RESEARCH ARTICLE

Citizen science approach reveals groundwater fauna in Switzerland and a new species of *Niphargus* (Amphipoda, Niphargidae)

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Abstract

Knowledge on the diversity and distribution of subterranean organisms is still scattered, even in faunistically relatively well-researched countries such as Switzerland. This is mostly due to the restricted access to these subterranean habitats. Better knowledge on these organisms is needed, because they contribute substantially to overall biodiversity of a region, often contain unique elements of biodiversity, and can potentially be indicative of the ecological status of subterranean ecosystems that are providing important ecosystem services such as drinking water. Past research on subterranean organisms has often used highly specialised sampling techniques and expert knowledge. Here, we show that inclusion of non-professionals can be an alternative and highly promising sampling strategy. We retrieved citizen science-based samples from municipal groundwater wells across Switzerland, mainly from the Swiss Plateau. Opportunistic samples from 313 sites revealed a previously undocumented groundwater fauna including organisms from different major invertebrate groups, with a dominance of crustaceans. Here, we studied amphipods of the genus *Niphargus*. Among all 363 individuals sampled, we found in total eight nominal species. Two of them, namely *N. fontanus* and *N. kieferi*, are reported for Switzerland for the first time. We also found four further phylogenetic lineages that are potentially new species to science. One of them is here formally described as *Niphargus arolaensis* **sp. nov.** The description is based on molecular and morphometric data. Our study proves the suitability of citizen science to document subterranean diversity, supports groundwater conservation efforts with data, and raises awareness for the relevance and biodiversity of groundwater amphipods among stakeholders.

Introduction

Groundwater is among the most essential resources for human well-being (Zektser and Everett 2004; Griebler and Avramov 2015). Groundwater is also the largest freshwater habitat on earth (Gibert et al. 1994) and harbours unique and diverse obligate groundwater dwellers (Deharveng et al. 2009), referred to as stygofauna. Very few ecosystems have a comparable history of stable conditions, making groundwater ecosystems evolutionary unique (Culver and Pipan 2009). Subterranean fauna contributes local and unique elements to a region's overall biodiversity (Mammola et al. 2019). However, conservation and management of groundwater is often neglecting its fauna and its role as an ecosystem (Gibert et al. 2005). Given the many anthropogenic threats to groundwater (Burri et al. 2019), this aspect needs to be better considered to understand state and changes of groundwater in the context of climate change, groundwater depletion, or chemical pollution.

For many regions of the world the knowledge about the diversity and distribution of groundwater organisms – or subterranean organisms in general – is mostly lacking (Ficetola et al. 2019). Whereas there exist plethora of monitoring programs and conservation legislations for aboveground biodiversity (Scholes et al. 2012; Proença et al. 2017), their subterranean counterparts are rarely monitored and knowledge is still scattered (Gibert and Culver 2009). This can be largely attributed to both the restricted access to subterranean habitats that requires specialised sampling techniques and the subsequent expert knowledge needed to identify the groundwater organisms (Dole-Olivier et al. 2009). The existing knowledge on subterranean biodiversity therefore stands in stark contrast to its relevance for overall biodiversity.

A key part of groundwater diversity is composed by invertebrates, of which crustaceans, and especially amphipods, are among the most common ones (Sket 1999; Stoch and Galassi 2010). In many countries or biogeographic regions, the diversity and distribution of epigean amphipods is often better documented than their hypogean counterparts. Switzerland can be seen as a typical example thereof (Altermatt et al. 2014, 2019): Only very recently, data about groundwater amphipods were collected and systematically analysed (Fišer et al. 2017, 2018). While half of the known amphipod species in Switzerland live in subterranean habitats (Altermatt et al. 2019), they still contribute to just a tiny fraction of all available records. With four endemic species (*Niphargus luchoffmanni* Fišer et al., 2018; *Niphargus murimali* Fišer et al., 2017, *Niphargus muotae* Fišer et al., 2017, *Niphargus styx* Fišer et al., 2017), however, their proportion of endemics is higher than in any other organismal group in Switzerland (Tschudin et al. 2017), indicating to a potential further undocumented species diversity awaiting its scientific exploration.

Here we addressed this knowledge gap on groundwater organisms, with a focus on the genus *Niphargus* Schiödte 1849, focussing on the Swiss plateau. To cover a fine spatial resolution, we decided to deploy a citizen science approach (Lewenstein 2004), where we built on knowledge and collaboration with local drinking water well managers. About 80% of drinking water in Switzerland originates from groundwater (Freiburghaus 2012), which is collected in wells that are usually maintained and run on a municipal level. Many of these wells present an opportunity to collect and analyse water that is passively collected from the respective aquifer. An intriguing question is whether citizen science could foster biological sampling of these hardly accessible habitats and prompt the study of groundwater fauna. The inclusion of non-professionals in a scientific context, especially in data collection, dates back several hundred years, but gained increased significance in science recently in order to tackle questions at a larger spatial or temporal extent or with a wider scope (Miller-Rushing et al. 2012). Consequently, some of the most extensive datasets in ecology originate from citizen science projects and they can stir interest for a specific topic in a wider public. In our citizen science study we considered well managers as "members of the general public", and sample collection by them required gaining them as participants, distributing sampling kits with detailed instructions and maintain close contact to them. We revealed previously undocumented groundwater diversity for Switzerland, reporting three species of *Niphargus* for the first time for Switzerland, one of which is new to science and we provide a detailed description. We provide genetic barcodes for all found *Niphargus* species and discuss what we perceived as key elements for establishing and maintaining participation by citizen scientists.

Materials and methods

Sampling procedure

We focused on the Swiss plateau, a region where only very little previous data on groundwater amphipods were available (Altermatt et al. 2019), particularly on four cantons (Aargau, Basel-Landschaft, Solothurn, Zürich) in the Northern part of the Swiss Plateau (Fig. 1, grey shading). Within the study area of these four cantons (4,441 km²), we contacted the municipal drinking water well managers (hereafter referred to as well managers) to explain the project and to ask for participation in our study. We did so by sending out standard letters (Suppl. material 2) and making complementing phone calls in March 2019. Additionally, we presented the study plans and aims during the annual meeting of drinking water providers of Switzerland in April 2019. These efforts resulted in a few hundred informed well managers, also from some municipalities outside the four target cantons (Fig. 1).

The sampling of groundwater wells by the well managers followed a predefined protocol, fostering comparability. We sent the sampling material, instructions, and
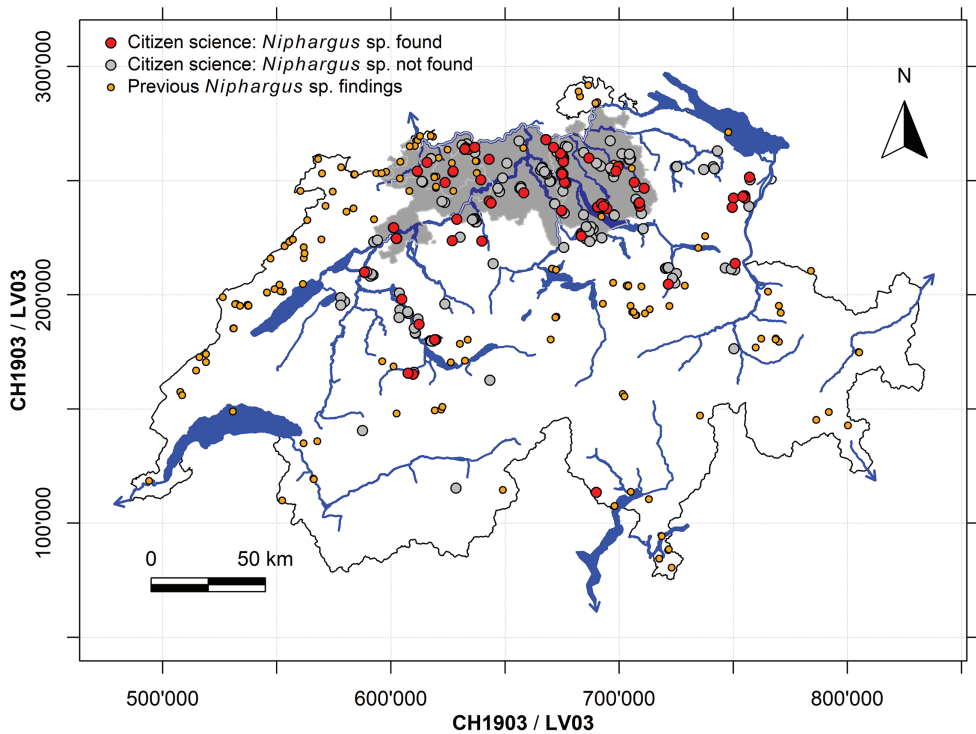
Figure 1. The study area (Switzerland) with the four focus cantons highlighted in grey. The map shows all sites sampled within the current citizen science project in which *Niphargus* sp. were either found (red circles) or not found (grey circles). All previously known findings of *Niphargus* sp. from Switzerland are indicated as orange circles. Geodata from Federal Office of Topography.

data sheets (Fig. 2A, Suppl. materials 3–8) to well managers that had agreed to pursue sampling. The sampling material provided included food-safe filter bags, cable ties, a small aquarium net, sample tubes prefilled with 80% molecular grade Ethanol, forceps, and labels. The food-safe filter bags (monofilament, nylon thermo-setting, polypropylene, polyester, PEEK; mesh size 800, diameter 100 or 180 mm; Sefiltec AG, Höri, Switzerland) were fixed by the well managers to the (piezo-)pipe draining groundwater from the aquifer to the drinking water well (Fig. 2B). This sampling method excluded pumped waters and relied on passively collected aquifers. The filter bags collected all material that was washed from the aquifer for a week. The local well managers checked the contents for organisms after this sampling period. They transferred all organisms observed to the provided sample tubes containing Ethanol (molecular grade, 80%) with provided forceps. Additionally, or if sampling procedure was not possible according to the above protocol, the overflow chamber (Fig. 2C) could be sampled with a small aquarium net (Tetra Fish-Net, 10.0 × 8.0 cm; mesh size 0.5 × 1.0 mm; Tetra GmbH, Melle, Germany). We specifically asked to report also if there were no organisms found. Samples and completed data sheets were sent back via postal service.
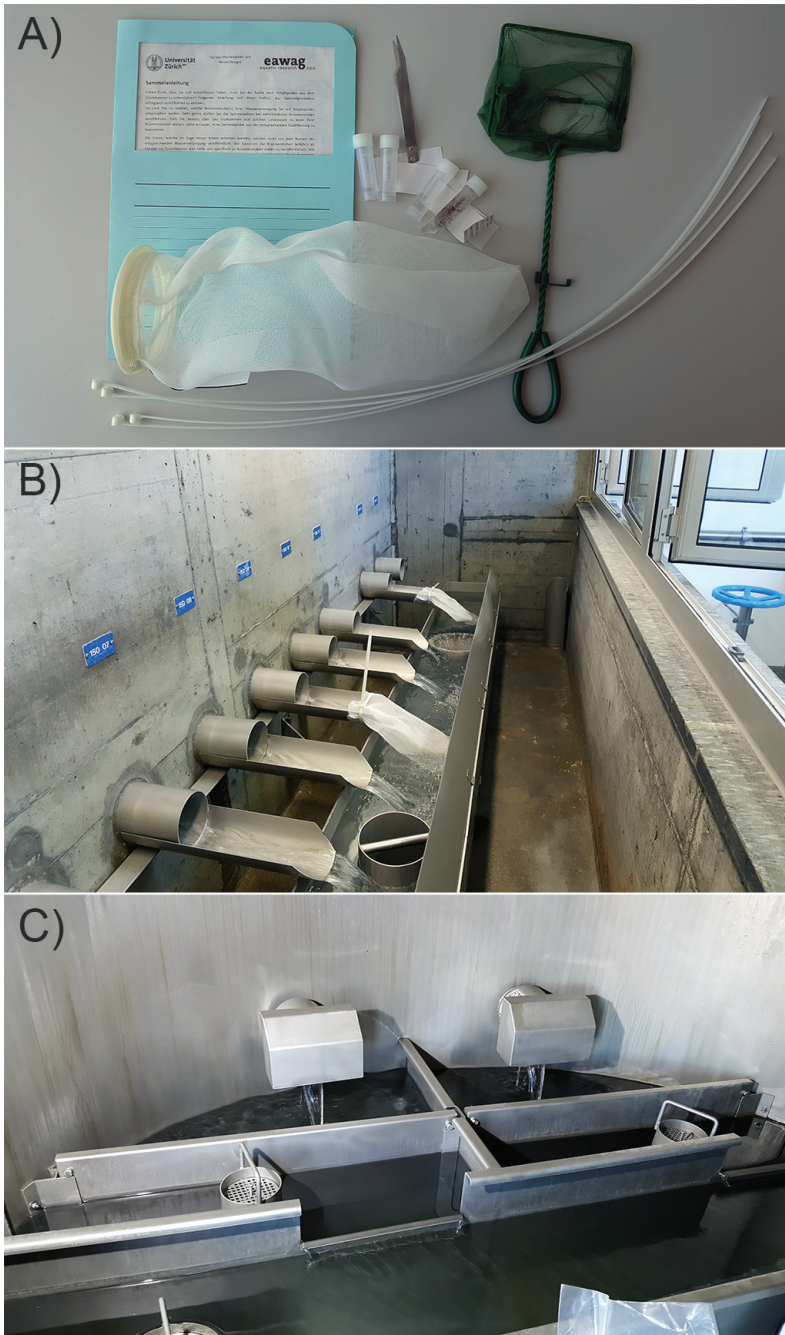
Figure 2. A Sampling material, instructions, and data sheets that were provided to well managers **B** the filter bags were fixed to groundwater draining pipes and collected all washed organisms larger than 0.8 mm **C** the overflow chambers were sampled with a small aquarium net (here: type locality of *Niphargus arolaensis* sp. nov.).

Morphological analysis and identification

After receiving back the samples, we separated all amphipods from organic matter and other macroinvertebrates, using a sorting plate and a stereomicroscope (Nikon SMZ1500, 0.75–11.25×). We identified the *Niphargus* specimens to species level with a stereomicroscope (Olympus SZX9) and a light microscope (Zeiss Primo Star). For detailed analysis, we dissected a few specimens in glycerol, and mounted them on glass slides in glycerol gelatine. We performed morphometric measurements using the program cellSense (Olympus) according to the landmarks detailed in Fišer et al. (2009). We prepared morphological illustrations using digital inking (Coleman 2003, 2009) in Adobe Illustrator 2020. We took template pictures on a Leica M205C with a mounted Canon EOS 5D Mark III. All other invertebrates were identified to relatively coarse taxonomic levels using various determination literature (Freude et al. 1981; Sartori and Landolt 1999; Schminke et al. 2007; Waringer and Graf 2011; Lubini et al. 2012; Bährmann and Müller 2015; Pfeifle and Decker 2019; Stresemann et al. 2019).

Molecular and phylogenetic analysis

For *Niphargus* specimens, we sequenced from each site at least one specimen of each morphologically distinct *Niphargus* species, resulting in 120 specimens from 68 sites. We isolated genomic DNA from one of the pereopods using the GenElute Mammalian Genomic DNA (Sigma-Aldrich, United States). We amplified the two nuclear DNA gene fragments: part of 28S rRNA gene (28S), histone H3 (H3) and the mitochondrial cytochrome oxidase I (COI) gene. We used primers from Colgan et al. (1998) for H3 fragment, primers from Verovnik et al. (2005) for 28S fragment and primers LCO 1490 and HCO 2198 (Folmer et al. 1994) for COI fragment. PCR cycling conditions for 28S and H3 are described in Fišer et al. (2013). For COI we followed the protocol of KAPA2G Robust PCR Kit (Sigma-Aldrich, United States). PCR products were purified using Exonuclease I and FastAP (Thermo Fisher Scientific Inc., United States) according to the manufacturer's instructions. Bidirectional sequencing was performed by Macrogen Europe (Amsterdam, Netherlands), using PCR amplification primers. We assembled and edited chromatograms in Geneious 11.0.3 (Biomatters, New Zealand).

We aligned the sequences with MAFFT 7.388 (Katoh and Standley 2013), using E-INS-I algorithm with scoring matrix 1PAM/k=2 and with the highest gap penalty. For 28S we eliminated poorly aligned positions and divergent regions with Gblocks (Talavera and Castresana 2007). We concatenated and partitioned alignments by codon position for H3 and COI and one partition for 28S.

We ran molecular phylogenetic analyses to assess the phylogenetic position of new *Niphargus* species within the genus. The dataset comprised six specimens of newly described species and 163 *Niphargus* taxa from different phylogenetic lineages with emphasis on potentially closely related species, each represented by one specimen. We used *Microniphargus leruthi* Schellenberg, 1934 and two species from genus *Pseudoniphargus* Chevreux, 1901 as an outgroup. We used available sequences from previous studies (Altermatt et al. 2014; Esmaeili-Rineh et al. 2015; Fišer et al. 2017, 2018,

2019, and references therein) and newly obtained sequences for seven specimens. The list of studied species, the origin of samples, and GenBank accession numbers are available in Suppl. material 9: Table S1 and Suppl. material 10: Table S2.

We reconstructed the phylogenetic relationships with Bayesian inference (BA) in MrBayes v3.2.6 (Ronquist et al. 2012) and maximum likelihood (ML) in IQ-TREE 1.6.6 (Nguyen et al. 2015). For BA we chose the optimal substitution model using Partition Finder 2 (Guindon et al. 2010; Lanfear et al. 2017), under corrected Akaike information criterion (AICc) (Suppl. material 11: Table S3). We ran two simultaneous independent runs with four chains each for 20 million generations, sampled every 1000th generation. Convergence was assessed through average standard deviation of split frequencies, LnL trace plots and PSRF, and the effective sample size. We analysed results in Tracer 1.7 (Rambaut et al. 2018). We discarded the first 25% of trees and calculated the 50% majority rule consensus tree. In ML analysis we used an option to simultaneously determine the best-fit substitution model (Suppl. material 11: Table S3) and run phylogenetic inference analysis, with ultrafast bootstrap approximation (UFBoot) and SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010; Minh et al. 2013). Phylogenetic analyses were run on the CIPRES Science Gateway (Miller et al. 2010) and IQ-TREE web server (Trifinopoulos et al. 2016). The corresponding NEXUS files are available on Zenodo (10.5281/zenodo.4770187). Finally, we calculated the average uncorrected pairwise genetic differences (e.g. p-distance) for the COI fragment between the *Niphargus arolaensis* sp. nov. and all other species using Geneious 11.0.3.

Results

Our citizen science approach proofed successful and had high return rates. First response rate to an initial letter asking for participation was 21% (40 out of the 191 contacted well managers). Subsequent talking to the well managers in person at the annual meeting resulted in more than two thirds of positive feedback. Some of the participants that initially received the letter volunteered only after meeting in person. We sent the sampling kit (Fig. 2A) and instructions to 130 well managers. 82 of those well managers participated in our study during spring and summer 2019.

The well managers provided either samples or information about null findings. Many well managers sampled multiple sites, resulting in 313 unique sites sampled (pipes draining different aquifers but collected in the same water well were considered separate sites). Additionally, some sites were sampled repeatedly, resulting in 491 samples that were sent back to our lab. 56% (274) of the samples contained organisms, totalling to over 1,900 specimens. The samples contained overall 18 different orders of macroinvertebrates. These were: Amphipoda (Crustacea, Malacostraca), Araneae (Arachnida), Chordeumatida (Diplopoda), Coleoptera (Insecta), Diptera (Insecta), Entomobryomorpha (Entognatha, Collembola), Ephemeroptera (Insecta), Hemiptera (Insecta), Hymenoptera (Insecta), Isopoda (Crustacea, Malacostraca), Julida (Diplopoda), Littorinimorpha (Gastropoda), Plecoptera (Insecta), Poduromorpha (Entognatha, Collembola), Polydesmida (Diplopoda), Pseudoscorpiones (Arachnida), Opiliones (Arachnida), and
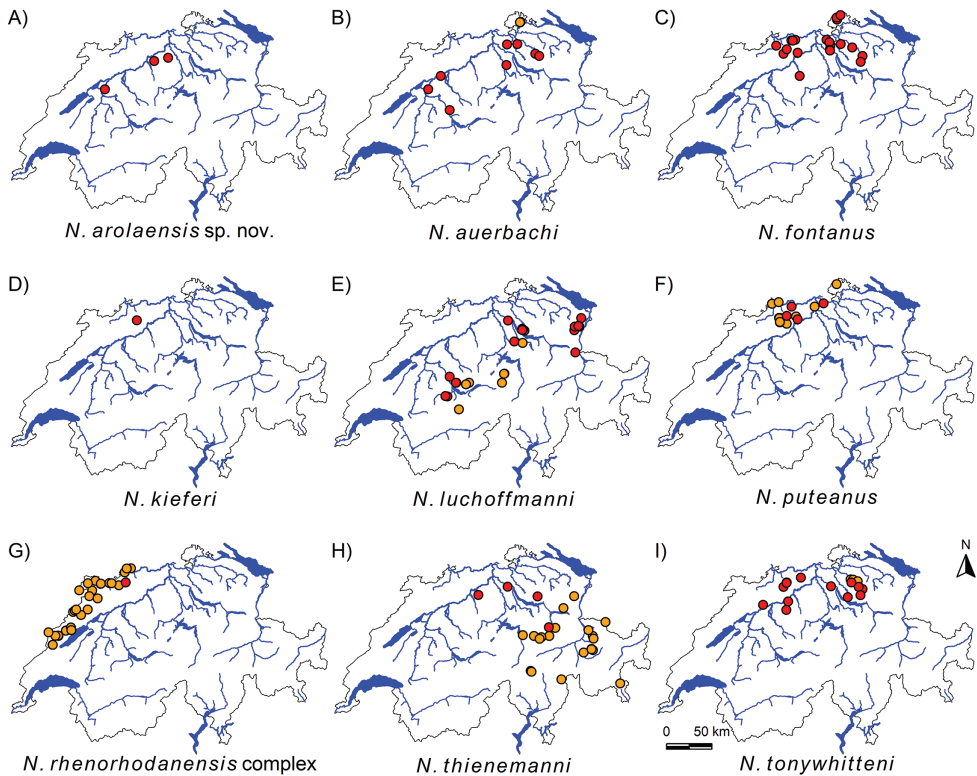
Figure 3. Maps depicting all *Niphargus* sp. findings and their respective taxonomic assignment from the current citizen science project (red circles) as well as all previously known findings of the respective species (orange circles). One species is new to science (**A**) and two species are reported for the first time for Switzerland (**C, D**). Six species (**B, E–I**) were previously reported from Switzerland. Map **G** shows all cryptic *N. rhenorhodanensis* species, including the newly found specimen of lineage H in red. Geodata from Federal Office of Topography.

Trichoptera (Insecta). Some of these organisms were not groundwater inhabitants, but may have been washed in from surface waters, or even of terrestrial origin. Amphipods were the most common and most widespread groundwater organisms in the samples, with in total 424 individuals collected from 74 sites. The majority of those (363 individuals from 63 sites) belonged to the genus *Niphargus*, while the remaining were epigean *Gammarus fossarum* that either had been washed from surface waters or colonised the water-wells from downstream sites. Here, we only focus on *Niphargus* species.

Species identity determination using the COI fragment and subsequent alignment to existing barcodes revealed 13 different phylogenetic lineages of *Niphargus*. Nine of them could be ascribed to eight nominal species. These were: *Niphargus auerbachi* Schellenberg 1934, *Niphargus fontanus* Spence Bate, 1859 (belonging to the clade A sensu McInerney et al. 2014), *Niphargus kieferi* Schellenberg, 1936, *Niphargus luchoffmanni* Fišer et al., 2018 (2018), *Niphargus puteanus* (Koch, 1836), *Niphargus rhenorhodanensis* Schellenberg, 1937 (lineages H and JK sensu Lefébure et al. 2007; lineage JK was found far outside its known range and will not be treated here further because of an ongoing revision of this species complex), *Niphargus thienemanni* Schellenberg, 1934, and *Niphargus tonywhitteni*

Fišer et al., 2018 (Fig. 3, Suppl. material 10: Table S2). We recognized four further lineages as potentially new species to science. One of them we here describe as *Niphargus arolaensis* sp. nov. (see section "Species description" below). For few *Niphargus* specimens found in nine samples, we do not yet draw further taxonomic conclusions, either due to immature stages/low number of individuals only, and/or inconclusive results from the sequencing, and we here treat them only at the genus level (*Niphargus* sp.).

Six of the found *Niphargus* species had been previously reported from Switzerland (Fig. 3A–F), namely *N. auerbachi*, *N. luchoffmanni*, *N. puteanus*, *N. rhenorhodanensis*, *N. thienemanni*, and *N. tonywhitteni* (Altermatt et al. 2014, 2019; Fišer et al. 2017, 2018). However, for *N. auerbachi* (Fig. 3F) the last records date back to the 1930s (Schellenberg 1934a; Altermatt et al. 2019).

Two species are herewith reported from Switzerland for the first time, namely *Niphargus fontanus* and *Niphargus kieferi*. We found *N. fontanus* in 19 sites in the Aare drainage area and the Rhine drainage area (Fig. 3G). Our data show that it is a widely distributed and common species in Switzerland. *Niphargus kieferi* we only retrieved from one site near Oberdorf in the canton of Baselland (Fig. 3H).

Twenty-five specimens sampled from three water wells in the cantons of Aargau and Bern, all in the Aare drainage area (Fig. 3J), belong to a new species that we here formally describe as *Niphargus arolaensis* sp. nov. (see section "Species description" below). The species appeared as a unique monophyletic lineage on the multilocus phylogeny (Fig. 4 and Suppl. material 1: Fig. S1), with p-distance on COI of at least 6% to its closest relatives, namely a lineage of probably two species, labelled *Niphargus* cf. *thienemanni* in previous publications (Fišer et al. 2017, 2018). On the other hand, all specimens of *N. arolaensis* sp. nov. closely resemble each other, with p-distances less than 1%. These results support its species status (Lagrue et al. 2014). The molecular data of the six barcoded specimens are deposited on GenBank (Accession numbers are in Suppl. material 9: Table S1 and Suppl. material 10: Table S2).

Both newly constructed phylogenetic trees showed a congruent topology. Swiss amphipods classify into few well-defined lineages. The relationship between these lineages is incompletely resolved. While ML recovered a relatively well supported clade that comprised most species reported from Switzerland (Fig. 4), BA analyses recovered parts of this clade (Suppl. material 1: Fig. S1). In either case, the newly discovered species falls into a well-defined clade comprised of Swiss species.

We submitted all newly generated COI sequences of *Niphargus* species to GenBank. All accession numbers are listed in Suppl. material 9: Table S1. The sampling localities are listed in Suppl. material 10: Table S2.

