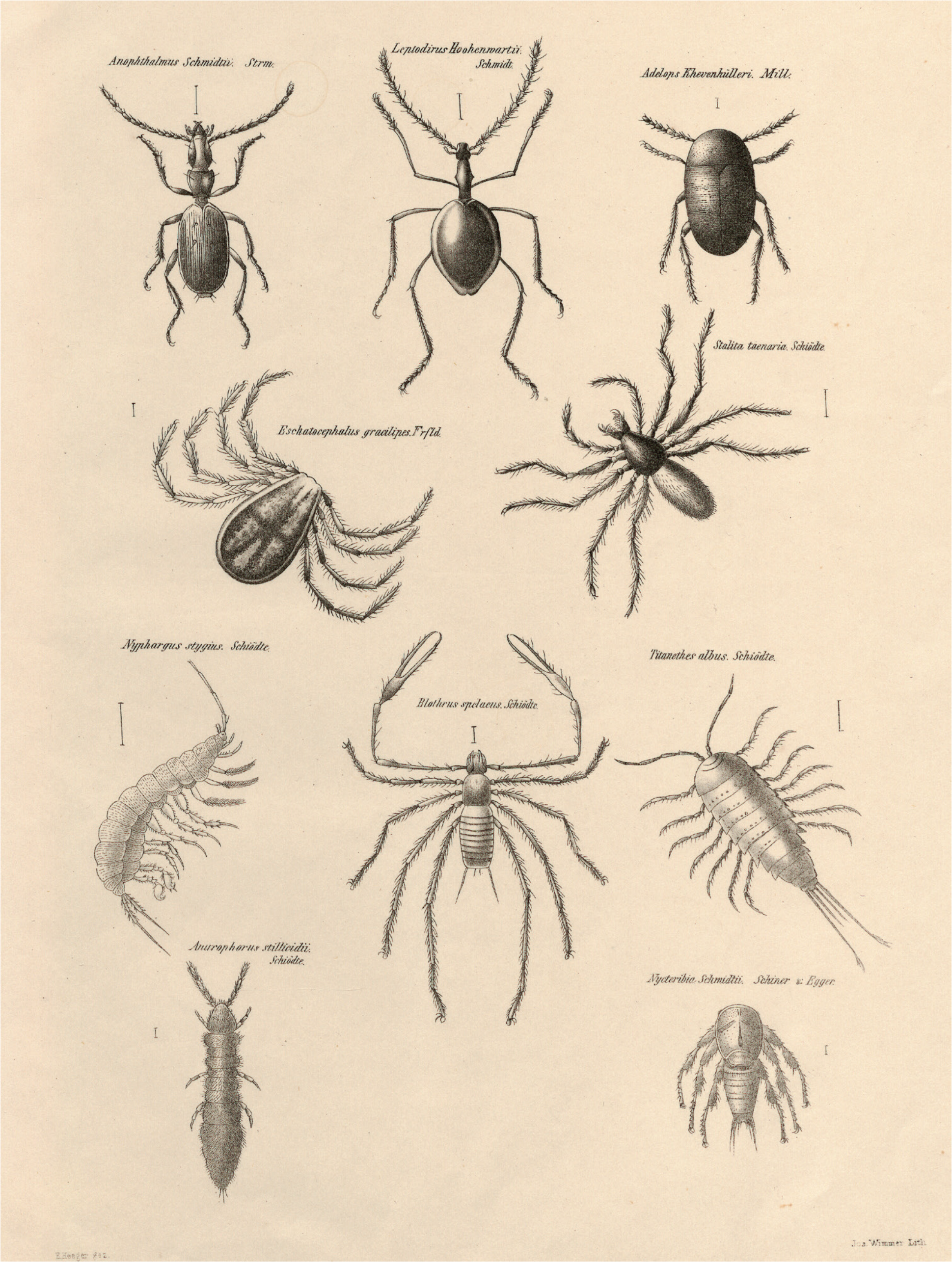

Figure 5. Iconography of the earliest known subterranean insect fauna. Artist: Ernst Heeger. Source: Schmidl (1854, Tab. 15), Austrian National Library, Bildarchiv (ALB Vues 08765).

organisms in the Americas outside the United States (Howarth 2023). Particularly noteworthy was his striking comparison with the renowned Postojnska jama, which served as an epilogue on the last page of his paper. Probably influenced by Khevenhüller-

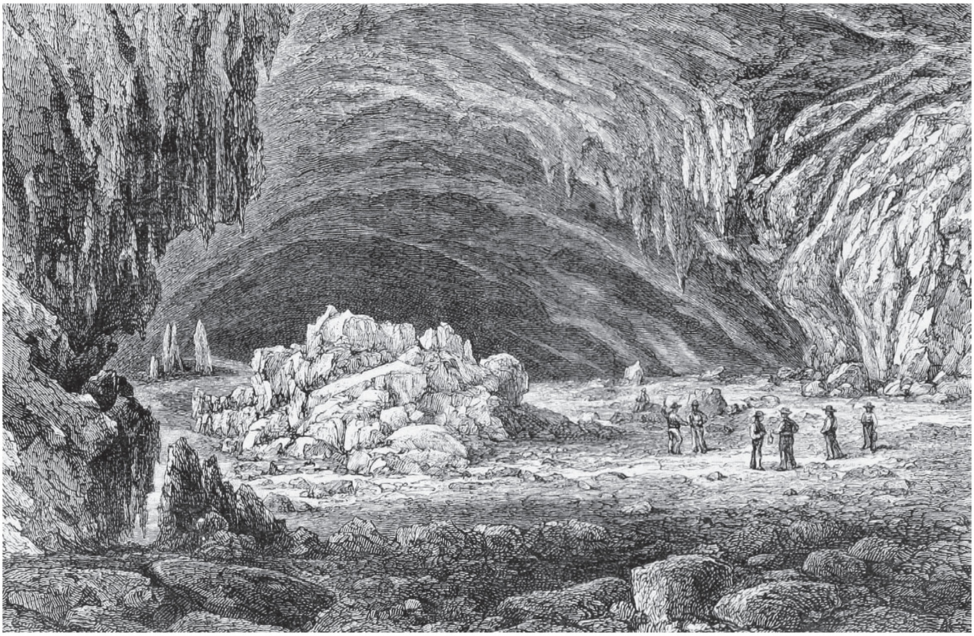

Figure 6. Entrance to the Grutas de Cacahuamilpa in Guerrero, Mexico. Source: Brocklehurst (1883, Tab. 38).

Metsch's (1850–1851) early ideas on the interconnectedness of cave fauna, Bilimek (1867: 908) noted the absence of "wingless flies" in the Grutas de Cacahuamilpa and attributed this to the lack of bats and the sparse number of visitors, resulting in insufficient "food and wood" entering the cave. This comparative analysis led Bilimek to argue that an ecological interdependence exists among the subterranean fauna, somewhat akin to a food chain model: "The presence of these species in the grotto," he contended, "is mutually conditioned." Beetles thrive on plant and animal matter, spiders prey on flies dependent on moisture, and woodlice and silverfish subsist on food particles in the cave soil. "As water reservoirs are known to be found deeper inside [the cave], it is likely that these are also inhabited." In his quest to find unity in diversity, Bilimek moved beyond collectors' individual observations to elucidate the co-existence of species as a symbiotic relationship, highlighting the intricate and interdependent nature of cave ecosystems.

Conclusion

This article examines the emergence of subterranean zoology as a research field in mid-nineteenth-century Central Europe, focusing on the multifaceted activities of Dominik Bilimek and his fellow collectors. While historical studies of biospeleology, often referring to Anglo-American and French scholarship, have examined major advances in species discovery and scientific thought, this paper centers on the sociopolitical frameworks that shaped early understandings of cave fauna. Tracing the origins of cave entomology within the Habsburg monarchy has opened avenues for examining research practices in specific historical contexts, shedding light on imperial agendas and the interplay between science and society.

Mid-nineteenth-century studies of (cave) fauna differed methodologically from contemporary geoscientific fieldwork in its less coordinated and systematic approach, influenced by collectors' varying focuses and access to sites. Bilimek's visit to the Grutas de Cacahuamilpa exemplifies how such ventures, under individuals' own initiative, were nonetheless integrated into broader natural history inquiries. The collection, classification, and publication of findings involved multiple contributors and relied on transregional networks. While local stakeholders, supported by family members or hired indigenous workers, often captured and prepared the specimens, "professionals"—especially curators in Vienna or provincial capitals—subsequently documented these findings. The Vienna Zoological-Botanical Society was the sole association of its kind in Central and Southeastern Europe until the 1870s. It formalized these cooperative networks, facilitating the exchange of cave insect specimens and related knowledge, particularly concerning sites and environmental conditions. Furthermore, through its journal, the Society provided a platform for accrediting and internationalizing findings.

Thus, by exploring the social and political contexts of the emergence of subterranean zoology, I have highlighted the significance of lesser-known actors like Bilimek in producing, accumulating, and disseminating knowledge on cave fauna. However, their profile gradually diminished with the professionalization of research. Cooperation within these early networks relied on mutual recognition, evident through publications; the acknowledgment of both authors and finders in naming new specimens; and the prestige associated with private collections. Troglobites, valued for their scarcity and the exoticism of their habitats, occupied a prominent position in specimen circulation, which was driven by their purchase, exchange, or donation. Initiated by the discovery of *Leptodirus hochenwartii*, collecting cave insects became a fashion in mid-nineteenth-century Vienna that transcended class and political divisions. Empire-wide initiatives, aiming to pool sources and specimens from the crown lands and centralize them in Vienna, paved the way for the expansion of faunistic research from Carniola to the monarchy's other karst regions. Until the discovery of extensive fauna in Pyrenean caves by Charles Delarouzée (1857) and Charles Lespès (1857), the Habsburg monarchy's learned societies dominated the emerging field of cave entomology.

Overall, Bilimek's impact on early subterranean biology extends beyond the discovery of two species, which had placed him in the community of zoologists, naturalists, and collectors exploring the entomological "Eldorado" of the Carniolan caves in the 1840s and 1850s. The extensive geographical scope of his work, facilitated by his service to two empires, allowed for collecting specimens on a significant scale (and without ethical concerns). However, Bilimek's (1867) description of food chains in the Grutas de Cacahuamilpa and the Postojnska jama did not address the fundamental questions raised by Charles Darwin (1809–1882) in his recently published book "On the Origin of Species" (1859). Furthermore, Bilimek refrained from engaging in the heated debates on Darwin's theory that unfolded within the Vienna Zoological-Botanical Society in the 1860s, which ultimately led to new advances in cave biology. Nevertheless, his contributions laid the groundwork for later European researchers to embark on collecting expeditions to the Americas, marking a significant, if complex, legacy.

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Temporal consistency and spatial variability in detection: implications for monitoring of macroinvertebrates from shallow groundwater aquifers

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Abstract

Implementing and optimizing biodiversity monitoring is crucial given the current, worldwide biodiversity decline. Compared to other ecosystems, monitoring of biodiversity is lagging behind in groundwater ecosystems, both because of sparse taxonomic knowledge and methodological constraints. We here assessed temporal variation in the occurrence and abundance of macroinvertebrates collected systematically from shallow groundwater aquifers of Switzerland to establish general principles on seasonality and repeatability of assessment outcomes. We found no seasonal abundance pattern for obligate groundwater amphipods and isopods, indicating temporal consistency. In contrast, other macroinvertebrates (predominantly stygophiles and stygoxenes) showed pronounced seasonality in their detection rate. However, we found variability in detection rates across groundwater amphipod species and especially across sampling sites. For groundwater communities, characterized by narrowly-distributed and rare species, our results highlight the need for tailored and extensive sampling strategies. When setting up monitoring programs on groundwater fauna, detection probability, temporal autocorrelation, and standardization of sampling effort should be carefully considered. Applying novel, systematic approaches, can offer promising methodologies for understanding and conserving groundwater ecosystems.

Keywords

Citizen science, detection, *Niphargus*, occurrence, seasonality, stygofauna, subterranean

Introduction

Biodiversity decline is one of the most pressing environmental challenges of our time, with profound implications for ecosystem function and services, human well-being, and global sustainability (Rockström et al. 2009; Dirzo et al. 2014). In response to this crisis, numerous efforts have been initiated worldwide to halt and reverse the decline in biodiversity, and there is a need for standardized, robust biodiversity monitoring programs. Such programs are essential for accurately assessing the status and trends of biodiversity, for evaluating the effectiveness of conservation management, and to inform policy decisions (Yoccoz et al. 2001; Gonzalez et al. 2023b). To be effective, optimizing monitoring efforts and their implementation across diverse ecosystems and representatively covering organism groups remains crucial.

Signals in biodiversity data can only be meaningfully detected with adequate sampling design (Yoccoz et al. 2001; Gonzalez et al. 2023a). Particularly, the temporal sampling scheme must be adapted to the specific characteristics of the system which is to be monitored, to distinguish a signal from inherent system variability (noise). This includes for example determining the optimal sampling frequency and the overall duration of the time series (Magurran et al. 2010), as well as considering heterogeneity in species detection (Yoccoz et al. 2001). The challenge is to find a balance between monitoring effort and the comprehensiveness of the data collected (Francomano et al. 2021). This task becomes even more complex in the absence of pre-existing knowledge about the temporal dynamics of a system, where the lack of baseline data can limit the identification of trends and signals in biodiversity indices (Mihoub et al. 2017).

One ecosystem with limited baseline knowledge on temporal dynamics is groundwater. Despite being the largest freshwater reservoir on earth and a keystone ecosystem (Ferguson et al. 2021; Saccò et al. 2024), it is one of the least studied ecosystems, mainly due to difficulties in accessibility (Mammola et al. 2019; Mammola et al. 2021). Yet, it harbors a unique range of organisms that are adapted to the dark and energy deprived conditions (Culver and Pipan 2019). These obligate groundwater dwellers (stygobites) exhibit a high rate of endemism, and some of the species originate from ancient adaptive radiations (Trontelj et al. 2009; Borko et al. 2021). Despite their hidden nature, groundwater organisms are not completely sheltered from human influence. Anthropogenic pressures extend well beyond surface environments, increasingly affecting also groundwater ecosystems (Couton et al. 2023b; Nanni et al. 2023; Vaccarelli et al. 2023).

Generally, groundwater and other subterranean environments are more buffered from fluctuating environmental conditions than aboveground systems. Yet, especially shallow groundwater habitats exhibit some temporal patterns, as they are closely linked to aboveground ecosystems through hydrologic flows (Culver and Pipan 2011). This, in turn, can drive temporal dynamics in groundwater communities. For example, groundwater recharge and discharge can be linked to rainfall and snow melt, affecting groundwater communities through altered nutrient inputs, temperature, and groundwater flow regimes (Di Lorenzo et al. 2005; Opalički Slabe 2015; Hutchins et al. 2021; Saccò et al. 2021). While the aforementioned studies have detected some temporal patterns in groundwater faunal communities, others have not found such evidence (Pacioglu and Robertson 2017), or suggest that seasonality might primarily influence microbial assemblages (Korbel and Hose 2015).

Over the past decades, there has been a growing effort to develop sampling tools for monitoring groundwater fauna (Koch et al. 2024). Apart from traditional sampling, new approaches such as environmental DNA (Couton et al. 2023b; van der Heyde et al. 2023) and citizen science approaches (Alther et al. 2021; Knüsel et al. 2024b; Korbel and Hose 2024) have emerged. However, there is still a lack of temporal data for many parts of the world (Koch et al. 2024) and simultaneously, protected areas fail to represent subterranean biodiversity (Fišer et al. 2022; Colado et al. 2023; Mammola et al. 2024). The tendency to overlook subterranean ecosystems and the scarcity of temporal baseline data hinder the development of comprehensive monitoring programs, as well as the implementation of effective conservation and management strategies (Sánchez-Fernández et al. 2021; Mammola et al. 2022; Nanni et al. 2023).

We here temporally assessed macroinvertebrate communities in shallow groundwater aquifers of Switzerland. For this region, spatial distribution and diversity patterns are relatively well known (Altermatt et al. 2014; Altermatt et al. 2019; Alther et al. 2021; Schneider et al. 2023; Knüsel et al. 2024b) through eDNA and classical organismal sampling (Studer et al. 2022; Couton et al. 2023a, 2023b). Yet, knowledge about temporal variability of detection is still limited and groundwater fauna is generally not yet included in any of the national biodiversity monitoring programs (BAFU 2023). We here close knowledge gaps concerning temporal aspects of groundwater fauna sampling, specifically the effect of seasonality and further time-varying covariates on stygobite and non-stygobite occurrence, temporal autocorrelation of their presence–absence, and detection rate. Based on these aspects, we discuss considerations for the development of monitoring strategies.

Materials and methods

Sampling procedure

Data was collected as part of a large citizen science project across Switzerland (Knüsel et al. 2024b). Specifically, drinking water providers were asked to sample groundwater for macroinvertebrates. The sampling was conducted in spring catchment boxes, where groundwater is captured passively from horizontal drainage pipes (Fig. 1). At the pipe inlets into the respective spring catchment box, filternets (mesh size 0.8 mm) were attached for one week and then all captured organisms were collected and stored in 80% ethanol. We used two datasets covering different temporal and spatial resolutions for this study.

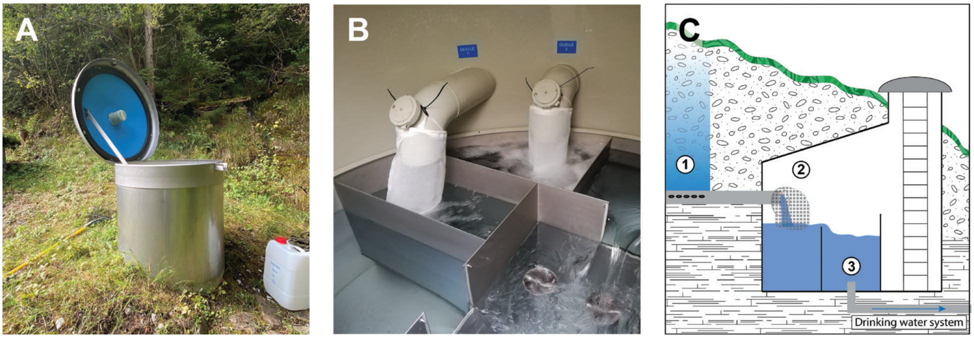

Figure 1. Methodology for sampling groundwater macroinvertebrates at spring catchment boxes (groundwater extracted for drinking water usage). Open spring catchment box from outside (**A**) and from inside with filter nets attached to the inlets of the drainage pipes (**B** both modified from Knüsel et al. 2024b). Schematic representation (**C** modified from Couton et al. 2023a) of the passive groundwater collection (1), inlet into spring catchment box with filter net installed (2), and injection of the water into the local drinking water supply system (3).

Monthly dataset

Firstly, we used data from 17 inlets spread across Switzerland (Fig. 2A) that were sampled approximately monthly over one year between May 2021 and April 2022. The filtering was conducted during one week per month. The collected macroinvertebrates were morphologically identified to major taxa (Schneider et al. 2023) and then split into three groups. We used two groups covering the most commonly found, obligate groundwater macroinvertebrates (stygobites), namely groundwater amphipods (genera *Niphargus* and *Crangonyx*) and groundwater isopods (including genus *Proasellus*) (Schneider et al. 2023). All other collected macroinvertebrates were combined as a third group (hereafter named ‘other macroinvertebrates’). It consists predominantly of organisms that are occasionally (stygophiles) or accidentally (stygoxenes) entering groundwater ecosystems. For each sample, we calculated the filtering duration based on the given start and end date (using 7 days as default when the start date was not specified by the participant). If the outflow rate was available for each sample, we calculated the total volume of water filtered for the respective samples. In cases where only one measure of outflow rate was missing for a particular sampling date, but the previous and subsequent measurements were available, we used the mean between the two. We additionally extracted data on precipitation, as this affects groundwater recharge (Stoll et al. 2013) and thereby might have an effect on groundwater fauna wash out at spring catchment boxes (see also Di Lorenzo et al. 2018). Therefore we calculated each sampling sites’ local precipitation sum (mm, equivalent to liters per square meter) over two weeks prior to the end date of each filtering period from the RhiresD data provided by the Federal Office of Meteorology and Climatology MeteoSwiss (https://www.meteoschweiz.admin.ch/klima/klima-der-schweiz/raeumliche-klimaanalysen.html). For longer-term seasonal precipitation trends, we calculated the same 2-week precipitation sums from a measuring station in Bern

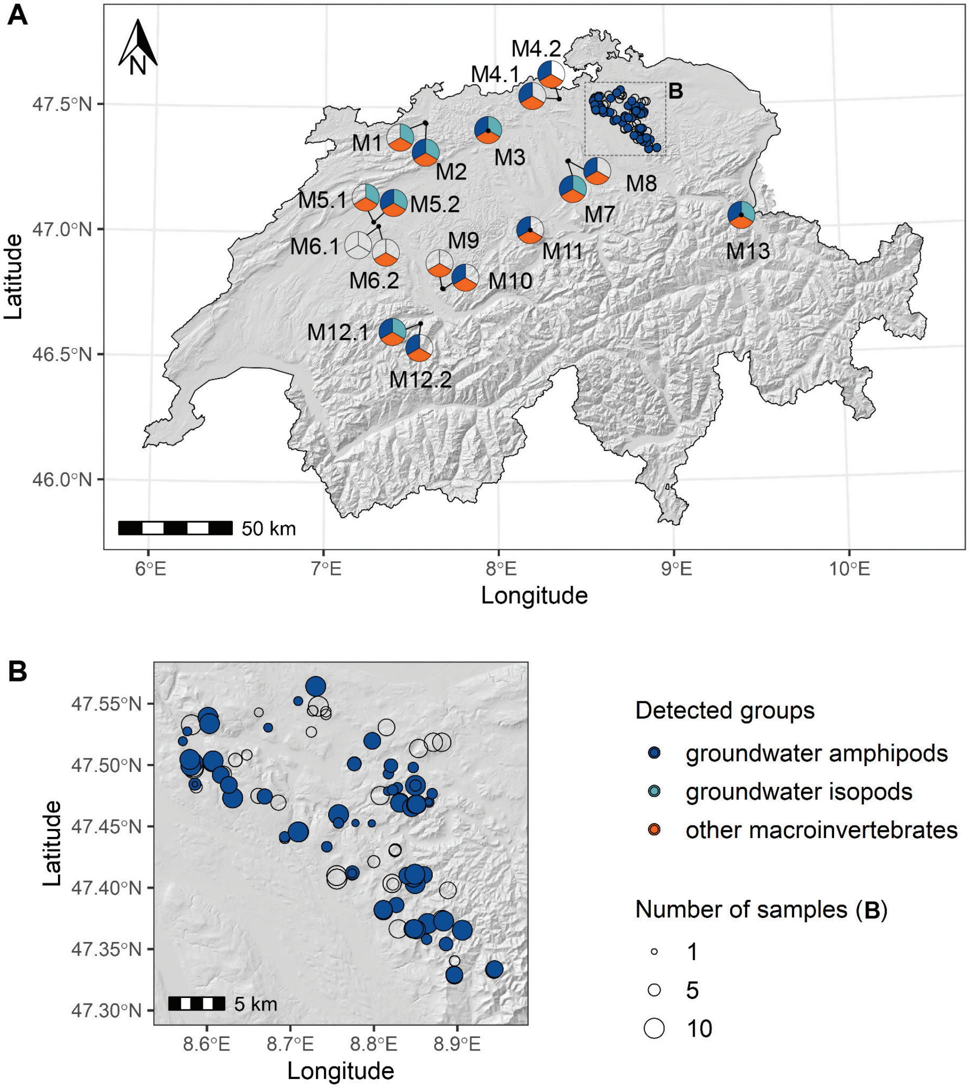

Figure 2. Sampling sites across Switzerland. Pie charts depict sampling inlets from the monthly dataset with presence of groundwater amphipods, groundwater isopods and other macroinvertebrates marked in dark blue, turquoise, and orange, respectively (**A**). Absences of the corresponding organism group are marked as empty sectors in the pie charts. Sampling sites from the weekly dataset are shown enlarged (**B**), filled circles mark inlets with groundwater amphipod detection and empty circles without (remaining macroinvertebrate groups were not considered). The point size represents number of samples. Geodata from swisstopo (permission JA100119).

(Federal Office of Meteorology and Climatology MeteoSwiss, https://data.geo.admin.ch/ch.meteoschweiz.klima/nbcn-tageswerte/nbcn-daily_BER_previous.csv) and then compiled the mean and standard deviation per day of year across the years 1990–2020.

Weekly dataset

In the catchment area of the river Töss, 143 inlets were sampled 1–10 times each in subsequent weeks (mean 6.8 weekly samples per inlet) (Fig. 2B). The sampling was conducted in spring 2021 (Studer et al. 2022). All groundwater amphipods were sorted and identified to the species level by sequencing the Folmer fragment of the mtDNA COI gene (Folmer et al. 1994). A detailed protocol of the molecular identification can be found in Studer et al. (2022) and Knüsel et al. (2024b). Organisms other than groundwater amphipods were not further identified in this dataset.

Statistical analyses

All analyses were performed in R (ver. 4.2.2; R Core Team 2022b). We used a Generalized Additive Modeling (GAM) approach to assess effects of seasonality, outflow, and precipitation on the abundances of groundwater amphipods, groundwater isopods, and other macroinvertebrates in the monthly dataset. Both, outflow and precipitation were found to affect groundwater community assemblages over time in previous studies (Hutchins et al. 2021; Saccò et al. 2021). Hereafter, we refer to the pattern across the sampled year as "seasonality" for simplicity, yet we acknowledge that seasonal and event-based effects cannot be clearly distinguished from 12 monthly samples. For each organism group we included data from all inlets where the respective group was found at least once. Seasonality was modeled as day of year using cyclic cubic regression splines (to ensure matching ends for continuity, Wood 2023), while outflow [L/sec] and precipitation [mm] were modeled with thin plate regression splines (default, Wood 2023). We used splines varying by organism group for each of these covariates and the default 10 knots. Further, we added the inlet identifier as random effect, the organism group as a factor, and an offset to account for varying sampling effort. We ran two models using different log-transformed offsets, one using the sampling duration (in number of sampling days) and one using the total discharge volume (in megaliters). Models were fitted in the R package 'mgcv' (Wood 2023) using restricted maximum likelihood (REML) and negative binomial response distribution (Suppl. material 1: R-code). Abundances were then predicted for sampling efforts of one week (representing the commonly used filtering duration) and one megaliter, using varying levels of either seasonality, outflow, or precipitation while keeping the other parameters at their median value. Additionally to the GAM, we calculated temporal autocorrelation and corresponding 95% confidence intervals based on presence–absence of the three organism groups in consecutive samples grouped per inlet using the function acf() from R package 'stats' (R Core Team 2022a). We used the type 'correlation' and set lag.max to 11 (maximum number of sampling occasions per inlet is 12).

Using the weekly dataset, we calculated detection rates of groundwater amphipods based on their capture history per inlet:

$$p_{i,j} = \frac{x_{i,j}}{n_j} \qquad \text{(Eq. 1)}$$

where $p_{i,j}$ is the detection rate of species i at inlet j (given presence), $x_{i,j}$ is the number of samples in which the species i was detected at inlet j, and n_j is the total number of sampling occasions at inlet j (see e.g., McArdle 1990; MacKenzie et al. 2002; MacKenzie 2018). We computed inlet-specific detection rates for four amphipod species, each of which was found at minimally 10 inlets (to ensure sufficiently large sample size for reliable estimates). Additionally, we calculated inlet-specific detection rates for all groundwater amphipods combined. We then calculated the mean detection and corresponding standard deviation across all inlets where the given species or combined amphipods were found at least once by:

$$\bar{p}_i = \frac{\sum_{j=1}^{N_i} p_{i,j}}{N_i} \tag{Eq. 2}$$

where $\bar{p}_i$ is the mean detection of species i across inlets (given presence) and N_i the number of sampled inlets (given presence of species i). Higher sampling effort is expected to yield more precise detection rate estimates compared to lower sampling effort including few repeated samples per inlet. In a last step, we thus assessed how the detection rate estimates change under varying sampling effort and if they stabilize upon sufficient sampling. Therefore, we calculated the detection rates repeatedly based on the formula above, but using a subset of inlets that were sampled a certain minimum number of times by:

$$\bar{p}_{i,k} = \frac{\sum_{j=1}^{N_{i,k}} (p_{i,j} \mid n_j \geq k)}{N_{i,k}} \tag{Eq. 3}$$

where k is the threshold of sampling occasions per inlet, ranging from 1 (all inlets included) up to 10 (inlets with 10 sampling occasions included only) and $N_{i,k}$ corresponds to the number of sampled inlets that fulfill the given threshold criterion.

Results

Macroinvertebrates sampled monthly during one year (monthly dataset)

The sampled inlets showed pronounced differences in faunal composition with respect to the presence/absence of the two stygobite groups and other macroinvertebrates (Fig. 2A). Also, there was a large variety of measured outflow rates and their temporal patterns between inlets (Fig. 3A). Precipitation patterns were comparable across inlet locations, with a large peak in July and a smaller peak between December and February (Fig. 3B). Generally, precipitation in summer 2021 was higher than the long-term average for many areas, while in autumn 2021 it was lower than the long-term average (Fig. 3B, MeteoSchweiz 2022). The outflow rates of some inlets reflected the precipitation peak(s) with certain lags, whereas other inlets had rather consistent outflow rates throughout the sampling period, irrespective of precipitation fluctuations.

We found no significant effect of seasonality on groundwater amphipod and isopod abundances in the GAM (Table 1, Fig. 4). In contrast, seasonality significantly affected

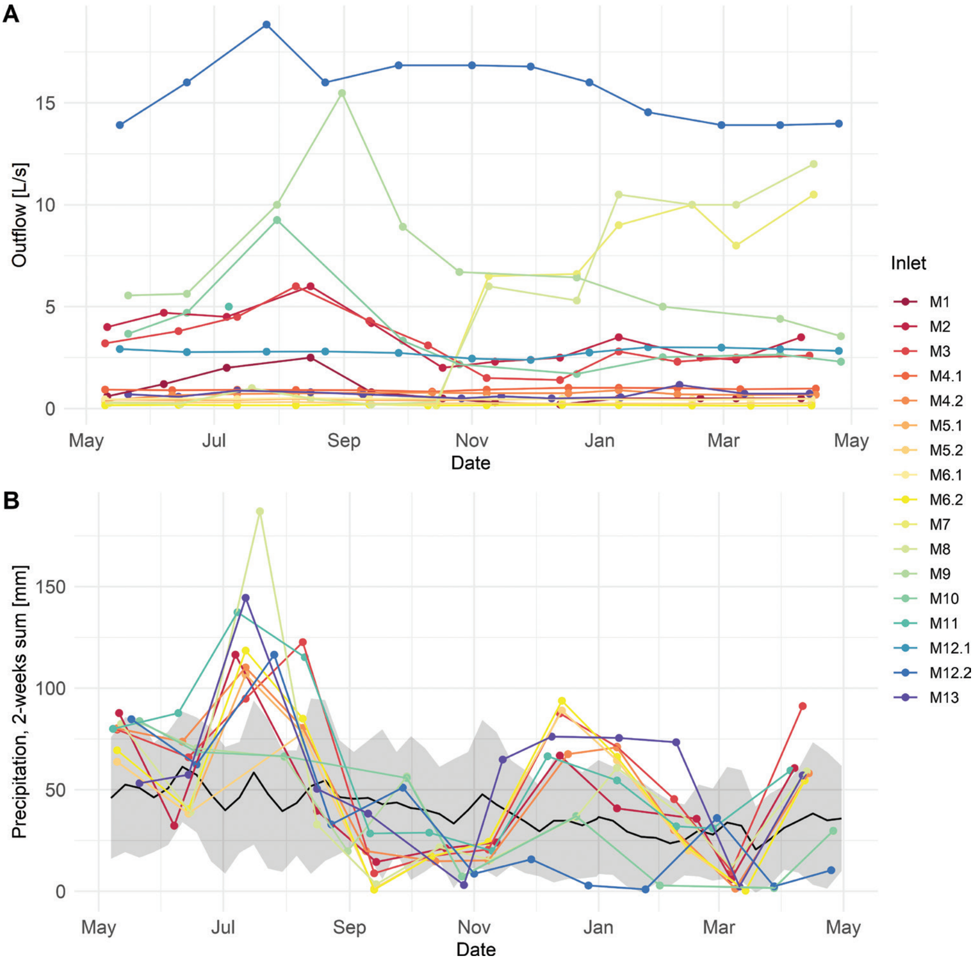

Figure 3. Environmental covariates along the sampling period (monthly dataset), plotted per inlet **A** outflow in L/s and **B** precipitation sum across two weeks prior to the sample collection. For comparison, long-term precipitation mean (black line) and standard deviation (grey shaded area) from Bern (1990 to 2020) are plotted in the background.

the other macroinvertebrates, with predicted abundances peaking in July (Fig. 4). This pattern was mostly driven by EPT (Ephemeroptera, Plecoptera, and Trichoptera) larvae (Suppl. material 1: fig. S1). The outflow rate did not have a significant effect when abundances where modeled per day, but was significant when abundances were modeled per megaliter (Table 1). In the latter case, abundances of all three organism groups per megaliter were predicted to be highest at low inlet outflow rates and to decrease with increasing outflow rates (Fig. 4). We did not identify any significant effect of precipitation on any of the organism groups' abundances. However, there might be a confounding effect between precipitation and seasonality. In both models, the random inlet effect was highly significant. Still using the monthly dataset, the model with

Table 1. GAM results for the abundances of groundwater amphipods (amphi), groundwater isopods (isopod), and other macroinvertebrates (macro). Model 1 was run with discharge volume as an offset and model 2 with number of sampling days. The "parametric coefficients" component refers to the linear (or parametric) part of the model, which includes the coefficients for the categorical variable "organism group".

Model 1 (per megaliter offset)

Component	**Term**	**Estimate**	**Std error**	**z-value**	**p-value**
A. parametric coefficients	(Intercept)	0.59	0.30	1.98	0.048
	Group: amphi	-1.09	0.20	-5.44	< 0.001
	Group: isopod	-1.85	1.34	-1.38	0.17
Component	**Term**	**Edf**	**Ref. df**	**Chi.sq**	**p-value**
B. smooth terms	s(precip:macro)	1.00	1.00	1.49	0.22
	s(precip:amphi)	1.00	1.00	1.61	0.20
	s(precip:isopod)	1.00	1.00	0.26	0.61
	s(outflow:macro)	3.49	4.28	49.47	< 0.001
	s(outflow:amphi)	2.63	3.24	50.48	< 0.001
	s(outflow:isopod)	3.45	4.17	33.11	< 0.001
	s(sesonality:macro)	2.21	8.00	10.35	0.0031
	s(seasonality:amphi)	0.97	8.00	1.60	0.18
	s(seasonality:isopod)	0.00	8.00	0.00	0.73
	s(inlet)	12.48	15.00	116.90	< 0.001

Deviance explained 62.4%, n = 399

Model 2 (per day offset)

Component	**Term**	**Estimate**	**Std error**	**z-value**	**p-value**
A. parametric coefficients	(Intercept)	-1.53	0.31	-4.98	< 0.001
	Group: amphi	-1.07	0.20	-5.42	< 0.001
	Group: isopod	-1.54	0.91	-1.69	0.092
Component	**Term**	**Edf**	**Ref. df**	**Chi.sq**	**p-value**
B. smooth terms	s(precip:macro)	1.00	1.00	2.47	0.12
	s(precip:amphi)	1.00	1.00	2.89	0.089
	s(precip:isopod)	1.00	1.00	0.41	0.52
	s(outflow:macro)	1.00	1.00	1.01	0.31
	s(outflow:amphi)	1.00	1.00	1.72	0.19
	s(outflow:isopod)	3.09	3.79	3.38	0.41
	s(sesonality:macro)	2.13	8.00	9.27	0.0054
	s(seasonality:amphi)	1.10	8.00	2.00	0.15
	s(seasonality:isopod)	0.00	8.00	0.00	0.83
	s(inlet)	12.94	15.00	122.44	< 0.001

Deviance explained 38.1%, n = 399

abundances per discharge volume had a higher deviance explained than the model with abundances per sampling duration (62.4% vs. 38.1%; Table 1).

Temporal occurrence varied between organism groups (Fig. 5A) and their capture histories (detection–nondetection) indicated temporal autocorrelation patterns (Fig. 5B). For groundwater amphipods, temporal autocorrelation was significant up to the third lag (= three months). In contrast, the autocorrelation for groundwater isopods was significant up to the tenth lag (Fig. 5B), in line with either very high or very low detection rates across sampling occasions (Fig. 5A). The other macroinvertebrates showed a similar pattern as the groundwater amphipods, with significant and decreasing autocorrelation up to the second lag.

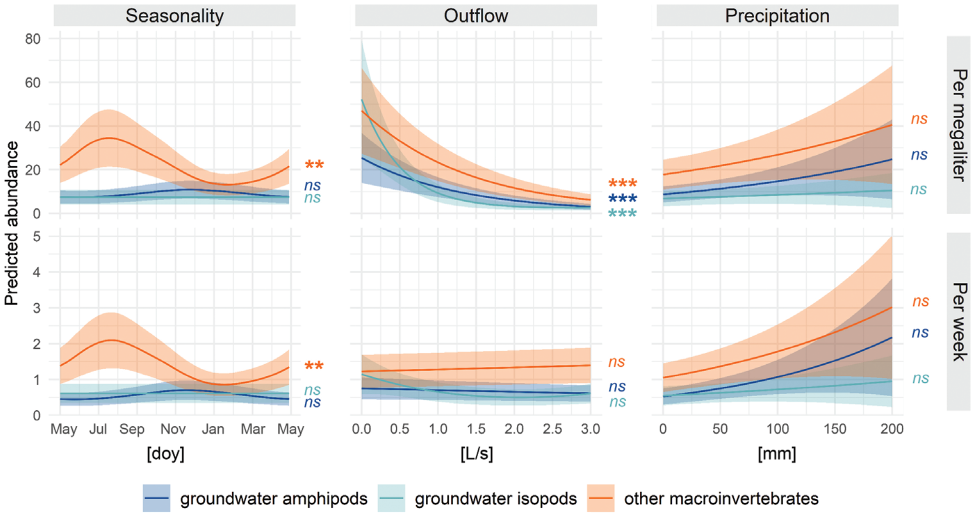

Figure 4. Predicted abundances (mean and 95% CI) of groundwater amphipods (dark blue), groundwater isopods (turquoise), and other macroinvertebrates (orange), plotted along gradients of seasonality [day of year, labelled in months], groundwater outflow [liters per second], and precipitation sum over 14 days preceding the sample collection date [millimeters]. GAM predictions were computed based on abundances per discharge volume (megaliter, upper plots) and per sampling duration (week, lower plots). Significance levels based on Table 1 are indicated (*ns* for p > 0.05, * for p ≤ 0.05, ** for p ≤ 0.01, *** for p ≤ 0.001).

Amphipods sampled repeatedly over consecutive weeks (weekly dataset)

Groundwater amphipods were detected in 55% of the 143 sampled inlets in the Töss catchment. In total, nine species were identified, of which five were only detected in 1–3 inlets each (Table 2). Detection rates varied substantially between species, both in terms of mean and variance across inlets (Fig. 6). Specifically, detection rates of *Niphargus fontanus* and *N. tonywhitteni* both indicated a very large heterogeneity across inlets. The same was found for groundwater amphipods combined; detection rates per inlet varied from 0.1 up to 1.0. In contrast, *Crangonyx* cf. *subterraneus* was found to have the lowest detection rate and the smallest variance across inlets.