Species description

Niphargus arolaensis sp. nov.
http://zoobank.org/DCC92744-5C5C-4540-B48F-BF6083A6C57B

Type material. *Holotype* (Figs 5–9): Female, 7.8 mm (tip of rostrum to tip of third urosomite). The sample is deposited in the collection of the Musée de

Figure 4. IQ-Tree: Phylogenetic hypothesis from Maximum Likelihood. Nodes are labelled with ultra-fast bootstrap support (UFBoot)/approximate likelihood ratio test (SH-aLRT) when values are higher than 95/80 respectively. Species that occur in Switzerland are in bold.
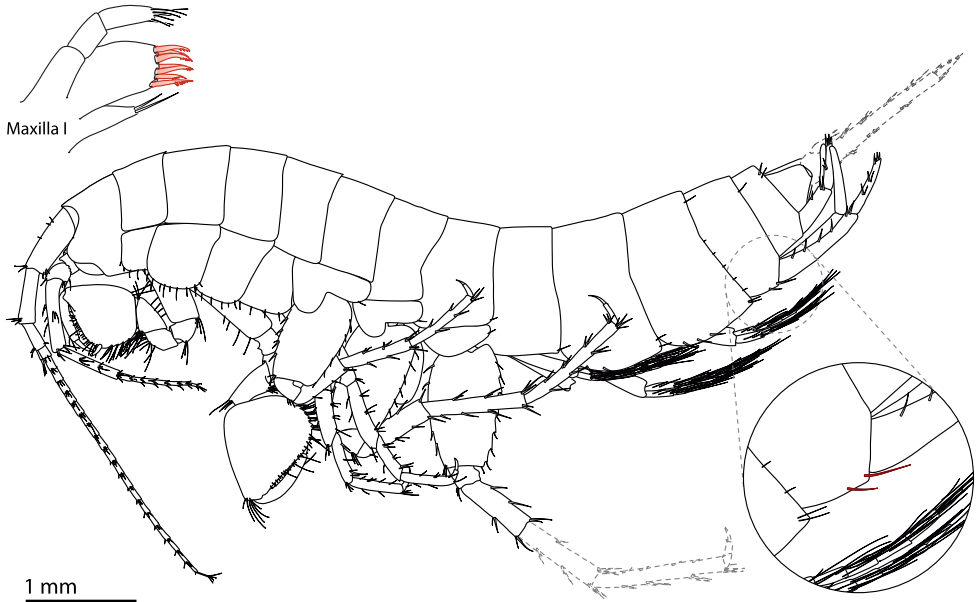
Figure 5. A new *Niphargus* species from Switzerland. *Niphargus arolaensis* sp. nov. (holotype, female 7.8 mm). The two diagnostic features (seven comb-like spiniform setae on the outer lobe of maxilla I and two spiniform setae on the lower distal part of the first urosomite near the insertion of uropod 1) are highlighted on the figure.

Zoologie, Lausanne, Switzerland under voucher number GBIFCH00602901 and GBIFCH00602902.

Paratypes: One male and three females of respective lengths 7.7, 7.8, 8.7 and 9.5 mm; specimens are partially dissected and mounted on slides with voucher numbers GBIFCH00602903, GBIFCH00602904, GBIFCH00602905, GBIFCH00602906, GBIFCH00602907.

Type locality. Stedliquelle (left inflow), Aarberg, Switzerland. CH1903: 588'518, 209'959 (WGS84: 47.04056°N, 7.28756°E), 478 m a.s.l.

Habitat and distribution. Only known from three drinking water wells: Stedtliquelle close to Aarberg, Stöckhof close to Egliswil and Lätzloch close to Kölliken, all in Switzerland.

Etymology. The name "*arolaensis*" is derived from the Latin name of the river Aare (Arola), since all findings were located in the drainage basin of the river Aare.

Diagnosis. Small and slender *Niphargus*, defined by combination of two traits. Two spiniform setae are located on the lower distal part of the first urosomite near the insertion of uropod I (Fig. 5). The outer lobe of maxilla I is armed with seven comb-like spiniform setae (Fig. 5).

Description. *Head and trunk* (Fig. 5): Body length up to 9.5 mm. Head length 6.6–7.4% of body length; rostrum absent. Pereonites I–VI without setae, pereonite VII with tiny seta close to ventro-posterior corner.

Pleonites I–III with up to three setae along the entire dorso-posterior margins. Epimeral plate II roughly perpendicular, posterior and ventral margins convex; ventro-postero-distal corner distinct; along ventral and posterior margins three spiniform and four to five thin setae, respectively. Epimeral plate III inclined, posterior and ventral margin slightly-distinctly concave and slightly convex, respectively; ventro-postero-distal corner distinct but not produced. Along ventral and posterior margin 3–4 spiniform seta; along posterior margin five thin setae.

Urosomite I postero-dorso-laterally with one slender, flexible seta; urosomite II postero-dorso-laterally with 2–3 strong setae among which at least one is strong and stout; urosomite III without seta. Ventrally on urosomite I, at the base of uropod I, are two strong spiniform setae in a row.

Telson (Fig. 9E) length : width ratio is 1 : [0.81–0.85]; cleft measures 0.61–0.75 of telson length; telson lobes margins straight and narrowing apically. Telson armature (per lobe, left-right lobe asymmetry commonly observed): 2–4 apical, 0–1 mesial, 1–2 lateral and no dorsal spiniform setae. Apical spiniform setae as long as 0.50–0.63 of telson length. Pairs of plumose setae inserted medially, along lateral margins.

Antennae (Fig. 6): Antenna I (A) measures 0.40–0.45 of body length. Flagellum with 18–22 articles; each article with one aesthetasc. Peduncle articles in ratio 1 : [0.70–0.82] : [0.35–0.40]. Accessory flagellum biarticulated, proximal : distal article in ratio 1 : [0.25–0.33].

Ratio of lengths antenna I : antenna II as 1 : [0.48–0.52]. Flagellum of antenna II (B) with 7–8 articles; each article with setae and elongated, thick sensilla of unknown function. Peduncle articles lengths 4 : 5 in ratio 1 : [0.89–0.95]; flagellum 0.57–0.66 of length of peduncle articles 4+5.

Mouthparts (Fig. 7): Labrum (A) and labium (B) typical of the genus; inner lobes of labium well visible.

Left mandible (C and D): incisor with five teeth, lacinia mobilis with four teeth; between lacinia and molar a row of serrated setae, molar triturative, at the base of molar long seta. Right mandible (E and F): incisor processus with four teeth, lacinia mobilis with several small teeth, between lacinia and molar a row of thick serrated setae, molar triturative. Mandibular palp article 3 articulated. Ratio of mandibular palp article 2 (middle) : article 3 (distal) is 1 : [1.2–1.37]. Proximal palp article without setae; the middle article with 7–8 setae; distal article with 3–5 A setae in a row; 3–4 B setae; 15–18 D setae and four E setae.

Maxilla I (G and H), distal palp article with 6–7 apical setae. Outer lobe of maxilla I with a row of 7 stout spiniform setae, each with many (>4) denticles (comb-like); inner lobe with two setae along medial and apical margin.

Maxilla II (I and K) inner lobe slightly smaller than outer lobe; both lobes setose apically and medially.

Maxilliped (L) inner lobe with three stout flattened and tooth-like setae apically and 6–11 setae along latero-apical margins; outer lobe with 7–11 stout and flattened, tooth-like setae mesially-subapically and 5–7 thick rounded and hairy setae apically. Maxilliped palp article 2 with 8–10 rows of setae along inner margin; dactylus with a dorsal seta, and few tiny setae at the socket.
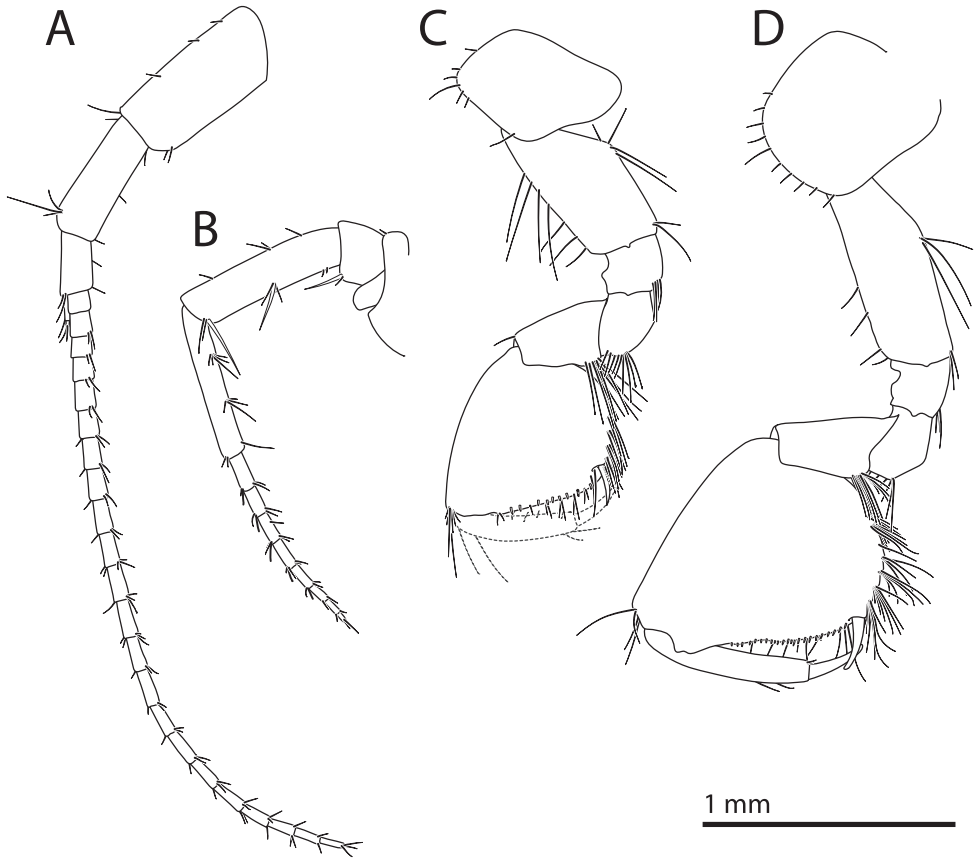
Figure 6. *Niphargus arolaensis* sp. nov. **A** antenna I **B** antenna II **C** gnathopod I **D** gnathopod II. The dactylus on gnathopod I (grey dashed) is added from the other body side.

Coxal plates, and gills (Figs 6 and 8): Coxal plate I in shape of flattened parallelogram; anterior and ventral margin of coxa I with 5–6 setae. Coxal plates II–IV width : depth ratios as [0.90–1.10] : 1, [0.85–0.94] : 1 and [1.00–1.13]: 1, respectively; anterior and ventral margins with 8–9, 6–11 and 6–7 setae. Coxal plate IV posteriorly shallowly concave. Coxal plates V–VI with well-developed anterior lobe, posterior coxal margin with one seta. Coxal plate VII half-circular with one posterior seta. Gills II–VI narrowly ovoid.

Gnathopod I (Fig. 6C): Ischium with up to 5 postero-distal setae in a single row. Carpus 0.70–0.73 of propodus length; broadened proximally. Carpus with only one distal group anteriorly, transverse rows of setae on a posterior bulk and a row of setae postero-laterally. Propodus quadratic with moderately inclined palm. Along posterior margin 4–5 rows of setae. Anterior margin with antero-distal group counting 5–10 setae and additional 8–10 setae in three groups. On the inner surface are several pairs of short setae. Palmar corner armed with one strong and stout palmar spine, a group of three long thin and simple setae anteriorly to palmar spine, one strong short and smooth "supporting" spine on the inner surface and three serrated spines
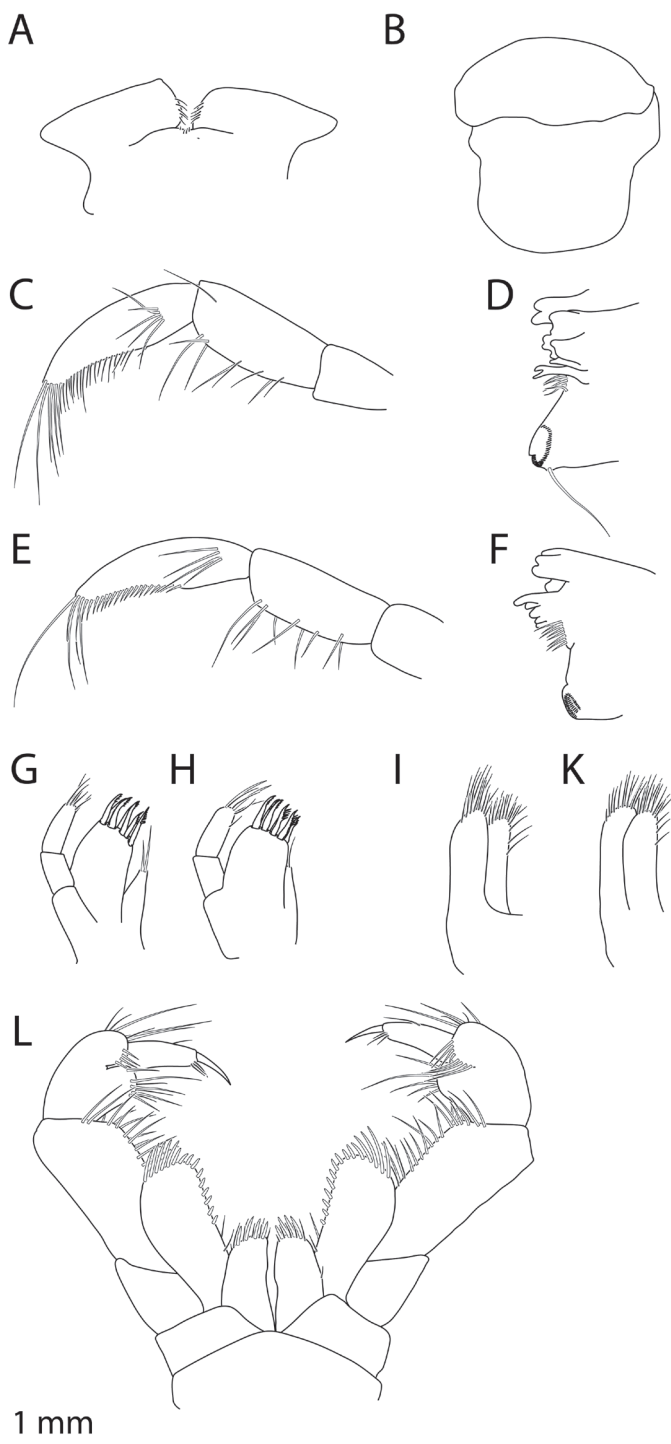
Figure 7. *Niphargus arolaensis* sp. nov., mouthparts **A** labrum **B** labium **C** left mandibular palp **D** left mandible **E** right mandibular palp **F** right mandible **G, H** maxilla I **I, K** maxilla II **L** maxillipeds.
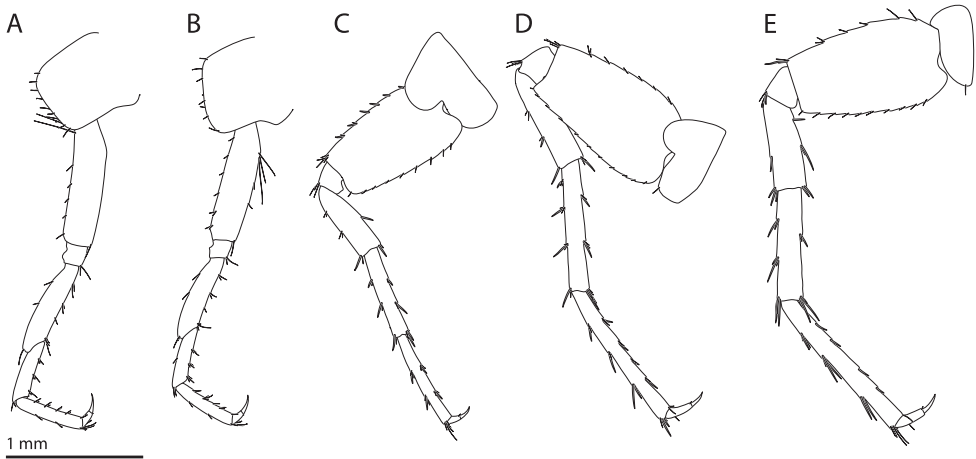
Figure 8. *Niphargus arolaensis* sp. nov. **A–E** pereopods III–VII.

behind the palmar spine. Palm with a row of short setae. Nail length 0.30–0.34 of total dactylus length; along anterior margin 2–4 single seta; along inner margin a row of short setae.

Gnathopod II (Fig. 6D): Ischium with 1–3 postero-distal setae in a single row. Carpus 0.66–0.84 of propodus length, proximally broadened. Carpus with a single groups of distal setae anteriorly; some transverse rows of setae on a posterior bulk and a row of setae postero-laterally. Propodus hoof-shaped with strongly inclined palm and large. Circumference measures up to 0.19–0.23 of body length; ratio between propodus I and II lenghts is [0.74–0.95] : 1. Along posterior margin six rows of setae. Anterior margin with antero-distal group counting 6–8 setae and additional 4–5 setae in 2–3 groups. On the inner surface are several pairs of short setae. Palmar corner armed with one strong and stout palmar spine, a group of 2–3 long thin and simple setae anteriorly to palmar spine, one strong short and smooth spine on the inner surface and 1–2 serrated spines behind the palmar spine. Palm with a row of short setae. Nail length 0.30–0.34 of total dactylus length; along anterior margin 2–4 single seta; along inner margin a row of short setae.

Pereopods III–IV (Fig. 8A and 8B): Lengths of pereopods III : IV as [0.90–0.97] : 1. Dactyli III–IV long and slender, dactylus IV measures 0.42–0.46 of propodus IV; nail length 0.56–0.65 of total dactylus length. Dactyli III–IV with 1 dorsal plumose seta; at the base of nail 1 tiny seta and one tiny spiniform seta.

Pereopods V–VII (Fig. 8C–8E): Lengths of pereopods V : VI : VII is 1 : [1.34–1.41] : [1.31–1.41]; pereopod VII measures 0.46–0.47 of body length.

Bases V–VII slender, respective length : width ratios as 1 : [0.57–0.64], 1 : [0.57–0.64] and 1 : [0.58–0.64]; posterior margins straight or slightly convex, distally ending with small to moderate-sized lobes; posterior margins armed with 8–10, 9–10 and 8–10 setae, respectively; anterior margins armed with 6–7, 6–7 and 5–6 groups of stouter setae, respectively. Dactyli V–VII with one dorsal plumose seta; at the base of
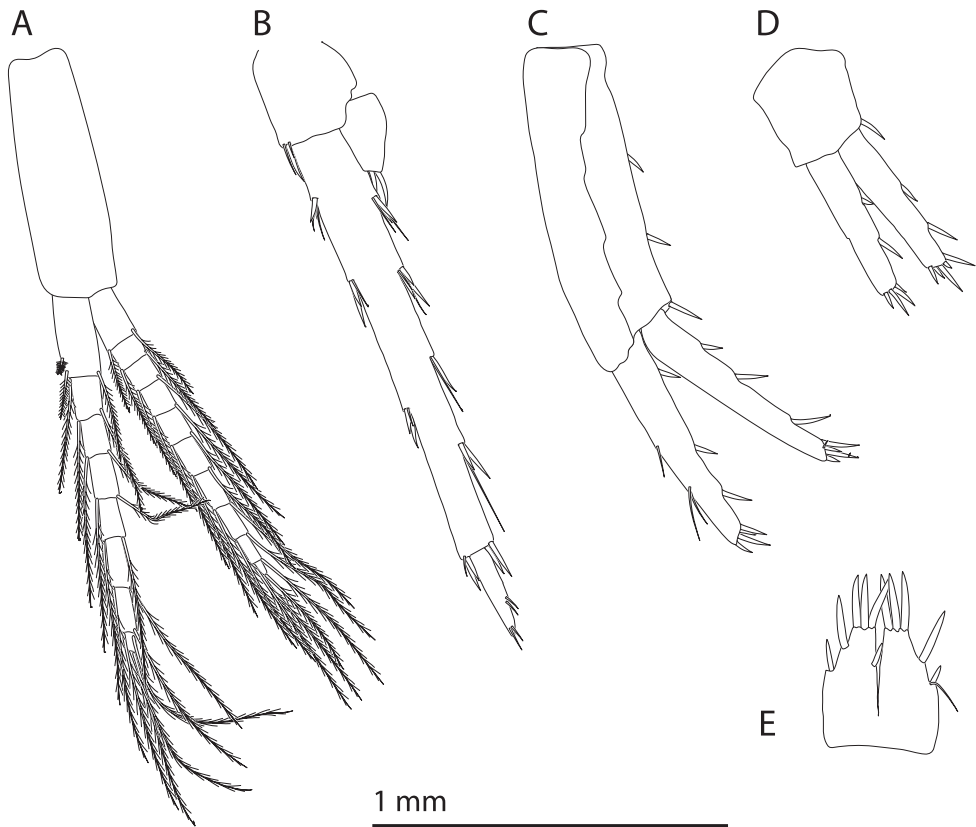
Figure 9. *Niphargus arolaensis* sp. nov. **A** pleopod II **B–D** uropods I–III **E** telson.

nail one tiny setae and one spiniform seta. Dactylus VII long and slender, its length measures 0.28–0.32 of propodus length; nail long, measuring 0.34–0.38 of total dactylus length.

Pleopods and uropods (Fig. 9): Pleopods I–III (A) with two hooked retinacles. Pleopod II inner and outer rami with 6–7 and 8–9 articles, respectively.

Uropod I (B) protopodite with six dorso-lateral spiniform setae and 2–3 dorso-medial spiniform setae. The ratio exopodite : endopodite lengths is 1 : [0.98–1.06]; rami straight. Endopodite with four individual spiniform setae laterally, rarely accompanied with a slender and flexible seta, and four spiniform setae apically. Exopodite with 2–6 spiniform setae alone or in groups; apically 4–6 spiniform setae.

Uropod II (C) exopodite : endopodite lengths ratio is 1 : [1.00–1.05].

Uropod III (D) rod-shaped, measuring 0.20–0.22 of body length. Protopodite elongated, sometimes with a single weak lateral seta and with 5–7 apical spiniform setae. Endopodite short, measures approximately 0.56–0.63 of protopodite length; laterally armed with 0–1 spiniform setae, apically armed with 3–4 spiniform setae, of which 1–2 are strong and spiniform. Exopodite of uropod III rod-shaped, distal article 0.16–0.22 of the proximal article length. Proximal article with five groups of spiniform

and plumose setae along inner margin and 4–5 groups of spiniform setae along outer margin. Distal article with 0–2 setae laterally and 1–4 setae apically.

Variability. We found no sexual dimorphism in proportions, females had oostegites on pereopods II–IV. Number of setae vary, smaller specimens had fewer setae.

Remarks and affiliation. The diagnosis is a combination two unambiguous traits. Two strong spiniform setae at the base of uropod I is a rare character, hitherto found only in *Niphargus bodoni* G. Karaman, 1985 (Italy, Karaman 1985), *Niphargus lindbergi* S. Karaman, 1956 (Borko et al. 2019; Greece, Karaman 2018), *Niphargus sertaci* Fišer, Çamur-Elipek & Özbek, 2009 (Western Turkey, Fišer et al. 2009), *Niphargus turcicus* Andreev & Kenderov, 2012 (Eastern Turkey, Andreev Kenderov 2012) and *Niphargus borisi* Esmaeili-Rineh, Sari & Fišer, 2015 (Iran, Esmaeili-Rineh et al. 2015). However, all these species have a different spiniform setae on outer lobe of maxilla I, i.e., the inner seta is multidenticulate and the remaining six setae have 1–3 denticles. By contrast, the herein described *N. arolaensis* sp. nov. has all these spiniform setae on outer lobe of maxilla I multidentate. To ease its identification in Europe, it is noteworthy that the species remarkably differs from *N. bodoni* in shape of its gnathopods. The Italian species has much smaller and more quadratic propods of gnathopods I–II, while the herein described species from Switzerland has relatively large propodi with a strongly inclined palm. Finally, it is worthy to warn that the newly described *N. arolaensis* sp. nov. superficially resembles *Niphargus forelii* Humbert, 1876 from the Alpine region. It is small, of relatively slender body, with large gnathopods, long and slender dactyli, a telson with no dorsal spiniform setae, but very long apical and marginal spiniform setae. Besides the diagnostic combination, the newly described species differs from *N. forelii* as its males apparently do not have an elongated uropod III (Karaman and Ruffo 1990).

Discussion

Unlike for the Swiss cave fauna (Strinati 1966), there is yet no general overview published about groundwater fauna for Switzerland. Here, we provide the means of tapping into this knowledge gap by applying a citizen science approach, with a focus on amphipods of the genus *Niphargus*. The opportunistic sampling campaign revealed organisms from 18 different orders. An important fraction of all individuals belonged to the genus *Niphargus*. We present a conclusive overview for those species across the Swiss Plateau, reporting 13 lineages belonging to eight nominal species, of which two are for the first time reported for Switzerland and one is even new to science. The results confirmed that a collaboration with local drinking water well managers could successfully generate data about groundwater fauna, data that would be hard to collect in a different manner (Thornhill et al. 2019) but is very valuable for biodiversity research and conservation (Theobald et al. 2015). The fraction of further subterranean species (other than *Niphargus* sp.) will be analysed and treated in a separate study.

The collaboration with well managers significantly increased the current knowledge about Swiss *Niphargus* species, raising the number of known sites of *Niphargus*

occurrence by about 22% (288, compared to 45 in Altermatt et al. 2013 and 225 in Altermatt et al. 2019). We also raised the known number of amphipod species from Switzerland to 43 species (Altermatt et al. 2019; https://www.amphipod.ch/en/resources/checklist/), adding three species to the Swiss Amphipoda checklist, namely *N. fontanus*, *N. kieferi* and *N. arolaensis* sp. nov.

The most spectacular finding of this citizen science project was the finding of a species new to science, here formally described as *N. arolaensis* sp. nov. (Figs 3J, 6–9). A total of 25 specimens were retrieved from three water wells in Aarberg (canton of Bern), Egliswil, and Kölliken (both canton of Aargau). These findings were all close to the Aare river, and fit into a biogeographic region that has been shaped by the Aare glacier (in the Chibanian). Morphologically, *N. arolaensis* sp. nov. is not very distinctive, and it is hard to align it with other ecologically distinct species. Being small, it resembles other groundwater inhabiting *Niphargus* species. Interestingly, while the gnathopods indicate that *N. arolaensis* sp. nov. might be a predator, the comb-like maxillar spines suggest that the species might feed on small particles.

While the adjacent mountainous regions (Jura Mountains and Alps) have been more intensely studied with respect to subterranean amphipods, these studies almost exclusively focussed on karstic regions (especially caves) or on interstitial habitats and less on inaccessible alluvial aquifers. Cave habitats are almost absent in the Swiss Plateau and many interstitial habitats, especially of the larger rivers, have been heavily modified by humans by river regulations and dams. Our study now shows that the groundwater habitats in the Swiss Plateau, geologically largely dominated by alluvial habitats shaped by glaciers, is (next to karstic caves and interstitial) another important habitat of *Niphargus* in Switzerland, encompassing a surprisingly high diversity of *Niphargus* species.

Swiss amphipods classify into few well-defined clades with different phylogenetic origin within *Niphargus* (Fig. 4 and Suppl. material 1: Fig. S1). However, most of the Swiss species aggregate into one phylogenetic lineage. This pattern emerged only after including new samples, obtained by this study. These new samples bring new views on the historical biogeography of species, indicating the putative presence of local radiations in groundwater. It is expected that future sampling of groundwater will reveal additional *Niphargus* species, will clarify the status of this potential Swiss radiation and enable us to explore its biogeographical and evolutionary history.