With increasing sampling effort, we found detection rate estimates to become more conservative (Fig. 7, Suppl. material 1: fig. S2). Because of the procedure how detection rate was estimated, detection rates at higher sampling effort are inevitably lower than estimations obtained at low sampling effort, but allegedly level off upon sufficient sampling. The detection rates of groundwater amphipods combined seemed to stabilize around a mean detection of 0.4, although standard deviations remained large (Suppl. material 1: fig. S3). The lowest mean detection of groundwater amphipods combined (i.e., 0.38, including inlets with at least 6 sampling occasions) would translate to a minimum of 6.3 samples necessary to infer the absence of groundwater amphipods with 95% probability (based on Kéry 2002). However, this calculation is based on the mean detection rate and does not account for the substan-

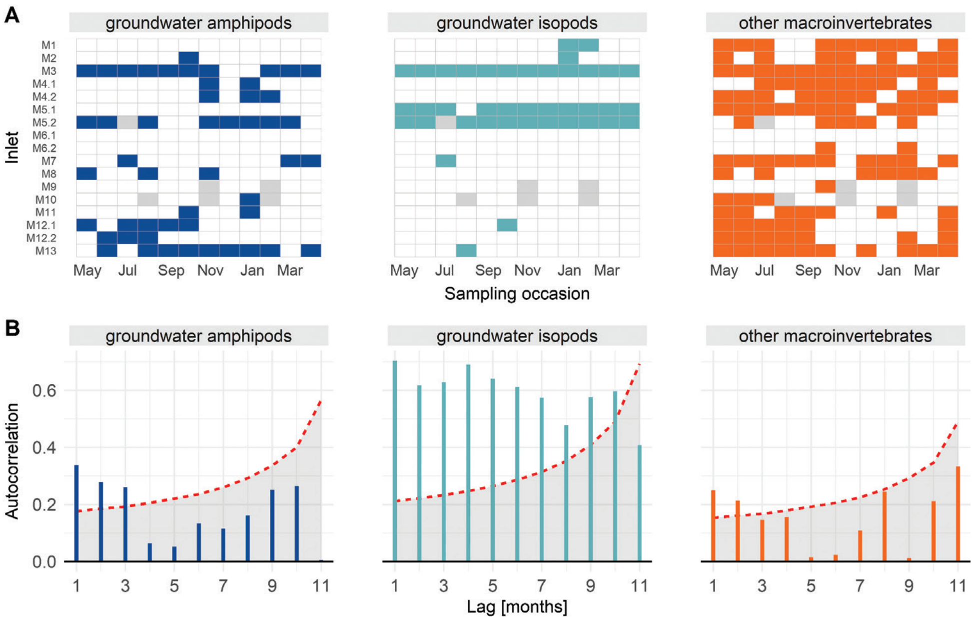

Figure 5. A capture histories across the sampling occasions for the three organism groups. Filled tiles mark presence and empty tiles mark absence of the corresponding group, while tiles marked in light grey depict occasions where no sample was taken. Sampling occasions (x-axis, approximated by month for comparability to other plots) consist of monthly one-week filtering periods **B** temporal autocorrelation of presence–absence of the organism groups. The lag is based on subsequent, monthly samples. The grey area (confined by red dashed line) marks 95% confidence band, autocorrelations larger than the band are significant.

Table 2. Groundwater amphipod occurrence (in number of inlets and number of specimens) in the weekly dataset using the filternet method. Specimens that could not be identified to the species level are listed as *Niphargus* sp.

Species	Number of inlets	Number of specimens
Niphargus tonywhitteni	46	177
Niphargus auerbachi	39	77
Niphargus fontanus	24	153
Crangonyx cf. *subterraneus*	14	37
Niphargus puteanus	3	83
Niphargus arolaensis	2	10
Niphargus thienemanni	2	4
Niphargus sp. Elgg	1	1
Niphargus ruffoi	1	1
Niphargus sp. (undet.)	20	36
Groundwater amphipods (combined)	78	579

tial variation in groundwater amphipod detection rates among different inlets. At the species level, we noticed considerable variation in whether detection rate estimates appeared to stabilize or not, given the sampling effort used in this study. Detection

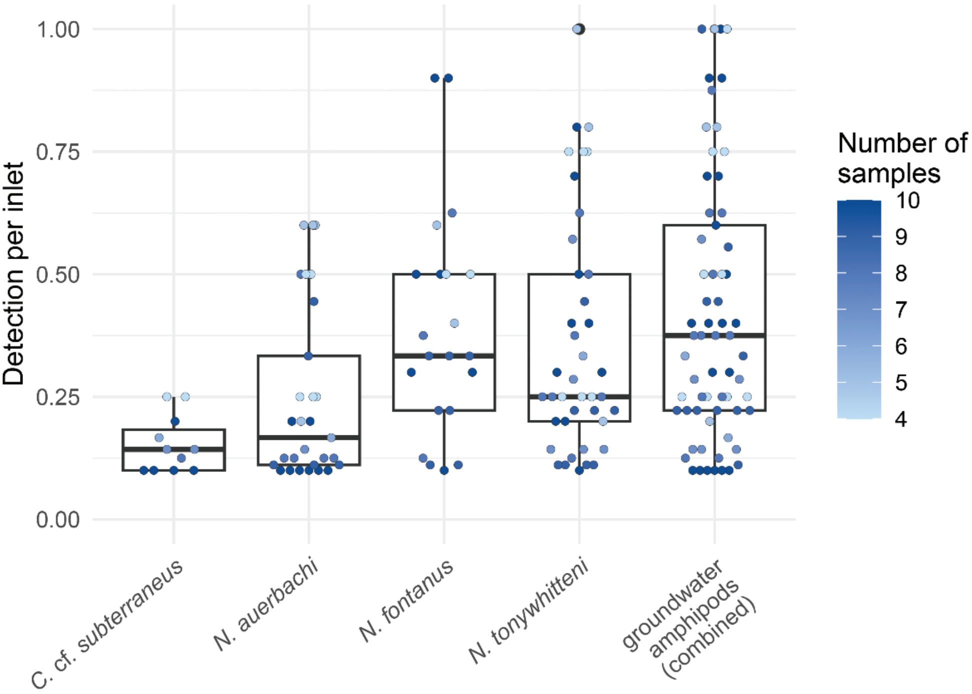

Figure 6. Detection rates of amphipods at groundwater extraction sites. Data is shown for four species and for all groundwater amphipods combined. Each point marks the detection rate of a given species at a certain inlet. The filling indicates how many samples were available from the respective inlet to calculate the detection rate, with darker filling indicating more samples. Inlets with at least four sampling occasions were plotted. Boxes give the median and interquartile range (IQR, hinges at 25% and 75% quantiles), with whiskers extending from the box hinges to ±1.5 * IQR.

rates for *N. fontanus* and *N. tonywhitteni* stabilized around a detection of 0.37 and 0.3, respectively, at threshold levels set to 6 samples or higher. However, when setting a too restrictive threshold and thus including very few inlets per species, detection rates seemed to destabilize again. The detection rate for *N. auerbachi* did not stabilize and instead decreased almost linearly with increasing samples per inlet. As the maximum number of sampling occasions was 10 per inlet, we could not identify detection rates smaller than 0.1. The mean detection rate of *C.* cf. *subterraneus* converged towards this lower detection limit (Fig. 7).

Discussion

We assessed temporal variability in the occurrence and abundance of macroinvertebrates detected from shallow groundwater aquifer samples. While no seasonal pattern was found for obligate groundwater amphipods and isopods, we found a seasonal pattern in the remaining macroinvertebrates (consisting predominantly of stygophiles and

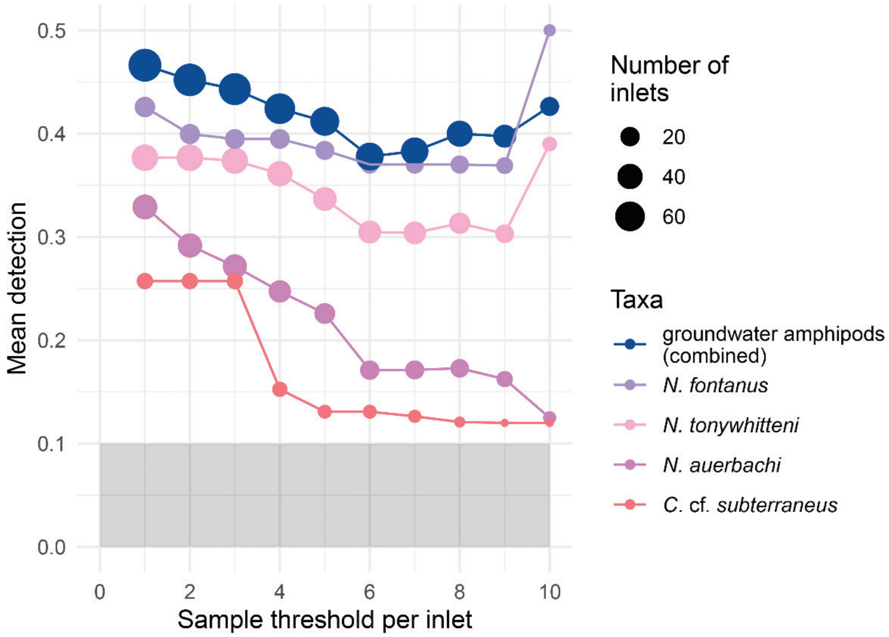

Figure 7. Detection rates of groundwater amphipods depending on number of samples collected per inlet. Detection was calculated as mean over all inlets where the given species or group occurred and then repeated reducing the data to inlets with a given threshold of minimal sampling occasions. Standard deviations are reported in Suppl. material 1: fig. S3. Point sizes show how many inlets were included for calculating the mean. The area below a mean detection rate of 0.1 is shaded, because the maximum number of samples per inlet is 10, and thus smaller detection rates per species could not be captured.

stygoxenes), suggesting differing seasonal effects of environmental conditions on the detectability of obligate and facultative groundwater organisms. Detection rates for individual groundwater amphipod species were highly variable, with a generally high heterogeneity among inlets. Some species had very low detection probabilities, implying that a substantial number of samples are required to distinguish true from false absence at a given inlet.

Temporal consistency in obligate groundwater macroinvertebrates

Organisms from the surface are exposed to strong environmental fluctuations and many of them thus show seasonal patterns in detectability. We identified a peak in macroinvertebrates' abundances (predominantly shaped by EPT larvae) around July, which may partly reflect the seasonal life cycle of these insects (see also Burgherr et al. 2002; Lubini et al. 2012). Their occurrence might indicate a close hydrological connection between the groundwater and nearby surface waters (Stanford and Ward 1988). In contrast, no seasonal effect was found for groundwater amphipod and isopod abundances, suggesting temporally more consistent detectability in these organisms, possibly reflecting the longer development time of stygobites (Hose et al. 2022).

Precipitation did not have any significant effect on detectability of either of the studied organism groups, despite being identified as a key driver of community dynamics and energy flows in other groundwater systems (Saccò et al. 2020; Saccò et al. 2021; Saccò et al. 2022). However, a possible effect might be confounded by the inclusion of a seasonality covariate along with precipitation in the models, particularly as the main peak of EPT detection and (higher than the long-term average) precipitation coincide in July. Since our data is limited to only one year, some effects may be concealed. The non-significant precipitation result might also arise from varying time lags among aquifers, at which precipitation affects groundwater ecosystems (determined by factors such as geology). Generally, the models explained a moderate proportion of the deviance, suggesting that while the included covariates and smooth terms captured some of the variability in the organisms' abundances, other factors not included may also play a role.

Groundwater amphipods: low detection rates and high variability among inlets and species

Out of nine groundwater amphipod species found in the Töss catchment, five species occurred at only 1–3 inlets each, a pattern that is characteristic for groundwater communities (Hahn and Fuchs 2009; Trontelj et al. 2009; Knüsel et al. 2024b). The other four species occurred at 10 or more sites each, allowing the calculation of detection rates with sufficient spatial replicas. The mean detection rates of *N. fontanus* and *N. tonywhitteni* stabilized when the effort consisted of six or more sampling occasions per inlet, indicating that detection might be overestimated when less repeated samples are available per inlet. In contrast, the detection rate of *N. auerbachi* did not stabilize and the one of *C.* cf. *subterraneus* showed an asymptotic shape towards the lower detection limit of 0.1. For both species, we expect true detection to be lower than 0.1. Results from Knüsel et al. (2024b) using an occupancy modeling approach based on a Swiss-wide dataset confirm the expected low detection probabilities of *N. auerbachi* and *C.* cf. *subterraneus.* Considering all groundwater amphipods combined, the lowest mean detection rate would translate to a minimum of 6.3 samples necessary to infer their absence with 95% probability. In comparison, findings from southwestern Germany indicated that 2–17 samples are necessary to collect 95% of species in vertical wells (boreholes) (Gutjahr et al. 2013). However, we found a high heterogeneity in detection rates between sampled inlets. Consequently, any inlet with an amphipod detection rate below the mean would require more than the computed 6.3 samples to infer the absence of groundwater amphipods. For example, an inlet with a detection rate of 0.1 would require 29 samples to infer the absence with 95% probability (Kéry 2002). However, it is not completely resolved yet to what extent the heterogeneity in detection rates reflects underlying environmental factors and community characteristics, or is an artefact of the sampling methodology (Knüsel et al. 2024b).

We acknowledge that we here only analyzed data from inlets where the respective species were present. Because the occurrence process was not modeled, neglecting false absences where the species is present but not detected likely results in overestimating true detection rates (see e.g., Kéry and Royle 2016; MacKenzie 2018; Knüsel et al. 2024b). Further, one must be careful to apply conclusions on detection rates and sampling effort from this region to other regions, as amphipod occurrence and likely also detection rates might be different, for example in mountainous areas (Knüsel et al. 2024a). The occurrence data analyzed here encloses a specific region in Switzerland, that exhibits a rather high amphipod occurrence and richness (Studer et al. 2022) and might have served as refugium during the Last Glacial Maximum (Knüsel et al. 2024a). If the necessary sampling effort based on detection rates is to be estimated, we advise to carefully consider the sampling area and species expected to occur, the heterogeneity in detection rates among sampling sites, and variability in environmental conditions among sites. Generally, when detection probabilities are low but spatially heterogeneous, monitoring programs should cover a relatively large time frame with sufficient spatial and temporal replicates and take into account possible heterogeneity in detection among species to ensure accurate assessments (Boulinier et al. 1998; MacKenzie et al. 2002).

Considerations on standardization and sampling design

Studies of groundwater communities in natural springs commonly standardize abundances by discharge volume (Di Lorenzo et al. 2005; Mori et al. 2015). Since the inlets sampled here were built by humans, the effects of outflow rate on organism abundances might not be directly comparable to that of natural springs. We here found a negative effect of outflow when abundances were standardized per discharge volume, which indicates a possible 'dilution effect' of the organism density. In other words, the density of organisms might be independent of an inlet's discharge rate, and standardizing by volume could introduce a bias in the analysis. Hence, it may be sufficient to standardize sampling effort based on filtering duration, independent of the discharge volume of the given inlet, or to add the mean outflow rate as a random effect.

When occurrences are correlated across time, subsequent samples might be biased. Here, we detected temporal autocorrelation in groundwater amphipod occurrence up to three months apart, which is expected given the slow life history and limited mobility of stygobites (e.g., Hose et al. 2022). For standardized monitoring of groundwater amphipods collected at drinking water extraction facilities, given a limited number of sampling occasions per year, we recommend to place sampling occasions evenly throughout the year and to avoid clustering them in a short timeframe, as the latter could lead to correlated false absences. Generally, one could also consider extending the filtering periods from one up to multiple weeks, which would increase the chance of collecting groundwater organisms (e.g., Di Lorenzo et al. 2018). However, organisms will start to decompose at some point, which could hinder the identification of those organisms that remained in the filter nets for longer periods. When deciding on the passive sampling periods, it is important to consider whether the goal is to assess occurrence or abundance, as well as the desired level of taxonomic resolution and the identification method (as decomposition likely affects the accuracy of both, morphological and molecular identification).

Choosing the optimal sampling approach

Using citizen science data from drinking water providers has shown to be an effective approach to study groundwater macroinvertebrates (Alther et al. 2021; Knüsel et al. 2024b). However, the generally low abundances and low detection probabilities despite high sampling efforts, in combination with large spatial variability in detection, pose challenges. The question remains how monitoring strategies could be scaled up to cover regional to national scales, in order to assess groundwater ecosystem health. One approach might be to combine multiple sampling strategies and to focus on bioindicator species (Koch et al. 2024 and references therein). Apart from citizen science, one novel methodology that is being applied to study groundwater communities is eDNA metabarcoding (Deiner et al. 2017; Couton et al. 2023b). It is fast, less work-intensive, and provides information on the whole groundwater community. While eDNA samples generally integrate biodiversity of larger spatial and temporal scales than traditional samples (Deiner et al. 2016; Carraro et al. 2020), Couton et al. (2023a) found eDNA to be (yet) less effective in detecting groundwater amphipods than directly filtering the organisms. Nevertheless, combining multiple approaches might give complementary insights into the state and dynamics of groundwater ecosystems and could thus be one way forward.

Conclusion

Our study highlights the temporal consistency of obligate groundwater macroinvertebrate occurrence patterns, contrasting with the seasonal variability observed in other macroinvertebrates (predominantly stygophiles and stygoxenes). Based on the low detection probabilities for many groundwater amphipod species, our findings emphasize the importance of tailored and extensive sampling strategies. For effective monitoring, standardizing sampling effort based on filtering duration rather than discharge volume and ensuring evenly spaced sampling occasions throughout the year is recommended. High variability in detection rates across groundwater amphipod species and sampling sites indicates the need for region-specific approaches.

Open research statement

Data supporting the results are publicly available on Zenodo (DOI https://doi.org/10.5281/zenodo.13828713). Due to sensitivity of the drinking water provider data, coordinates of the spring catchment boxes will not be published (please contact the corresponding authors for requests).

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Supplementary material 1

Supplementary information

Authors: Mara Knüsel, Roman Alther, Marjorie Couton, Florian Altermatt

Data type: pdf

Explanation note: Contains additional images and model specifications.

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Two new species of Collembola (Arthropoda, Hexapoda) from Cova Urbana, a cave under a city

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Abstract

The Collembola fauna of the Cova Urbana located under the city of Tarragona (Spain) is studied, and the climatic characteristics of the cave, its partial geomorphology and its planimetry are presented. For the first time in this cave, some specimens of Collembola have been captured and studied, and two new species for science belonging to the genera *Coecobrya* and *Pygmarrhopalites* are described and illustrated. In addition, a specimen of the genus *Arrhopalites* probably belonging to another new species has been studied, but it could not be described because only one specimen was available. The presence of so few specimens and only four species is attributed to the absence of organic matter from percolation and detritus (oligotrophic cave).

Keywords

Arrhopalitidae, cave fauna, Entomobryidae, new species, troglobionts, urban cave

Introduction

The “Cova Urbana de Tarragona” (Fig. 1) is a cavity located in the basement of the city of Tarragona. It was discovered accidentally, when laying the foundations of a new building in 1996, although there was already news of its existence in ‘Tarragona

Figure 1. One of the cave galleries of Cova Urbana, showing its character flooded by the water table. Author: Víctor Ferrer Rico, a professional photographer specialized in caves.

Monumental', a book from 1849 (Albiñana et al. 1849), when investigating a Roman water conduction gallery. An opening in this gallery leads to a natural cavity with wells, lakes, large rooms, and a highly developed underwater network, not fully explored. The cavity can be visited, with a prior request to the "Societat d'Investigacions Espeleològiques de Tarragona (SIET) (Arnabat Castilla 1998; Ferrer 2014).

Methods

Characteristics of studied cave

This cavity, excavated in a small core of dolomites from the Jurassic period, has developed through a stratification joint, expanded by the corrosion of water from the phreatic level marked by the base level of the Mediterranean. There are also some samples of water circulation under hydrostatic pressure. Subsequent graviclastic processes have created rooms of notable volume, with the consequent chaos of resulting blocks. The chemolithogenic process is very small, taking into account the development of the cavity: it is limited to the base of the entrance shafts and first rooms. The enormous volume of water in the cavity is of phreatic origin and the analyzes carried out by the "Societat d'Investigacions Espeleológiques" show a very low level of chlorides and sodium, so it is ruled out that there may be contact or leaks of seawater, given its proximity (Llopis Lladó 1970; IGME 1986). The karstifiable dolomite massif, where the cavity is developed, is isolated by alluvial terrain and Quaternary foothill areas and without contact with other nearby massifs with the same characteristics (Musara, Montsant) (ICGC 2023). Towards the east, there is an outcrop of Miocene reef calcarenites, weakly karstifiable, in contact with the dolomites of the cavity. The absence of forms of absorption from the outside may suggest the possibility that it is a cavity of hypogenic origin (Miñarro et al. 2021). A detailed geomorphological study of the cavity would be necessary to accurately determine this provenance.

The climatic conditions of the cavity are exceptional, with a very high-temperature range along the cave (between 20.1 °C and 21.9 °C.) and a relative humidity that is also very high (HR 97.9), a logical consequence of the evaporation of water from the cavity. The concentration of ambient oxygen varies according to the area: from 18.7% to 20.5%, although in one of the Roman galleries half-clogged by debris, 17.2% was recorded. The measurements were carried out with a Hibok C-315 digital thermo-hygrometer and a Hibok P2-250 digital oximeter.