Next to *Niphargus arolaensis* sp. nov., we also report two additional species new to the Swiss fauna. The first one belongs to the *Niphargus fontanus* species complex, originally described from the United Kingdom, but also found in continental Europe. Its lineages may not be told apart based on morphology alone, and formal revision of the complex is pending. Our specimens found belong to the lineage *N. fontanus* A, that was reported from France, Belgium, Germany and parts of Austria (Hartke et al. 2011; McInerney et al. 2014). *Niphargus fontanus* A had been sampled in our pilot study (Fig. 3G) in the canton of Schaffhausen in 2018 (Rodrigues, unpublished). Subsequent sampling across the Swiss Plateau in 2019 added many more findings over an area covering a few thousand km². *Niphargus fontanus* turned out to be a widely

distributed and frequently found species, with findings scattered across the Swiss Plateau, both within the Rhine drainage area as well as within the Aare drainage area. The fact that this seemingly widely distributed species had not been found before is highlighting the need for further investigations of the groundwater fauna in Switzerland.

The second species reported for the first time for Switzerland is *Niphargus kieferi* (Fig. 3H). This species was first described as subspecies *Niphargus jovanovici kieferi* by Schellenberg from a well near Gündlingen (Germany) in the Upper Rhine plain (Schellenberg 1936), but later raised to full species by Karaman (Karaman 1980). The species is distributed in the Upper Rhine plain in France and Germany. In 2001 and 2002, the species was reported from several sites in Baden-Württemberg (Fuchs 2007), relatively close to Switzerland. We found a single specimen near Oberdorf (500 m a.s.l.) in the canton of Baselland within the Rhine drainage area and the finding fits well into the previously known distribution.

We also increased the knowledge on the distribution of six *Niphargus* species hitherto already known from Switzerland, mostly for the Swiss Plateau, but also beyond. Specifically, for the recently described species *N. luchoffmanni* (Fig. 3A), previous findings were restricted to the Central Alps in Switzerland (Fišer et al. 2018; Altermatt et al. 2019). We here report more findings around Lake Thun (Aare drainage area), in the Alpine Rhine valley in Eastern Switzerland (Rhine drainage rea), and also around Lake Zurich (Limmat catchment). This suggest that *N. luchoffmanni* may be representative for prealpine regions or lower elevations of the Swiss northern alpine regions. *Niphargus thienemanni* (Fig. 3D) was hitherto only reported from springs and groundwater habitats in alpine regions above 1000 m a.s.l., and up to 2560 m a.s.l., specifically the Eastern Alps (Altermatt et al. 2019; Austria, Germany, Switzerland; Schellenberg 1942). Here we show that it also occurs at elevations around 500 m a.s.l., which is interesting from an ecological point of view, and indicating a wide ecological (elevational) niche. *Niphargus tonywhitteni* (Fig. 3E), in its original description, was only reported from the Töss catchment in North-eastern Switzerland, and exclusively from interstitial samples (Fišer et al. 2018) as well as from a few sites in Austria and South-western Germany (Fišer et al. 2018). Here, we expand the known distribution to the whole Swiss Plateau, showing that the species is much more widespread and likely more common than initially thought (Altermatt et al. 2019). It is now also reported from the Aare drainage area, the High Rhine drainage area, and the Limmat catchment. *Niphargus puteanus* and *N. rhenorhodanensis* (represented with two phylogenetic lineages, namely H and JK sensu Lefébure et al. 2007) (Fig. 3B) were the only species in our study without a major increase in their known distribution. All findings matched well with the previously known distribution in northern and western Switzerland, respectively (Altermatt et al. 2019). Finally, we confirmed the presence of *N. auerbachi* (Fig. 3F) in Switzerland after almost a century without records. After its original description based on samples from Schaffhausen, Northern Switzerland (Schellenberg 1934a) in the 1930ies, it had never been found again (Altermatt et al. 2019). A putative finding from the Hölloch cave by Moeschler (1989) was classified as a misidentification (Fišer et al. 2017). We rediscovered *Niphargus auerbachi* during a

pilot study in Schaffhausen in 2018 (Rodrigues, unpublished), using the same citizen science approach, which was then complemented with various additional findings of the species in the greater Zurich area (Rhine and Limmat catchment) and in the Aare catchment around Bern, indicating that *N. auerbachi* is much more widespread across the Swiss plateau than initially thought (Fig. 3F).

Altogether, our study reveals that the *Niphargus* fauna of Switzerland has distinct patterns of biodiversity and distribution. A community of species inhabiting karstic areas (especially caves) is predominantly found in the Jura mountains in (North)Western Switzerland (*N. puteanus*, *N. rhenorhodanensis*, but also *N. virei*). Another community of species is predominantly inhabiting the Northern (pre)Alps, in a wide range of habitats such as caves, interstitial and groundwater, including *N. luchoffmanni*, *N. muotae*, *N. murimali*, *N. styx*, and *N. thienemanni*. Geographically in between, in the Swiss Plateau, we now report a third community cluster of species predominantly inhabiting interstitial and (alluvial) groundwater habitats, including *N. auerbachi*, *N. fontanus*, *N. kieferi*, and *N. tonywhitteni*. Further research is needed especially in the Southern and Western part of Switzerland, especially those falling into the Rhone, Ticino and Adda drainage basins.

Next to the increase in faunistic knowledge on amphipods in Switzerland, our study also showed how a generalizable citizen science approach targeting well managers could be exceptionally fruitful for gaining access to an otherwise hardly accessible ecosystem. There are debates what qualifies to be considered a citizen science project (Heigl et al. 2019), but the potential of these approaches is considerable (Thornhill et al. 2019). A key aspect of our success was that the citizen science approach targeted a well-defined group of people who access groundwater ecosystems for their use and provisioning of drinking water. While the well-defined group of people contacted may have contributed to high response rates, establishing a collaboration between local stakeholders still required a few key elements to be considered also for other similar projects. Firstly, direct personal contact and interaction with the well managers was a main factor for successfully starting and maintaining our collaboration (Evans et al. 2005). Whereas the first contact was a letter, participation rate massively increased after a direct contact (from 25% to 60% of contacted people responding positively). This required many phone calls and meetings in person. The additional time effort to do so, however, paid back in gaining further participants and samples. However, even in our short sampling scheme that required a one-time investment from the volunteers, some well managers initially agreed to take a sample but never provided any data. There are several explanations why people drop out (Marsh and Cosentino 2019). We did not investigate specifically why this was the case in our study, but a targeted community management that takes care of the volunteers might dampen some of the dropouts (Rotman et al. 2012). Secondly, being a native speaker helped a lot in fostering a common basis for collaboration, especially when explaining the goals and implications of the project concisely. Being able to show value for groundwater protection and the benefit for science could be a main motivator (Domroese and Johnson 2017). Thirdly, providing the necessary sampling kit with easy-to-follow guidelines lowered the threshold to participate and guaranteed some standardization among participants. Fourthly,

we were relatively flexible with respect to the implementation of the sampling protocol, which seemed to be an important aspect motivating well managers to participate. The local well managers are experts on their drinking water well and they often already knew about the presence of groundwater fauna and where or how to sample it best. Tapping into this knowledge, and not prescribing too strict sampling protocols likely contributed to our high rate of success. Allowing for some flexibility does not automatically increase noise in the data but may improve them (Schmeller et al. 2009). Fifthly, after the samples were sent back, providing feedback was an imperative (Rotman et al. 2012). In our case, the benefit of participating in the study was the information about the local fauna that was returned to each volunteer. Additionally, the results were published in a stakeholder oriented journal (Alther et al. 2020). Regular updates about the project, e.g., using a web blog or newsletter, asking for feedback on the scientific results, or involvement in the data analysis typically increase identification with the project (Heigl et al. 2019). The present study had an exploratory character, making these additional participatory measures hard to be implemented in time. Finally, we assured and communicated data protection from start, for example clarifying which data and how they would be published. This lowered the threshold to participate, as some drinking water managers expressed concerns about a possible release of names and specific localities of their drinking water wells. We therefore agreed that the data collected are only published in specific scientific journals, without highlighting single well managers or municipalities (or only after consultation with the respective well manager).

Overall, our approach proved highly successful. However, there are still some possible limitations associated to the approach and methods chosen. Since the groundwater was sampled in a passive way and not pumped, most retrieved samples were in a good state. However, the collected organisms may not be representative of the overall diversity in the respective localities, since some types of organisms might get washed out more easily than others. The discharge differed considerably between the sampled localities and could change depending on the surface conditions (personal communication by the well managers). Additionally, only organisms bigger than 0.8 mm were collected due to the chosen mesh size. All these circumstances make the approach a rather qualitative assessment, likely to underestimate the true diversity of groundwater fauna, highlighting the need of further and more intense sampling. This should not only cover different seasons, but all biogeographic regions of Switzerland. The herein described citizen science approach offers the potential of sampling an extended timescale and to capture potential seasonal patterns (Dickinson et al. 2010; Gouraguine et al. 2019). This is especially needed since data series or seasonal data about groundwater fauna are generally very scarce and temporal dynamics only poorly understood (Mammola et al. 2020). Consequently, little is known about the ecosystem services provided by these organisms (Griebler and Avramov 2015), such as drinking water provisioning, and if groundwater communities could be indicative of the ecological status of subterranean ecosystems (Griebler et al. 2014; Mammola et al. 2020). We thus expect that citizen science approaches may be generally valid and useful for gaining access to an unprecedented number of samples for hitherto largely understudied ecosystems such as groundwater.

Conclusion

Our study showed the feasibility of a citizen science approach in collecting data on groundwater fauna on a large spatial scale. This concept hasn't been applied at this extent to study groundwater fauna. Collaboration with local well managers resulted in groundwater samples from 313 sites, mainly across the Swiss Plateau. They included different major invertebrate groups, mainly crustaceans. We focused on the genus *Niphargus*, with 363 individuals the most common taxa in the available samples. We report eight nominal species (*N. auerbachi*, *N. luchoffmanni*, *N. puteanus*, *N. rhenorhodanensis*, *N. thienemanni*, *N. tonywhitteni*, *N. fontanus* and *N. kieferi*), with the latter two being reported for Switzerland for the first time. Additionally, we discovered four phylogenetic lineages that are potentially new species to science. One of them we describe as *Niphargus arolaensis* sp. nov. Our study is a proof-of-concept, showing that a citizen science approach could increase spatial coverage substantially, but could also raise awareness about groundwater biodiversity among stakeholders.

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Supplementary material 1

Figure S1
Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: image (.tiff file)
Explanation note: MrBayes: Phylogenetic hypothesis from Bayesian inference. Nodes are labelled with posterior probabilities, when higher than 0.80. Lower values are reported in light-grey for focal Swiss clade. Species that occur in Switzerland are in bold.
Link: https://doi.org/10.3897/subtbiol.39.66755.suppl1

Supplementary material 2

Supporting file 1
Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Standardized information letter to well managers, in German.
Link: https://doi.org/10.3897/subtbiol.39.66755.suppl2

Supplementary material 3

Supporting file 2
Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling instructions to well managers, in German.
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Link: https://doi.org/10.3897/subtbiol.39.66755.suppl3

Supplementary material 4

Supporting file 3
Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling instructions to well managers, in French.
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Link: https://doi.org/10.3897/subtbiol.39.66755.suppl4

Supplementary material 5

Supporting file 4
Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling protocol to well managers, in German.
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Link: https://doi.org/10.3897/subtbiol.39.66755.suppl5

Supplementary material 6

Supporting file 5

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling protocol to well managers, in French.
Link: https://doi.org/10.3897/subtbiol.39.66755.suppl6

Supplementary material 7

Supporting file 6

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling labels to well managers, in German.
Link: https://doi.org/10.3897/subtbiol.39.66755.suppl7

Supplementary material 8

Supporting file 7

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt
Data type: pdf document
Explanation note: Sampling labels to well managers, in French.
Link: https://doi.org/10.3897/subtbiol.39.66755.suppl8

Supplementary material 9

Table S1

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt

Data type: excel table

Explanation note: List of species used for phylogenetic analyses, with the origin of samples, and GenBank accession numbers.

Link: https://doi.org/10.3897/subtbiol.39.66755.suppl9

Supplementary material 10

Table S2

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt

Data type: excel table

Explanation note: List of discovered Niphargus individuals from the citizen science approach with GenBank accession numbers.

Link: https://doi.org/10.3897/subtbiol.39.66755.suppl10

Supplementary material 11

Table S3

Authors: Roman Alther, Nicole Bongni, Špela Borko, Cene Fišer, Florian Altermatt

Data type: table (docx. file)

Explanation note: Results of substitution model selection for Bayesian inference (Partition Finder 2) and maximum likelihood (IQ-TREE) analyses.

Link: https://doi.org/10.3897/subtbiol.39.66755.suppl11

RESEARCH ARTICLE

A new subterranean species of *Anillinus* Casey (Carabidae, Trechinae, Anillini) from Florida

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Abstract

A new species of blind subterranean ground beetle in the genus *Anillinus* Casey is described from Florida. *Anillinus albrittonorum* **sp. nov.** (type locality: 6 miles NW High Springs, Columbia County, Florida) has a unique structure of female genitalia and occupies an isolated position within the genus. This new species is illustrated with images of the habitus, body parts, and male and female genitalia. Relationships of *A. albrittonorum* to other members of the genus are discussed.

Keywords

Column trap, distribution, new species, soil fauna

Introduction

The genus *Anillinus* Casey is one of the most speciose North American genera of blind coleopterans. It currently includes more than 55 species, distributed across the eastern and central parts of the United States (Bousquet 2012; Sokolov 2020). Of these, up until now, only two species, *Anillinus dohrni* (Ehlers) and *Anillinus kovariki* Sokolov and Carlton, are reported from Florida. *Anillinus dohrni*, described more than 130 years ago (Ehlers 1884), was the third anilline species reported from North America

at that time. Its description was based on a single female specimen originating from the collection of Carl August Dohrn, a German entomologist from Stettin (type now deposited in Szczecin in Poland). This specimen, according to the description (Ehlers 1884, p. 36), was collected in "Florida", but no precise locality was provided. The species was originally placed in the genus *Anillus* Jacquelin du Val and its description written in Latin. This description was only 11 lines long and does not contain currently useful diagnostic species-specific characters. Despite its long history, *A. dohrni* remains one of the "mysterious" species of the genus. One concept of this species was suggested by Jeannel (1937, 1963a), who, doubting the origin of the type specimen, based his re-description on specimens from Georgia, identified as *A. dohrni* by G.H. Horn (Sokolov et al. 2004). Another concept was suggested by Sokolov et al. (2004), who claimed that Ehlers' *A. dohrni* and *A. dohrni* sensu Jeannel (1937, 1963a) represented two different species. The second species from Florida, *A. kovariki*, is currently known from a single specimen from near Tallahassee (Fig. 1), where it was collected in a pocket gopher burrow. This species is well described (Sokolov et al. 2004) and its interpretation does not cause any difficulties.

It appears that these two Floridian species of *Anillinus* are only a part of the anilline fauna of the state. One explanation for the low number of species recorded from Florida, in addition to climatic, ecological, and physiographic factors, could be insufficient sampling of the appropriate habitat. A recent investigation of soil fauna conducted in the state resulted in the collection of a series of anilline specimens, which, after examination, proved to be a new species of *Anillinus*. The description of this new species forms the major content of this paper.

Materials and methods

This study is based on the examination of 23 specimens of *Anillinus* collected near High Springs in Florida. Type material of *Anillinus albrittonorum* is deposited in the following collections:

CUAC   Clemson University Arthropod Collection, Clemson, SC, USA;
FSCA   Florida State Collection of Arthropods, Gainesville, FL, USA;
KESC   Kyle E. Schnepp Collection, Gainesville, FL, USA;
NMNH   National Museum of Natural History, Washington, DC, USA.

Terms used in this paper follow Sokolov and Carlton (2008) and Sokolov et al. (2014).

Extractions and processing of genitalia were made using standard techniques as described by Sokolov and Kavanaugh (2014).

Photographs of the external features of specimens were taken with a Macropod Pro photomacrography system (Macroscopic Solutions, LLC). Digital images of genitalia were taken with a Nikon Eclipse N*i*-U light microscope supplied with DS-Fi2 camera and DS-LR3 camera control unit.

All specimens were measured using tpsDig 2.17 (Rohlf 2013) software on digital photographs. Measurements for various body parts are encoded as follows:

ABL	apparent body length, from clypeus to apex of elytra;
WH	width of head at level of first orbital setae;
WPm	maximum width across pronotum;
WPa	width across anterior angles of pronotum;
WPp	width across posterior angles of pronotum;
LP	length of pronotum from base to apex along the midline;
WE	width of elytra at level of 2nd discal seta;
LE	length of the elytra, from the apex of the scutellum to the apex of the left elytron.

Apparent body length (ABL) measurements are given in mm, others are presented as ratios: mean widths – WH/WPm and WPm/WE; body parts – WPa/WPp, WPm/WPp, WPm/LP, WE/LE, LP/LE, LE/ABL, and WE/ABL. All values are given as the mean ± standard deviation.

Results

Order Coleoptera Linnaeus, 1758
Family Carabidae Latreille, 1802
Subfamily Trechinae Bonelli, 1810
Tribe Anillini Jeannel, 1937

Genus *Anillinus* Casey, 1918

Anillinus Casey, 1918: 167. Type species: *Anillus* (*Anillinus*) *carolinae* Casey, 1918, by original designation.
Micranillodes Jeannel, 1963a: 57. Synonymy established by Bousquet (2012: 699) and confirmed by Sokolov et al. (2014: 83). Type species: *Micranillodes depressus* Jeannel, 1963a, by original designation.
Troglanillus Jeannel, 1963b: 147. Synonymy established by Barr (1995: 240). Type species: *Troglanillus valentinei* Jeannel, 1963b, by original designation.

Anillinus albrittonorum Sokolov & Schnepp, sp. nov.
http://zoobank.org/87B4A499-1E9A-46C5-9EC7-3373DFDFF2E4
Figs 1–4

Type material. *Holotype*: male (NMNH), dissected, labeled "FLORIDA: Columbia Co., 6mi NW High Springs, 29.8674°N, 82.6664°W, May 6 – August 5, 2020, underground column trap, Kyle E. Schnepp".
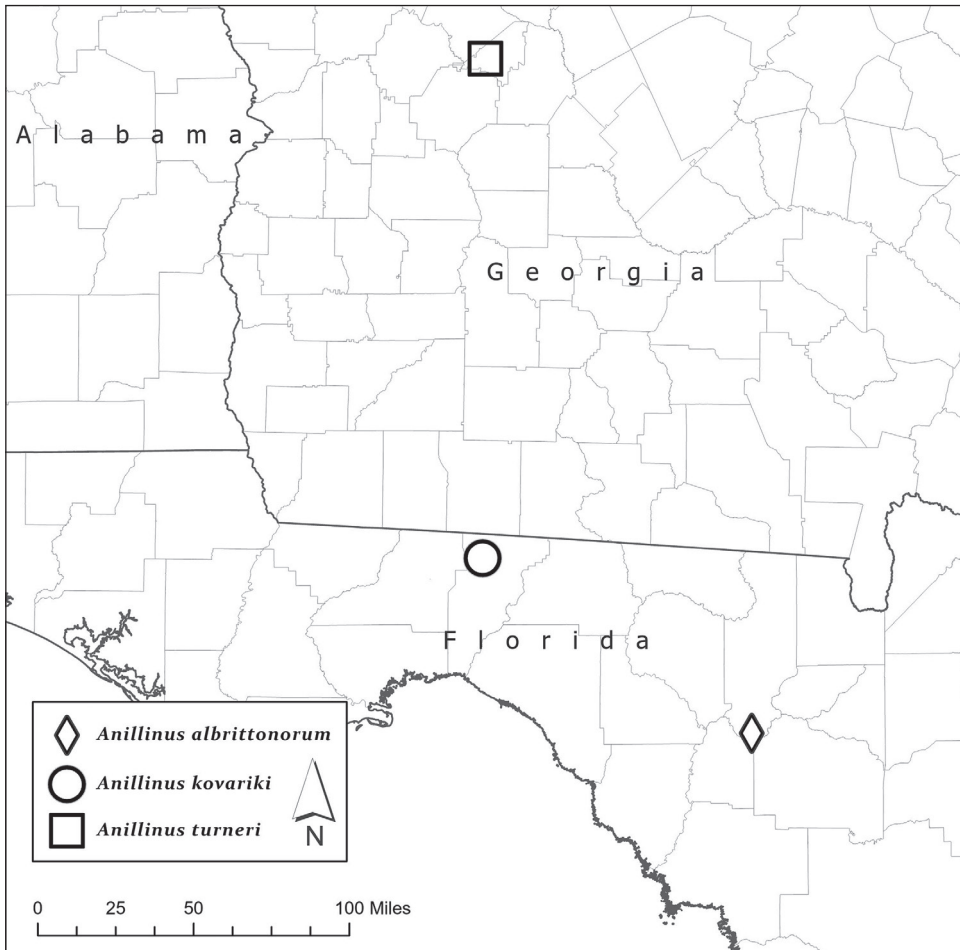
Figure 1. Map of Florida and Georgia showing positions of the known locality records for three species of *Anillinus*.

Paratypes (22 specimens). Same data as holotype [1 male, CUAC; 2 males, KESC; 1 male, 1 female, NMNH]; same data except November 11, 2019 – March 8, 2020 [1 female, FSCA]; March 8 – May 6, 2020 [2 females, CUAC, FSCA]; August 5 – September 25, 2020 [2 males, 3 females, KESC]; September 25 – October 16, 2020 [1 male, 5 females, FSCA]; October 16 – December 3, 2020 [1 male, 2 females, KESC].

Etymology. This species is named in honor of the Albritton family, Matthew, Pam, Rowan, and Henry, whose interest and assistance in collecting brought about the discovery of this beetle.

Type locality. USA, Florida, Columbia County, 6 miles northwest High Springs, 29.8674°N, 82.6664°W (Figs 1, 2).

Diagnosis. Adults of *A. albrittonorum* can be distinguished from both Florida species of *Anillinus* by its subparallel, elongate, only slightly convex habitus. *Anillinus kovariki*

Figure 2. Habitat and type locality of *Anillinus albrittonorum* sp. nov. Underground traps indicated by a pair of flags in foreground and background.

and *A. dohrni* belong to the group of species with ovoid and convex habitus (cf. description of *A. dohrni* "Testaceus, robustus ovatus supra convexus…", Ehlers 1884, p. 36). Additionally, adults of the new species can be distinguished from those of other subterranean members of *Anillinus* by details of the microsculpture of the head and pronotum. The presence of a smooth frons with completely microsculptured vertex of the head and a smooth pronotal disc with a distinctively microsculptured base is distinctive. Males and females of *A. albrittonorum* can also be distinguished from congeners by the structure of their genitalia.

Description. Moderate-sized for the genus (ABL 1.56–1.92 mm, mean 1.71±0.094 mm, n = 17). Males (ABL 1.70–1.92 mm, mean 1.78±0.098 mm, n = 5) slightly larger than females (ABL 1.56–1.88 mm, mean 1.69±0.082 mm, n = 12).

*Habitus***:** Body form (Fig. 3A) slightly convex, subparallel, elongate (WE/ABL 0.33±0.006), head moderately large in comparison to pronotum (WH/WPm 0.77±0.017), pronotum large relative to elytra (WPm/WE 0.88±0.021).

*Integument***:** Body color brunneo-rufous, appendages testaceous. Microsculpture (Fig. 3B, C) present on vertex, base of pronotum, and on elytra where it is represented by isodiametric polygonal sculpticells; and absent from clypeus and frons on head, and from disc of pronotum. Body surface shiny, surface sparsely and finely punctate,
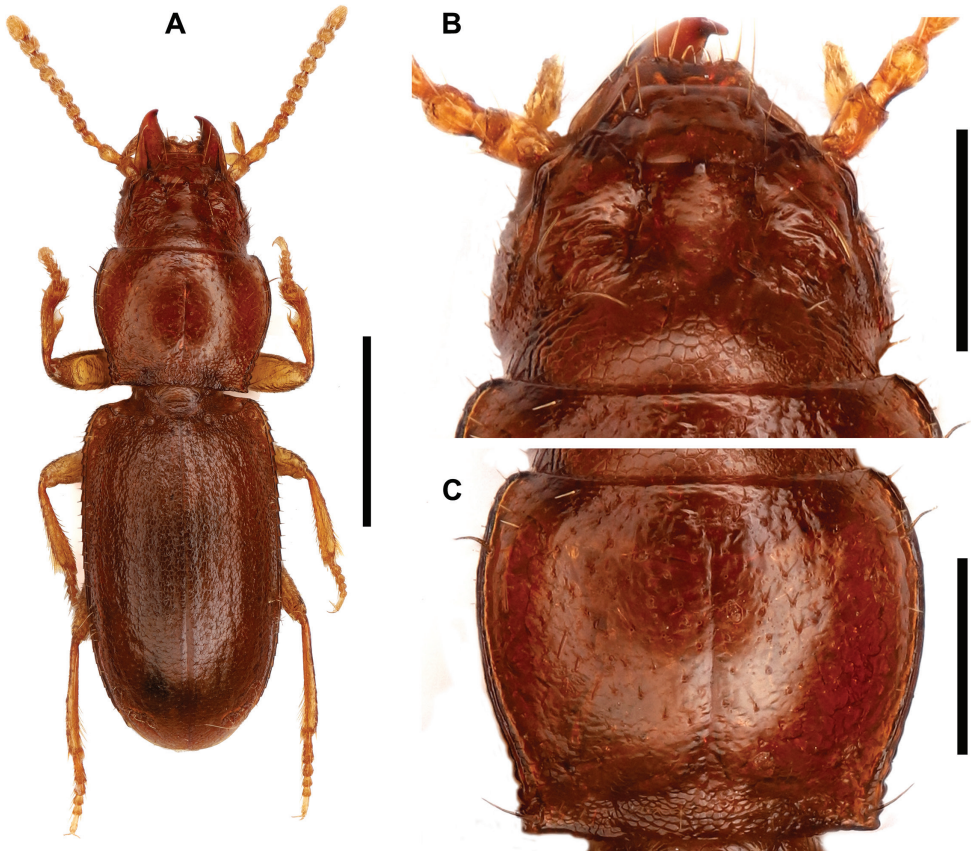
Figure 3. Digital images of external features of *Anillinus albrittonorum* sp. nov. (female, 6 mi NW High Springs, Columbia County, Florida) **A** habitus, dorsal aspect **B** head, dorsal aspect **C** pronotum, dorsal aspect. Scale bars: 0.5 mm (**A**); 0.2 mm (**B, C**).

covered with sparse, yellowish, short setae. Vesiture of elytra short (~0.3× the length of discal setae).

Prothorax: Pronotum (Fig. 3C) moderately convex, of moderate size (LP/LE 0.40±0.012) and moderately transverse (WPm/LP 1.24±0.024), with lateral margins almost rectilinearly and moderately constricted posteriorly (WPm/WPp 1.26±0.025). Anterior angles indistinct, posterior angles almost rectangular (89–100°). Width between posterior angles equals the width between anterior angles (WPa/WPp 1.00±0.023). Basal margin slightly concave in middle.

Scutellum: Externally visible, triangular, with pointed apex.