Biocenosis and sampling

A priori, the biological condition of this cavity could be described as almost azoic in terms of troglobitic fauna, considering the faunal richness of relatively nearby cavities. The Cova Urbana is a very mineral cavity, that is, being without contact with the outside, there is no organic, vegetal, or animal matter inside, which could facilitate the formation of a trophic chain: the scarcity of fauna may be motivated due by the poor ecological conditions (limited availability of food) of this underground habitat and the geological isolation of the cavity (Racovitza 2004; Sendra 2023). Despite the numerous visits and the pitfall and aquatic traps, only a few terrestrial isopods (possibly *Catalauniscus* sp.) located at the base of the entrance wells; a specimen of 'very small shrimp', possibly an amphipod, accidentally captured when taking one of the water samples for analysis (J.M. Plana, personal communication). If we count the trogloxene and troglophile fauna, the relationship becomes a little more extensive, although their habitat is the Roman galleries and entrance wells, which maintain a certain communication with the outside environment: spiders from various families (some under study), some Blattidae, very abundant in the upper levels, Diptera (*Limonia* sp.) and a Myriapoda, possibly *Lithobius* sp. that could not be captured.

Springtails are the most representative troglobitic fauna of the cavity, and for this reason, sampling has focused on their capture. They have only been found –and after many attempts– in the Maginet Room (Fig. 2), near the main siphon of the cavity, in a piece of wood abandoned by the cave conditioning works. The subsequent placement of attraction baits at this point facilitated the collection of springtails, but only *Folsomia candida* Willem, 1902 was captured.

Figure 2. Original diagram of the topography of Cova Urbana elaborated by the Societat d'Investigacions Espeleològiques de Tarragona team.

Material processing

After the preliminary sorting to separate the Neelipleona and Symphypleona from other Collembola, some representative specimens of each species were selected and mounted in Hoyer's medium for observation under a microscope, and some specimens were cleared in Nesbitt's fluid. The remaining samples were stored in 70% ethyl alcohol. The slides were observed under two microscopes: an Olympus BX51-TF (Olympus Group, Tokyo, Japan) with multiple viewing and phase contrast and a Zeiss model Axio Imager.A1 with differential interference contrast (DIC). For measurements, a U-DA drawing attachment UIS (Universal Infinity System) and a scale calibrated with a slide by Graticules Ltd., Cambridge, UK (1 mm divided in 100 parts) were used. For SEM (Scanning Electron Microscopy) the specimens were dehydrated using a series of ethyl alcohol followed by critical-point drying in CO_2, then mounted on aluminum SEM stubs, and coated in an Argon atmosphere with 16 nm gold in a sputter-coater Emitech Ltd., Strovolos, Chipre, model K550. SEM observations were made with a FE-SEM Zeiss model Sigma 300 VP (Zeiss, Oberkochen, Germany).

Nomenclature

The terminology for *Pygmarrhopalites* Vargovitsh, 2009 used in descriptions follows Fjellberg (1984) for the outer maxillary palp; Christiansen and Bellinger (1996) for Ant III sensory organ; Bretfeld (1999) for Abd VI; Christiansen (1966) and Christiansen and Bellinger (1998) for empodium; and Vargovitsh (2009, 2017), for head, body, and legs. The material has been deposited at MZNA—Museum of Zoology at the University of Navarra (Pamplona, Spain). The terminology for *Coecobrya* follows Jordana and Baquero (2005) and Zhang and Deharveng (2015) for dorsal chaetotaxy.

Abbreviations used in the description

Abd	abdomen or abdominal segment I–VI
al	anterolateral s-chaeta
as	anterosubmedial s-chaeta
Ant	antennal or antenna/ae
a.s.l.	above sea level
Mc	macrochaeta/ae
mes	mesochaeta
mic	microchaeta
ms	microsensillum/a
PAO	postantennal organ
psp	pseudopore
Th	thorax, or thoracic segments

Institutions

MZNA	Museum of Zoology at the University of Navarra, Pamplona, Spain

Results

Faunistic study

Throughout three samplings, four species belonging to the Collembola group have been found, and one mite specimen (possibly due to accidental presence). Regarding the Collembola, in addition to the two species described, a single specimen of *Arrhopalites* sp., and 17 specimens of *Folsomia candida*, a cosmopolitan species present in many biotopes in addition to in caves, where it sometimes causes problems due to transporting fungi from the outside that alter the ecosystem were also collected.

Taxonomic part

Class Collembola Lubbock, 1873
Order Entomobryomorpha Börner, 1913
Family Entomobryidae Schäffer, 1896 sensu Godeiro et al. 2023
Subfamily Entomobryinae Schäffer, 1896 sensu Godeiro et al. 2023
Genus *Coecobrya* Yosii, 1956

Coecobrya urbana **Jordana & Baquero, sp. nov.**

https://zoobank.org/18825AE8-013D-42A0-9A18-30994FDAF0FA

Figs 3–8

Type material. ***Holotype*** • female on slide, Spain: Tarragona city, Tarragona province, Cova Urbana, geographic coordinates 41.114193, 1.248222, 15 m a.s.l. (the entrance is in an urban area), 6/vii/2022, dark zone of the cave, by aspirator (MZNA code 782957). ***Paratypes*** • same data as holotype, three females on two slides (MZNA codes 782958, 782972 to 782973), 10 in ethyl alcohol (MZNA codes 782961 to 782970) and one mounted on stub for SEM observation (MZNA code 782971) • 29/vii/2022, two females on slide (MZNA codes 782972 to 782973), and two specimens in ethyl alcohol (code samples 782987 to 783988) • 1/vii/2023, 10 specimens in ethyl alcohol (code samples 782975 to 783985) and one mounted on stub for SEM observation (MZNA code 782971). All Floren Fadrique leg (BIOSP, Associació Catalana de Biospeleologia). Specimens deposited in MZNA.

Description. ***Habitus*** as in Fig. 3. Body length (without antennae) up to 1.7 mm (holotype 1.6 mm). No scales. Eyes absent. Color whitish in alcohol, without any pigment. Antennae 1.41–1.85 times the length of the head (n = 4; lower proportion in smaller specimens). Abd IV 3.4 times as long as Abd III.

Antennae (Figs 4, 5). Antennal segments not subdivided nor annulated. Ant I dorsally with two Mc type one (capitate), numerous Mc type two (acuminate) with different sizes (Christiansen 1958), and three small mic at basis, forming a triangle, with the distal one bigger; ventrally with 9–10 basal smooth spiny mic of various sizes; one distal pseudopore. Ant II dorsally without paddle-like chaetae in its distal part, with small sensilla; two distal pseudopores. Ant III sensory organ as usual, with five sensillae and the special ones swollen. Ant IV 1.55–1.83 times longer than Ant II or Ant III, not subdivided, without apical bulb. Subapical organite slightly knobbed, with a basal accessory special small mic.

Head. Clypeal and labral areas in Fig. 6A; prelabral and labral chaetae (4/5, 5, 4) all smooth, central chaetae of row a thicker, and external on small papillae. Maxillary outer lobe with one apical and one basal chaeta, and three sublobal hairs (Fig. 6B). Papillae E of labrum in Fig. 6C. Chaetae of labial basis all smooth (-mrel$_1$l$_2$) similar in length except for 'r' short. One or two special 'x' chaetae. On each side of linea ventralis, 5–6 smooth and 1–2 ciliate chaetae (Fig. 6D).

Legs. Legs devoid of scales, covered with ordinary ciliated chaetae of various lengths, mic not seen. Coxa of leg I with one proximal psp and two chaetae posteriorly; coxa of leg II with eight chaetae in anterior row, two chaetae in posterior row and one proximal psp; coxa of leg III without proximal psp. Trochanteral organ with 15 smooth, straight, unequal spine-like chaetae (Fig. 7A). Tenent hair spatulated (22–30 µm). Tibiotarsi I–III with a single row, interior, of appressed chaetae, the most distal on tibiotarsus III smooth. Claw slender (Fig. 7B–D); claw I and II subequal, claw III slightly longer. All claws with a pair of basal inner teeth at approx. 35−40% and two unpaired inner teeth at 55% and 80–90% of inner edge from basis respectively. Empodium approx.

Figure 3. *Coecobrya urbana* sp. nov. habitus at SEM. Scale bar: 0.1 mm.

0.8 times as long as inner edge of claw, slightly swollen baso-internally, truncated apically, devoid of inner tooth, with a big outer tooth at 1/2 of its length.

Ventral tube (Fig. 7E). Anterior basal with 4 + 4 ciliated Mc; anterior distal flaps with 7 + 7 smooth chaetae; posteriorly with eight smooth chaetae, two of them as mic.

Furcal complex. Tenaculum with four large teeth and one ciliated chaeta. Manubrium covered with ciliated chaetae, and seven smooth chaetae on two rows on posterior side. Manubrial plate with two pseudopores and two ciliate chaetae, one each side. Dens without spines, annulated and covered with ciliated chaetae on both sides, and 2 + 2 basal smooth posterior chaetae. Distal smooth part of dens similar in length to mucro. Mucro falcate, basal spine long, nearly reaching the tip of the mucronal tooth (Fig. 7F).

Dorsal chaetotaxy (Fig. 8A, B). Head: dorsal cephalic chaetotaxy with four antennal (An_{1-3}, An_{3a1}), four anterior (A_0, A_{2-3}, A_5), four median (M_0, M_{1-2}, M_4) and seven sutural (S_0, S_{1-5}, S_{4i}) Mc. Th II with three (m_{1-2}, m_{2i}) medio-medial (area T1) (Jordana and Baquero 2005), three (a_5, m_4, m_{4p}) medio-sublateral (T_2) and 15–18 posterior Mc, 1 + 1 ms and 2 + 2 sensillae laterally. Abd II without Mc above internal bothriotrichum (area A1), three (m_3, m_{3e} and m_{3ea}) central (A2) and one (m_5) lateral Mc. Abd III without Mc on areas A3 and A4, only one (m_3) on area A5, and three (a_7, pm_6 and p_6) lateral Mc. Bothriotrichum not surrounded by modified chaetae. Abd IV with four central Mc (A_3, A_6, and B_{4-5}), four (E_{2-4}, D_3) lateral Mc and about nine long sensilla. Abd V with five Mc, and 3 + 3 sens. The S-chaetae formula is the usual for the family.

Ecology. The specimens were captured in the dark area of the cave (Sala Maginet), in the deep and isothermal area of the cavity, very close to the aerial end of the cave (from here, the route is underwater), on wood with fungus. After placing a cheese bait during 15 days on that same wood, only specimens of the *Folsomia candida* species were captured.

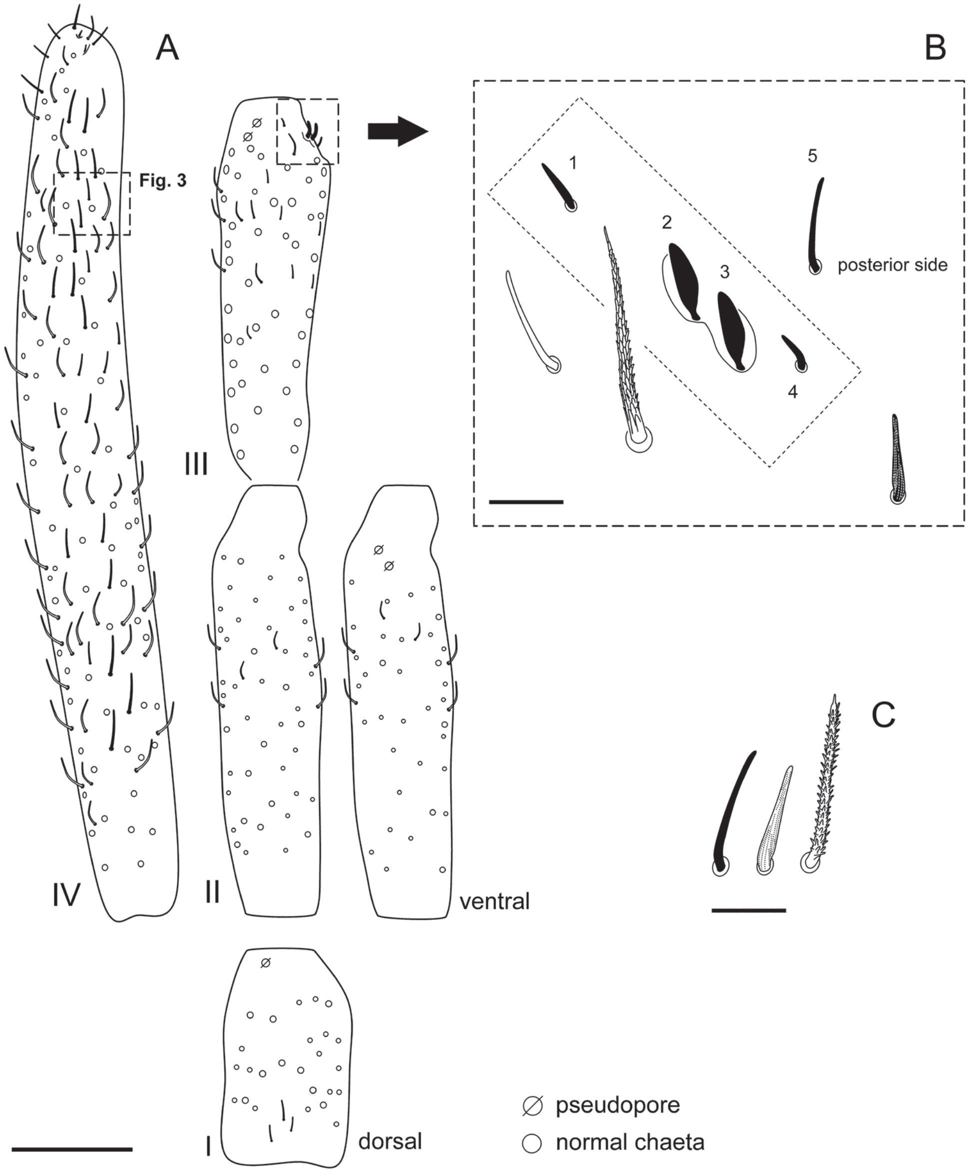

Figure 4. *Coecobrya urbana* sp. nov. **A** antennal segments I–IV (dorsal view except for Ant II, both) **B** detail of the Ant III sensory organ, and **C** the three types of sensilla on Ant IV. Scale bars: 0.05 mm (**A**); 0.005 mm (**B–C**).

Etymology. The new species is named after the name of the cavity where it was found, Cova Urbana, which in turn is so named because its entrance is in the heart of a city.

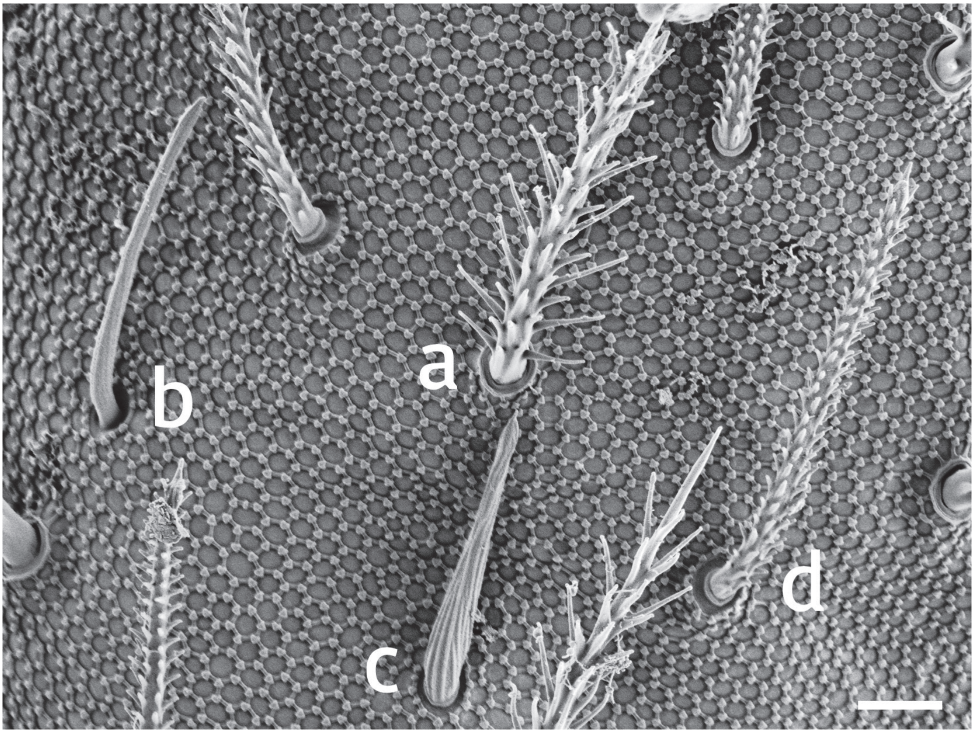

Figure 5. *Coecobrya urbana* sp. nov.: antenna, detail of Ant IV and different types of Mc (**a**) and sensilla (**b–d**). Scale bar: 0.002 mm.

Remarks. The new species differs morphologically from other known *Coecobrya* species by presenting four internal teeth of claw. To the best of our knowledge, *Coecobrya montana* (Imms, 1912) *sensu* Zhang, Deharveng & Chen, 2009 from India, *C. submontana* Stach, 1960 from Afghanistan, *C. troglobia* Jantarit & Nilsai, 2021 (in Nilsai et al. 2021) from Thailand and *C. decemsetosa* Jordana & Baquero, 2023 (in Baquero et al. 2023) from Portugal are the only four described species of *Coecobrya* with four teeth on the internal claw. Unfortunately, the chaetotaxy of *C. montana* and *C. submontana* are unknown (the type material was observed by the authors, and the conservation of the specimens made no possible to observe the chaetotaxy), but have small differences with *C. decemsetosa* and the new species described here in the morphology of the claw, a character used traditionally for differentiate the species. In addition, *C. decemsetosa* poses large differences in number of Mc on tergite from Th II–Abd V too. *C. submontana*, which has been studied after requesting some specimens deposited in Stach's collection of PAN (Polska Akademia Nauk, Warszawa, Poland), from a cave near Kabul, has a short basal spine on the mucro, and the dorsal tooth of the claw in an intermediate position between the base and the paired teeth of the inner edge. *Coecobrya troglobia* can be differentiated from the new species because it has longer antennae

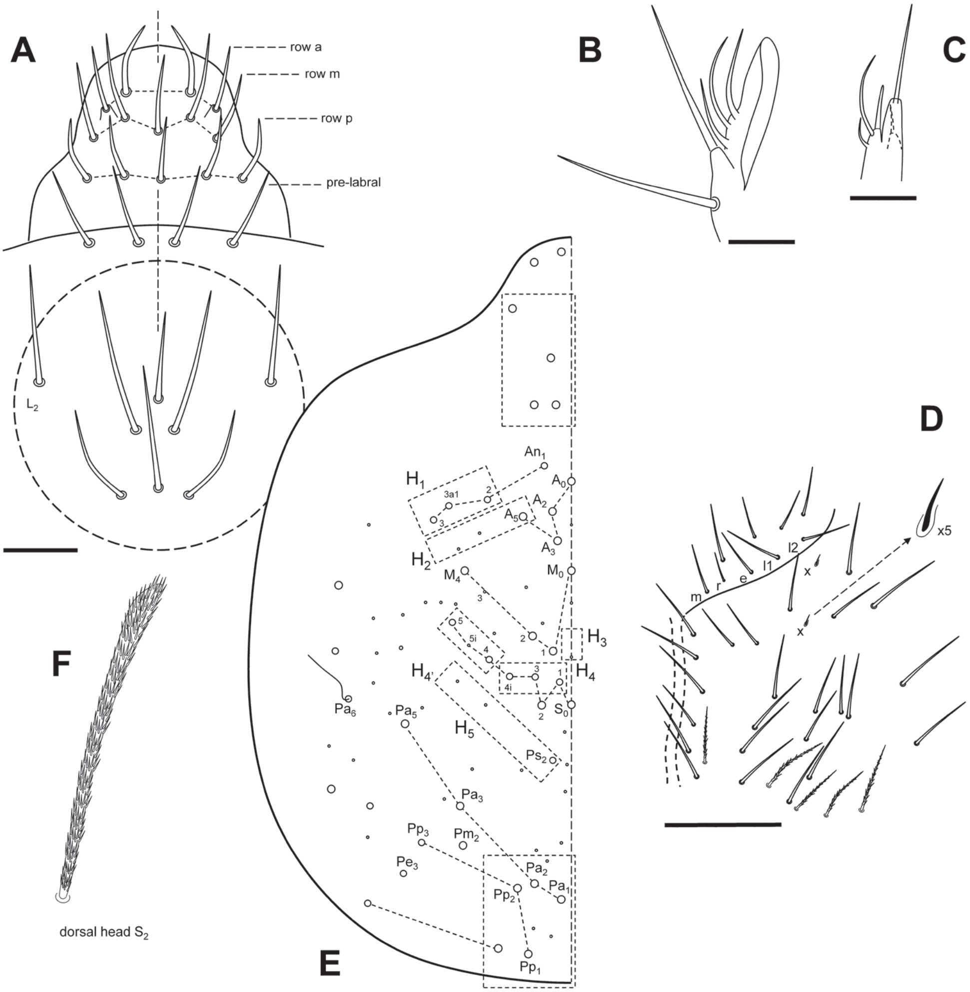

Figure 6. *Coecobrya urbana* sp. nov. **A** clypeal, prelabral and labral areas **B** maxillary outer lobe **C** papilla E of labrum **D** labial basis **E** head chaetotaxy. Scale bars: 0.02 mm (**A–C**); 0.075 mm (**D**).

(4–6.8 times the length of the head), the paired teeth on inner claw closer from the basis (30%), the external empodium serrated at its posterior half, only two Mc on dorsal Abd II, five central Mc (each side) on Abd IV and manubrium without smooth Mc.

Order Symphypleona Börner, 1901 sensu Bretfeld 1986
Suborder Appendiciphora Bretfeld, 1986
Superfamily Katiannoidea Bretfeld, 1994
Family Arrhopalitidae Stach, 1956
Genus *Pymarrhopalites* Vargovitsh, 2009

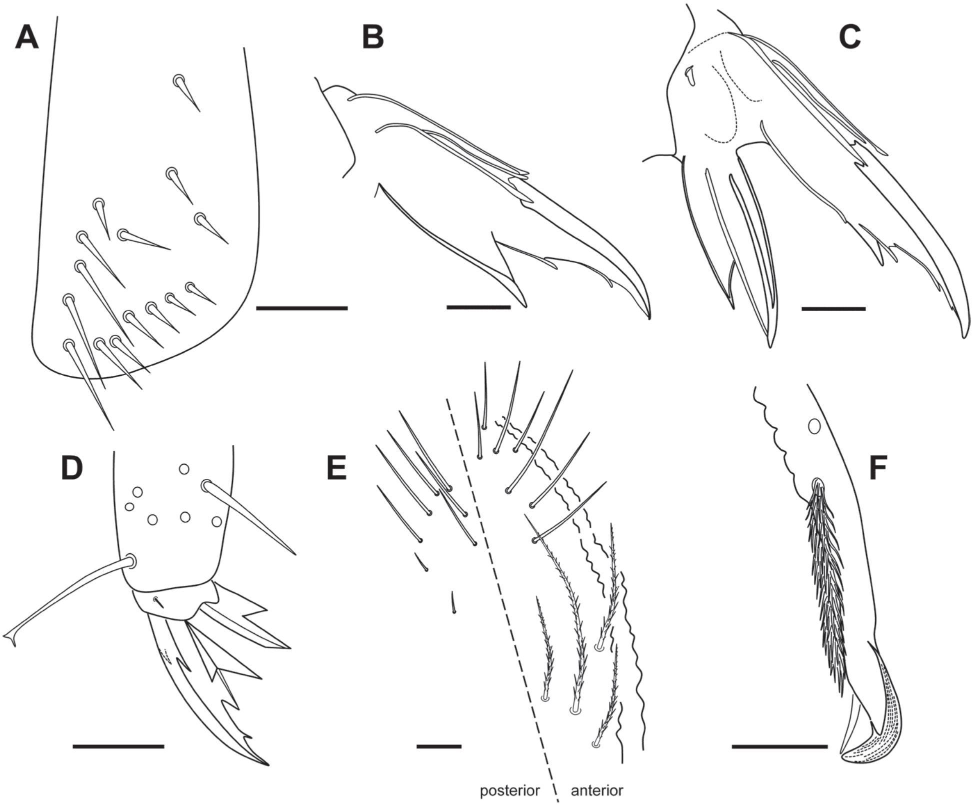

Figure 7. *Coecobrya urbana* sp. nov. **A** trochanteral organ **B** claw 1 **C, D** claw 3; **E** ventral tube **F** tip of dentes and mucro. Scale bars: 0.02 mm (**A, D, E**); 0.005 mm (**B–C**); 0.01 mm) (**F**).

Pygmarrhopalites sietae Baquero & Jordana, sp. nov.

https://zoobank.org/43E780DE-E64A-43B6-A2DD-B8BE6FACEC8D
Figs 9–14

Type material. ***Holotype*** • female on slide, Spain: Tarragona city, Tarragona province, Cova Urbana, geographic coordinates 41.114193, 1.248222, 15 m a.s.l. (the entrance is in an urban area), 6/vii/2022, dark zone of the cave, by aspirator (MZNA code 782954). ***Paratypes*** • same data as holotype, three specimens on slide (MZNA codes 782955 and 782986), and one mounted on stub for SEM observation (MZNA code 782953). All Floren Fadrique leg (BIOSP, Associació Catalana de Biospeleologia). Specimens deposited in MZNA.

Description. Female. ***Body*** not pigmented; length (holotype): head, 0.23 mm; body 0.62 mm.

Head. Eyes 1 + 1, unpigmented. Clypeal area, row a: 2 + 2 and an axial chaeta; row b: 2 + 2; row c: 2 + 2; row d: 4 + 4; row e: 5 + 5; row f: 4 + 4. Inter-antennal area, row α: 2 + 2;

Figure 8. *Coecobrya urbana* sp. nov. **A** Th II–Abd II **B** Abd III–VI **C** detail of two Mc and a long sensilla of Abd IV. Scale bar: 0.05 mm (**A–B**); 0.01 mm (**C**).

row β: 1 + 1 and an axial chaeta; rows A and C: 2 + 2 and an axial chaeta; row B: 1 + 1 and an axial chaeta; row D: 2 + 2 chaeta. Lateral chaetae of rows C and D are not spine-like (Fig. 9A). Chaetotaxy of the mouth region. Labrum: pre-labral/labral chaetotaxy: 6/554. 2 + 2 chaetae near the ventral groove. Maxilla: apical chaeta of the maxillary outer lobe with a short and thin subparallel branch at the base; sublobal plate with three sublobal hairs.

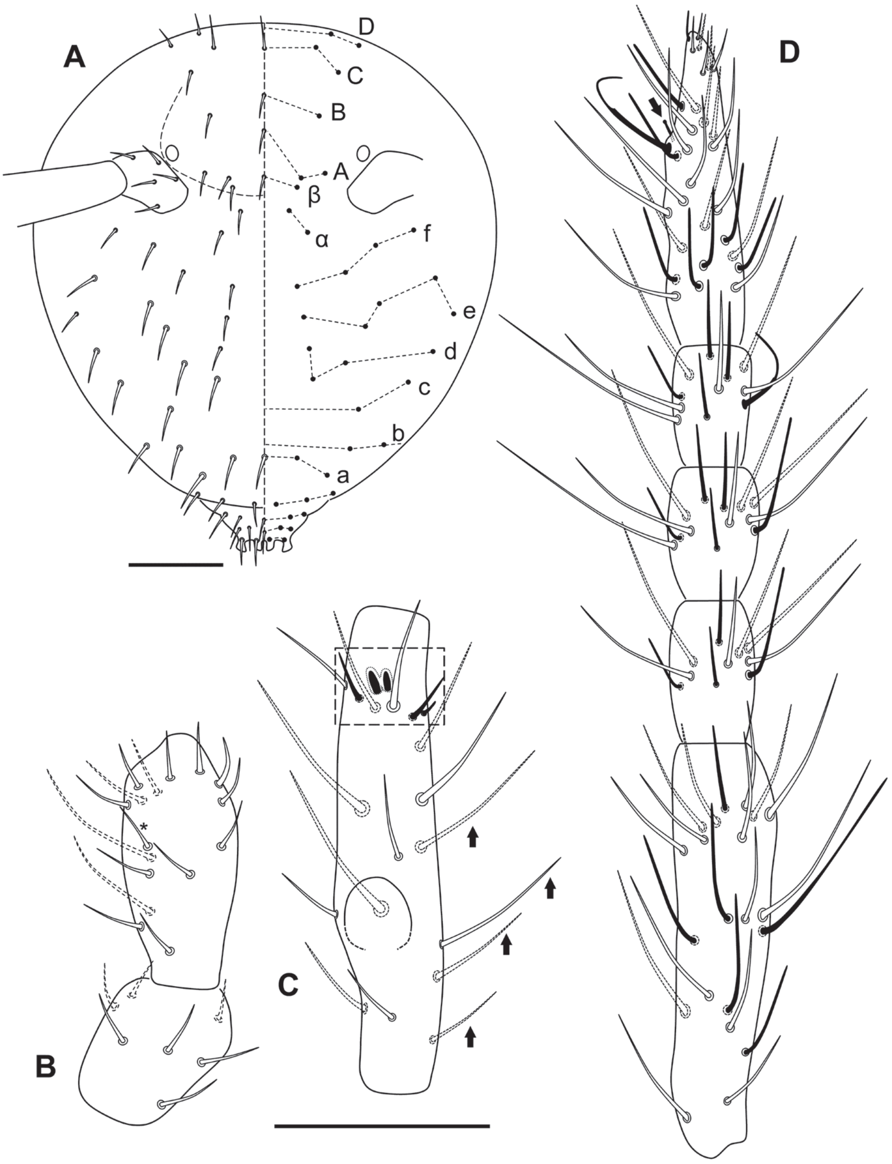

Figure 9. *Pygmarrhopalites sietae* sp. nov. **A** head chaetotaxy **B** Ant I–II (* means that this chaeta has been observed only in one specimen) **C** Ant III (the arrows point to chaetae that are noticeably thinner than the others and the sensory organ are included in a rectangle with the chaetae in black) Ant IV (the arrow points to the knobbed subapical organite). Scale bar: 0.05 mm.

Antenna (Fig. 9B–D): I/II/III/IV, 0.030/0.058/0.106/0.090-0.032-0.32-0.030-0.070; shorter than the body (ratio 0.72) and ratio Ant/head as 1.95; basal subsegment of Ant IV 0.84 times longer than Ant III. Ant I with seven chaetae, any as mic. Ant II

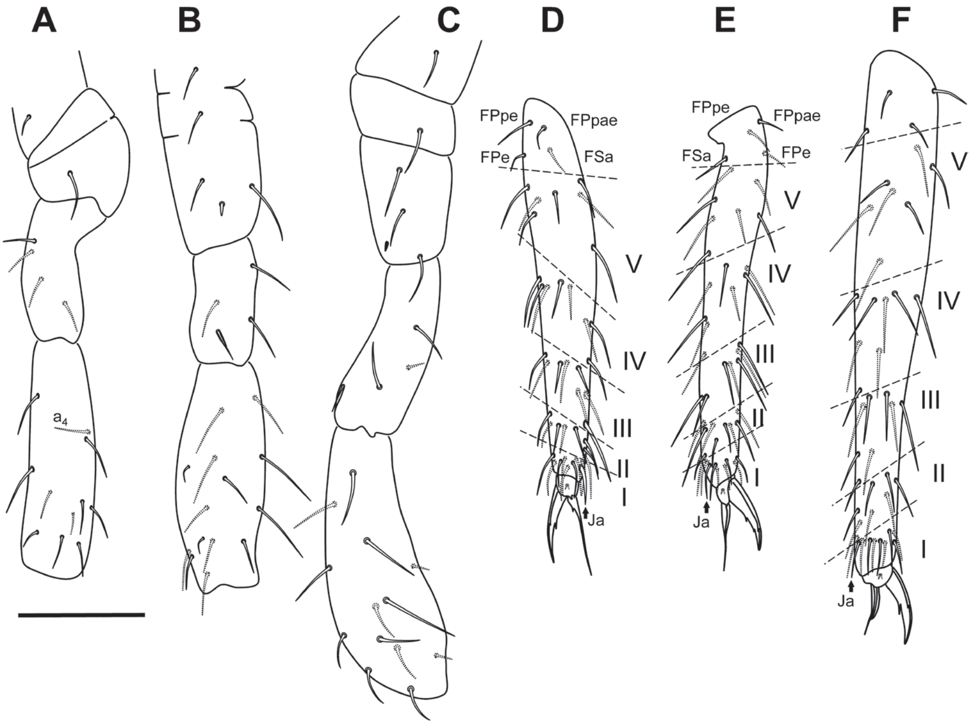

Figure 10. *Pygmarrhopalites sietae* sp. nov. **A–C** precoxa 1–2 and coxa of legs 1 to 3 respectively **D–F** tibiotarsi, claw and empodium of legs 1 to 3. Scale bar: 0.05 mm.

with 15 chaetae, two interior ones longer than others (16 because an additional one in the second whorl on one antenna of one specimen). Ant III with a conspicuous papilla, 15 chaetae, the two sense rods, and two thin setae and one short lateral sensilla; Ant IV with five distinct subsegments, with four evident whorls: one at the end of the first and three on the second to fourth subsegments. Apical subsegment with knobbed subapical organite; one of the chaetae on this area has a narrowing since the terminal half.

Legs. (Figs 10A–F, 11): Foreleg pre-coxae 1, 2, and coxa with 1, 0, 1 chaetae respectively. Trochanter with three anterior and one posterior chaetae. Femur with 11 chaetae, a_4 turned perpendicularly to the longitudinal axis of the segment. Tibiotarsus: whorl I with nine chaetae among which Ja curved and somewhat thickened, II–V with 8, 8, 8, and 7 chaetae respectively; region F with three primary FP chaetae (e, ae, pe) and secondary chaeta FSa. Pre-tarsus with one anterior and one posterior chaetae. Foot complex: claw thin, without tunica evident, with inner tooth and two pairs of indistinct lateral teeth (25 and 60% from claw basis); empodium thin, with corner tooth (Fig. 11), and long apical filament surpassing the tip of the claw. Mid leg pre-coxae 1 and 2 with 1, 1 chaetae respectively, pre-coxal process present, coxa with two chaetae and a microsensillum. Trochanter with three chaetae and a trochanteral

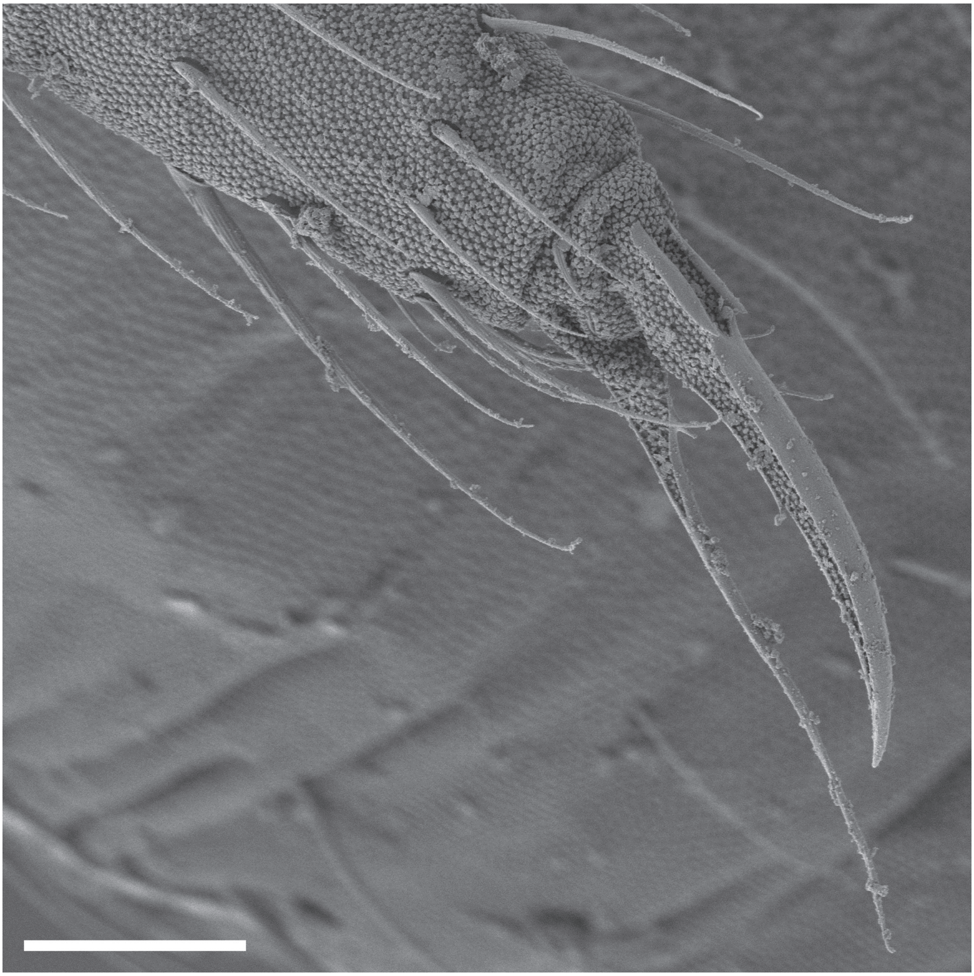

Figure 11. *Pygmarrhopalites sietae* sp. nov. Claw and empodium of leg 1. Scale bar: 0.001 mm.

organ. Femur with 14 chaetae, p_1 and p_3 very small. Tibiotarsus: whorl I with nine chaetae, whorls II–V with 8, 8, 8, and 7 chaetae respectively; region F with three FP chaetae and FSa chaeta. Foot complex: claw wider than foreleg claw, with tunica not evident, inner tooth, and two pairs of small lateral teeth; empodium without corner tooth but with a long apical filament surpassing the tip of the claw. Hind leg pre-coxae 1 and 2 with 1, 1 chaetae respectively, process on pre-coxa 1 present, coxa with three chaetae and a microsensillum. Trochanter with three chaetae and a trochanteral organ. Femur with 13 chaetae, p_1 and p_3 as mic. Tibiotarsus: whorl I with nine chaetae, whorls II–V with 8, 8, 8, and 7 chaetae respectively; region F with three FP chaetae and FSa chaeta. Foot complex: claw wider than foreleg claw, with tunica not evident,

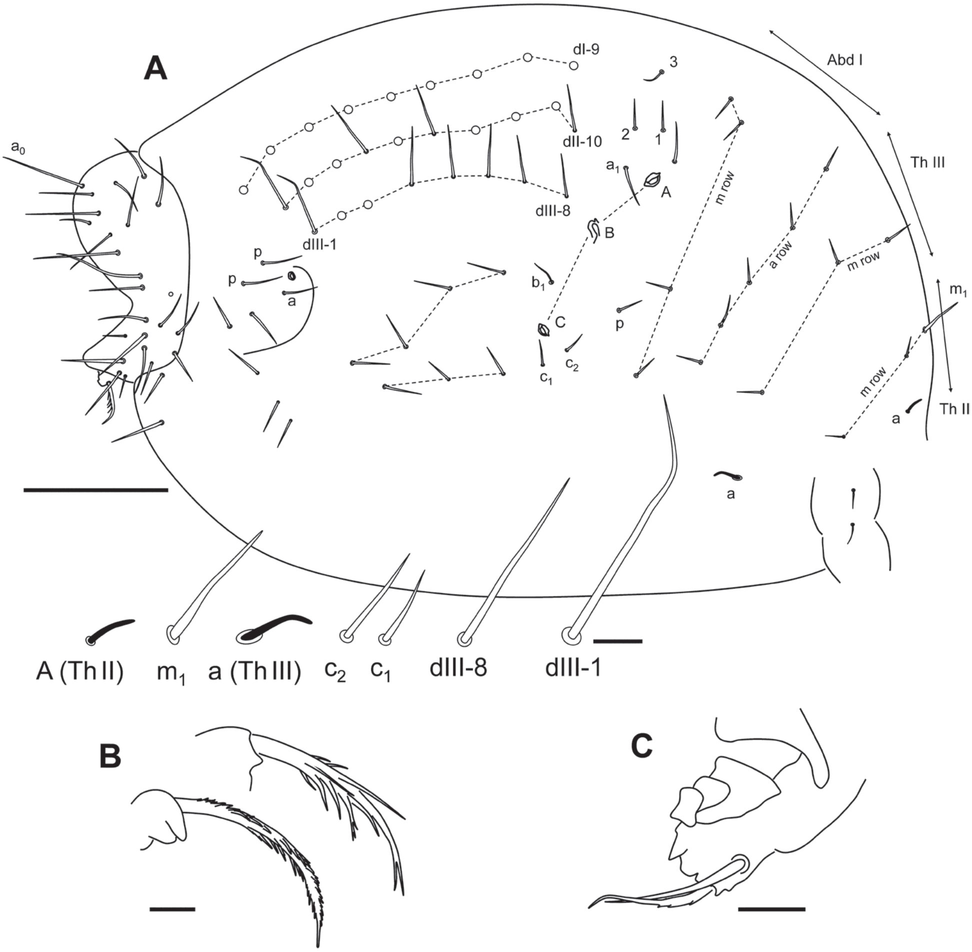

Figure 12. *Pygmarrhopalites sietae* sp. nov. **A** great abdomen, with detail of the shape of some chaetae **B** two some different morphologies of the anal appendage **C** tenaculum. Scale bars: 0.05 mm and 0.001 mm for detail of the chaetae (**A**); 0.005 mm (**B**).

inner tooth, and two pairs of small lateral teeth; empodium without tooth, and a short apical filament.