Elytra: Slightly convex, of average length (LE/ABL 0.58±0.006) and width (WE/LE 0.57±0.011) for the genus, with traces of 5–6 striae. Humeri distinct, rounded, in outline forming an obtuse angle with longitudinal axis of body. Lateral margins subparallel in middle, slightly convergent at basal fifth, evenly rounded to apex at apical third, with shallow subapical sinuation. Basal margination distinct.
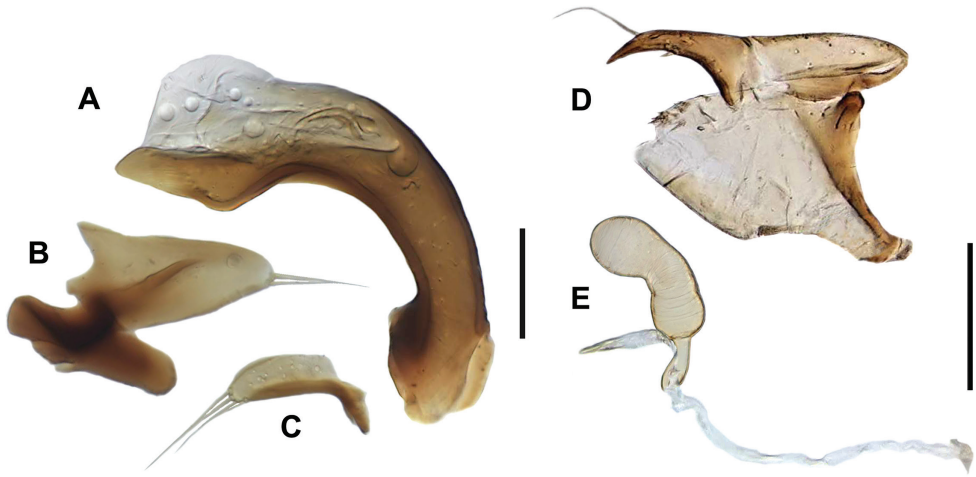
Figure 4. Digital images of male and female genitalia of *Anillinus albrittonorum* sp. nov. (6 mi NW High Springs, Columbia County, Florida). Male genitalia: **A** median lobe, right lateral aspect; apex to upper left and basal bulb to lower right **B** left paramere, left lateral aspect **C** right paramere, right lateral aspect. Female genitalia: **D** ovipositor sclerites **E** spermatheca. Scale bars: 0.1 mm.

***Legs*:** Protarsi of male with moderately dilated tarsomere I. Profemora moderately swollen.

Males with metafemora modified; each bearing a small projection with small tubercles at posterior margin. Females metafemora unmodified.

***Male genitalia*:** Median lobe (Fig. 4A) of aedeagus anopic, moderately arcuate and moderately twisted. Shaft slightly dilated in apical half, enlarged trianguloid apex with sides almost rectangularly tapered to narrowly rounded tip. Apical orifice long, occupies almost half of the shaft length. Ventral margin of median lobe curved, most strongly bent at the middle of shaft, with abrupt enlargement before apex, without poriferous canals. Dorsal copulatory sclerites short, fused to form slightly curved blade-like structure. Spines and scaled membranous folds of internal sac absent. Left paramere (Fig. 4B) of shape common in the genus, paramere apex with two long setae. Right paramere (Fig. 4C) of moderate length, bearing four long setae, which are approximately the length of paramere.

***Female genitalia*:** Spermatheca (Fig. 4E) slightly sclerotized, formed from two compartments of different width and shape. The distal compartment of a bean-like shape, wide and long, occupies two-thirds of the spermatheca length, and presumably corresponds to the cornu of other species of the genus. Proximal part cylindrical, short and narrow, presumably corresponds to the fused ramus and nodulus of other species of the genus (cf. Fig. 4E with the spermatheca of *A. cherokee* Sokolov and Carlton on fig. 11 in Sokolov and Carlton 2008, p. 43). Length of spermathecal gland shorter than length of spermatheca. Spermathecal duct long and uncoiled. Gonocoxite II slightly falciform, more than 2× longer than it is wide basally, with acute ensiferous setae (Fig. 4D). Laterotergite with 7–8 setae (Fig. 4D).

Geographic distribution. This species is known only from the type locality in the High Springs area of Columbia County, Florida (Fig. 1).

Habitat. All specimens of this species were collected from deep sand soil using underground column pitfalls. The underground traps used are comprised of ½ inch hardware cloth tied into a cylinder with PVC plastic pipe on each end. Each section of pipe is 10 inches in length and the hardware cloth is two feet long. The cloth overlaps with each pipe approximately 2 inches, resulting in a trapping length of 20 inches. The effective trapping depth is from 10 inches to 30 inches below the soil surface. A plug of soil the size of the trap is removed from the ground and the trap installed in the hole. A jar containing propylene glycol with a funnel on top the same diameter as the pipe is lowered to the bottom and is used to collect and preserve insects burrowing through the sand. These traps were placed in an area of deep sand on the north end of the northern Brooksville ridge, one of many "islands" of elevated karst and sand that cover Florida. There are numerous ridge systems in Florida, generally running north to south, that were beach dunes formed by past fluctuations in ocean levels (Bousquet and Skelley 2010, 2012). The type locality and surrounding area is mostly disturbed sandhill with secondary growth and pastureland (Fig. 2). Other carabid species collected in the same traps that are regarded as interesting, rare, and indicators of the subterranean habitat include *Clivina choatei* Bousquet and Skelley and *Scarites stenops* Bousquet and Skelley. *Anillinus albrittonorum* is a true endogean species and has never been found in litter samples.

Relationships. The new species belongs to group VII of the endogean *Anillus* species (Sokolov et al. 2004), characterized by a combination of the partly microsculptured head and a smooth disc of the pronotum. However, *A. robisoni* Sokolov and Carlton from Arkansas and species of the *Anillinus moseleyae* group from North Carolina that form group VII of the endogean species have only a superficial similarity to the new species. Within this group, as well as within other groups of the endogean and litter species, *A. albrittonorum* differs in the structure of its spermatheca from all *Anillinus* species where the spermathecae have been examined. Among endogean species with similar habitus, the range of *A. albrittonorum* is geographically (Fig. 1) close to the range of *A. turneri* Jeannel, described from the Atlanta area (Peach County) in Georgia. Externally, both species can be distinguished by the structure of the frons, completely microsculptured in specimens of *A. turneri* but smooth in the specimens of *A. albrittonorum*. Both species can also be distinguished based on the male and female genital structures. The spermatheca of females of *A. turneri* (Peach Co., Georgia, NMNH) have a question-mark shape, typical for *Anillinus*, and thus shows no similarity to the spermatheca of females of *A. albrittonorum*. As it was mentioned above, two Florida species, *A. dohrni* and *A. kovariki*, exemplify ovoid and convex species, i.e. belong to other morphological groups of species, and in comparison with *A. albrittonorum* demonstrate quite dissimilar genital structures. The male median lobes of both Florida species have simple, not enlarged apices, and shafts of different shapes (cf. Fig. 4A with the male median lobe of *A. dohrni* on fig. 64 in Jeannel 1963a, p. 75, and the male median lobe of *A. kovariki* on fig. 28 in Sokolov et al. 2004, p. 194).

Discussion

This new finding increases to three the total number of *Anillinus* species recorded from Florida. Thus, in relation to anilline diversity, Florida occupies the third position among the Gulf States (after Alabama and Texas), and several considerations suggest that additional new species remain to be discovered in the state.

The Florida peninsula has a rather complicated geological history, involving changing ocean levels, isolation from other areas, and the indirect impact of glaciation with periodic multiple marine transgressions, and fluvial and rainfall erosion (Howden 1963, 1966; Bahtijarević and Faivre 2016). All these events have shaped the specific topography of the peninsula characterized by evident north-south monotonous decrease in the absolute altitude of lands above sea level. The northern part of the peninsula is represented by a continuous broad belt of uplands, which become discontinuous southward. In central Florida, the local highlands are mostly represented by sub-parallel ridges separated by broad valleys and in south Florida all the highlands are eventually replaced with lowland (White 1970). This complicated topography is accompanied by a diversity of underlying geologic features, particularly karst relief (Scott et al. 2006) and various terrestrial ecological habitats which result in almost twenty different ecological communities for north and central Florida (Soil and Water Conservation Service 1989). It is no surprise that the northern and central regions are known for their numerous endemic plants (Sorrie and Weakley 2001), vertebrates (Neill 1957), and terrestrial arthropods (Deyrup 1990), including those whose immature stages demonstrate a subterranean way of life (Woodruff 1973; Skelley 2003). Additionally, from a biogeographic point of view, the terrestrial fauna of the Florida Panhandle bears features of a genetic discontinuity, known as the Apalachicola River discontinuity (Soltis et al. 2006). Altogether, the topographical, geological, ecological, and biogeographical patterns of central and northern Florida may impact not only terrestrial but also subterranean fauna and, presumably, the soil fauna of Florida still hides an uninvestigated and unknown diversity. Thus, there are likely a substantial number of Anillini yet to be reported from Florida. Additional trapping and investigation of soils are required to expand distributions and identify new species.

Acknowledgements

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RESEARCH ARTICLE

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Chaimowiczia: a new Iuiuniscinae genus from Brazil (Oniscidea, Synocheta, Styloniscidae) with the description of two new troglobitic species

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Abstract

A new genus of Styloniscidae, *Chaimowiczia* **gen. nov.**, is described with two new species: *Chaimowiczia tatus* **sp. nov.** from Gruta do Padre cave (Santana, Bahia) and *Chaimowiczia uai* **sp. nov.** from Lapa d'água do Zezé cave (Itacarambi, Minas Gerais). The new genus and species were allocated into the subfamily Iuiuniscinae, hitherto monotypic, by the pronounced rectangular-shaped lateral pereonites epimera, dorsal surface smooth, body outline continuous without a gap between pereon and pleon, and pleonites 3 to 5 developed forming tips. The two species of *Chaimowiczia* gen. nov. differ in the shape of cephalon antennal lobes, pereonite 1 epimera, pleonite 5 posterior margin and uropod exopod and endopod proportion.

Keywords

Introduction

The family Styloniscidae is currently composed of 16 genera (Boyko et al. 2020), grouped into four subfamilies: Styloniscinae Vandel, 1952, Notoniscinae Vandel, 1952, Kuscheloniscinae Strouhal, 1961 and Iuiuniscinae Souza, Ferreira & Senna,

2015. Styloniscinae are the most representative, including 12 genera, some with pantropical distribution, while others are endemic to a single location (Dana 1852; Graeve 1914; Arcangeli 1930; Paulian de Félice 1950; Vandel 1952; Andersson 1960; Dalens 1989; Taiti and Xue 2012; Campos-Filho et al. 2014; Taiti and Montesanto 2020).

Most Styloniscidae species known in Brazil inhabit subterranean ecosystems, except for *Pectenoniscus angulatus* Andersson, 1960 and *Styloniscus spinosus* (Patience, 1907) (Lopes et al. 2005; Magrini et al. 2010; Campos-Filho et al. 2018). Styloniscidae species recorded in Brazilian caves belong to the subfamily Styloniscinae (*Spelunconiscus* Campos-Filho, Araujo and Taiti, 2014, *Xangoniscus* Campos-Filho, Araujo and Taiti, 2014, *Pectenoniscus* Andersson, 1960 and *Cylindroniscus* Arcangeli, 1929). Only one species of Iuiuniscinae has been recorded so far.

We present a new genus of Styloniscidae allocated into the subfamily Iuiuniscinae, with the description of two new species found in Brazilian caves. In addition to the taxonomic descriptions, this paper provides ecological and conservation information related to the new species and the subterranean ecosystems where they were found.

Materials and methods

The specimens were manually collected and fixed in 70% ethanol. They were measured and photographed with a ZEISS Axio ZoomV16 stereomicroscope coupled with an Axio Cam 506 Color camera, dissected and mounted in slides using Hoyer's medium in the Center of Studies on Subterranean Biology of the Federal University of Lavras (CEBS–UFLA, Lavras, Brazil). Drawings were made either from photographs or with the aid of a camera lucida coupled with the microscope Leica DM750. Illustrations were prepared using the software GIMP (v. 2.8) (Montesanto 2015, 2016). For analysis of the dorsal cuticular structures, pictures were taken using the scanning electron microscope Hitachi TM4000. Holotype and paratypes of the new species were deposited in the Subterranean Invertebrate Collection of Lavras (ISLA) in the Federal University of Lavras.

Taxonomy

Family Styloniscidae Vandel, 1952

Genus *Chaimowiczia* gen. nov.
http://zoobank.org/1650BE9A-CE55-4CF1-BB7B-B10A7E840318

Type species. *Chaimowiczia tatus* sp. nov.

Diagnosis. Body non-volvational. Cephalon with antennal lobes, distinct supraantennal line bent in middle, vertex with lateral grooves. Body outline continuous with pereonites epimera well developed, widely separated, pleonites 1 and 2 bridge the gap

between pereon and pleon, pleonites 3–5 with epimera well developed. Telson with subtriangular distal half depressed with rounded apex. Antennula of three articles covered with setae, distal article with two apical aesthetascs. Antenna with flagellum of three distinct articles covered with setae. Mandibles *pars molaris* large and projected. Maxillula outer ramus with entire teeth and two long and thick setose stalks; inner ramus with three penicils at apex. Maxilla inner lobe wider than outer lobe. Maxilliped basis trapezoidal; endite bearing one penicil between two strong teeth. Pereopods with unbranched dactylar setae. Genital papilla lanceolate. Male pleopod 1 exopod and endopod subequal in length, endopod two-jointed, with flagelliform distal article. Male pleopod 2 endopod with two thickset articles, distal one tapering apically.

Etymology. The genus is named after Dr Flavio Chaimowicz, a physician who provided important contributions for the Brazilian speleology. Gender feminine.

Remarks. The diagnosis of Styloniscinae, Notoniscinae and Kuscheloniscinae has been presented in old publications that unfortunately include few characters of their members (Vandel 1952; Strouhal 1961). Meanwhile, more details have been provided for Iuiuniscinae (Souza et al. 2015). According to Vandel (1952: 95), Styloniscinae exhibit *i.* body smooth or tuberculated, without longitudinal ribs, and *ii.* pleon-epimera 1–5 narrow, with a gap between the pereon and pleon. For Notoniscinae, Vandel (1952: 95–96) noted *i.* pereonites dorsum tuberculated or with longitudinal ribs (sometimes with conspicuous protuberances also on the pleonites); *ii.* pleon-epimera 3–5 or 4–5 well developed, reducing the gap between the pereon and pleon; *iii.* genital tract of styloniscid type; *iv.* eyes with 3 ommatidia. For Kuscheloniscinae Strouhal (1961: 217) indicated the following: *i.* outline of pleon continuous with that of pereon; *ii.* pleon-epimera 3–5 very reduced; *iii.* anterior pereonites with protuberances and lateral ribs. Finally, Iuiuniscinae are characterized by *i.* dorsal integument smooth or without ribs or large protrusions; *ii.* enlarged epimera; *iii.* pereopod 1 much shorter than the others flanking the head; *iv.* pleon-epimera forming acute tips; *v.* telson distal half lower than the proximal half, and *vi.* habit to build mud shelters to molt and to protect juveniles (Souza et al. 2015). *Chaimowiczia* gen. nov. can be promptly distinguished from all the already described Styloniscidae by the pronounced rectangular-shaped lateral projections of pereonites, which is not observed in other members of this family. Moreover, tubercles are absent and the body outline is continuous without a gap between pereon and pleon. Epimera are developed in pleonites 3 to 5 forming tips, and telson distal half is narrower than the proximal half. Based on these characters, *Chaimowiczia* gen. nov. was allocated into the subfamily Iuiuniscinae.

Chaimowiczia gen. nov., as well as *Iuiuniscus*, occurs in the São Francisco River Basin and the caves are in the limestone plateaus of the Bambuí Group (Auler et al. 2001) (Fig. 1). The new genus resembles *Iuiuniscus* by the widely separated pereonites 1–7 epimera directed outwards, pleonites 3–5 epimera well developed; mandibles *pars molaris* large and projected. However, *Chaimowiczia* gen. nov. is not able to build mud shelters as *Iuiuniscus.* These genera also differ in the number of aesthetascs in antennula distal article (*Iuiuniscus* 12 *versus* 2 in *Chaimowiczia* gen. nov.), in the number of articles in antennal flagellum (*Iuiuniscus* 8 *versus* 3 in *Chaimowiczia* gen. nov.), teeth morphology in
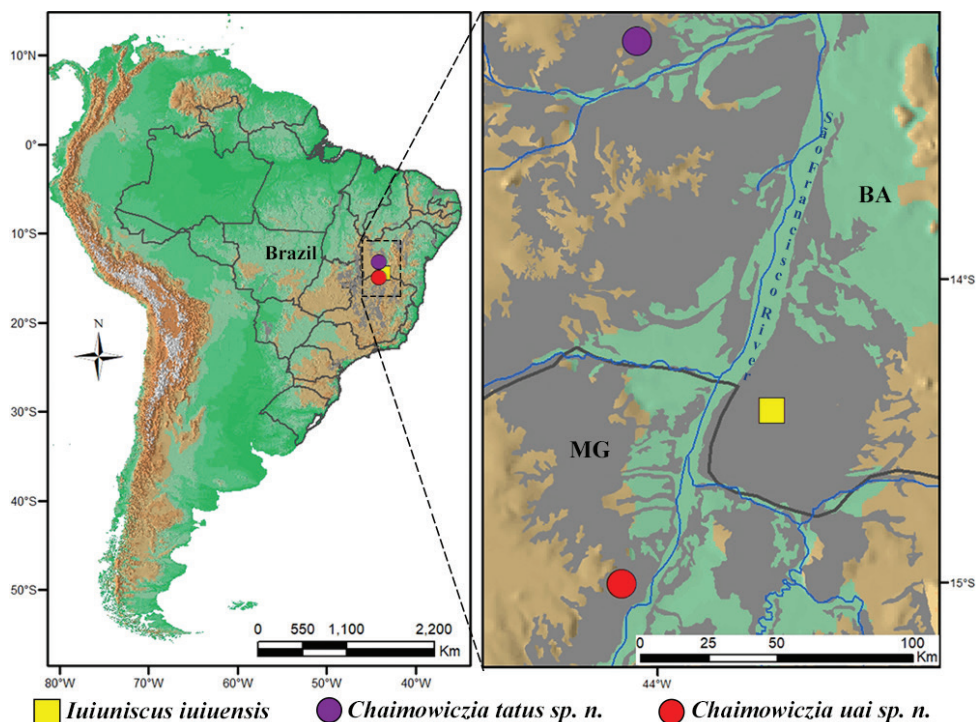
Figure 1. South America with the distribution of *Chaimowiczia* gen. nov. and *Iuiuniscus*. Delimited area in the states of Minas Gerais and Bahia states with the karst area of the Bambuí Group are represented in gray, rivers from São Francisco River Basin are represented in blue.

the maxillula outer ramus (outer group with curved teeth in *Iuiuniscus versus* straight in *Chaimowiczia* gen. nov.; inner group with two longer teeth in *Iuiuniscus versus* subequal in *Chaimowiczia* gen. nov.), male pleopod 1 exopod and endopod proportion (exopod longer than endopod in *Chaimowiczia* gen. nov. *versus* the opposite in *Iuiuniscus*), shape of male pleopod 1 exopod, shape of male pleopod 2 exopod (triangular in *Iuiuniscus versus* semicircular in *Chaimowiczia* gen. nov.), and notably by the morphology of pereon and pleon (with very prominent and very acute tips in pereon and pleon epimera in *Iuiuniscus versus* not so prominent nor so acute tips in *Chaimowiczia* gen. nov.).

Chaimowiczia tatus sp. nov.

http://zoobank.org/C9F67EAC-3104-44A1-B835-6580D9C83BFF
Figs 2–5

Material examined. *Holotype.* • 1 Male; Bahia, Santana, Gruta do Padre cave, -13.216325°, -44.065194°, 11 July 2014, leg. R. L. Ferreira, ISLA78105. ***Paratypes.*** • 1 female, same data as for holotype, ISLA78106; • 1 male 1 female, same locality as for holotype, 18 July 2019, ISLA78107.
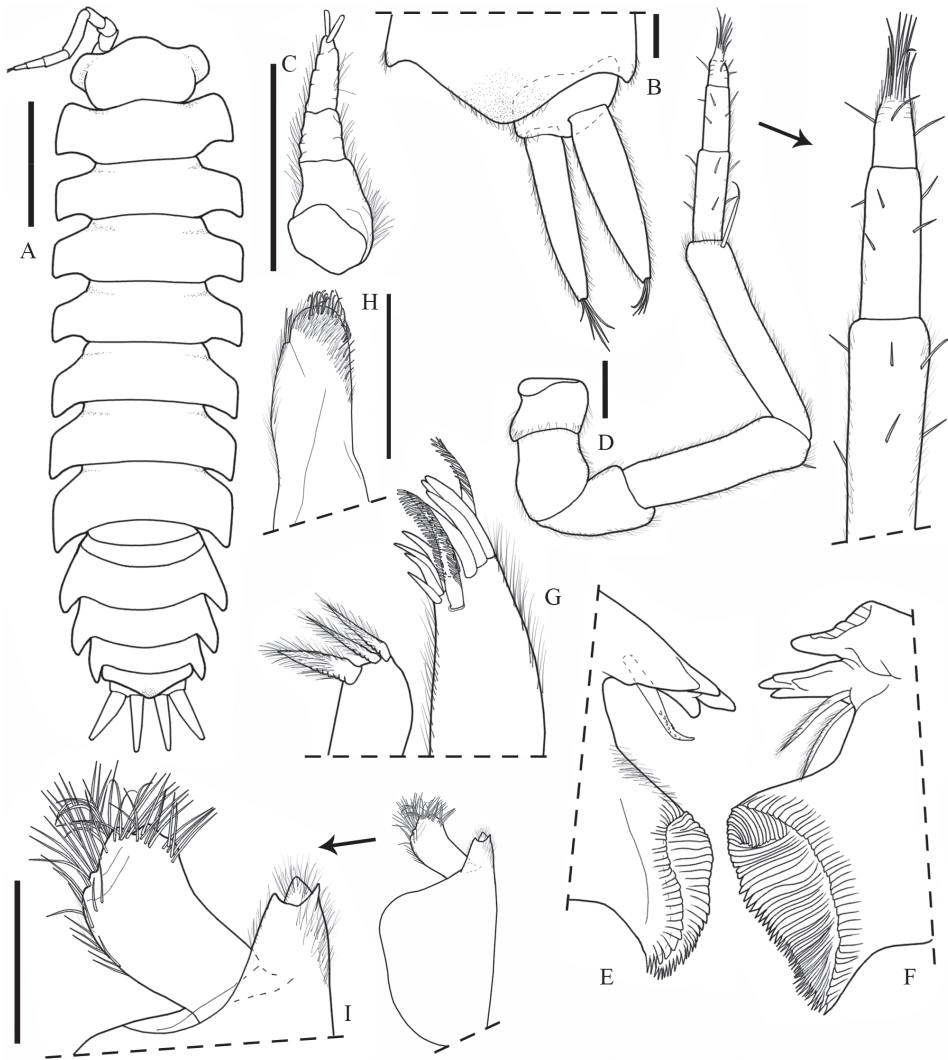
Figure 2. *Chaimowiczia tatus* sp. nov. Male **A** habitus, dorsal view **B** telson and uropod, dorsal view **C** antennula **D** antenna **E** right mandible **F** left mandible **G** maxillula **H** maxilla **I** maxilliped. Scale bars: 1 mm (**A**); 0.2 mm (**B–I**).

Diagnosis. *Chaimowiczia tatus* sp. nov. is characterized by pereonite 1 epimera directed sideways; quadrangular antennal lobes; pleonites 3–5 epimera tips well developed, pleonite 5 short, not surpassing the apex of telson; and uropods endopod and exopod subequal in length.

Description. Maximum length: male, 9 mm. Colorless, eyes absent (Figs 2A, 3A, B). Dorsal surface smooth covered with scale setae with short triangular base and long sensory sheathed hair (Fig. 3C). Cephalon (Fig. 3A, B) frons with distinct suprantennal line, downward and truncate in middle, quadrangular antennal
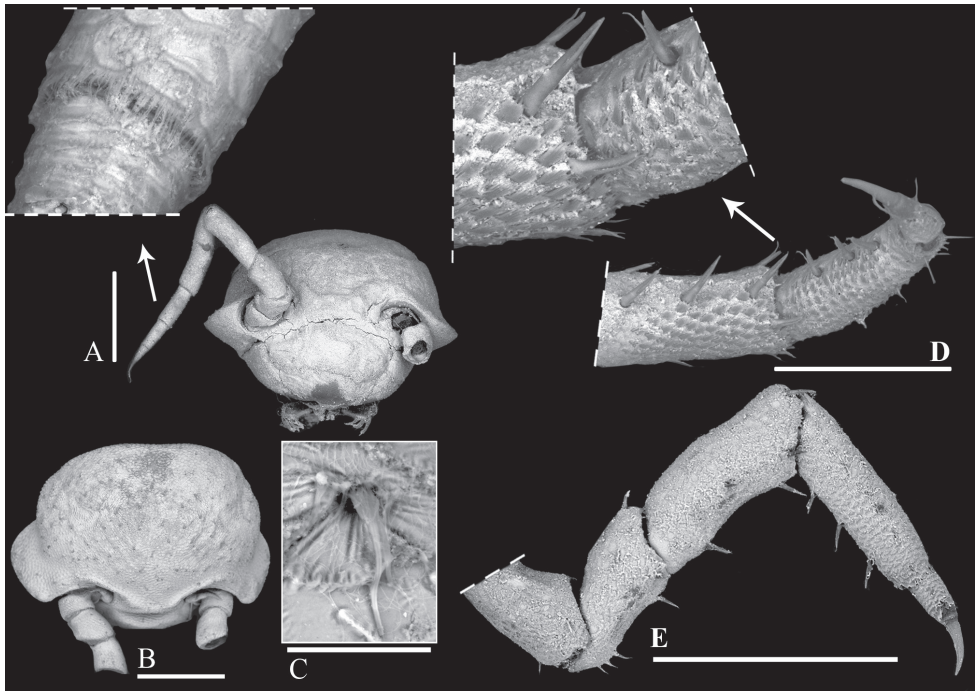
Figure 3. *Chaimowiczia tatus* sp. nov. Male **A** cephalon, frontal view **B** cephalon, dorsal view **C** pereopod 1 propodus, carpus and dactylus. Scale bar: 500μm (**A**); 200μm (**B**).

lobes. Body convex, pereonites 1–7 epimera quadrangular, widely separated and outwardly extended, pereonites postero-lateral corners progressively directed backward; pleon epimera 3–5 well developed (Fig. 2A). Telson (Fig. 2B) distal half subtriangular depressed with round apex. Antennula (Fig. 2C) with three articles covered with thin setae, distal article longer than second article, with two apical aesthetascs. Antenna (Figs 2D, 3A) surpasses pereonite 1 when extended backward, fifth article of peduncle as long as flagellum; flagellum with three articles. Right mandible (Fig. 2E) with one penicil; left mandibles with two penicils (Fig. 2F). Maxillula (Fig. 2G) outer ramus with 4 + 5 teeth, apically entire, and two thick plumose stalks; inner ramus with three penicils, proximal one stout. Maxilla (Fig. 2H) bilobate, inner lobe wider than outer lobe, with several thin and thick setae. Maxilliped (Fig. 2I) basis trapezoidal, distal portion slightly wider than basal; palp apex with tufts of setae; endite shorter than palp, setose, apex with one conic penicil between two strong teeth, inner tooth long. Pereopod 1 antennal grooming brush composed by pectinate scales longitudinally on frontal face of carpus and propodus (Fig. 4A), dactylus with one claw; pereopod 7 with water conducting scale rows. Uropod (Fig. 2B) protopod surpasses distal margin of telson; endopod and exopod subequal in length, inserted at the same level, covered with pectinate scales.