Great abdomen (Fig. 12A): Th II with a sensillum in row a, and three chaetae in row m (m_1 bigger, thickened at the base). Th III with a sensillum in row a and three chaetae in row m. Abd I row a with five chaetae, row m with four, and four p chaetae, above bothriotrichal complex. Bothriotrichal complex: ABC almost linear; bothriotrichum A with one posterior accessory short chaeta; bothriotrichum B with one posterior accessory short chaeta; bothriotrichum C with two anterior accessory short chaetae. Posterior lateral complex with 4 + 3 chaetae. Posterior dorsal complex with three rows with 8, 10, and 9 long chaetae each.

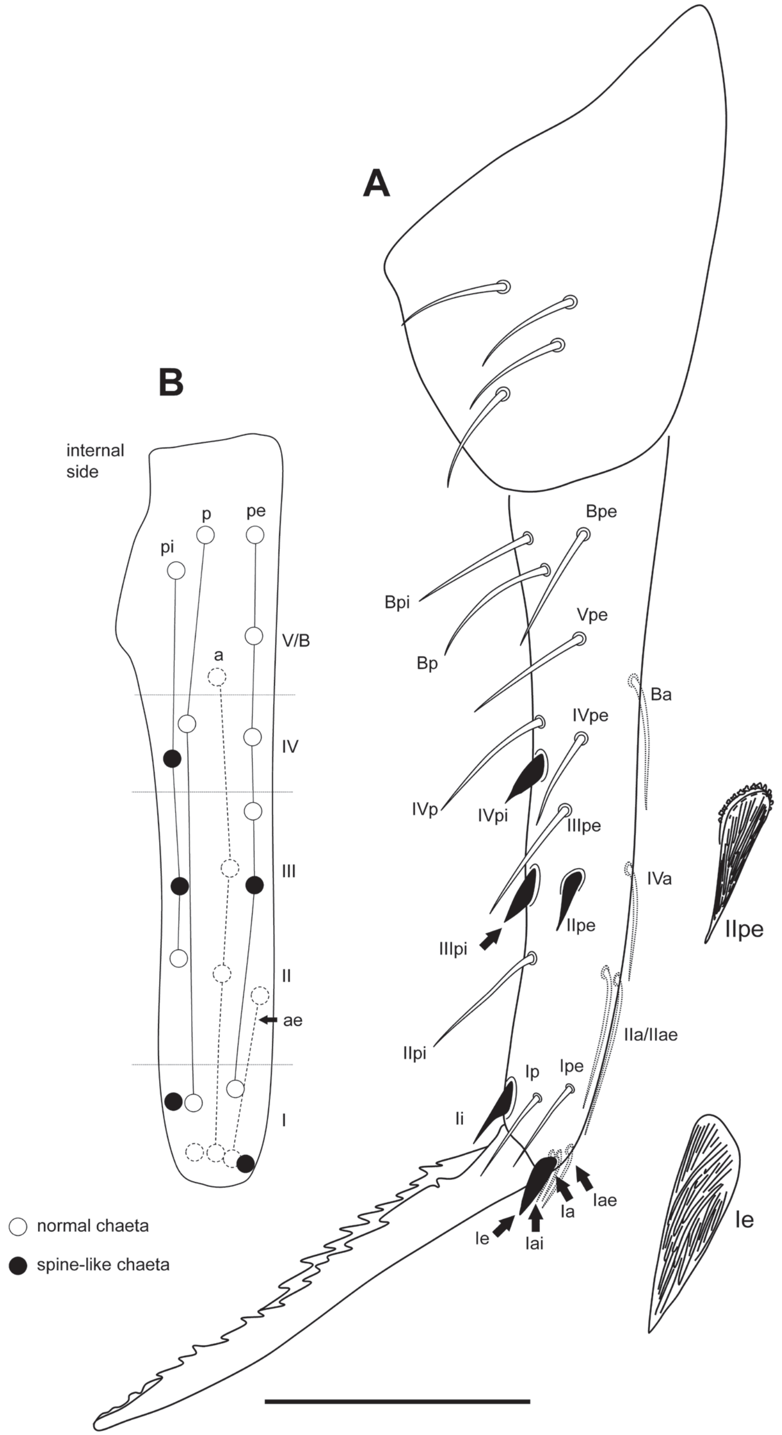

Figure 13. *Pygmarrhopalites sietae* sp. nov.: furcal complex, with detail of some spine-like chaetae. Scale bar: 0.05 mm.

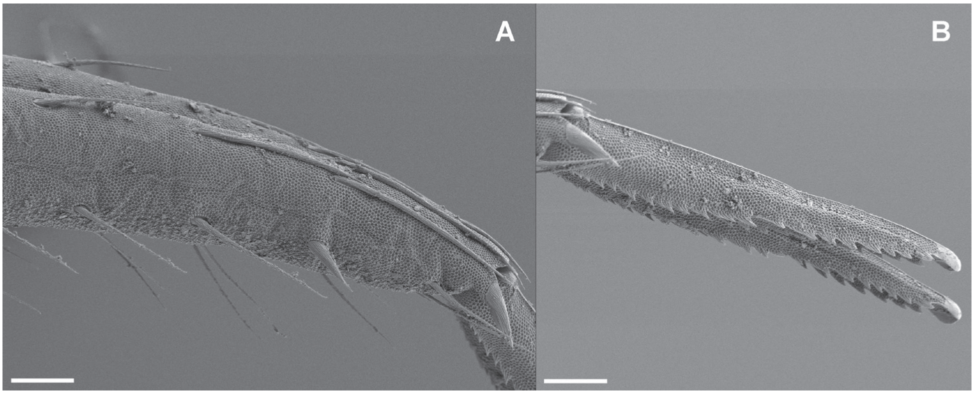

Figure 14. *Pygmarrhopalites sietae* sp. nov. **A** distal area of dentes, external view **B** mucro, external view. Scale bars: 0.001 mm.

Abd VI. Sixth abdominal segment: a_0 not bifurcate, and none of the other circum-anal chaetae broadened, winged or serrated; anal appendage with long fringes or brush-like along final two thirds (Fig. 12B).

Ventral tube with 1 + 1 subapical chaetae.

Tenaculum with two apical chaetae on the corpus, three teeth, and a basal process on each ramus (Fig. 12C). Furca (Fig. 13A, B): manubrium with 4 + 4 posterior chaetae. Dens: anterior side with 3, 2, 1, 1 chaetae; externally Ie as massive spine, and IIpe as a big spine-like chaeta; internally Ii, IIIpi and IVpi moderately spinous, but with alveoli (Fig. 14A). Mucro: both lamellae serrated forming a channel at the end. Dens about 1.4× as long as mucro (Fig. 14B).

Ecology. The specimens were only captured near the main siphon of the Maginet Room (Fig. 2).

Etymology. The name of the new species is dedicated to the association that maintains the cavity and organizes the visits, the Societat d'Investigacions Espeleològiques de Tarragona (SIET).

Remarks. There are only two previously described species that share the presence of only one eye, absence of spine-like chaetae on posterior head, presence of papilla on Ant III, the number and position of spines/spine-like on dens (203 for external/anterior/internal) and a similar shape of anal appendage: *P. furcatus* (Stach, 1945) and *P. ornatus* (Stach, 1945). *P. furcatus* has not tooth on the claw of leg 1, a long filament on empodium of leg 3, and the anal appendice simply bifurcate; *P. ornatus* has not tooth on empodium of leg 1 and the filament on empodium of leg 3 is longer than in the new species; both species have the proximal spine (IIpe) on external dens strong and articulate, while in the new species is only a spine-like chaeta. The three descriptions of *P. ornatus* (Stach 1945; Cassagnau-Debouteville 1953; Dallai 1972) do not coincide with each other; Cassagnau-Delamare and Dallai say that their specimens coincide with the original description, but in the first case the anal appendage is branched, and in the second case the circumanal chaetae are broadened (in Stach one of the three drawings of the appendage is shown branched and the circumanal chaetae are not thickened). Bretfeld (1999) says that none of the post-Stach descriptions have consulted types (we haven't either). In future, if more specimens ar found in this cave, this species should be compared with the types of *P. ornatus*, since they are very similar.

Arrhopalites sp.

Fig. 15

Material examined. One female dehydrated on stub (for SEM observation), Spain: Tarragona city, Tarragona province, Cova Urbana, geographic coordinates 41.114193, 1.248222, 15 m a.s.l. (the entrance is in an urban area), 6/vii/2022, dark zone of the cave, by aspirator (MZNA code 782956). Floren Fadrique leg (BIOSP, Associació Catalana de Biospeleologia). Specimen deposited in MZNA.

Sixth abdominal segment description: three spines per side on the dorsal valve and one on each of lateral; broadened, winged, and sometimes serrated circumanal chaetae (A_{1-3}, $Av_{1'}$, Av_1 and Av_3) (Fig. 15A, B); anal appendage with denticulation/serration at its tip, and some serrated along (Fig. 15C).

Remarks. All descriptions of the small abdomen of *A. caecus* mention 2 + 2 cuticular spines per side (two in dorsal valve and two in lateral valves) (e.g. Stach 1945; Zeppelini 2006; Fjellberg 2017) (Vargovitsh per. comm.). In our specimens, there are three spines per side on the dorsal valve and one on each of lateral (a second spine could be hidden under the broad seta in the view of Fig. 15A). Considering the spines and arrangement/shape of circumanal chaetae we consider that belongs to an undescribed species of *Arrhopalites*, but we have only one specimen and more are necessary to complete a description.

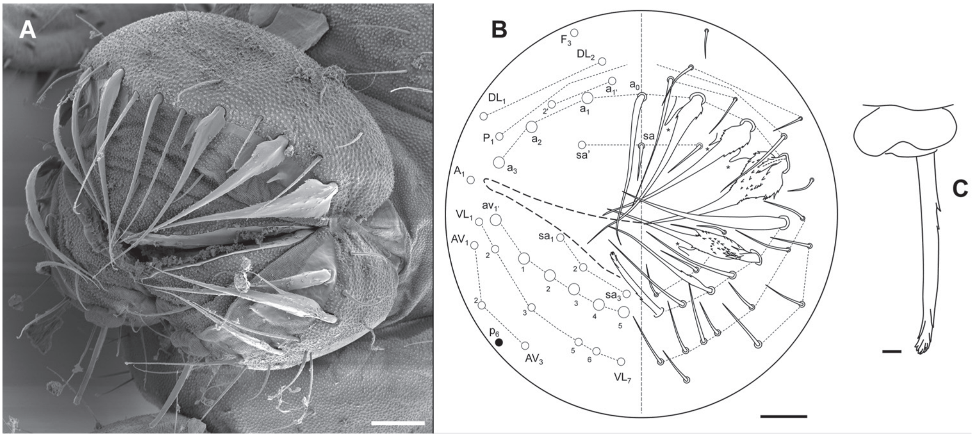

Figure 15. *Arrhopalites* sp., anal valves and circumanal chaetae **A** SEM microphotograph **B** drawing with chaetotaxy **C** detail of anal appendage. Scale bars: 0.02 mm (**A–B**); 0.003 mm (**C**).

Discussion

This work demonstrates the need for collaboration between different institutions, on this occasion two associations related to caving and natural or cultural heritage, and a university. The interdisciplinary work has made it possible to make available to those interested the topography of an interesting cave located in the center of a large city (135 000 inhabitants) belonging to the Catalonian Community, advances in the knowledge of the biocenosis of this cave, and the description of two new species for the fauna. It is once again demonstrated that the isolation of Collembola species that live in relatively close caves has caused their speciation into different species. Also that the low availability of organic matter means that the abundance of fauna in the caves is low and, as on this occasion, that human beings can be important in directing the distribution of fauna in the caves.

Acknowledgments

We are deeply indebted to Jakub Sternalski (Department of Invertebrate Zoology, Institute of Systematics and Evolution of Animals, Polish Adademy of Sciences) by the loan of the specimens of *Sinella montana* and *Sinella submontana* (Stach Collection) for comparation. The third author would like to express his deep gratitude to Josep Lluís Almiñana and Josep Maria Plana (SIET) for their collaboration in carrying out this study, especially for their work as guides during the sampling in the cavity. We acknowledge the constructive comments made by Robert S. Vargovitsh and an anonymous reviewer; their suggestions helped considerably to improve the present contribution.

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RESEARCH ARTICLE

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Siamcyclops isanus sp. nov. (Copepoda, Cyclopoida, Cyclopidae), a new cave-dwelling species from northeastern Thailand

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Abstract

Siamcyclops isanus **sp. nov.** has been found from a cave in northeastern Thailand. It differs from the other member of its genus by having: 1) smooth free margin of anal operculum, 2) a different armature on the second endopod of the first and fourth legs, 3) the armature of the second endopod of the male third leg, and 4) the shape of spermatophore. The discovery of the second species from the genus *Siamcyclops* thus leads to the proposal for the generic diagnosis. The morphological differences between the genus *Siamcyclops* and other closely related genera is added.

Keywords

Groundwater, Indo-Burma region, limestone cave, taxonomy, unsaturated zone

Introduction

Thailand is a part of the Indo-Burma region, a hotspot for biodiversity in Asia (Myers et al. 2000). The country has about 6,000 caves and about 90% of those are in limestone (Dunkley et al. 2017; Jantarit and Ellis 2023). The limestone caves of northeastern Thailand are primarily located in Loei, Chaiyaphum and Nong Bua Lam Phu provinces (all are located in the North Phetchabun mountain range) (Choenkwan et al. 2014). Thirty-six species of cave fauna have been described from Loei Province: dipterans (1 species), diplopods (2 species: *Glyphiulus mongkon* Golovatch, Geoffroy, Mauriès & VandenSpiegel, 2011 is known only from its type locality), copepods (5 species), reptiles (3 species: *Cyrtodactylus kunyai* Pauwels, Sumontha, Keeratikiat & Phanamphon, 2014 is known only from its type locality), and mammals (25 species) (Watiroyram 2021a; Jantarit and Ellis 2023). While copepod sampling in Loei Province was carried out, *Bryocyclops maholarnensis* Watiroyram, Brancelj & Sanoamuang, 2015 was found from Maholarn cave; it was the first stygobiont found in northeastern Thailand (Watiroyram et al. 2015). At present, there are 14 stygobiotic and stygophylic copepods recorded from caves in northeastern Thailand including two calanoids, three cyclopoids and nine harpacticoids (Watiroyram 2021a; Boonyanusith et al. 2024).

The genus *Siamcyclops* Boonyanusith, Sanoamuang & Brancelj, 2018, a monospecific genus, was described from Ratchaburi and Prachuap Khiri Khan provinces and its distribution appears to be restricted to the central part of Thailand (Boonyanusith et al. 2018; the present study). Among the most similar genera, *Siamcyclops* more closely resembles *Palaeocyclops* Monchenko, 1972, *Thalamocyclops* Fiers & Van Damme, 2017, and *Bryocyclops* Kiefer, 1927. The unknown species, collected along with *B. maholarnensis* from the Maholarn cave in Loei province, is proposed as a new species of the genus *Siamcyclops*.

Materials and methods

Study area

The Maholarn cave is located in Nonghin subdistrict, Nonghin district, Loei province, northeastern Thailand (Fig. 1A, B). The cave is situated in the local temple, one of the famous limestone caves visited by tourists and assumed to be at least 265 million years old (Fontaine et al. 2005). It is about 220 m long, 3–4 m wide and 6–7 m high, with a single passage. Near the entrance, there is a small pool on the rock filled by dripping water from a stalactite, which always dries out during the dry season (March – early May). During the rainy season, the rimstone pool at the end part of the cave receives a heavy flow of water dripping from the stalactites. In addition, a small opening in the cave roof allows water to flow in from outside. The cave is flooded from the far end up to the middle of its length, and mud is always found on the floor throughout the passage.

Sampling and taxonomic study

The samples were collected from a small pool near the entrance and a large rimstone pool in the innermost part of the cave (Fig. 1C, D) using a bottle with mesh size 60 µm and was fixed immediately in 70% ethanol (Brancelj 2004). Adult males and females

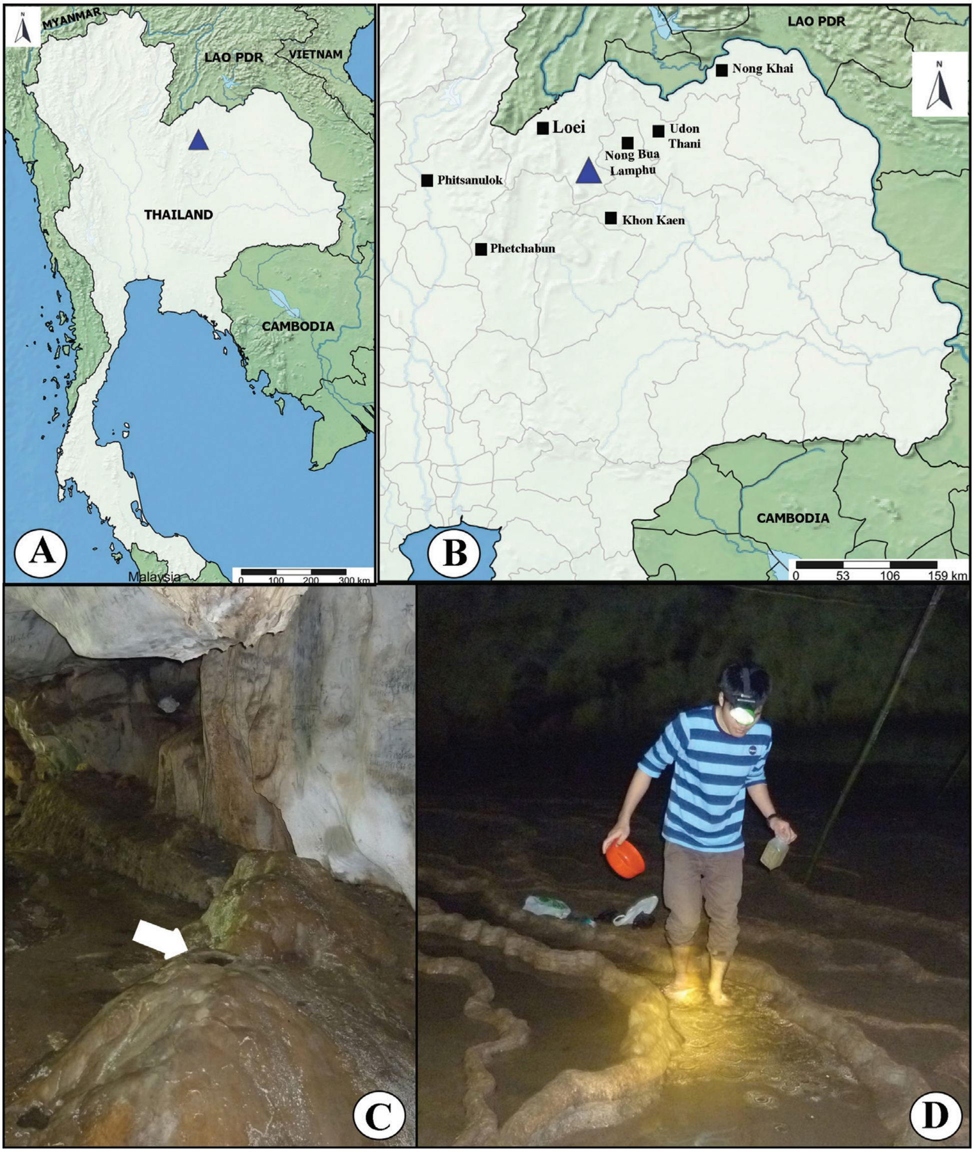

Figure 1. Map of the sampling site of *Siamcyclops isanus* sp. nov. **A** map of Thailand [triangle = location of the Moholarn Cave] **B** location of Loei Province [triangle = location of the Moholarn Cave; square = capital city nearby] **C, D** photo of the sampling site [white arrow indicates small pool at the entrance].

of copepods were sorted and preserved in 70% ethanol. Specimens were dissected under an Olympus SZ51 stereomicroscope in a mixture of glycerol and 70% ethanol (ratio ~ 1:10 v/v). Dissected specimens were mounted in pure glycerol on slides sealed with transparent nail polish and examined using an Olympus compound microscope (CX31) at 1,000 × magnification. Body length of specimens was measured using Zeiss compound microscopy software (Axio Imager 2). The pencil drawings were made using a drawing tube (Olympus U-Da) mounted on the compound microscope. The final drawings were made using CORELDRAW® X7 graphic software (https://www.coreldraw.com/en/). Specimens for scanning electron microscopy (SEM) were dehydrated through a series of ethanol concentrations (50%, 70%, 80%, 90%, 95%, 100%, and 100% abs.) for 15 min at each concentration. Specimens were dried in a critical point dryer and mounted on stubs. Mounted specimens were coated with gold in a sputter-coater. SEM photographs were carried out using a LEO 1450VP scanning electron microscope.

Type specimens were deposited at the Thailand Natural History Museum in Pathum Thani, Thailand (THNHM) and at the Faculty of Science in Nakhon Phanom University, Thailand (NPU).

The morphological terminology follows Huys and Boxshall (1991). The abbreviations used in the text and figures are: **A** = aesthetasc; **S** = spine; **Enp** = endopod; **Exp** = exopod; **Exp-n** or **Enp-n** = exopodal segment n or endopodal segment n; **P1–P6** = swimming legs 1–6.

Results

Taxonomic section

Order Cyclopoida Burmeister, 1834
Family Cyclopidae Rafinesque, 1815

Genus *Siamcyclops* Boonyanusith, Sanoamuang & Brancelj, 2018

Type species. *Siamcyclops cavernicolus* Boonyanusith, Sanoamuang & Brancelj, 2018

Other species. *Siamcyclops isanus* sp. nov.

Emended diagnosis of the genus. Body cyclopiform, small size, with the greatest width at the cephalothorax. Pseudosegment present anterior to the genital double somite. Genital double-somite enlarged laterally, shorter than wide. Genital pore located near middle of somite length. Seminal receptacle small; anterior expansion longer and wider than posterior part. Anal operculum well developed or not, with a smooth or serrated distal margin. Antennule 11-segmented in female, 15-segmented in male. Antenna without Exp. Mandibular palp reduced, represented by three setae. P1–P3 coxa with inner seta but absent on P4. P1–P4 intercoxal sclerite with round prominences. P1 basis with inner seta. P1–P4 with 2-segmented Exp and Enp, subequal in size; setal and spines formula of Exp-2 as 5.5.5.4–5 and 3.3.3.2, respectively. P5 fused to pediger 5, with lateral seta and two ventral setae on small lobe. P6 of female with slender seta and two spiniform setae, located dorso-laterally on the simple plate of the genital double-somite; three slender setae in male. P3 Enp-2 of male with a transformed apical spine and inner subterminal seta.

Siamcyclops isanus **sp. nov.**

https://zoobank.org/7B285B72-0665-4AE7-8F0F-0F40F5452BDB
Figs 2–6

Type locality. The Maholarn cave, Nonghin Subdistrict, Nonghin District, Loei Province, northeastern Thailand: 17°06'23.04"N, 101°52'48.54"E, 315 m altitude. Sample collected from a rimstone pool in the innermost zone, filled with dripping water.

Etymology. *Siamcyclops isanus* sp. nov. is named after 'Isan', the local name for northeastern Thailand. The species epithet is a masculine, singular adjective.

Type specimens. ***Holotype*** • one adult female dissected and mounted on one slide, THNHM-IV-20465. ***Allotype*** • one adult male dissected and mounted on one slide, THNHM-IV-20466. ***Paratypes*** • one adult female and male dissected and mounted on two separate slides, (NPU 2024-01–02) • four adult females and nine adult males stored in a 1.5 ml microtube with 70% ethanol, (NPU 2024-01–02) • one adult female and male dissected and mounted on two separate slides, NPU 2020-003 • one adult male dissected and mounted on one slide, THNHM-IV-20467. All samples were collected by the first author on 5 July 2013.

Description of adult female (holotype). Habitus (Fig. 2A) cyclopiform. Body length, excluding caudal setae, 550 µm, with prosome/urosome ratio of 1.5. Body surface ornamented with refractile points (not figured). Nauplius eye indiscernible. Posterior margin of prosomites with smooth free margin of hyaline fringe. Cephalosome completely fused with first thoracic somite, forming cephalothorax. Urosome length 212 µm; length/width ratio of 1.1. Pseudosegment present between pediger 5 and genital double-somite. Genital double-somite (Fig. 2B–D) enlarged; anterior part slightly wider than posterior part; 1.2 times as wide as long; with pair of sclerotised semi-rounded structures laterally and P6 dorsolaterally; posterior margin with irregularly serrated free hyaline fringe. Urosomites 3–4 (Fig. 2B–D) with about 1.6 times as long as wide; posterior margin with irregularly serrated free hyaline fringe. Anal somite (Fig. 2A–D) short, 1.6 times as long as wide; with a pair of dorsal sensilla at base of anal operculum. Anal operculum (Fig. 2B, D) reaching slightly beyond distal end of anal somite; semicircular; free margin smooth. Caudal rami slightly divergent (Fig. 2A–C). Caudal ramus rectangular, about 2.5 times as long as wide, without dorsal longitudinal keel. Anterolateral accessory seta (I) reduced. Lateral seta (II) pinnate, slightly shorter than 1/2 length of caudal ramus, inserted at 1/3 length of caudal ramus. Posterolateral seta (III) pinnate, strong, spine like, shorter than caudal ramus, with spinules at insertion point on ventrolateral side. Outer terminal seta (IV) pinnate, about 2.5 times as long as caudal ramus, without fracture plane. Inner terminal seta (V) pinnate, about 4.0 times as long as caudal ramus, without fracture plane. Innermost terminal seta (VI) bare; shortest. Dorsal seta (VII) pinnate, articulated, longer than caudal ramus.

Antennule (Fig. 3A). 11-segmented, not reaching posterior margin of cephalothorax. Armature formula as follows: 7.2.5.2.0+S.2.3.2+A.2.2+A.7+A. Aesthetascs cylindrical.

Antenna (Fig. 3B). 4-segmented; coxobasis smooth, with seta on distal inner corner. Enp 1–3 with small spinular rows on outer margin, Enp-1 with seta on distomedial margin; Enp-2 with medial seta and four distal outer setae; Enp-3 with seven apical setae, two of them geniculated. All setae smooth.

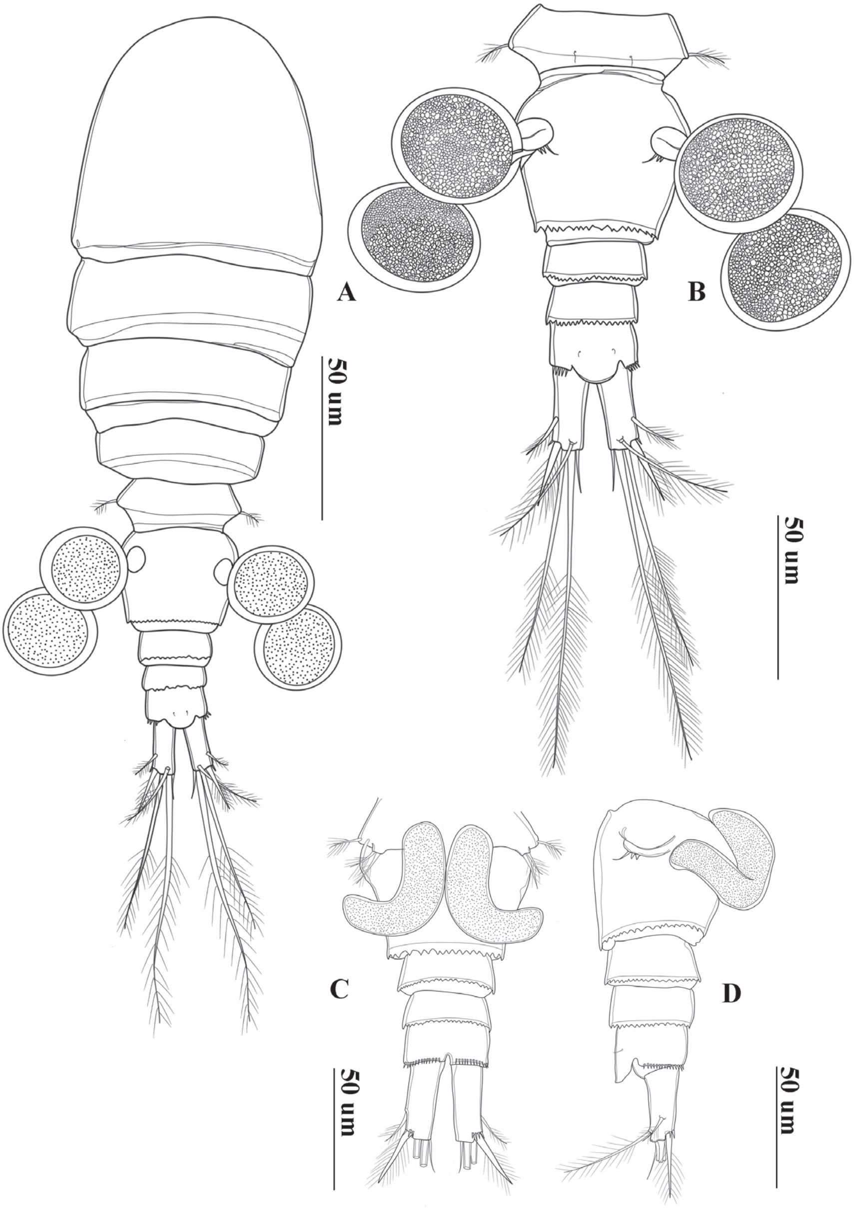

Figure 2. *Siamcyclops isanus* sp. nov., holotype female (**A, B**) and paratype female (**C, D**) **A** habitus, dorsal view **B** urosome with a pair of egg sacs, dorsal view **C** urosome with spermatophore, ventral view **C** urosome with spermatophore, lateral view.

Mandible (Fig. 3C). Gnathobase with eight strongly chitinized teeth, and a small seta dorsally. Basis very reduced, with two long bipinnate setae and a short smooth seta, representing Exp and Enp.

Maxillule (Fig. 3D). Precoxal arthrite with strongly chitinized teeth, one tricuspidate and two bicuspidate; four strong smooth setae and one spinulate seta on inner

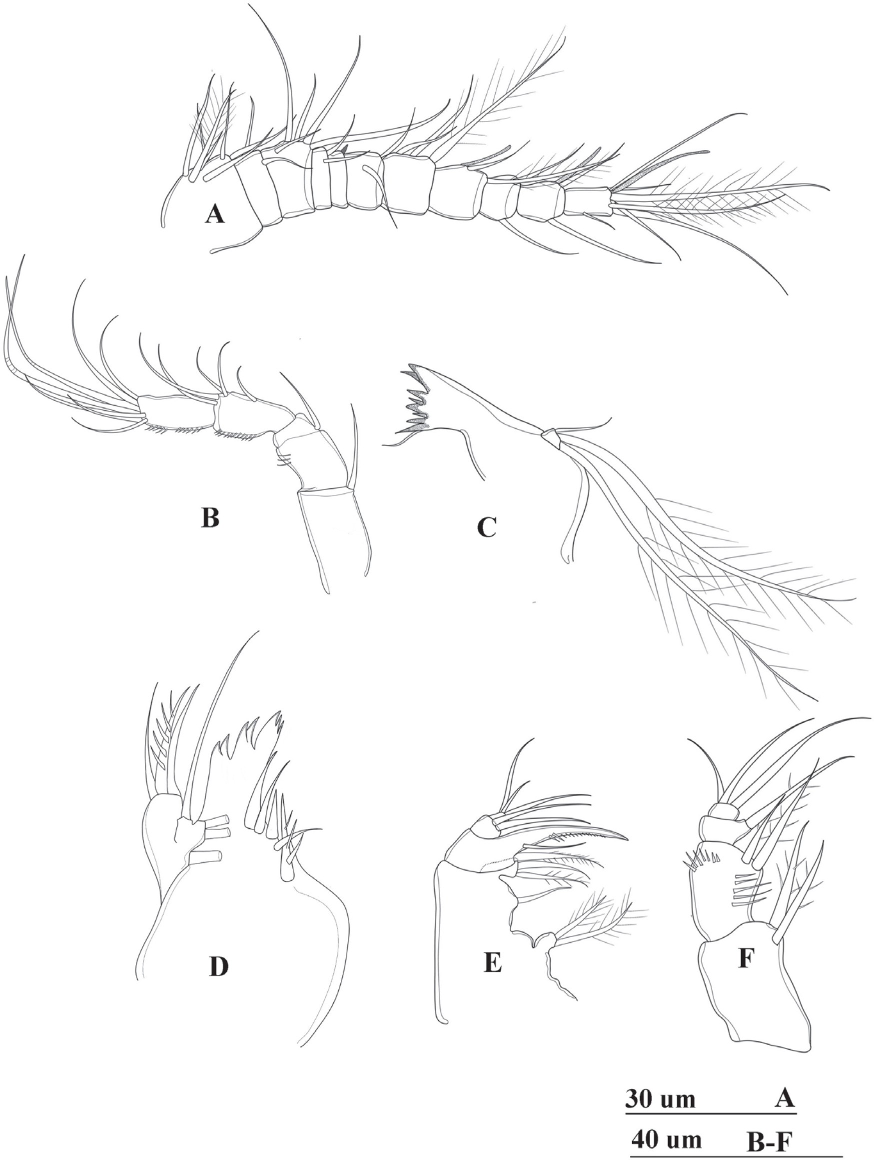

Figure 3. *Siamcyclops isanus* sp. nov., holotype female **A** antennule **B** antenna **C** mandible **D** maxillule **E** maxilla **F** maxilliped.

margin. Coxobasis with spinulate seta and two smooth setae distally. Exp completely reduced, represented by a smooth seta. Enp with three smooth setae.

Maxilla (Fig. 3E). Precoxal endite with two pinnate setae. Coxa with two endites: proximal endite with smooth seta; distal endite with smooth seta and pinnate seta. Basis drawn out into claw-like expansions, with slender seta on proximal inner margin. Two-segmented Enp: Enp-1 with two smooth setae; Enp-2 with three smooth setae.

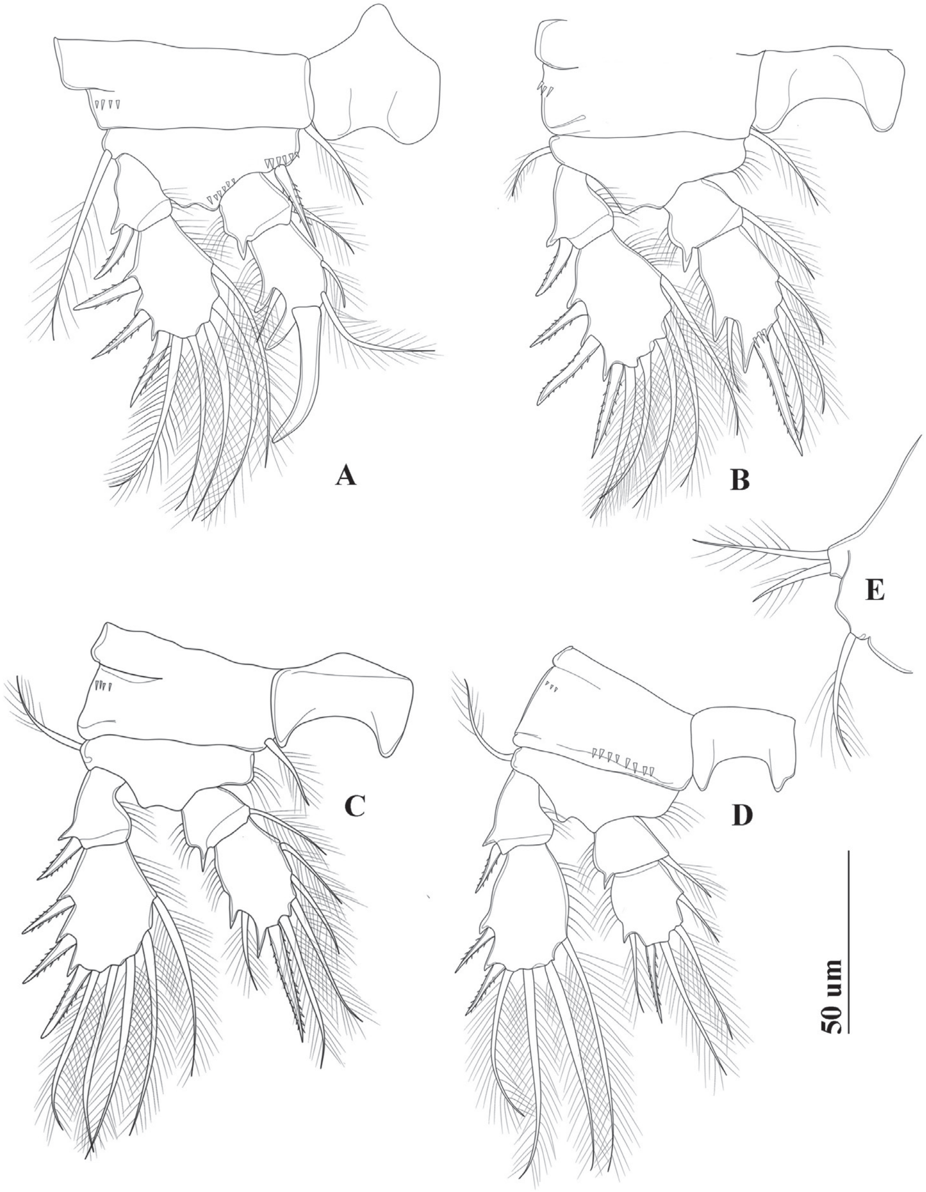

Figure 4. *Siamcyclops isanus* sp. nov., holotype female **A** P1 **B** P2 **C** P3 **D** P4 **E** P5.

Maxilliped (Fig. 3F). Syncoxa with two spinulate setae on distal inner margin. Basis with two spinulate setae on distal inner margin, accompanied by two spinule rows on its surface. Enp-1 with inner seta; Enp-2 with three apical setae; all setae smooth.

P1–P4 (Fig. 4A–D). P1–P4 with 2-segmented Exp and Enp. Exp and Enp ornamented with setules on its inner and outer margin, respectively. No blunt seta on Exp-2, all setae being equal. Armature formula (setae in Arabic numerals and spines in Roman numerals from outer-inner or outer-apical-inner margins) as follows:

	Coxa	Basis	Exp	Enp
P1	0-1	1-I	I-0; III-2-3	0-1; 1-I-2
P2	0-1	1-0	I-0; III-2-3	0-1; 1-I+1-2
P3	0-1	1-0	I-0; III-2-3	0-1; 1-I+1-3
P4	0-0	1-0	I-0; II-2-2	0-1; 0-I+1-2

P1 (Fig. 4A). Basis with small spinules near its insertion. Exp slightly larger than Enp. Exp-1 much smaller than Exp-2. Exp-2 about twice as long as wide. Enp-2 larger than Enp-1, about 2.0 times. Enp-2 with robust apical seta, as long as Enp-1 and Enp-2 combined, its tip bent outward; distal inner seta as long as apical spine, the two remaining setae shorter.