Male. Pereopods 1, 6 and 7 (Figs 4A–C) covered with setae; merus sternal margin with proximal tuft of setae. Pleopod 1 (Fig. 4D) protopod trapezoid, apex tapering;
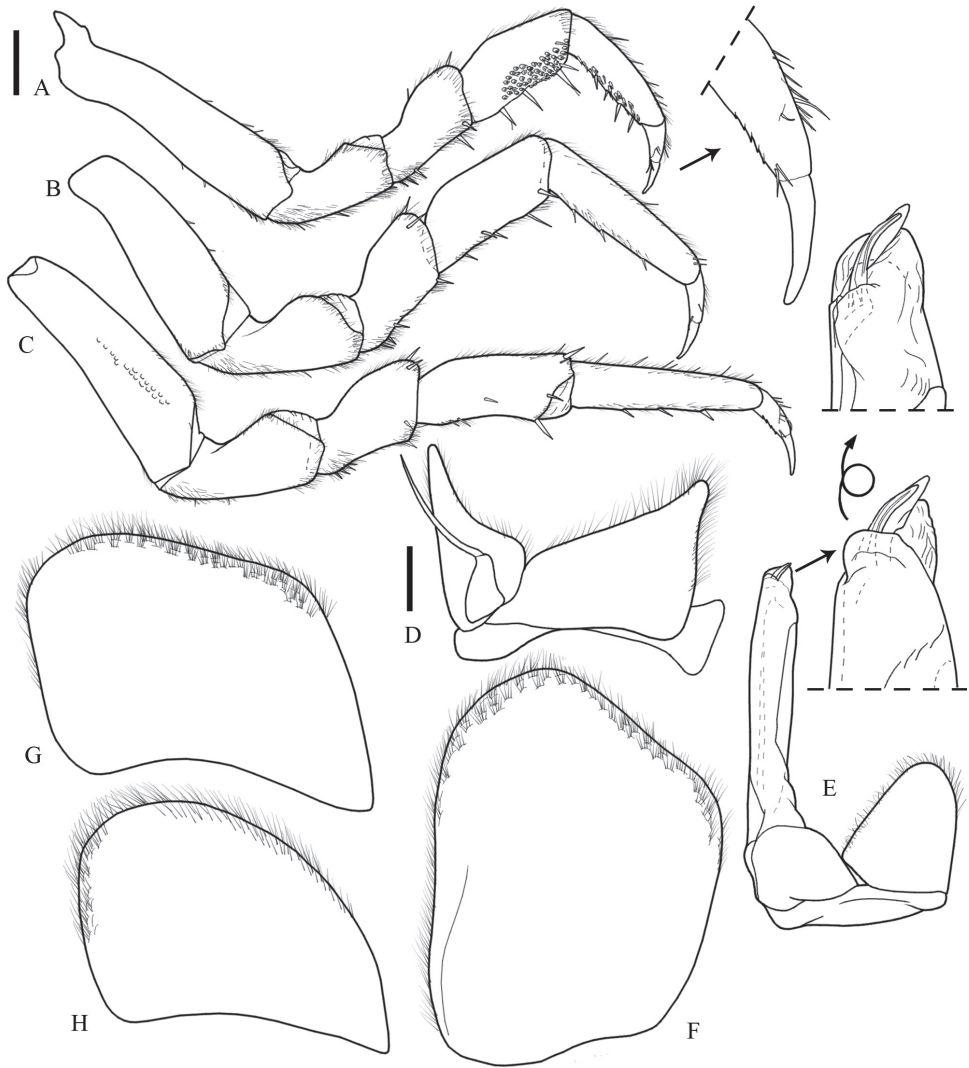
Figure 4. *Chaimowiczia tatus* sp. nov. Male **A** pereopod 1 **B** pereopod 6 **C** pereopod 7 **D** pleopod 1 **E** pleopod 2 **F** pleopod 3 exopod **G** pleopod 4 exopod **H** pleopod 5 exopod. Scale bars: 0.2 mm (**A**–**H**).

exopod covered with setae, triangular with sinuous external margin; endopod as long as exopod, with narrow basal article and flagelliform distal article. Pleopod 2 (Fig. 4E) exopod semi-oval, rounded distal margin, covered with setae; endopod of two articles, basal article quadrangular, shorter than exopod, distal article stout, apex with acute lobe directed outward. Pleopod 3 exopod (Fig. 4F) trapezoid, covered with thin setae on the distal portion and along the inner margin. Pleopod 4 exopod (Fig. 4G) rhomboid, wider than long, covered with thin setae. Pleopod 5 exopod (Fig. 4H) ovoid, wider than long, covered with thin setae.

Etymology. The epithet "*tatus*" refers to the "Tatus II project", an experiment of human permanency inside a cave held in 1987, conducted in Gruta do Padre cave.
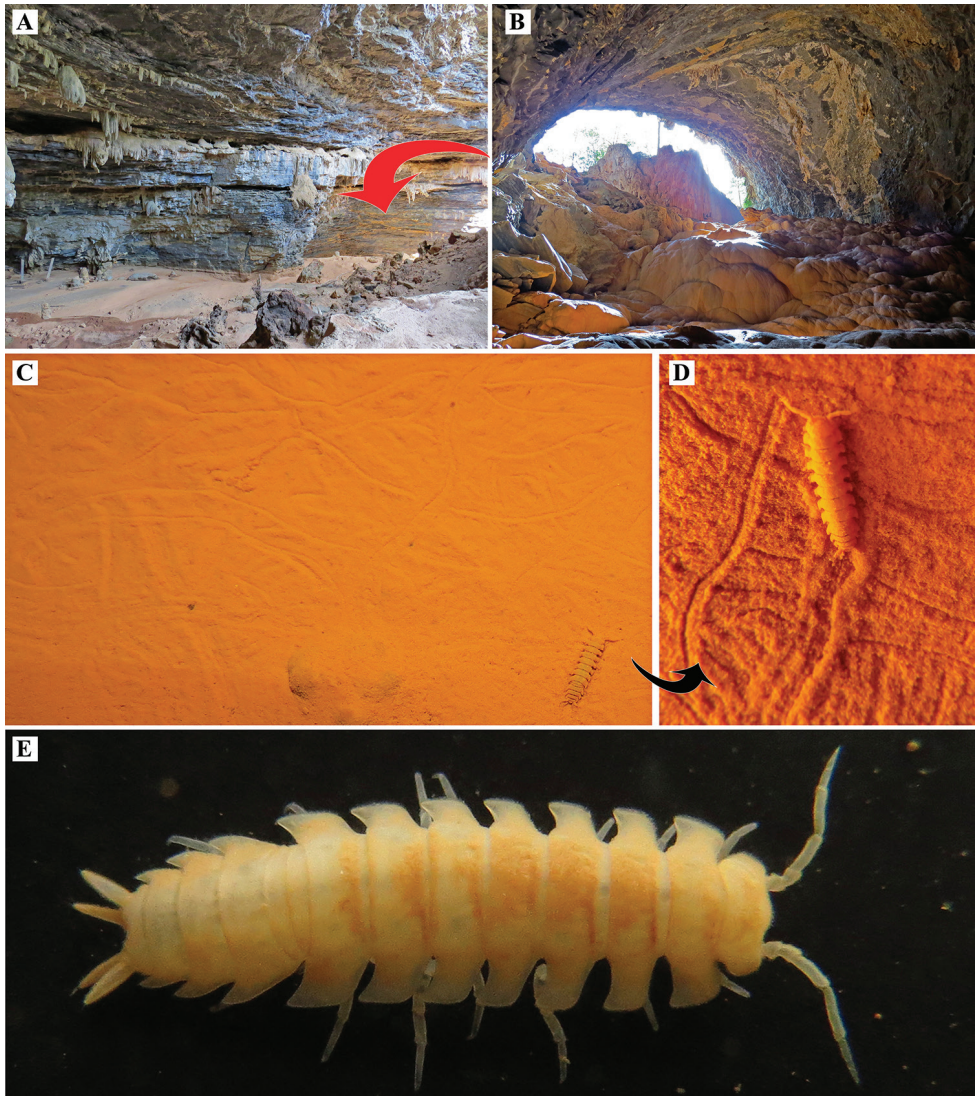
Figure 5. A Gruta do padre cave main entrance **B** rock shelter leading to cave main entrance **C** Pond where the species was collected **D** Substrate marked by the species activity **E** Living specimen of *Chaimowiczia tatus* sp. nov. with approximately 9 mm.

During the experiment, a group of speleologists stayed for 21 days inside the cave performing topographic and speleology surveys (Chaimowicz, 1987).

Ecological remarks. Gruta do Padre comprises an extensive cave with 16,400 m of horizontal projection and is currently considered the fifth longest cave in Brazil (Rubbioli et al. 2019). It presents two entrances and three distinct levels. A river flows in the lowest level, which is the most extensive. The main entrance comprises a huge rock shelter (Fig. 5A) that connects to a descending set of flowstones (Fig. 5A, B).

Specimens of *Chaimowiczia tatus* sp. nov. were observed in a single chamber in the second level (ca. 500 m from the main cave entrance), in clayish sediment pools (Fig. 5C–E). Two other troglobitic styloniscid species occur in this cave: one terrestrial (*Pectenoniscus santanensis* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020a) and one new amphibious species. A peculiar condition is observed regarding the distribution of the two styloniscid species. While one species is amphibious, occurring in both aquatic and moist terrestrial habitats, *C. tatus* sp. nov. was observed exclusively underwater. The ponds where *C. tatus* sp. nov. occurs are devoid of the amphibious species, suggesting they might avoid each other. There are dozens of ponds along the lower conduit formed by the river overflow or by percolating water (especially in the case of travertine pools), where hundreds of individuals of the amphibious species were observed. However, no specimens of *C. tatus* sp. nov. were observed in the lower level coexisting with the other styloniscid. The ponds in which specimens of *C. tatus* sp. nov. occur usually present the substrate full of traces made by these individuals (Fig. 5C) indicating their high motility and activity. Since no visible organic matter was observed within the ponds (like bat guano or vegetal debris), they may be feeding on the substrate itself, which might be rich in microorganisms. Gruta do Padre Cave presents other troglobitic species: the beetle *Coarazuphium tessai* (Godoy & Vanin, 1990), the amphipod *Spelaeogammarus santanensis* Koenemann & Holsinger, 2000, and the millipede *Phaneromerium cavernicolum* Golovatch & Wytwer, 2004. All of them were discovered during the Tatus II experiment, demonstrating the relevance of this cave regarding the biota. Although some alterations were caused during the Tatus II experiment (in both the cave interior – a camping area was established inside the cave - and the external area), no impacts from past actions are currently visible. The external environment surrounding the cave was altered by the replacement of the native vegetation by pastures or crops. On the other hand, the inner portion of the cave is well preserved. Since the huge extension of the cave and the fact that only a few speleologists visit it each year (especially due to the difficult access), *C. tatus* sp. nov. does not seem to be currently threatened.

Chaimowiczia uai sp. nov.
http://zoobank.org/94FB3A0F-1209-44E6-B2B4-033E95C1872C
Figs 6–9

Material examined. *Holotype.* • Male, Minas Gerais, Itacarambi, Lapa d'água do Zezé cave, -15.006745°, -44.117087°, 15 July 2019, leg. R. L. Ferreira, ISLA78108. *Paratypes.* • 2 males 1 female, same data as for holotype, ISLA78109; • 2 male 2 females, same locality as for holotype, 12 December 2014, ISLA78110.

Diagnosis. *Chaimowiczia uai* sp. nov. is characterized by the concave shape of pereonites epimera, with pereonite 1 epimeron directed frontward; round antennal lobes; pleonites 3–5 epimera with tips well developed, pleonite 5 surpassing apex of telson; and uropods endopod longer than exopod.
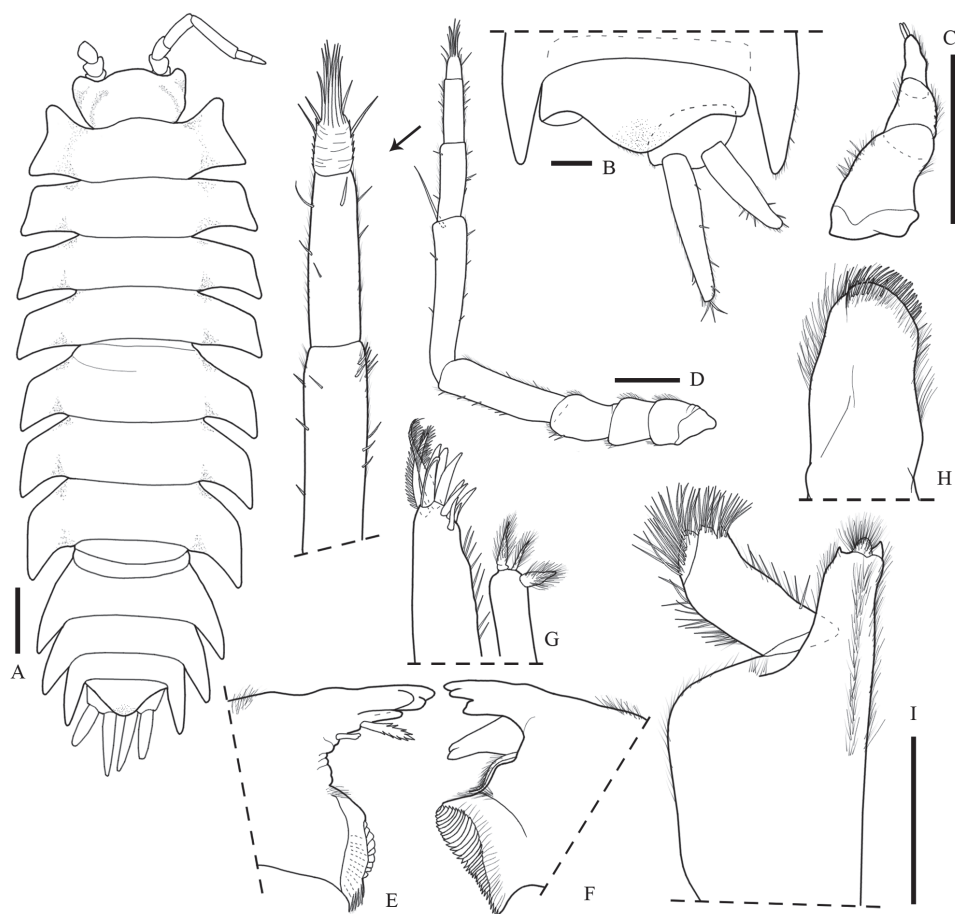
Figure 6. *Chaimowiczia uai* sp. nov. Male **A** habitus, dorsal view **B** pleonite 5, telson and uropod, dorsal view **C** antennula **D** antenna **E** right mandible **F** left mandible **G** maxillula **H** maxilla **I** maxilliped. Scale bars: 1 mm (**A**); 0.2 mm (**B, D–J**); 0.1 mm (**C**).

Description. Maximum length: male, 8 mm. Colorless, eyes absent (Fig. 6A, 7A, B). Dorsal surface smooth covered with scale setae with long base (reaching half the total length) and free sensory hair (Fig. 7C). Cephalon (Fig. 7A, B) vertex with lateral grooves; frons with distinct suprantennal line, downward in middle; round antennal lobes. Body convex; pereonite 1 postero-lateral corners well developed and projected forward, lateral margin concave; pereonite 7 slightly surpassing distal margin of pleonite 2; pleon 3–5 epimera well developed, pleonite 5 surpassing telson apex (Fig. 6A). Telson (Fig. 6B) with distal half subtriangular depressed, rounded apex. Antennula (Fig. 6C) with three articles covered with setae, distal article as long as second article, with two apical aesthetascs. Antenna (Fig. 6D) surpasses pereonite 1 when extended backwards, fifth article of peduncle shorter than flagellum; flagellum with three articles. Left mandible with two penicils (Fig. 6E); right mandible with one penicil (Fig. 6F). Maxillula (Fig. 6G) outer ramus with 5 + 5 teeth, apically entire, and
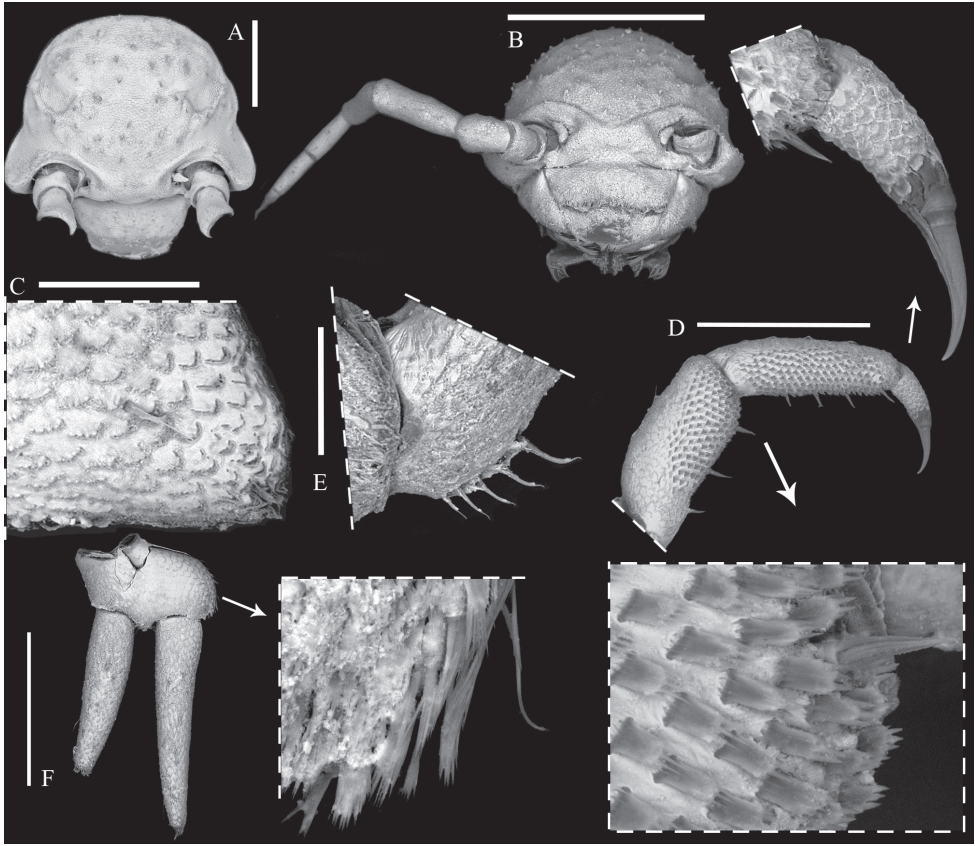
Figure 7. *Chaimowiczia uai* sp. nov. Male **A** cephalon, dorsal view **B** cephalon, frontal view **C** epimeron 1, dorsal view **D** pereopod 1 merus, propodus, carpus and dactylus **E** pereopod 2 merus **F** Uropod. Scale bars: 1 mm (**A**); 100μm (**B**); 400μm (**C**); 500μm (**D**).

two thick plumose stalks; inner ramus with three penicils, two of them stout. Maxilla (Fig. 6H) with bilobate apex, inner lobe wider than outer lobe with several setae on distal margin. Maxilliped (Fig. 6I) basis distal portion slightly wider than basal; palp apex with tufts of setae; endite rectangular, shorter than palp, setose, apex with one rounded penicil between two strong teeth, inner tooth longer. Pereopod 1 (Fig. 8C) antennal grooming brush composed by pectinate scales longitudinally on frontal face of propodus and carpus, dactylus with one claw; pereopod 7 basis with water conducting system scale rows. Uropod (Figs 6B, 7F) protopod surpasses distal margin of telson, covered with pectinate scales; endopod longer than exopod, inserted at the same level.

Male. Pereopods 1, 2 and 7 (Figs 7C, E; 8A, B) covered with setae; merus sternal margin concave with proximal hairy tuft of setae. Genital papilla (Fig. 8C) lanceolate. Pleopod 1 (Fig. 8C) exopod triangular with sinuous outer margin, covered with setae; endopod shorter than exopod, basal article narrow and flagelliform distal article; protopod trapezoidal, rounded apex. Pleopod 2 (Fig. 8D) exopod semicircular, rounded distal margin, covered with setae; endopod of two articles, basal article rectangular,
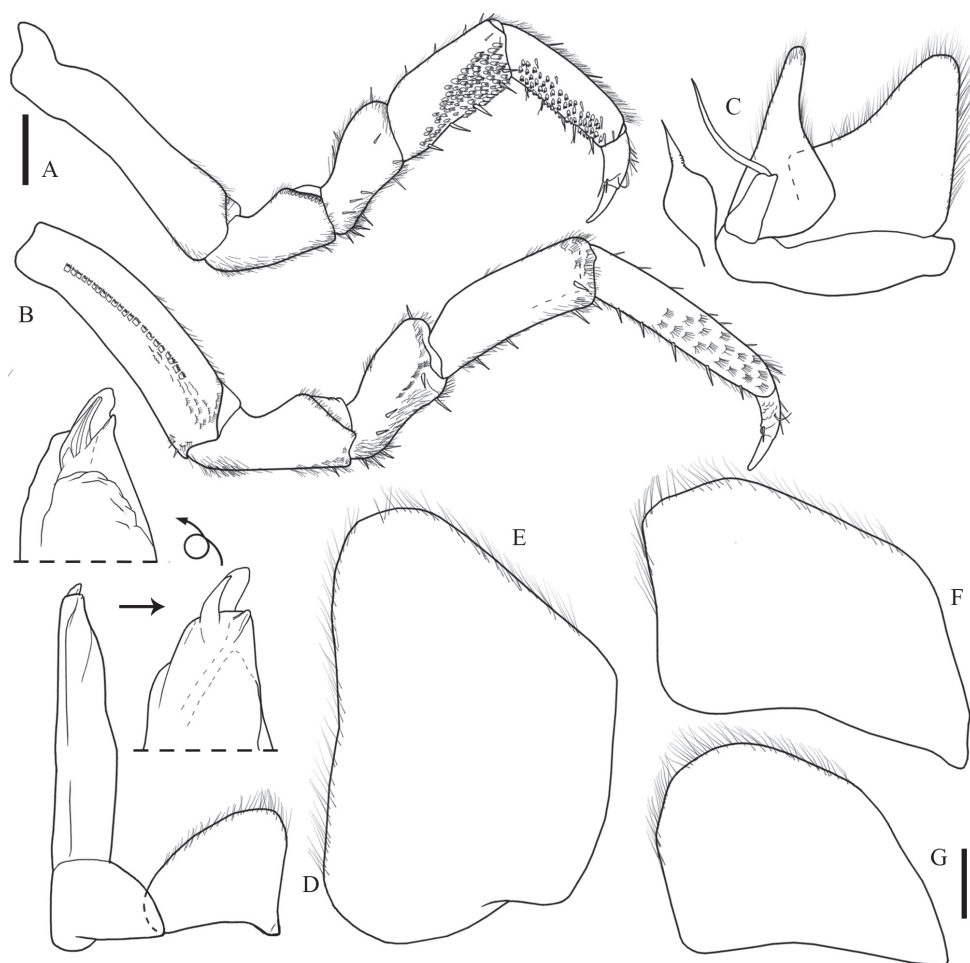
Figure 8. *Chaimowiczia uai* sp. nov. Male **A** pereopod 1 **B** pereopod 7 **C** genital papilla and pleopod 1 **D** pleopod 2 **E** pleopod 3 exopod **F** pleopod 4 exopod **G** pleopod 5 exopod. Scale bars: 0.2 mm (**A–H**).

shorter than exopod, distal article slender, directed backward, apex with distal projection. Pleopod 3 exopod (Fig. 8E) trapezoidal, distal margin straight covered with setae. Pleopod 4 exopod (Fig. 8F) rhomboid, wider than long. Pleopod 5 exopod (Fig. 8G) ovoid, wider than long.

Etymology. The epithet "*uai*" refers to the word often used by people from the state of Minas Gerais, Brazil, to express doubt, astonishment or surprise.

Ecological remarks. Lapa D'Água do Zezé cave is located at the border of Cavernas do Peruaçu National Park. Although most of the outcrop where the cave is inserted within the limits of the park, the cave entrance is outside the park's limit. The external landscape is composed of a well-preserved deciduous forest on the limestone outcrop and surroundings (Fig. 9A), which is inserted in a transition between two phytogeographic domains, Cerrado (Brazilian savannah) and Caatinga (mesophytic and xeromorphic forests). Lapa D'Água do Zezé is a labyrinthine cave with one horizontal
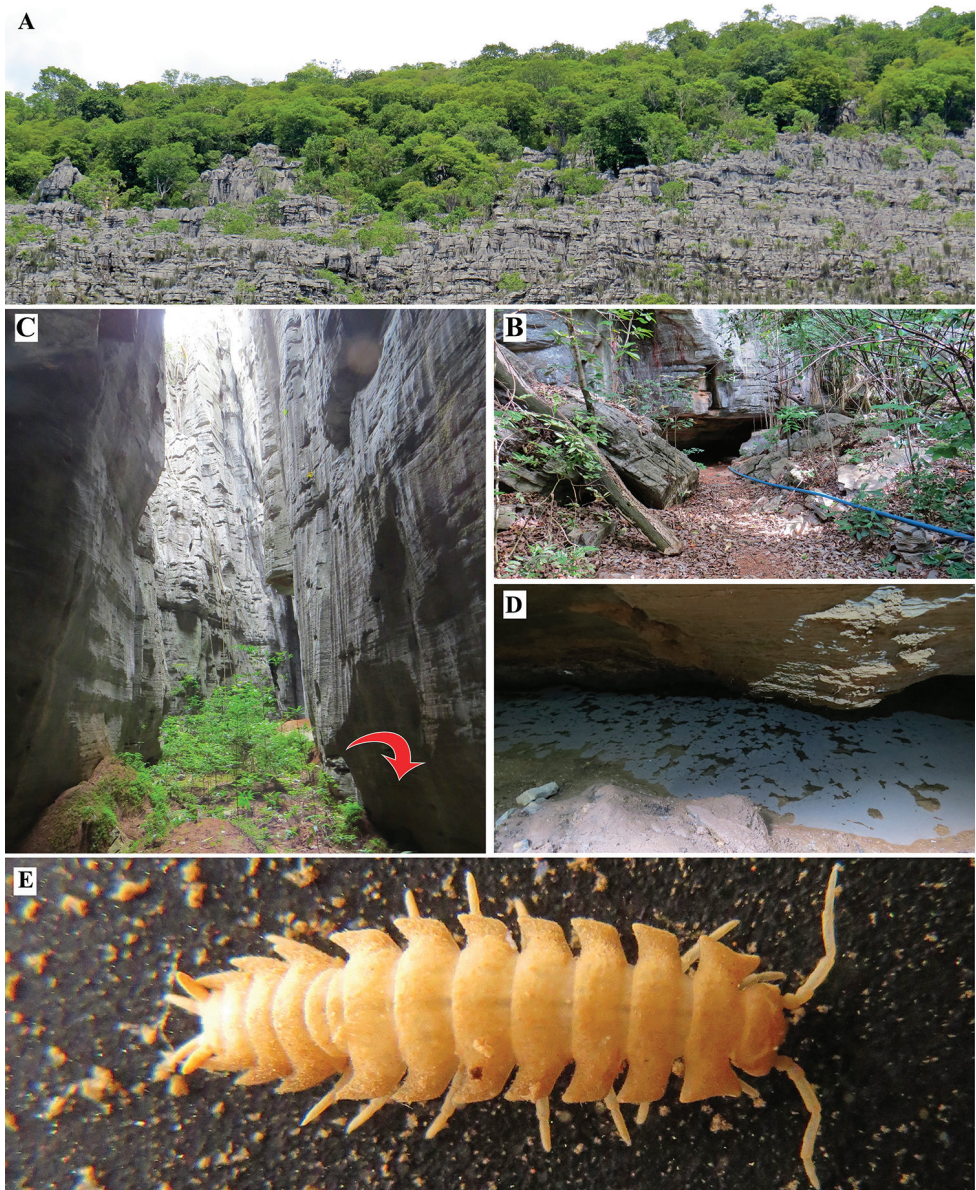
Figure 9. A external landscape of Lapa d'água do Zezé cave **B** vertical entrance of the cave **C** narrow passage inside the cave with a skylight, red arrow indicates the collection site **D** water table where the species was collected **E** living specimen of *Chaimowiczia uai* sp. nov. with approximately 8 mm.

entrance (main entrance, Fig. 9C) and at least two vertical openings. The cave presents perennial water bodies with different conditions. The first one comprises the only accessible part of the water table, a narrow passage in the base of a diaclasis (Fig. 9B) close to one of the cave's vertical openings (Fig. 9C). The second area comprises a very small drainage, apparently originated by the water table overflow. Some physical and chemical parameters of the water were measured during one visit (January 2015): dissolved

oxygen 3.46 mg/L, temperature 25.35 °C, pH 8.45, electrical conductivity 0.565 µS/cm, total dissolved solids 0.359 g/L. This cave also harbors two other stygobitic species and one troglobitic species: the amphipod *Spelaeogammarus uai* (Bastos-Pereira & Ferreira, 2017), which is easily observed in the water table (accessible through the small passage) and seldom at the small drainage; the isopod *Xangoniscus santinhoi* Cardoso, Bastos-Pereira, Souza & Ferreira, 2020b, which is only observed in the drainage; and the hydrometrid *Spelaeometra gruta* Polhemus & Ferreira, 2018. Considering the presence of the amphipod on the drainage, it is possible to infer that both water bodies are connected. Each species seems to present specific preferences. Only a few amphipods were observed in the drainage during several visits to the cave. They seem to avoid this area due to the water flow. Interestingly, specimens of *C. uai* sp. nov. were only found in the water table, sharing the habitat with amphipods, while no specimens were observed in the drainage (Fig. 9D, E). As mentioned for *C. tatus* sp. nov., *C. uai* sp. nov. seems to avoid other styloniscid isopods, which are quite abundant along the drainage and very rare at the water table. This apparent avoidance may have resulted from competition between species, and this certainly deserves further investigation. Organic debris is seasonally transported to the water table (during the rainy periods) due to the proximity to the vertical entrance. Accordingly, the observed organic matter is mainly composed of vegetal debris.