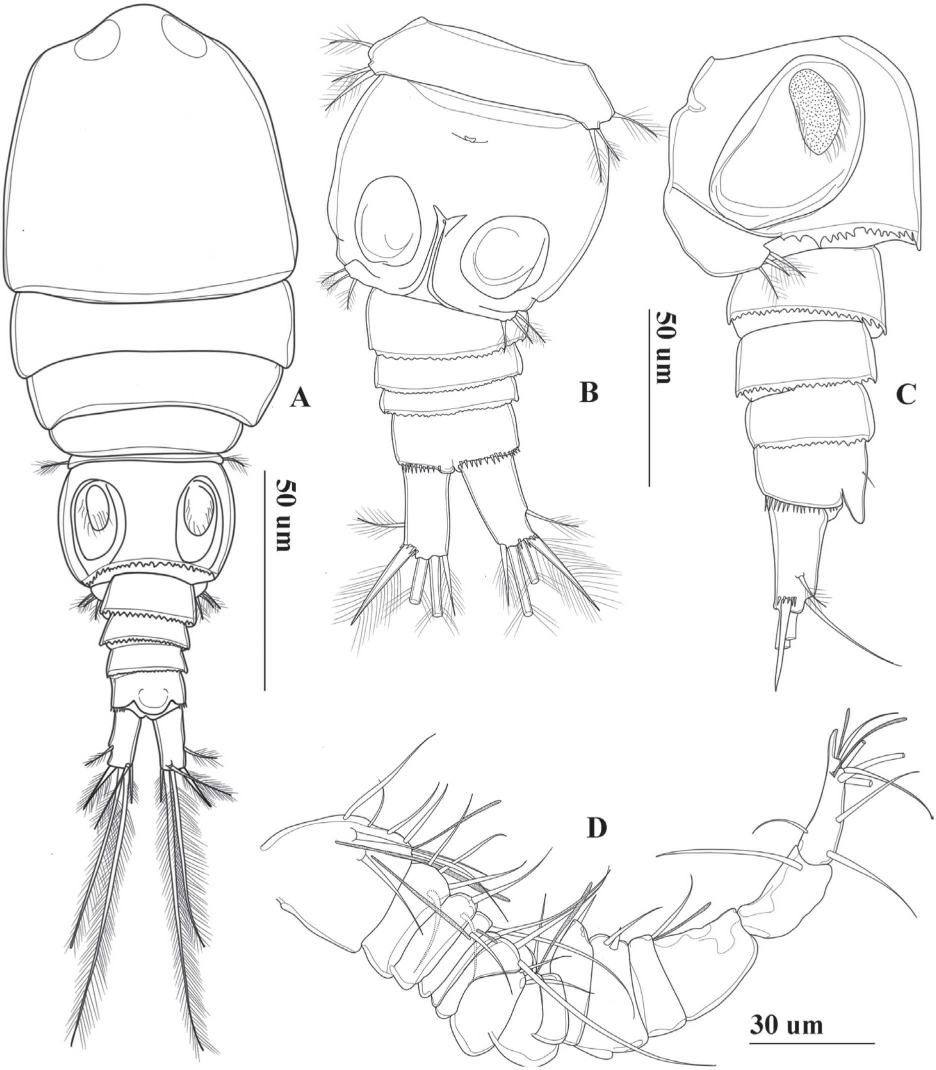

Figure 5. *Siamcyclops isanus* sp. nov., allotype male **A** habitus, dorsal view **B** urosome, ventral view **C** urosome with urosomite 1, lateral view **D** antennule.

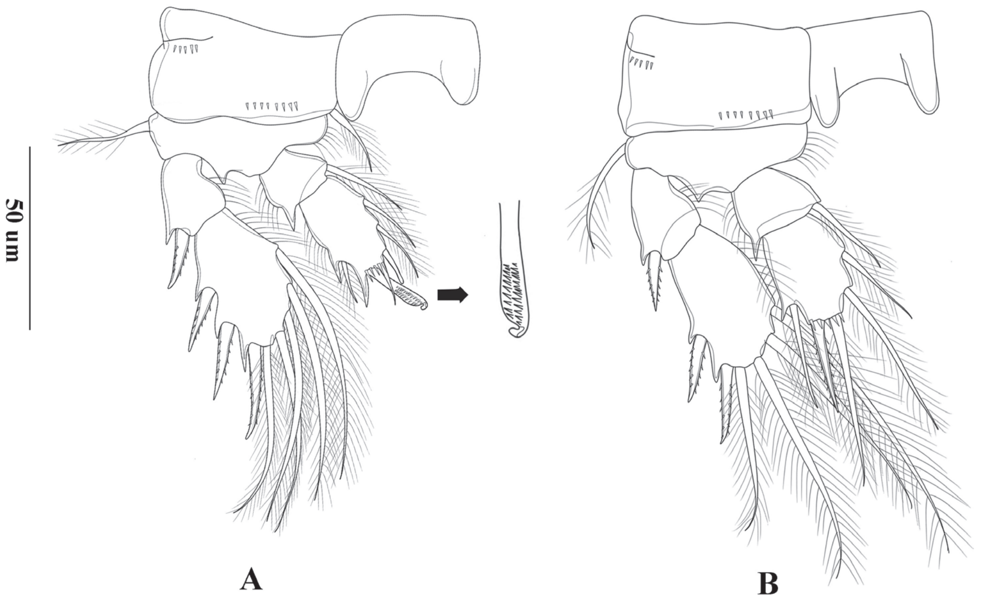

Figure 6. *Siamcyclops isanus* sp. nov., allotype male **A** P3 **B** P4.

P2 (Fig. 4B). Similar to P1 but lateral seta of basis shorter. Enp-2 with strong spinulated apical spine, with strengthened tip; two setae as long as apical spine but two remaining setae shorter.

P3 (Fig. 4C). Similar to P2 but lateral seta on basis longer, Enp-2 with slightly less robust apical spine. Enp-2 with two setae longer than apical spine, three remaining setae shorter.

P4 (Fig. 4D). Exp-1 and Exp-2 with weakly spinulated outer spines. Exp-2 twice as long as wide; three inner setae longer than Exp-1 and 2 combined, outermost seta shortest. Enp-1 slightly shorter than wide, Enp-2 about 1.3 times as long as wide. Enp-2 with slender apical spine, short as long as segment bearing it; outer and innermost seta shorter than apical spine, two remaining setae longer than apical spine.

P5 (Fig. 4E). Completely fused to pediger 5, proximal segment represented by lateral seta, and distal segment retains two pinnate apical setae on a small lobe. Lateral seta slightly longer than apical inner seta; apical inner seta about 1.3 times as long as apical outer one.

P6 (Fig. 2D). Reduced to a semi-circular plate, with three short elements: dorsal seta thin and short, two ventrally positioned elements spiniform.

Egg sac (Fig. 3A, B). Each sac with two large eggs; each egg about 70 µm in diameter.

Spermatophore (Fig. 3C, D). Three-dimensional C-shaped.

Description of adult male (allotype). Body length (Fig. 5A), excluding caudal rami, 540 µm; smaller than female. General segmentation and ornamentation (Fig. 5A–C) similar to female except genital somite and antennules. Genital somite (Fig. 5B, C) not fused with urosomite 3 like in female, enlarged laterally; 1.3 times as wide as long; with pair of sclerotised rounded structures dorso-laterally; posterior margin with irregularly serrated free hyaline fringe. Urosomites 3–5 (Fig. 5A–C) with about 1.5 times as long as wide; posterior margin with irregularly serrated free hyaline fringe.

Antennule (Fig. 5D). 15-segmented, geniculate. Armature formula as follows: 6+3A.4.2. 2+A.1.2.2.2+A.0.1.1+S.1+A.0.1.8+2A.

Antenna, mouthparts, P1, P2 and P5 similar to female.

P3 (Fig. 6A). Exp similar to female. Enp-2 shorter than wide, Enp-2 about 1.5 times as long as wide. Enp-2 with transformed spine and smooth seta apically: transformed spine bent inward, with round tip bent outward, serrated by two rows of spinules on distal half of it. Inner subterminal seta transformed to spine-like seta, strong, bare, with blunt tip.

P4 (Fig. 6B). Exp and Enp similar to female but Enp with slightly longer, about 1.5 times as long as wide.

P6 (Fig. 5B, C). Reduced to simple plate, represented by three subequal pinnate setae.

Variability. One of female (out of five) has P6 with two elements.

Distribution. Known only from the type locality.

Differential diagnosis. The new species is similar to *S. cavernicolus* Boonyanusith, Sanoamuang & Brnacelj, 2018 in the following characteristics: 1) mandibular palp with three setae; 2) Exp and Enp of P1–P4 are subequal; 3) spine formula of P1–P4 Exp-2 is 3.3.3.2; 4) P4 lacks coxal seta; 5) P4 intercoxal sclerite with round distal margin; 6) P5 fused to pediger 5, remaining small unsegmented lobe; 7) P6 with three elements (see Table 1). However, the new species differs from *S. cavernicolus* in: 1) anal operculum smooth and unproduced over segment bearing it (serrated free margin and well-developed in *S. cavernicolus*); 2) P1 Enp-2 with four elements (five elements in *S. cavernicolus*); 3) P3 Enp-2 with six elements in male (five elements in *S. cavernicolus*); 4) P4 Exp-2 with four setae (five setae in *S. cavernicolus*); 5) spermatophore is three-dimensional C-shaped (L-shaped in *S. cavernicolus*).

Table 1. Morphological differences between the genus *Siamcyclops* and the closely related genera.

Genera characteristics	*Bryocyclops*	*Thalamocyclops*	*Palaeocyclops*	*Siamcyclops*
Both sexes				
Mandibular palp	With 1 seta	With 1 seta	Unknown	With 3 setae
Annal operculum	Well-developed	Well-developed	Well-developed	Normal – Well-developed
Size of Exp versus Enp on P1–P4	Larger	Larger	Subequal	Subequal
Spine formula of P1–P4 Exp-2	3.3.3.3(4)	3.3.3.3	2.3.3.3	3.3.3.2
Setal formula of P1–P4 Exp-2	5(4).5.5.4	5.5.5.4	5.5.5.4	5.5.5.5(4)
Coxal seta of P1–P4	1(0).0.0.0	1.0.0.0	1.1.1.0	1.1.1.0
Intercoxal sclerite of P4	Acute	Acute	Acute	Round
P5 segment	Absent –prominence (shape **a**)	Large lobe (shape **c**)	Absent (shape **a**)	Small lobe (shape **b**)
Female				
Number of segments on P4 Enp-2	1 or 2	1	2	2
Number of setae and spines on P4 Enp-2	4	4	5	5
Male				
Number of setae and spines on P6	3	3	2	3

The SEM photos of P5 show the degree of reduction in *Siamcyclops* and *Bryocyclops* species. The distal segment of P5 is reduced to a small lobe on pediger 5 in *Siamcyclops* (Fig. 7C, D), while it is more reduced in *Bryocyclops*, where it is represented by a small prominent knob (Fig. 7A, B). In addition, the setae tend to be shorter and stronger, resembling spiniform setae in some species (Fig. 7B), which is advantageous for species living in the confined spaces of the epikarst.

Key to species of the genus *Siamcyclops*

1 Anal operculum well-developed, with serrated free margin; P4 Exp-2 with five setae; the female P1 Enp-2 with five elements; the male P1 & P3 with five elements each .... ***S. cavernicolus***

2 Anal operculum short, with smooth free margin; P4 Exp-2 with four setae; the female P1–Enp-2 with four elements; the male P1 & P3 Enp-2 with four and six elements, respectively .... ***S. isanus* sp. nov.**

Discussion

In terms of oligomerization, many characters were used for species and generic determination which are apomorphic traits developed for living in semiterrestrial and subterranean habitats (Watiroyram 2018a, 2021b; Hołyńska et al. 2024). However, characters under strong evolutionary selection should be used with caution for taxonomic purposes because they may represent parallel evolution (and sometimes convergence). The subterranean Cyclopinae have much reduced mandibular palp, presenting a tiny distinct segment until a reduction to 1–3 setae only; small body size (usually less than 550 µm), short antennule (10 or 11 segments, usually reaching two-third of cephalothorax length), and reduced swimming legs (Fiers et al. 1996; Pesce 1996; Fiers and van Damme 2017). Pesce (1996) noted that the lineage of the *Bryocyclops* group (including *Siamcyclops*) shows the most reduced P5 form of the family Cyclopidae (the sixth morphological group sensu Pesce 1996) in having P5 completely fused to the somite, represented by a prominence of the remaining distal segment or absent; P1–P4 has 2-segmented Exp and Enp (some species have 1-segmented Exp and Enp; for example, *B. fidjiensis* with 1-segmented Exp, while *B. jayabhumi*, *B. maholarnensis*, *B. muscicola*, *B. muscicoloides*, *B. trangensis* with 1-segmented Enp in females). The setae and spines of their legs are also reduced in number, size and structure. For example, *Siamcyclops* species lack P4 coxal seta, whereas some species of *Bryocyclops* lack this seta on all P1–P4 swimming legs; setae tend to be shorter, and stronger than those from epigean habitats, or the setae are transformed to be spiniform (see fig. 4D in Watiroyram et al. 2012; Figs 6A, 7B in the present study).

The genus *Siamcyclops* has a transformed apical spine on P3 Enp-2 in the male, coxal seta on P4 and P5 segment absent in both sexes, indicating that it closely resembles the genus *Palaeocyclops* Monchenko, 1972 [*Bryocyclops* (*Palaeocyclops*) *jankowskajae* Monchenko, 1972 is an alternative name used by Walter and Boxshall (2024)],

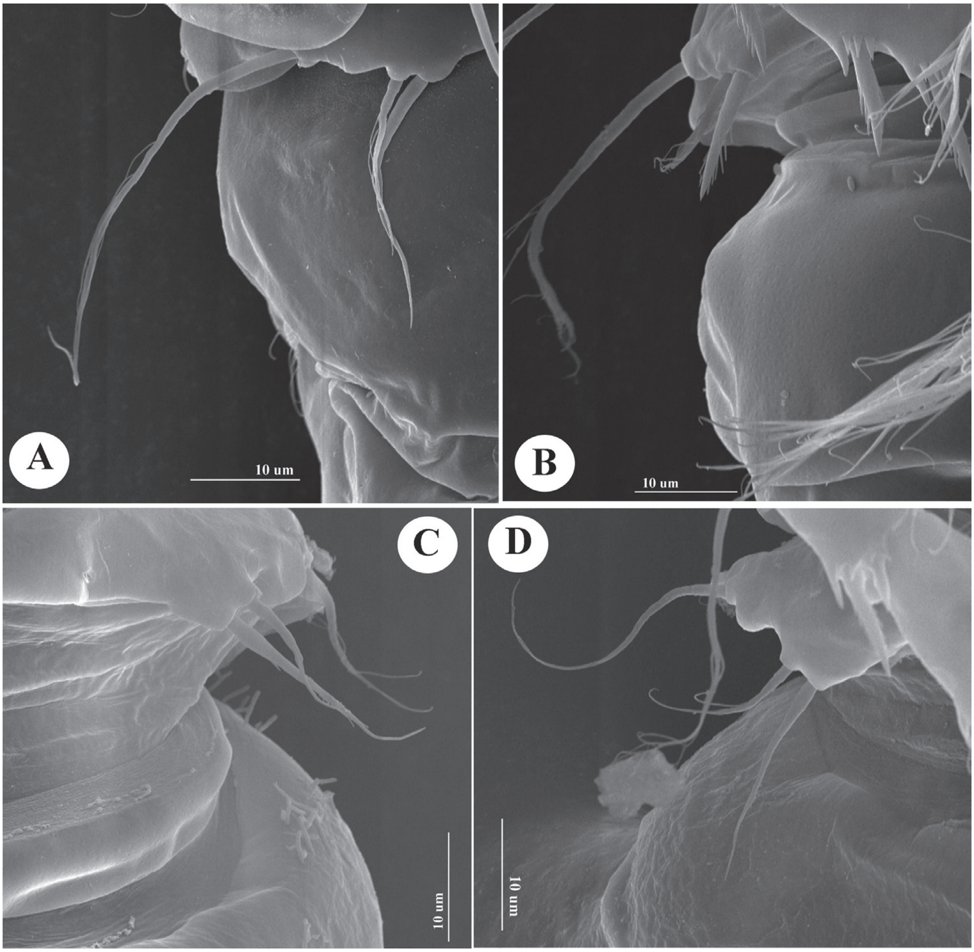

Figure 7. Photographs taken using scanning electron microscope **A** *Bryocyclops asetus* Watiroyram, 2018 **B** *B. jayabhumi* Watiroyram, 2021 **C** *Siamcyclops cavernicolus*, male **D** *S. cavernicolus*, female.

Thalamocyclops Fiers & Van Damme, 2017, and *Bryocyclops* Kiefer, 1927 (Fiers and van Damme 2017; Watiroyram 2021b). The transformed apical spine of the male P3 Enp-2 is also present in the genus *Itocyclops* Reid & Ishida, 2000 but P4 bears a coxal seta and P5 has a distinct segment in *Itocyclops*. After re-examining the P5 of *Siamcyclops* and *Bryocyclops* genera collected from Thailand, we classified the P5 of the *Bryocyclops* group sensu Pesce (1996) into three shapes based on the degree of reduction in the distal segment (absent or with a prominence/knob, small lobe, or large lobe): shape a with dorsal seta and two ventral setae (usually spiniform) fused to somite or inserted on a small prominence, versus shapes b and c with dorsal seta and two ventral setae on a lobe of an ancestral distal segment. The genus *Thalamocyclops* retains the vestigial P5 distal segment (shape c; with large lobe) more than the genus *Siamcyclops* (shape b; with small lobe; Figs 4E, 5B, 7C–D) followed by *Bryocyclops* (shape a; absent or with small prominence; Fig. 7A, B) and *Palaeocyclops* (shape a; prominence absent). Among closely related genera, *Siamcyclops* shows the least reduced form (i.e., a plesiomorphic trait) in contrast to *Bryocyclops* s. str. such as the mandibular palp with three setae, an unproduced anal operculum and P4 intercoxal sclerite, subequal size of exopodal and endopodal segments, presence of coxal seta on P2–P3, and a number of segments and setae on P4 (see Table 1).

In the *Bryocyclops* group sensu Pesce (1996), the anal operculum is well-developed in the genus *Bryocyclops* s. str., whereas it is incompletely developed, semicircular or subquadrate and with a smooth distal free margin in the remaining genera. Both scenarios are also present in the genus *Microcyclops*, being probably due to parallel evolution or convergence, or within the genus *Siamcyclops* (*S. cavernicolus* has a well-developed anal operculum but the new species does not). Based on our knowledge of the genera *Bryocyclops* and *Siamcyclops*, the shape of the spermatophore and refractile points is varied and not specific to these genera (Watiroyram 2021b; the present study). For example, the new species has C-shaped spermatophore versus L-shaped in *S. cavernicolus*, while most species of the genus *Bryocyclops* have a pair of bean-shaped spermatophore, whereas other shapes are present in *B. asetus* Watiroyram, 2018 (inverted V-shaped) and *B. trangensis* Watiroyram, 2018 (a pair of spermatophores is arranged in a straight line). Refractile points on the body surface are also not a unique apomorphy for the genera *Bryocyclops* and *Siamcyclops*: the new species has such cuticular pits but *S. cavernicolus* has not (Watiroyram 2018a, 2018b; the present study).

Our findings indicate that the genus *Siamcyclops* is endemic to Thailand, with two species having a well separated distribution area: *S. cavernicolus* in the central part and the new species in the northeastern part of the country. Regarding copepod samples taken by the first author, *S. cavernicolus* is also distributed southwards to Prachuap Khiri Khan province (Watiroyram 2021a). They are likely epikarstic species, well-adapted to a benthic lifestyle, thriving in both the confined spaces of the epikarst and the larger pools within caves as shown by the presence of the pseudosegment anterior of the genital double-somite, like in *Bryocyclops* species (Fig. 7B, C; Watiroyram 2018a). The pseudosegment is not present in *Thalamocyclops*, conforming to its habitats, which features a large space and a water volume, suitable for good swimmers. Fiers and Van Damme (2017) collected many adults and different stages of juveniles of *Thalamocyclops pachypes* from weathered limestone crevices, indicating that they inhabit these crevices for growth and breeding. In contrast, species of *Siamcyclops* and *Bryocyclops* collected from Thailand typically have a pseudosegment for living in small spaces, as indicated by the fact that adults are usually collected rather than juveniles (i.e. they accidentally drop from the ceiling of the cave, especially in the rainy season). Observations over many years (2013–present) allowed to collect *B. moholarnensis* only in the small pool near the entrance (Fig. 1C), while *S. isanus* sp. nov. was found in a large pool at multiple points in the inner section of Maholarn Cave (Fig. 1D).

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