Local farmers have installed a gravitational pump inside the cave in order to drag water from the cave for consumption and irrigation (Fig. 9C) (Bastos-Pereira and Ferreira 2017). Hence, the drainage was partially altered and is disturbed by farmers, who periodically remove the sediment to allow water flow. Such intervention occurs with low frequency (once in a year, according to the farmer), and only in a few parts of the drainage. It does not seem to affect the cave communities, especially considering that a great part of the populations may be in inaccessible areas of the cave. Lastly, although the vegetation seems well preserved in the surroundings of the cave entrance, the original forests were severely altered in many areas around the outcrops and the landscape is mainly composed of pastures and crops.

Discussion

Chaimowiczia uai sp. nov. differs from *C. tatus* sp. nov. in having rounded antennal lobes on cephalon (*vs.* quadrangular in *C. tatus* sp. nov.), anterior portion of pereonite 1 epimera directed frontward (*vs.* outwards in *C. tatus* sp. nov.), pleonite 5 posterior margin surpassing distal margin of telson (*vs.* shorter than distal margin in *C. tatus* sp. nov.), and uropod endopod longer than exopod (*vs.* endopod as long as exopod in *C. tatus* sp. nov.).

Chaimowiczia gen. nov. was allocated into the subfamily Iuiuniscinae. Iuiuniscinae was created to include *Iuiuniscus iuiuensis* Souza, Ferreira & Senna, 2015, a species with unique behavior in Oniscidea: it builds semi-spherical shelters using clay. This behavior represents an evolutionary novelty that probably could support the subfamily as a clade (or support a least inclusive group in which this characteristic has arisen),

even if other possible species of Iuiuniscinae, such as the new species of *Chaimowiczia* gen. nov. described here, do not exhibit this characteristic.

Good character interpretation is essential to achieve more robust results in phylogenetic analysis. In taxonomy, primary homology hypotheses are made when taxa are comparatively described. It is not possible to start a phylogenetic analysis without resorting to descriptive works. Improvement of descriptive works such as Campos-Filho et al. (2019) did for *Iuiuniscus* and Taiti and Montesanto (2020) did for *Thailandoniscus* Dalens, 1989 is important. Morphological characters can indicate kinship, which may be investigated in future phylogenetic analyses. Thus, hypotheses of primary homologies provided in taxonomic works can be tested and confirmed (or not) as synapomorphies through phylogenetic analyses. Therefore, evolutionary reasonings should be developed in taxonomic works, in addition to character description. However, based on the premises mentioned, it is necessary to amend some arguments provided by Campos-Filho et al. (2019). The illustration provided by these authors for the male pleopod 2 endopod confirms what was established by Souza et al. (2015: 10): "the morphology of the distal part pleopod 2 endopodite of male... is in part similar to *Spelunconiscus*". Campos-Filho et al. (2019) suggested that such similarity might indicate kinship. This could invalidate Iuiuniscinae along with the similarity between the male exopod 3 of *Xangoniscus*, *Spelunconiscus,* and *Iuiuniscus*. Such similarity might be symplesiomorphic instead. The character states have not been established yet, so such similarities can be considered superficial until future phylogenetic analyses are carried out.

An important morphological trait observed in both species of *Chaimowiczia* gen. nov. are the rectangular-shaped lateral projections of pereonites epimera and somewhat acute in pleonites. These projections of pereonites and pleonites may be, another synapomorphies of Iuiuniscinae, in addition to the behavioral characteristic already mentioned. These lateral projections differ from the lateral projections in *Iuiuniscus*, especially considering the pleonites. The presence of morphological modifications (as some sort of spines) in subterranean crustaceans is well documented, and evidences associate them to mechanical defense mechanisms preventing predation (Jugovic et al. 2010; Souza et al. 2015). In some cases, exaggerated spines can be observed, like in the stenasellid *Acanthastenasellus forficuloides* Chelazzi & Messana, 1985 from Somalia, which shares the habitat with the troglobitic cyprinid predator fish *Phreatichthys andruzzii* Vinciguerra, 1824 (Messana et al. 2001). The distinct morphology observed in *Chaimowiczia* gen. nov. species may be related to this tendency. However, no potential predators were observed in their habitats. Hence, a question rises on the origin of these body lateral expansions.

Connell (1980) proposed the term "ghost of competition past" to describe one possible reason for observed niche differentiations among species. The theory suggests that competing species may present a lower fitness compared to species that avoids competition by occupying non-overlapping niches. As such, natural selection would favor the non-competing species since their population could increase in contrast to the competing species population. The observed differentiation might be the result from a past competition, the called ghost of competition past. Further studies tested such concept, observing that natural selection reduced interaction strength among co-occurring species, facilitating coexistence and population persistence (Steiner et al.

2007). Sheriff et al. (2010) proposed that the lack of recovery of reproductive rates of the snowshoe hare (*Lepus americanus* Erxleben, 1777) during the early low phase of the reproductive cycle may be a result from impacts of intergenerational, maternally inherited stress hormones caused by high predation risk during the population decline phases. Following the idea firstly presented by Connell (1980) and posteriorly corroborated by other authors, past predation could have selected some traits in a given population along a time period, but later this selective force may have ceased by the predator disappearance from the habitat. Despite the lack of any direct evidence (as a fossil record, for example), the morphology observed in *Chaimowiczia* gen. nov. may be a product of a "ghost predation past" in a period when the ancestor populations could be under a predator selective pressure. After ceased the selection, the morphology was kept in an intermediate state, which is currently observed as the pereonite and pleonites 3–5 epimera well developed in *Chaimowiczia* gen. nov. It is important to stress that such hypothesis deserves further investigation.

The here described genus raises to 17 the number of Styloniscidae living genera in the world, nine of them with occurrence in Brazil. Brazilian caves currently shelter 20 described species of Styloniscidae, while five other are found in epigean habitats (Campos-Filho et al. 2018; Cardoso et al. 2020a, b). The subterranean species deserves special attention regarding conservation actions due to their short-ranged geographical distribution (most of them are restricted to a single cave) and surrounding landscape being frequently threatened by anthropic activities.

Acknowledgments

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RESEARCH ARTICLE

Host-parasite associations in a population of the nectarivorous bat *Anoura geoffroyi* (Phyllostomidae) in a cave in a Brazilian ferruginous geosystem

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Abstract

Parasitic relationships between Neotropical bats and their ectoparasites are not well known, even though parasitism is one of the factors that can affect the fitness of a host population. This study characterized parasite-host relationships in relation to sex, age, body size and reproductive status in a population of *Anoura geoffroyi* using the indices of Prevalence, Mean Intensity and Mean Abundance. Total prevalence for 93 sampled bats was 94.6%. Two species of streblid flies that are considered primary parasites of *A. geoffroyi*, *Exastinion clovisi* (n = 203) and *Anastrebla modestini* (n = 152), were the most abundant ectoparasites, followed by *Trichobius* sp. (n = 7). Two mite species, *Periglischrus vargasi* (Spinturnicidae) (n = 98) and *Spelaeorhynchus praecursor* (Spelaeorhynchidae) (n = 11), were also found. We recorded higher mean abundance and intensity of parasitism in pregnant females compared to reproductive males and reproductively inactive females, for different specific associations of ectoparasites. Host age and body condition had no effect on the parasitological indices. Even with high rates of parasitism, parasitic load did not influence host body condition, but infestation rates by mites were higher in reproductive males and higher by flies in reproductive females, showing that ectoparasites can have variable influences between the different stages of the life history of these host bats. Thus, the reproductive activity of the hosts could be an adverse factor for resistance to parasite infestations.

Introduction

Ectoparasitism can have a strong influence on host populations (Brown and Brown 2004; Møller and Saino 2004). Its diversity is directly influenced by several factors such as geographic distribution, roost environment and by host biology, morphology and behavior (Rui and Graciolli 2005; Postawa and Nagy 2016). Pressures resulting from parasitism can increase the rate of predation on hosts, weaken host physical condition, and increase disease incidence, which can result in decreases in survival and reproduction (Neuhaus 2003; Brown and Brown 2004; Ter Hofstede and Fenton 2005). Ectoparasites may have more significant influence on bat populations, as most bats are gregarious, that can form colonies of thousands of individuals and often roost in places with limited space, such as caves (Bredt et al. 1999; Lourenço and Palmeirim 2007, 2008; Guimarães and Ferreira 2014), which is the case for *Anoura geoffroyi* Gray, 1838 (Guimarães and Ferreira 2014; Farias et al. 2018; Reis 2018).

Anoura geoffroyi (Chiroptera: Phyllostomidae) is a nectarivorous bat (13–18 g) (Koopman 1994; Reid 1997) with a wide geographic distribution in the Neotropical region, from Mexico to Peru, Bolivia, and Brazil (Simmons 2005). The species has a strong association with natural cavities and preferably uses caves as diurnal roosts (Arita 1993; Guimarães and Ferreira 2014), where colonies of hundreds of individuals can form (Bredt et al. 1999; Farias et al. 2018; Reis 2018). The species presents seasonal population dynamics with a monoestrous reproductive pattern and reproductive activity occurring during the rainy season (Zortéa 2003; Farias et al. 2018).

Among the species of ectoparasites found on bats dipterans and mites are the most common. Dipterans of the family Streblidae are obligatory blood-sucking ectoparasites of bats, they are viviparous and have three larval stages that develop in the female's uterus, the pupa that develops in the host's roost and the adult, which is the hematophagous ectoparasite (Dick and Patterson 2006). Likewise, mites of the family Spinturnicidae are exclusive parasites of bats and complete their entire life cycle on the host's body. The dispersion of these mites requires direct contact between hosts, which is facilitated by the social behaviors of bats, such as copulation, birthing of young, parental care and the habit of living in groups. They are commonly found on the patagium of the host (Rudnick 1960; Lourenço and Palmeirin 2007; Almeida et al. 2015). In temperate region, it was observed that sex, age, and reproductive status of the host strongly influences the reproductive activity of parasites, and, according to the authors, reproduction of ectoparasites of many temperate cave-dwelling bats is mostly regulated by the reproductive cycle of their bat hosts (Lourenço and Palmeirim 2008).

Studies related to different aspects of the association between ectoparasites and bats in the Neotropical region are still scarce. Thus, there is a knowledge gap about parasitological relationships resulting from the numerous factors that influence ectoparasitism in bats such as sex, age and reproductive status of the host, among others (Rui and

Graciolli 2005; Patterson et al. 2007; Patterson et al. 2008; Postawa and Nagy 2016). The present study aimed to understand the ecological aspects involved in the interaction between ectoparasites and the phyllostomid bat *A. geoffroyi* in a colony that uses a ferruginous cave as a diurnal roost and has been present at the site for many years. The effects of bat sex, age, reproductive status, and body condition on the rate of ectoparasite infestation were investigated. Considering that the species exhibits seasonal monoestric reproductive activity during the rainy season, we hypothesize here that ectoparasites may have variable influences between the different stages of the hosts' life history, as well as in relation to their body condition.

Methods

Study area

The studied colony of *A. geoffroyi* lives in the ferruginous cave named Piedade (19°49'20"S, 43°40'33"W, 1,414 m altitude). The colony is formed by groups of varying sizes (5 to 20 individuals). Maximum abundance is observed in the reproductive period with a few hundred of individuals (Farias et al. 2018). The cave is situated in Serra da Piedade, located in Monumento Natural Estadual Serra da Piedade (state park), state of Minas Gerais, Brazil, and has a horizontal projection of about 360 m (Pereira et al. 2012). Serra da Piedade is situated in the Quadrilátero Ferrífero ferruginous geosystem (Bueno 1992), which harbors the largest iron ore reserve in Brazil (Souza and Carmo 2015). At present, there are 46 open pit mines reported from the region, which may pose a threat to local fauna and flora (Souza and Carmo 2015). The vegetation in the region varies with altitude, with semi-deciduous forest in the lower parts and altitudinal fields or rupestrian field at higher altitudes (Bueno 1992). The climate of the region is subtropical of altitude (Cwa), according to the Köppen classification. There are two well defined seasons with a rainy season from October to March (corresponding to spring-summer months) and a dry season from April to September (corresponding to the autumn-winter months) (Bueno 1992).

Data collection

Diurnal campaigns to the roost of *A. geoffroyi* (cave environment) were carried out on September 9, 2017; January 24, 2018; and September 18, 2018 to capture bats and collect ectoparasites. Bats were captured with a mist-net (12 × 3 m) installed inside the cave about 50 m from the colony, from 8:00 h to 14:00 h, which was checked every 20 minutes. To minimize the disturbance of the colony, the researchers remained outside waiting for the capture of bats in flight. With at least three openings inaccessible, the placement of mist nets outside the cave was not possible. The use of a pole net was also not possible due to the great height of the cave. Due to the difficulties imposed by local conditions, diurnal collections were used, which proved to be viable following the protocol of Farias et al. (2018). Information related to sex, age, body mass (grams), forearm

length (millimeters) and reproductive status was obtained for each captured animal. Bats were released without marking, shortly after the collection of ectoparasites and biometric data. The degree of ossification of the metacarpal epiphyses was evaluated to determine age (Anthony 1988). Reproductive status was analyzed by observing secondary reproductive characteristics (Farias et al. 2018). Inactive males had poorly developed testes, while active males presented fully developed scrotal testes. Pregnant or lactating females were considered reproductively active, while others were considered inactive.

Ectoparasites were collected by inspecting the pelage of the bats with the naked eye and using fine-tipped forceps to transfer them to individual containers (containing 70% ethanol) for each bat (Graciolli and Carvalho 2001). The ectoparasites were prepared as described in DeBlase and Martin (1980). Samples of each ectoparasite species were chosen under a stereomicroscope, and then these samples were placed on a microscope slide and cleared in Hertwig's solution (chloral hydrate). This solution is a clearing agent for microscopic examination and is commonly used in the diet analysis of small mammals (DeBlase and Martin 1980; Talamoni et al. 2008). After this process, the ectoparasites were mounted on a slide in a drop of glycerin and covered by a coverslip. Dipterans and mites were then identified under an optical microscope (10×), using dichotomous keys (Graciolli and Carvalho 2001; Herrin and Tipton 1975; Wenzel et al. 1976; Peracchi 1990).

Parasitological indices were calculated to analyze the infestation in the population and the association with each parasite, except for the species *Spelaeorhynchus praecursor* Neuman, 1902 (Spelaeorhynchidae), which was poorly sampled in the present study. Prevalence (P; number of infested hosts/number of hosts examined × 100) expressed as a percentage, Mean Intensity (MI; number of parasites/number of infested hosts) and Mean Abundance (MA; number of parasites/total number of hosts examined) were calculated (Bush et al. 1997). The influence that host sex, age (adult, non-adult) and reproductive status have on the parasitological indexes was investigated. All young and sub-adult animals of both sexes were included in the non-adult class, being differentiated by the presence of cartilaginous epiphyses and all being sexually immature (Farias et al. 2018). Mean intensities and mean abundances were compared by Student's t-test with randomization and 2000 replicates. The influence of host biological parameters on prevalence was evaluated by Fisher's exact test. Analyses were performed using Quantitative Parasitology 3.0 software (Rózsa et al. 2000). The Body Condition Index (BCI) (body mass/forearm length; Reichard and Kunz 2009) was used to investigate the relationship between host body condition and parasitic load through Spearman correlation tests. All tests were run using Bioestat 5.3 software (Ayres et al. 2007) with a significance level of 5%.

Results

A total of 93 bats were captured, 88 of which were infested, resulting in a prevalence rate of 94.6% (0.8–1.0, CI 95%). The total of 471 ectoparasites collected included flies of three species of the family Streblidae – *Anastrebla modestini* Wenzel, 1966
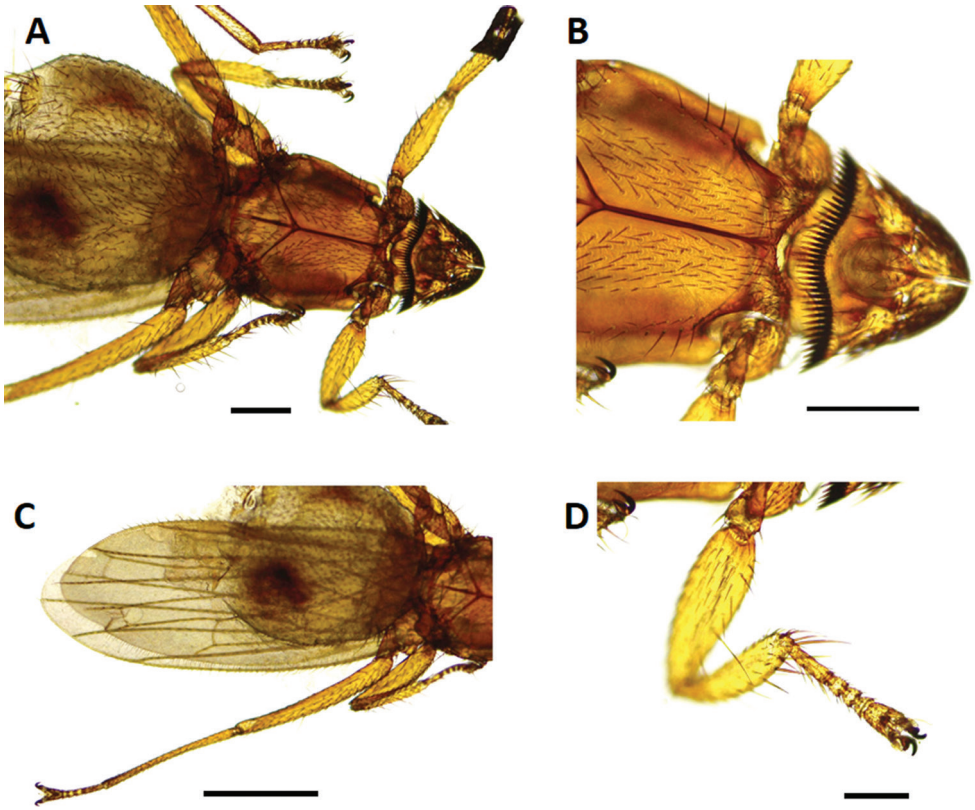
Figure 1. A ventral view of *Anastrebla modestini*, 40× magnification **B** detail of head, 100× **C** detail of wing and leg, 100× **D** detail of femur and setae, 100×. Leica DM500 optical microscope. Scale bars: 0.5 mm (**A–D**).

(n = 152, Fig. 1), *Exastinion clovisi* (Pessoa & Guimarães, 1936) (n = 203, Fig. 2) and *Trichobius* sp. (n = 7, Fig. 3). Specimens of two species of mites were also collected – *Periglischrus vargasi* Hoffmann, 1944 (Spinturnicidae, n = 98, Fig. 4) and *S. praecursor* (n = 11, Fig. 5). Mean Intensity (MI) was 5.35 (± 3.47) ectoparasites per host while Mean Abundance (MA) was 5.06 (± 3.58) ectoparasites per host.

Infestation analysis for all ectoparasites (Table 1) revealed that host sex did not affect P (p = 1.00), MA (t = 1.14; p = 0.24) or MI (t = 1.31; p = 0.18). Reproductively active and inactive males showed no differences in the indexes (P: p = 1.0; MI: t = -1.31, p = 0.21; MA: t = -1.52, p = 0.16), as was also the case for pregnant and inactive females (P: p = 0.55; MI: t = 0.30, p = 0.75; MA: t = 0.83, p = 0.39). On the other hand, pregnant females had higher MA than reproductively active males (t = 2.12, p = 0.03) (Table 1).

For the specific association between *A. geoffroyi* and *E. clovisi* (Table 2), female hosts had higher MA (t = 2.31, p = 0.02) than did male hosts, while for the association between *A. geoffroyi* and *A. modestini* (Table 2), pregnant females had higher P (p = 0.02) and higher MA (t = 2.87, p = 0.006) than did inactive females. Pregnant
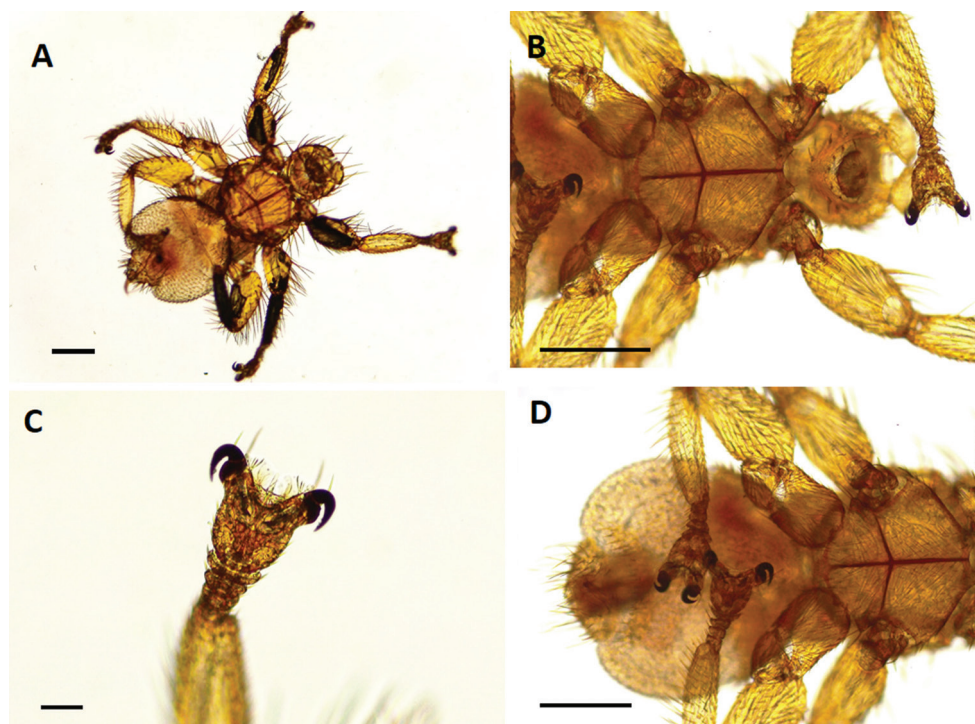
Figure 2. A dorsal view of *Exastinion clovisi*, 40× magnification **B** detail of thorax, 100× **C** distal detail of the leg, 100× **D** detail of abdomen, 100×. Leica DM500 optical microscope. Scale bars: 0.5 mm (**A, B, D**); 0.1 mm (**C**).
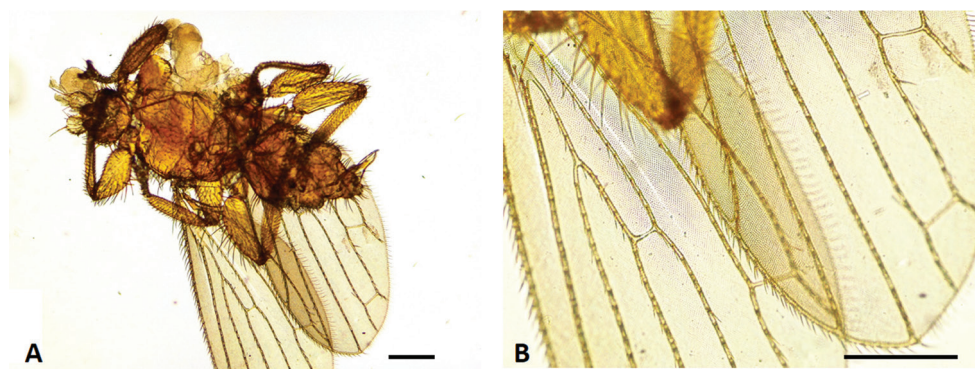
Figure 3. A dorsal view of *Trichobius* sp., 40× magnification **B** detail of wing, 100×. Leica DM500 optical microscope. Scale bars: 0.5 mm (**A, B**).

females were also more parasitized than reproductive active males, with significant differences in P (p = 0.005), MA (t = 3.32, p = 0.006) and MI (t = 1.99, p = 0.04). For the association between *A. geoffroyi* and the mite *P. vargasi* (Table 2), MA and MI were
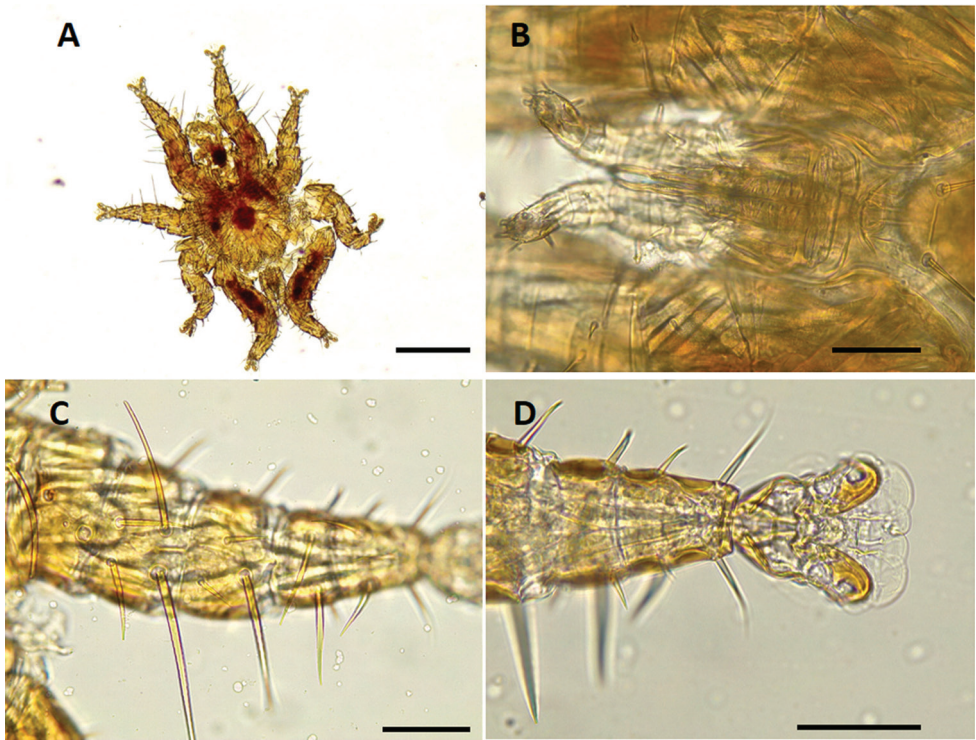
Figure 4. A specimen of *Periglischrus vargasi*, 40× magnification **B** detail of gnatossome, 400× **C** detail of setae insertion in the leg, 400× **D** distal detail of the leg, 400×. Leica DM500 optical microscope. Scale bars: 0.5 mm (**A**); 0.1 mm (**B–D**).

Table 1. Number of hosts examined (N), infected (in parentheses), Prevalence (P), Mean Intensity (MI) and Mean Abundance (MA), with Confidence Intervals (CI 95%), for ectoparasites of *Anoura geoffroyi* in Piedade cave, located in Serra da Piedade, state of Minas Gerais. Inactive males had poorly developed testes and inactive females were those who did not show evidence of pregnancy and lactation. * = Significant differences.

Host		N	P (%) (CI 95%)	MI (CI 95%)	MA (CI 95%)
Sex	Female	50 (47)	94.0 (0.8–0.9)	5.8 (4.8–6.8)	5.4 (4.4–6.4)
	Male	43 (41)	95.3 (0.8–0.9)	4.8 (3.8–6.0)	4.6 (3.6–5.7)
Reproductive status	Reproductive male	32 (30)	93.8 (0.7–0.9)	4.2 (3.3–5.4)	4.0 (3.0–5.0)*
	Inactive male	11 (11)	100 (0.7–1.0)	6.3 (3.9–9.3)	6.3 (3.9–9.3)
	Pregnant female	18 (18)	100 (0.8–1.0)	6.0 (4.5–7.5)	6.0 (4.5–7.5)*
	Inactive female	32 (29)	90.6 (0.7–0.9)	5.6 (4.5–6.9)	5.1 (3.9–6.4)
Age	Adult	52 (51)	98.1 (0.8–0.9)	5.1 (4.3–6.0)	5.0 (4.2–5.9)
	Non-adult	41 (37)	90.2 (0.7–0.9)	5.6 (4.5–6.9)	5.0 (3.9–6.4)

higher for pregnant females compared to inactive females (MA: t = -2.79, p = 0.009; MI t = − 2.93, p = 0.01), while reproductively active males were more parasitized than pregnant females with respect to MA and MI (MA: t = -2.34, p = 0.02; MI: t = -3.17, p = 0.01) (Table 2).
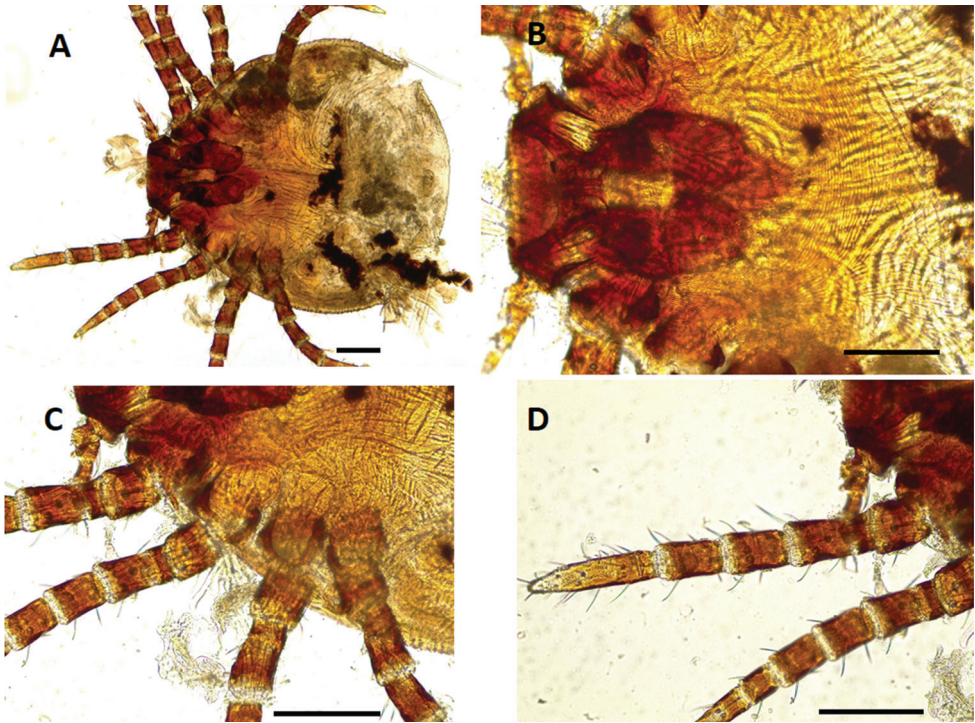
Figure 5. A specimen of *Spelaeorhynchus praecursor*, 40× magnification **B** detail of gnatossome, 100× **C** detail of legs, 100× **D** detail of leg and setae, 100×. Leica DM500 optical microscope. Scale bars: 0.5 mm (**A–D**).

Analyses found no correlation between host BCI and parasitic load for males in general (r_s = -0.05; n = 43; p = 0.70); for reproductively active males (r_s = -0.05; n = 32; p = 0.75); for inactive males (r_s = 0.19; n = 11; p = 0.75); for females in general (r_s = 0.002; n = 50; p = 0.98); for pregnant females (r_s = 0.17; n = 18; p = 0.740); for inactive females (r_s = -0.26; n = 32; p = 0.14); for adults (r_s = 0.13; n = 52; p = 0.33) and for non-adults (r_s = -0.21; n = 41; p = 0.17).

Discussion

The present study registered two common species of parasitic flies belonging to the family Streblidae, *A. modestini* and *E. clovisi*, both having already been registered as primary parasites of *A. geoffroyi* (Wenzel et al. 1976). Both species are widely distributed in the Neotropical region, as are their hosts (Komeno and Linhares 1999; Graciolli and Rui 2001; Bertola et al. 2005; Simmons 2005; Moras et al. 2013; Dornelles and Graciolli 2017; Trujillo-Pahua and Ibáñez-Bernal 2018). *Trichobius* spp. was not abundant in the present study, although some species of the genus have been previously registered on *A. geoffroyi*, such as *Trichobius tiptoni* (Graciolli and Rui 2001) and *T. propinquus* (De Vasconcelos et al. 2015), or *Trichobius* sp. (*dugesii* complex) (Reis 2018).

Table 2. Number of hosts examined (N), infected (in parentheses), Prevalence (P), Mean Intensity (MI) and Mean Abundance (MA), with Confidence Intervals (95% CI), for specific interactions of *Exastinion clovisi*, *Anastrebla modestini* and *Periglischrus vargasi* with *Anoura geoffroyi* in Piedade cave, located in Serra da Piedade, state of Minas Gerais. Inactive males had poorly developed testes and inactive females were those who did not show evidence of pregnancy and lactation. * = Significant differences.

Host-parasite relationship		N	P (%) (CI 95%)	MI (CI 95%)	MA (CI 95%)
Exastinion clovisi					
Sex	Female	50 (40)	80.0 (0.6–0.8)	3.3 (2.7–4.1)	2.6 (2.0–3.4)*
	Male	43 (27)	62.0 (0.4–0.7)	2.5 (2.0–3.2)	1.6 (1.1–2.1)*
Reproductive status	Reproductive male	32 (18)	56.3 (0.3–0.7)	2.5 (1.9–3.1)	1.4 (0.9–2.0)
	Inactive male	11 (9)	81.8 (0.4–0.7)	2.6 (1.6–4.1)	2.1 (1.0–3.5)
	Pregnant female	18 (15)	83.3 (0.5–0.9)	2.9 (2.0–3.9)	2.4 (1.6–3.3)
	Inactive female	32 (25)	78.1 (0.6–0.9)	3.5 (2.6–4.6)	2.7 (1.9–3.7)
Age	Adult	52 (39)	75.0 (0.6–0.8)	2.9 (2.3–3.5)	2.1 (1.6–2.7)
	Non-adult	41 (28)	68.3 (0.5–0.8)	3.2 (2.4–4.1)	2.2 (1.5–3.0)
Anastrebla modestini					
Sex	Female	50 (33)	66.0 (0.5–0.7)	2.73 (2.1–3.4)	1.80 (1.2–2.4)
	Male	43 (26)	60.5 (0.4–0.7)	2.38 (1.6–3.8)	1.44 (0.9–2.4)
Reproductive status	Reproductive male	32 (18)	56.3 (0.3–0.7)*	1.89 (1.3–2.5)*	1.06 (0.6–1.5)*
	Inactive male	11 (8)	72.7 (0.3–0.9)	3.50 (1.5–7.5)	2.55 (1.0–6.0)
	Pregnant female	18 (17)	94.4 (0.7–0.9)*	3.06 (2.1–4.0)*	2.89 (2.0–3.7)*
	Inactive female	32 (16)	50.0 (0.3–0.6)*	2.38 (1.6–3.6)	1.19 (0.6–1.9)*
Age	Adult	32 (16)	67.3 (0.5–0.7)	2.60 (2.0–3.1)	1.75 (1.2–2.3)
	Non-adult	11 (8)	58.5 (0.4–0.7)	2.54 (1.7–4.0)	1.49 (0.9–2.5)
Periglischrus vargasi					
Sex	Female	50 (24)	48.0 (0.3–0.6)	1.7 (1.3–2.0)	0.84 (0.5–1.1)
	Male	43 (22)	51.2 (0.3–0.6)	2.5 (1.8–3.3)	1.30 (0.8–1.9)
Reproductive status	Reproductive male	32 (14)	43.8 (0.2–0.6)	2.7 (2.0–3.7)*	1.22 (0.6–1.9)*
	Inactive male	11 (8)	72.7 (0.3–0.9)	2.1 (1.2–3.6)	1.5 (0.7–2.8)
	Pregnant female	18 (6)	33.3 (0.1–0.5)	1.1 (1.0–1.3)*	0.3 (0.1–0.6)*
	Inactive female	32 (18)	56.3 (0.3–0.7)	1.9 (1.5–2.2)*	1.0 (0.7–1.5)*
Age	Adult	52 (24)	46.2 (0.3–0.6)	1.9 (1.5–2.5)	0.9 (0.6–1.2)
	Non-adult	41 (22)	53.7 (0.3–0.6)	2.3 (1.7–3.0)	1.2 (0.7–1.7)

The mites *P. vargasi* and *S. praecursor* have reduced host specificity, commonly occurring on several Neotropical bat species (Herrin and Tipton 1975; Moras et al. 2013; Almeida et al. 2015). The present study found *S. praecursor* specimens to strongly attach to the tragus of the host, a behavior that has been regularly recorded for this species (Fain et al. 1967; Peracchi 1990).

Our data showed a high prevalence rate of most parasites. A study with *Artibeus lituratus* and *Sturnira lilium*, frugivorous phyllostomid bats that commonly roost in treetops and/or human buildings, found much lower prevalence rates, with 3.4% and 9.1% prevalences, respectively (Dornelles and Graciolli 2017). Environmental factors and host behavior can influence prevalence rates, while type of diurnal roost occupied by the host is one of the factors that indirectly influences prevalence rates (Kunz 1982; Ter Hofstede and Fenton 2005). A host habit of changing roosts can disrupt the life cycle of ectoparasitic flies, which spend part of their life cycle inside the roosts, while such movements would not affect mites, because they spend their entire life cycle on the host's body (Kunz 1982; Lewis 1995).

The studied bat population has used Piedade cave as a diurnal roost for at least a decade and the species is known to prefer caves (Guimarães and Ferreira 2014). Caves

represent favorable diurnal roosts for many bats, as they provide a stable microclimate and protection against predators and adverse weather (Kunz 1982; Lewis 1995). Because such roosts are confined spaces, the very habit of living in a group facilitates body contact and the host-switching activity of ectoparasites. Therefore, it is plausible to hypothesize the existence of an association between the high prevalence rate recorded in the present study (94.6%) and the type of shelter (cave) used, although further investigation is needed.

The present study registered an influence of host sex on the Mean Abundance in the association between *E. clovisi* and female hosts. Higher infestation levels for females have been frequently reported in the literature (Christie et al. 2007; Patterson et al. 2008; Presley and Willig 2008). By adding the reproductive status of females to the analysis, the results were able to reveal that pregnant females were more parasitized by flies than were non-pregnant females and males. Similar results were found by Reis (2018) in a study conducted in another cave inhabited by *A. geoffroyi*. The greater fly parasitism of pregnant females is consistent with observations for other bat species such as *A. lituratus* (Bertola et al. 2005), *Megaderma lyra* (Sundari et al. 2005), *Miniopterus schreibersii* (Lourenço and Palmeirim 2008), and *Cynopterus brachyotis* (Lee et al. 2018).

The Lourenço and Palmeirim (2008) study in temperate zone showed that four parasite species had a similar reproductive pattern, reproducing more intensively during the pregnancy and nursing seasons of *M. schreibersii*, mainly on pregnant and juvenile bats. The authors concluded that this may be an adaptive trait in which the reproductive cycles of the parasite species are adjusted to the cycles of their hosts in a seasonal environment. This hypothesis could explain our results and those of other studies with similar results. Further studies focusing on the synchrony of the reproductive cycles of ectoparasites and their hosts, in other regions with marked seasonality, may bring new clarifications about this relationship.

Thus, especially for pregnant female, a lower immunological defense during the reproductive phase, due to endocrine changes inherent to reproduction (Grossman 1985; Christie et al. 2000) can facilitate their greater infestation during a period of greater reproductive activity of their ectoparasites (Lourenço and Palmeirim 2008). Reproductively active males, on the other hand, were more parasitized by *P. vargasi*, than were pregnant females. *Anoura geoffroyi* males exhibit polygyny and the mating season is relatively short (3 months) (Farias et al. 2018). The energy expenditure directed by these animals for mating in a short period may lead to a more debilitated health condition, facilitating the infestation by mites. Thus, the reproductive activity of the hosts could be an adverse factor for resistance to parasite infestations (Christie et al. 2000), even though it does not affect their body condition. However, other factors acting together can contribute to this result (Lourenço and Palmeirim 2008), needing to be tested.

Even with high parasitism rates, the data of the present study demonstrated that parasite load did not influence host BCI. Although BCI is commonly used in studies involving parasites, it has also presented contradictory results in different studies about it being correlated or not with parasite load (Marshall 1982; Christe et al. 2000; Lučan 2006; Lourenco and Palmeirim 2007; Pearce and O´Shea 2007; Lee et al.

2018). According to Postawa and Nagy (2016), even if ectoparasite density varies, the health of the host is unlikely to be affected, as ectoparasites feed mainly on host lymph and blood and do not directly consume other resources such as fatty acids, so there is no direct impact on BCI.

Acknowledgements

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Refining sampling protocols for cavefishes and cave crayfishes to account for environmental variation

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Abstract

Subterranean habitats represent focal habitats in many conservation strategies; however, these environments are some of the most difficult to sample. New sampling methods, such as environmental DNA (eDNA), show promise to improve stygobiont detection, but sources of sampling bias are poorly understood. Therefore, we determined the factors affecting detection probability using traditional visual surveys and eDNA surveys for both cavefishes and cave crayfishes and demonstrated how detection affects survey efforts for these taxa. We sampled 40 sites (179 visual and 183 eDNA surveys) across the Ozark Highlands ecoregion. We estimated the detection probability of cave crayfishes and cavefishes using both survey methods under varying environmental conditions. The effectiveness of eDNA or visual surveys varied by environmental conditions (i.e., water volume, prevailing substrate, and water velocity) and the target taxa. When sampling in areas with average water velocity, no flow, and coarse substrate, eDNA surveys had a higher detection probability (0.49) than visual surveys (0.35) for cavefishes and visual surveys (0.67) had a higher detection probability than eDNA surveys (0.40) for cave crayfishes. Under the same sampling conditions, 5 visual surveys compared to 10 eDNA surveys would be needed to confidently detect cave

crayfishes and 9 visual surveys compared to 4 eDNA surveys for cavefishes. Environmental DNA is a complementary tool to traditional visual surveys; however, the limitations we identified indicate eDNA currently cannot replace visual surveys in subterranean environments. Although sampling designs that account for imperfect sampling are particularly useful, they may not be practical; thus, increasing sampling efforts to offset known detection bias would benefit conservation strategies.

Keywords

Detection probability, karst, Ozark Highlands Ecoregion, stygobionts

Introduction

Variable species detection probability (i.e., the probability of detecting a species if present) is a fundamental sampling challenge when conducting ecological studies (MacKenzie et al. 2018). Species detection can vary among habitats (Mollenhauer et al. 2018), sampling approaches (Pregler et al. 2015), species (McManamay et al. 2014; Mollenhauer et al. 2018), and over time (Hangsleben et al. 2013). The underlying species-environmental relationships of interest often do not emerge without consideration of variable sampling detection (Gwinn et al. 2016). Further, not accounting for variable detection can lead to incorrect estimates of extinction rates (Kéry et al. 2006; Pregler et al. 2015), species richness (Tingley and Beissinger 2013), and distributions (Chen et al. 2013; Lahoz-Monfort et al. 2014), largely due to false absences. For example, switching from seining to backpack electrofishing for sampling bridled shiner *Notropis bifrenatus* (Cope, 1867) in Connecticut led to underestimation of the species distribution due to differences in gear efficiencies (Pregler et al. 2015). As sampling design and statistics advances, ecologists had more options available to account for imperfect detection.

Variable species detection can be taken into account using appropriate study designs. Sampling standardization is useful for limiting some sampling variability (e.g., sampling at the same time of year; see also Bonar et al. 2009), but standardization alone does not account for environmental variability (i.e., flow or habitat) that is often of interest to ecologists (MacKenzie et al. 2004). For example, Mollenhauer et al. (2018) used standardized sampling to estimate the occupancy of Great Plains fishes and showed that sand shiner *Notropis stramineus* (Cope, 1865) occurrence was underestimated in one of two rivers due to differences in the environment (i.e., not controlled through standardization). Detection probability can be estimated by using a study design that uses repeated sampling while measuring environmental factors hypothesized to influence detection (MacKenzie et al. 2018). The concerns associated with sampling detection can be exacerbated in environments that are particularly difficult to sample or when species are rare.

Sampling difficulties in complex environments or where species are relatively rare create challenges for developing meaningful conservation actions. Large rivers, for example, are difficult to sample because of deep water, higher discharges (e.g., Detroit River, Lapointe et al. 2006), and vegetation cover (e.g., Niagara River, Crane and Kapuscinski

2018). Swampy streams, emblematic of complex habitat, make sampling using traditional approaches difficult (e.g., unconsolidated substrates, emergent vegetation, Jensen and Voukoun 2013). Even rivers with relatively homogenous substrates are difficult to sample when the target species is relatively rare (e.g., federally threatened Arkansas River Shiner *Notropis girardi* Hubbs & Ortenburger, 1929; Mollenhauer et al. 2018). In fact, many aquatic species are relatively rare or cryptic making adequate sampling problematic (e.g., bridle shiner, Jensen and Voukoun 2013; bull trout *Salvelinus confluentus*, Sepulveda et al. 2019). Despite advancements in sampling strategies, there remain notable examples of aquatic habitats that are difficult to sample but are considered especially important for both conservation and ecosystem services (e.g., subterranean environments, Mammola et al. 2019).

Sampling cavefishes and cave crayfishes can be difficult due to the challenges of traversing and sampling the subterranean environment. Cavefishes and cave crayfishes are typically surveyed by 1–3 people walking, crawling, or snorkeling slowly upstream in caves while recording the number of organisms observed (e.g., Graening et al. 2006a, 2010; Bichuette and Trajano 2015; Behrmann-Godel et al. 2017). Stygobiotic organisms (groundwater obligates, Sket 2008) may go undetected during visual surveys due to similar environmental factors as surface aquatic environments (e.g., water depth, turbidity), but also because researchers can only access limited portions of the underground ecosystem and accessible areas are often difficult to traverse (Mammola et al. 2020). Additionally, the biology of cave organisms (e.g., low density due to k-selected life history and uneven distributions within caves) makes them difficult to detect (Mammola et al. 2020). Several cave surveys may be needed to detect stygobionts; thus, estimates of species occupancy and richness are skewed toward commonly sampled locations (Culver et al. 2004; Krejca and Weckerly 2008).

Sampling using environmental DNA (eDNA) is a relatively new technique in ecology and conservation biology that may improve detection of cavefishes and cave crayfishes; however, sources of variable detection probability are poorly understood. Environmental DNA surveys document species presence via the collection of DNA from the environment (Ficetola et al. 2008), which is derived from sources such as waste products, shed hair and skin, the slime coat of fishes and amphibians, shed exoskeletons of arthropods, and decomposing individuals (Tréguier et al. 2014; Thomsen and Willerslev 2015). Many taxa have been surveyed via eDNA in surface habitats, including fishes (e.g., Jerde et al. 2011), crayfishes (e.g., Tréguier et al. 2014), mollusks (e.g., Egan et al. 2013), and reptiles (e.g., Piaggio et al. 2014). Studies in subterranean habitats are few to date (reviewed in Gorički 2019) but include stygobiotic *Proteus* salamanders (Gorički et al. 2017; Vörös et al. 2017), *Stygobromus* amphipods (Niemiller et al. 2018), and two *Cambarus* species of cave crayfishes (Boyd 2019). Environmental DNA surveys can improve species detection when compared to traditional survey methods (Jerde et al. 2011; Smart et al. 2015; Schmelzle and Kinziger 2016). Further, eDNA surveys make it possible to survey karst environments without sampling entire caves.

Understanding how our sampling approaches relate to our ability to detect a species is important to developing meaningful conservation actions. In many cases, particularly

with rare or cryptic organisms, sampling results in false absences (i.e., species was undetected when present). Therefore, our study objective was to determine some of the environmental factors associated with detection probability of cave crayfishes and cavefishes using both visual and eDNA surveys. Our overall goal was to assess how sampling bias related to the effort needed to adequately sample these taxa and obtain reliable presence or absence inferences. Results of this study will help managers choose the most efficient sampling approach for determining the presence of cavefishes and cave crayfishes and understand sources of detection error for both eDNA and visual surveys.

Methods

Study area

We conducted our study in the Ozark Highlands level-three ecoregion (hereafter referred to as the Ozark Highlands) of northeast Oklahoma, southwest Missouri, and northwest Arkansas (Figure 1). Average annual rainfall and air temperatures of the Ozark Highlands are 116 cm and 13.7 °C, respectively (30 yr climate normal for Springfield, Missouri; National Oceanic and Atmospheric Administration). The ecoregion was historically a mix of prairie, oak, hickory, and pine forests, but many lowland areas have been converted to agricultural uses (Woods et al. 2005). The lithology of the Ozark Highlands is primarily Mississippian limestone and Ordovician dolomite, which have been dissolved over time by groundwater, resulting in thousands of caves and springs (Unklesbay and Vineyard 1992).

Study species

We focused our study on two species of cavefishes, Ozark cavefish *Troglichthys rosae* (Eigenmann, 1898) and Eigenmann's cavefish *Typhlichthys eigenmanni* (Girard, 1859), and 5 species of cave crayfishes, Benton cave crayfish *Cambarus aculabrum* (Hobbs & Brown, 1987), bristly cave crayfish *C. setosus* (Faxon, 1889), Delaware county cave crayfish *C. subterraneus* (Hobbs, 1993), Oklahoma cave crayfish *C. tartarus* (Hobbs & Cooper, 1972), and Caney Mountain cave crayfish *Orconectes stygocaneyi* (Hobbs, 2001). The full distributions of many of our target species are unknown, though existing sampling data provide some insight. There is no known overlap in distributions among species within taxa (i.e., cave crayfishes or cavefishes; Figure 1). *Troglichthys rosae* is assumed to occur in the Springfield Plateau of northwest Arkansas, southwest Missouri, and northeast Oklahoma (Niemiller and Poulson 2010). *Typhlichthys eigenmanni* is considered endemic to the Ozark Highlands of central and southeast Missouri and northeast Arkansas (Niemiller et al. 2012); however, we only sampled along the western portion of the species estimated range. *Cambarus aculabrum* is known from only four locations in northwest Arkansas (Graening et al. 2006a). *Cambarus setosus* is the widest-ranging cave crayfish of the Ozark Highlands and has been docu-
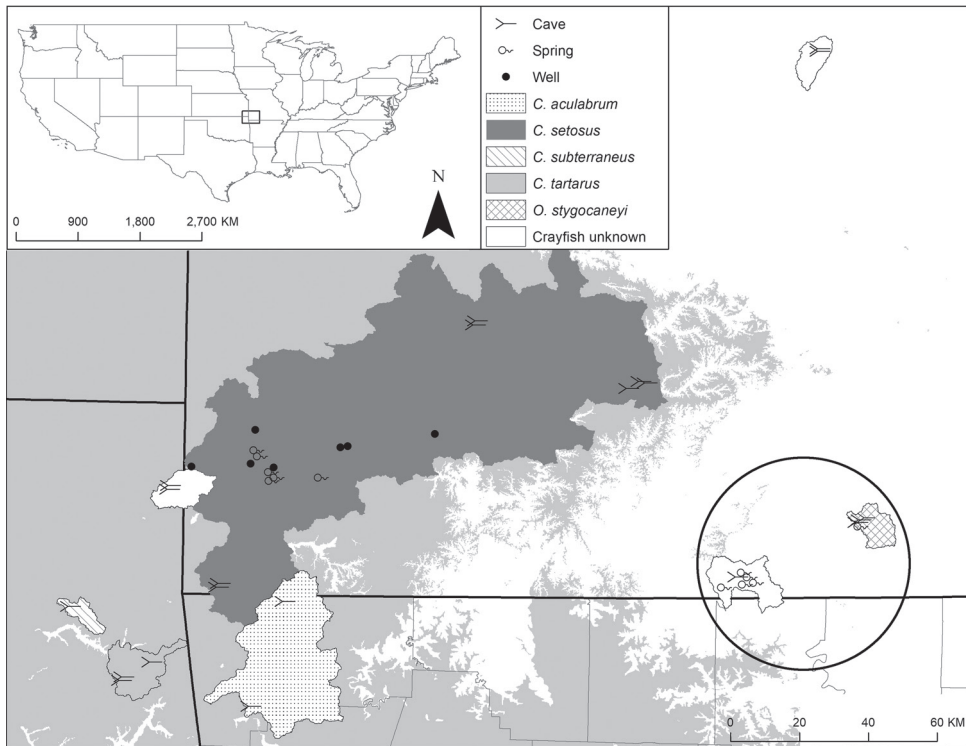
Figure 1. We conducted eDNA and visual surveys for cavefishes and cave crayfishes at 40 caves, wells, and springs across the Ozarks Highlands ecoregion. The estimated ranges of the cave crayfishes (i.e., *Cambarus aculabrum*, *C. setosus*, *C. subterraneus*, *C. tartarus*, *Orconectes stygocaneyi*) are outlined using United States Geological Survey 12-digit watersheds that encompass locations where these species have been observed. *Troglichthys rosae* is thought to restricted to the Springfield Plateau (light-grey outline); however, we detected the species at some of the sites enclosed by the circle. *Typhlichthys eigenmanni* was only surveyed at our two northern-most sites.

mented at 48 sites in southwest Missouri and two sites in Arkansas (Graening et al. 2006b). *Cambarus subterraneus* and *C. tartarus* have only been found in three and two caves in northeast Oklahoma, respectively (Graening and Fenolio 2005; Graening et al. 2006c). *Orconectes stygocaneyi* is assumed to be endemic to a single cave in south-central Missouri (Hobbs III 2001). Little is known about the biology and ecology of these organisms; however, descriptions for each species are quite similar due to convergent evolution (e.g., cryptic behavior, habitat generalists, albinistic, and reduced eyes).

Study design

We conducted both eDNA and visual surveys for cavefishes and cave crayfishes at 21 caves, 12 springs, and 7 wells (Figure 1, Suppl. material 1: Table S1). We sampled caves, springs, and wells (hereafter referred to as sites) because they allow access to the

groundwater habitat occupied by stygobionts. In fact, state agencies routinely sample hand-dug wells because they offer access to groundwater where caves may not be accessible and it is common to locate stygobionts at those locations (Doug Novinger, personal communication). We chose a combination of sites where some had previous documentation of cave crayfish and (or) cavefish occupancy ($n = 24$) and others had either never been sampled or no cavefishes or crayfishes had ever been identified ($n = 16$). Sites 15 and 16 occurred in the same cave but were considered different sites due to extreme differences in the hydrologic regime (Miller 2010). We selected 1–5 sampling units ($n = 61$) at each site based on presumed biological barriers (e.g., waterfalls or disconnected pools) and no sampling units were adjacent to one another. We chose to select multiple sampling units within caves because this allowed us to assess the spatial distribution of stygobionts. Sampling units were referenced by the site number, and then the sequential number of units within the site (e.g., 1.2 referred to the second sampling unit within site 1). For example, sampling unit 10.1 was a cave with a single pool of water and no discernible change in habitat, and sampling unit 16.1 was a pool within a cave bounded by a waterfall downstream and shallow riffle upstream. Sampling units were surveyed on 1–5 occasions (179 visual surveys and 183 eDNA surveys; Suppl. material 1: Table S2). Sampling was conducted during a relatively short time period (February–May 2017) to meet a closed-system assumption with respect to species occurrence (i.e., species neither colonize the sampling units nor go extinct during the survey period). Although there was some typical spring flooding at the end of our sampling period, we assumed there would be a lag between the initiation of high-water or low-water events before changes in species occupancy would occur (i.e., it would take time for species to recolonize when a sampling unit either became wet or dry again, Adams and Warren 2005). Further, defining our season to allow some changes in the physicochemical parameters at each sampling unit (Suppl. material 1: Table S1) was preferred to examine relationships between detection and a range of physicochemical parameters using both sampling methods.

eDNA surveys

We collected two water samples ($\approx$ 1-L each) for eDNA analysis at each sampling unit during each visit. We collected two water samples to provide a replicate in case of error or contamination in subsequent steps. We immersed sampling equipment in 50% bleach for at least 30 s between sites and then rinsed it in deionized water to avoid contamination. If possible, we sterilized gear between sampling units, but some caves were too difficult to navigate with more than a single equipment set. We filtered distilled water in the field on four occasions to provide negative controls, which were treated the same as field samples in subsequent steps. Water was collected in two 1-L sample bottles (312187-0032, ThermoFisher Scientific, Waltham, Massachusetts) from approximately 10 cm above the substrate, where possible, without disturbing the substrate. Water was collected just above the substrate when water depth was < 10 cm. We did not sample the substrate to both avoid inhibitors (e.g., humic acid) and possibly sampling older DNA

within the substrates that was not indicative of current occupancy. To collect water from wells, we lowered a Van Dorn sampler (3-1920-H62, Wildco, Yulee, Florida) to approximately 10 cm above the substrate, closed the sampler, returned it to the surface, and transferred the water to two 1-L sample bottles. We filtered the water immediately after collection, except for the samples collected from sampling units 4.1– 4.4 on 21 March 2017, which were frozen and filtered later in the laboratory. While wearing nitrile gloves, we placed a 0.45-µm cellulose-nitrate filter (14-555-624, Fisher Scientific, Waltham, Massachusetts) inside a filter funnel (09745, Fisher Scientific, Waltham, Massachusetts) attached to a vacuum flask via a rubber stopper (Figure 2). We used a hand pump (AC3310, Advance Auto Parts, Raleigh, North Carolina) to create a vacuum to pull water through the filter. Only one filter was typically needed to sample one L of water, but occasionally multiple filters (i.e., 2–6) were used due to clogging via sediment. Filters were stored at room temperature in vials of 900 µl of Longmire's buffer (Longmire et al. 1997), until extractions were completed (i.e., 1–18 mo after collection).

Visual surveys

Visual surveys for cavefishes and cave crayfishes occurred at most of the sampling units for later comparison to eDNA detection. We did not complete visual surveys at sampling units 10.1 and 18.1 on the last two survey dates due to local flooding. We did not visually survey the entirety of sampling units 5.1 and 6.2 due to sampling restrictions by the regulatory agency (i.e., safety concerns or concern for trampling crayfish). For springs and caves, two observers walked or crawled the entire sampling unit while carefully searching the whole wetted area for cave crayfishes or cavefishes by overturning rocks and examining crevices using headlamps to illuminate dark areas (e.g., Graening et al. 2006a, 2010). Hand-dug wells were surveyed in their entirety using a spotlight (QBeam Max Million III, The Brinkmann Corporation, Dallas, Texas) both before and after water samples were collected because disturbance from sampling sometimes caused stygobionts to emerge. We recorded the number of cavefishes and cave crayfishes observed and time spent observing (min).

Detection covariates

Our detection covariates were chosen based on *a priori* knowledge derived from the literature. We hypothesized that increased water turbidity (Thurow et al. 2006), greater water volume (Trajano 2001), flowing water (Thurow et al. 2006), and substrate (coarse or fine) (Albanese et al. 2011) would make it more difficult to detect stygobionts via visual surveys. The presence of light indicates surface connection and may affect detection via altered species abundance due to food availability (Simon et al. 2003) or predator abundance (Brown and Todd 1987). Increasing species abundance generally results in greater detection probability for various sampling methods (e.g., Pregler et al. 2015; Baldigo et al. 2017). For eDNA surveys, we hypothesized increased water turbidity would relate to more inhibitors in our samples (e.g., humic

Figure 2. Filtration setup for eDNA collection. While wearing gloves, a 0.45-µm microbial filter was placed inside a filter funnel that was attached to a vacuum flask via a rubber stopper. A hand pump was used to create a vacuum and pull water through the filter. Filters were stored at room temperature in vials of 900 µl of Longmire's buffer (Longmire et al. 1997).

acid, Jane et al. 2015), ultraviolet light could breakdown eDNA (Strickler et al. 2015), increased water volume would dilute eDNA (Rice et al. 2018), faster water would expel eDNA from the sampling unit (Jane et al. 2015), and fine substrates could easily be resuspended and lead to inhibition of eDNA PCR amplification (e.g., Buxton et al. 2017); all of which would decrease detection using eDNA surveys.

We estimated or measured (numbers in parentheses represent our measurement resolution): water turbidity (0.01 NTU), light (present or absent), water volume (1.0 m³), water-column velocity (hereafter water velocity, 0.01 m/s), and substrate (coded as either coarse; or fine or bedrock) at each sampling unit to explain variable detection of cave biota. We collected 250-ml water samples before the start of each visual survey to measure water turbidity using a turbidity meter (AQUAfast AQ4500, Thermo Fisher Scientific, Waltham, Massachusetts). Light was recorded as ambient light visible (present) or not visible (absent) at the water-sample location. The water volume of each sampling unit was estimated by multiplying survey length (1.0 m), wetted width (0.1 m), and maximum water depth (0.1 m). Wetted width and maximum water depth were measured at 3–5 points along the sampling unit to represent average conditions. Water velocity was visually estimated at the same locations where we measured wetted width and maximum water depth. We visually estimated water velocity because it was unreasonable to bring a flow meter into many of the caves we sampled (e.g., narrow crawl spaces and deep water). Prior to the study, we compared our visual water velocity estimates to values measured with a Marsh-McBirney flow meter (Marsh-McBirney Inc., Frederick, Maryland) to ensure that our estimates were relatively accurate (i.e., ± 0.1 m/s). We also distinguished between the prevalence of clay, silt, or bedrock substrates (hereafter "fine"), or pebble substrate, cobble substrates, or woody debris of similar size or larger (hereafter "coarse") at each sampling unit (see Wentworth 1922 for sizes of each substrate). Substrate was combined into these two categories based on our ability to observe stygobionts in these habitats. Stygobionts are relatively easy to observe on clay and bedrock substrates because they cannot conceal themselves within either as they can in cobble or woody debris.

Primer and probe development

Primers and probes were designed to amplify DNA for each of our study species (i.e., a species-specific quantitative PCR [qPCR] Taqman assay). We acquired template DNA for each of our study species from various sources (Suppl. material 1: Table S3). Genomic DNA was extracted from tissue samples using the Qiagen DNeasy Blood and Tissue kit (69504, Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cavefishes, a 500-bp fragment of the mitochondrial NADH dehydrogenase 2 (ND2) gene was PCR amplified using the forward primer MET: 5'-CAT-ACCCCAAACATGTTGGT-3' and reverse primer ND2B: 5'-TGGTTTAATCCGC-CTCAGCC-3' (Kocher et al. 1995). Each amplification reaction had a total volume of 30 μl, consisting of 1.0 μl of DNA, 2.4 μl of $MgCl_2$ (25mM), 4.8 μl of deoxynucleoside triphosphates (1 mM), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 2.4 μl of bovine serum albumin, 6.0 μl of GoTaq buffer, 0.12 μl of GoTaq DNA polymerase (M3001, Promega, Madison, Wisconsin), and 12.8 μl ddH_2O. The thermal profile consisted of an initial denaturation step of 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s and elongation at 72 °C for 5 min. For cave crayfishes, a 710-bp frag-

ment of the mitochondrial cytochrome c oxidase I (CO1) gene was amplified using the forward primer LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and the reverse primer HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). The amplification reaction consisted of the same reagents; however, the thermal profile was: an initial denaturation step of 94 °C for 5 min; 6 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min; and elongation at 72 °C for 5 min. We chose the CO1 and ND2 genes because they have a high copy number and are relatively easy to isolate and purify (Billington 2003), have rates of divergence that allow species to be distinguished (Billington 2003), and are commonly used to amplify DNA of cave crayfishes (e.g., Buhay et al. 2007) and cavefishes (e.g., Niemiller et al. 2012, 2013), respectively. PCR products were visualized on a 1.0% agarose gel then purified using a Wizard SV Gel and PCR Clean-Up System (A9281, Promega, Madison, Wisconsin). PCR products were Sanger sequenced and sequences were manually trimmed and aligned in Geneious (Version 11.1.5, Auckland, New Zealand) to generate a consensus sequence for the CO1 locus of each cave crayfish species and the ND2 locus of each cavefish species. The consensus sequences were entered in PrimerQuest (https://www.idtdna.com/primerquest/home/index) to generate species-specific qPCR Taqman assays (Table 1). Initial specificity of both the primers and probe was checked using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) searching against the *nr* database on GenBank.

We performed *in vitro* validation and quantified the lower limit of detection for our assays. The lower limits of detection for *C. setosus*, *C. subterraneus*, *C. tartarus*, *O. stygocaneyi*, and *T. rosae* DNA were 1.5×10^{-3} ng/μl, 3.9×10^{-4} ng/μl, 1.5×10^{-4} ng/ μl, 3.3×10^{-4} ng/μl, and 2.5×10^{-4} ng/μl, respectively. We were unable to test the assays *in vitro* for *C. aculabrum* and *T. eigenmanni* because we did not have genomic DNA for those species. We were unable to obtain samples of *C. aculabrum* DNA due to its rarity. We did not obtain samples of *T. eigenmanni* DNA because we only sampled a few sites, and many sequences were already available online. Not all assays developed were species-specific, but we confirmed species identity of field samples via Sanger sequencing of a subset of the positive samples.

eDNA extraction

We extracted eDNA from the filters using a DNeasy Blood and Tissue Kit by following the "purification of total DNA from crude lysates" protocol (Qiagen 2006) with the following modifications. We sterilized all laboratory surfaces and equipment with 10% bleach before extractions. DNA was initially extracted for only one filter collected at a sampling unit. Any additional filters were placed in fresh Longmire's buffer and set aside to use if the first filter was negative for the target species' DNA (see next section *Quantitative PCR amplification*). Using forceps, each filter was halved and torn into pieces. The pieces from each half were added to separate 2-ml microcentrifuge tubes. Forceps were sterilized between filters by immersion into 100% ethanol and

Table 1. Taqman assays were designed to amplify DNA for each of our target species. The 5' end of the probe was labeled with the fluorescent dye (6-FAM), the 3' primer end with a quencher (Iowa Black™ FQ), and there was an additional internal quencher (ZEN™). Probes were doubled quenched to reduce background fluorescence and increase signal intensity. All primer and probe sequences are reported 5' to 3'.

Species	Forward primer	Reverse primer	Probe
Cambarus aculabrum	CAA GAG GGA TAG TAG AGA GAG G	CCG GCT AAG TGC AAA GAA	ACC CAC CTT TAG CTT CAG CAA TTG CTC A
Cambarus setosus	CAG ACC AAA CAA ATA ATG GTA TCC	GCA CGG GAT GAA CTG TTT	AGC ATG AGC AAT TGC CGA AGC CAA
Cambarus subterraneus	GCA TTC GAT CCA TGG TCA TAC	CTT AGC TGG AGT GTC TTC TAT TT	CCG CCG CAC GTA TAT TAA TAG CTG TTG T
Cambarus tartarus	TCC GAT CCG TTA GTA GCA TAG	GTA CTG CAG GYA TGA CAA TGG	ATC TTT GCC TGT GCT AGC GGG AGC
Oreconectes stygocaneyi	CAT GAG CTG TCA CTA CCA CAT TA	TTT GGT ACT TGG GCT GGA ATA G	TCC GAT TAA CCT ACC TAC CTG GCC T
Troglichthys rosae	GGT GRT GYT GAT GAG CTA TG	ACC CWC TCA TCC TAG TAR CC	TTG CGA AGG TGA TAG TRG TGC CCA
Typhlichthys eigenmanni	CTG GCT ACT AGC ATG AAT GG	TTG CGC TGG CGA ATA AG	CCC GCG CAG TAG AAG CCA CAA CAA

flaming. The Longmire's buffer was split into two 1-ml tubes, and if the volume was < 360 µl, fresh buffer was added. The tubes of Longmire's buffer were then centrifuged at 8,000 g for 30 s. We then transferred 360 µl of the Longmire's buffer and the pellet to the respective tubes with the filter pieces. The above process resulted in a standard amount of filter pieces and buffer in each tube (i.e., 1 filter half and 360 µl). There were two tubes per sampling unit, and each tube was considered a subsample for that sampling unit. After samples were standardized, we followed the "purification of total DNA from crude lysates" protocol except we doubled the amounts of proteinase K, buffer AL, and 100% ethanol that were added to each tube. Further, we reduced the amount of buffer AE added in the final step to 125 µl. We stored our samples at 2 °C until amplification (i.e., up to 4 mo).

Quantitative PCR amplification

We amplified eDNA using quantitative Polymerase Chain Reaction (qPCR). Each amplification reaction had a total volume of 20 µl, consisting of 10 µl of TaqMan Environmental Master Mix 2.0 (4396838, ThermoFisher Scientific, Waltham, Massachusetts), 4.7 µl of ddH$_2$O, 0.9 µl of forward primer (20 µM), 0.9 µl of reverse primer (20 µM), 0.5 µl of probe (10 µM), and 3.0 µl of template DNA. Samples were run in 96-well optical plates (BC3496, ThermoFisher Scientific, Waltham, Massachusetts) on a LightCycler 480 (Roche, Pleasanton, California). The thermal profile consisted of an initial denaturation step of 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each subsample was run in triplicate, which resulted in an initial six pseudoreplicates for each sampling unit. If any pseudoreplicates amplified, then the sampling unit was considered positive for the species. If none of the pseodoreplicates amplified, we extracted eDNA from any re-

maining filters from the sampling unit and ran another qPCR. We repeated the above process until all filters were processed, or until any pseudoreplicates amplified. If only one subsample amplified from a single survey date, then we processed that subsample again. If the subsample still amplified, then the survey was considered positive for the species and if it was negative, then the survey was considered negative. We also ran three negative controls during each qPCR in which the template DNA was replaced by ddH$_2$O. If any of the negative controls amplified, then the qPCR run was discarded. A positive control was included that consisted of genomic DNA from the target taxa to ensure the reaction worked properly. We confirmed species identification of a subset of positive samples for each species using Sanger sequencing.

Statistical analysis

We modeled cave crayfish and cavefish detection probability when using both eDNA and visual surveys. Species of cave crayfishes and cavefishes tend to have narrow distributions (i.e., the species do not occur at all sites); thus, we modeled detection probability of all species of cave crayfishes as a single taxon and all species of cavefishes as a single taxon. Each taxon was either detected (1) or not detected (0) during each survey at a sampling unit, and the surveys were combined for sampling units to create capture histories for our response variable (i.e., a binomial response variable). For example, a capture history of 1010 would represent a taxon that was detected on the first and third surveys and undetected on the second and fourth surveys. Sampling units were included in the model twice if both taxa were detected, once if only one taxon was detected, and excluded if neither taxon was detected. Our approach required meeting the same assumptions for occupancy modeling with respect to the detection process: no false positives, sampling unit closure, and independent surveys. An alternative approach would be to make the individual surveys the outcome (i.e., 1 or 0, logistic regression), but we chose to use capture histories because it allowed us to evaluate model fit (see below). We assumed trait differences (e.g., morphology and behavior) among cavefish and cave crayfish species (i.e., within each taxon) would not influence detection probability. We excluded eDNA surveys for *C. setosus* because the assays did not amplify the subset of the field samples we tested with positive visual identification of the species. Our final model included 35 sampling units for cavefishes (105 visual surveys, 109 eDNA surveys) and 25 sampling units for cave crayfishes (77 visual surveys, 40 eDNA surveys).

We modeled detection probability of cavefishes and cave crayfishes in relation to light, substrate, water volume, water velocity, and water turbidity, where each environmental variable varied by both taxa and sampling method. The continuous variables water volume and water turbidity were natural-log transformed due to right-skewed distributions. Water volume and water turbidity were standardized to a mean of zero and a variance of one to improve coefficient interpretation. The correlation level between water turbidity and water volume was low, indicating independence of these variables (Pearson's pairwise correlation coefficient = 0.11). We made velocity a

category where 0 indicated no flow and 1 indicated flowing water. Light, substrate, and water velocity were treated as factors using a dummy variable approach (i.e., ambient light, no flow, and coarse substrate as the references). Independence between continuous and categorical variables was checked using point-biserial correlations and none were >0.22. Independence between categorical variables was checked using Cramer's V and none were >0.47. We also treated sampling method and taxa as factors using visual surveys and cave crayfish as the reference categories, respectively. The most complex model can be written as:

$$\text{logit}\left(p_{ij}\right) = \beta_0 + \beta_1 X_{1[i,j]} + \beta_2 X_{2[i,j]} + \beta_3 X_{3[i,j]} + \beta_4 X_{4[i,j]} + \beta_5 X_{5[i,j]} + \beta_6 X_{6[i,j]} + \beta_7 X_{7[i,j]} +$$
$$\beta_8 X_{1[i,j]} X_{2[i,j]} + \beta_9 X_{1[i,j]} X_{3[i,j]} + \beta_{10} X_{1[i,j]} X_{4[i,j]} + \beta_{11} X_{1[i,j]} X_{5[i,j]} + \beta_{12} X_{1[i,j]} X_{6[i,j]} +$$
$$\beta_{13} X_{1[i,j]} X_{7[i,j]} + \beta_{14} X_{2[i,j]} X_{3[i,j]} + \beta_{15} X_{2[i,j]} X_{4[i,j]} + \beta_{16} X_{2[i,j]} X_{5[i,j]} + \beta_{17} X_{2[i,j]} X_{6[i,j]} +$$
$$\beta_{18} X_{2[i,j]} X_{7[i,j]} + \beta_{19} X_{1[i,j]} X_{2[i,j]} X_{3[i,j]} + \beta_{20} X_{1[i,j]} X_{2[i,j]} X_{4[i,j]} + \beta_{21} X_{1[i,j]} X_{2[i,j]} X_{5[i,j]} +$$
$$\beta_{22} X_{1[i,j]} X_{2[i,j]} X_{6[i,j]} + \beta_{23} X_{1[i,j]} X_{2[i,j]} X_{7[i,j]}$$

for i = 1,2,…N, for j = 1,2,…J,

where p_{ij} is detection probability for survey j at sampling unit i, β_0 is the intercept, β_1 is the taxa main effect coefficient, β_2 is the method main effect coefficient, β_3 is the light main effect coefficient, β_4 is the turbidity main effect coefficient, β_5 is the velocity main effect coefficient, β_6 is the substrate main effect, β_7 is the volume main effect coefficient, β_8 is the taxa * method interaction term coefficient, β_9 is the taxa * light interaction term coefficient, β_{10} is the taxa * turbidity interaction term coefficient, β_{11} is the taxa * velocity interaction term coefficient, β_{12} is the taxa * substrate interaction term coefficient, β_{13} is the taxa * volume interaction term coefficient, β_{14} is the method * light interaction term coefficient, β_{15} is the method * turbidity interaction term coefficient, β_{16} is the method * velocity interaction term coefficient, β_{17} is the method * substrate interaction term coefficient, β_{18} is the method * volume interaction term coefficient, β_{19} is the taxa * method * light interaction term coefficient, β_{20} is the taxa * method * turbidity interaction term coefficient, β_{21} is the taxa * method * velocity interaction term coefficient, β_{22} is the taxa * method * substrate interaction term coefficient, β_{23} is the taxa * method * volume interaction term coefficient, X_1 is taxa, X_2 is method, X_3 is light, X_4 is turbidity, X_5 is velocity, X_6 is substrate, and X_7 is volume.

We fit our models using the program JAGS (Plummer 2003) called from the statistical software R (version 3.5.3; R Core Team 2019) using the package jagsUI (Kellner 2019). We used a broad uniform prior on the 0 to 1 scale for the detection probability intercept and broad uniform priors on the logit scale for other coefficients (Kéry and Royle 2016). Posterior distributions for coefficients were estimated using Markov chain Monte Carlo methods using 3 chains of 50,000 iterations each after a 10,000-iteration burn-in phase. We assessed convergence using the Brooks-Gelman-Rubin statistic (R̂, Gelman and Rubin 1992), where values < 1.1 for all model parameters indicates adequate mixing of chains (Kruschke 2015; Kellner 2019).

We used a three-step process to simplify our final model. We began by fitting the full model and simultaneously removed all three-way interaction terms with 95% highest density intervals (hereafter HDIs, Kruschke and Liddell 2018) that overlapped zero (i.e., were considered non-significant). The intervals are not interpreted in a traditional Frequentist sense (i.e., a 95% probability of containing the true value). Rather, the mean for the coefficient is the most plausible value, and the HDI contains credible values from the posterior distribution with a total probability of 95%. This use of a decision rule cut-off is analogous to hypothesis testing. However, an HDI that contains zero is not interpreted as failing to reject the null, but rather that an effect size of zero meets the minimum level of credibility. We then refit the model and used the aforementioned criteria to remove non-significant two-way interactions that were not retained in the three-way interactions. Finally, we repeated the above process to remove the main effects for environmental variables that were not significant. A model-selection process using HDIs has also been employed in similar studies (e.g., Kanno et al. 2015; Mihaljevic et al. 2015; White et al. 2020).

We examined model fit using posterior predictive distributions. The fit of the final model was assessed using a Bayesian p-value (Kéry and Royle 2016). A Bayesian p-value closer to 0.5 suggests adequate fit, and extreme values (i.e., > 0.90 or < 0.10) indicate a lack of fit (Hobbs and Hooton 2015; Kéry and Royle 2016; Conn et al. 2018).

Because our goal was to assess how sampling bias related to the effort needed to adequately sample these taxa, we interpreted our results via cumulative detection plots. Cumulative detection probability (p_c) was calculated as: $p_c = (1 - (1 - p)^k$ where k is the number of surveys. We plotted the cumulative detection probability of each taxa for each significant relationship with method and environmental covariate.

Results

eDNA and visual surveys

Environmental DNA surveys detected cavefishes at more sampling units than visual surveys, whereas visual surveys detected cave crayfishes at more sampling units compared to eDNA surveys. Environmental DNA surveys detected cavefishes at 33 of 61 sampling units, and visual surveys detected cavefishes at 14 of 61 sampling units. At 21 sampling units, we detected cavefish DNA but did not visually observe cavefishes. We detected cavefishes at six sites where they have never been detected using eDNA surveys, but did not detect any new populations using visual surveys. Environmental DNA surveys detected cave crayfishes at 10 of 61 sampling units, whereas visual surveys detected cave crayfishes at 17 of 61 sampling units. We detected cave crayfishes at one site where they have never been detected using eDNA surveys, but did not detect any new populations using visual surveys. Low eDNA detection could be the result of pseudogenes that we observed in the DNA of *C. setosus* and *O. stygocaneyi*. All of the negative controls collected in the field were negative, suggesting our decontamination protocol was adequate.

Detection covariates

The environmental factors we measured varied over the sample season (Suppl. material 1: Table S1). Water turbidity ranged from 0.20 to 41.50 NTU (mean ± SD = 2.97 ± 4.57 NTU). There was visible light at 26 sampling units, 34 sampling units were dark, and sampling unit 10.1 did not have visible light on the first 2 surveys but did on the last survey (i.e., we sampled at the cave entrance due to high water). We surveyed a range of water volumes across sampling units (0.06 m³–800.00 m³; mean ± SD = 61.21 ± 132.00 m³). Estimated water velocity ranged 0–0.53 m/s (mean ± SD = 0.06 ± 0.10 m/s), with 81 surveys classified as 0 (i.e., not flowing) and 102 surveys classified as 1 (flowing water). Substrate at 34 sampling units was classified as coarse substrate and 27 as fine substrate.

Statistical analysis

Detection probability of both cavefishes and cave crayfishes varied by survey method and was significantly related to water volume, substrate, and water velocity (Table 2). For cavefishes, detection probability at mean or reference levels of predictor variables was 0.35 (95% HDI: 0.19–0.55) using visual surveys and 0.49 (95% HDI: 0.32–0.67) using eDNA surveys. For cave crayfishes, detection probability at mean or reference levels of predictor variables was 0.67 (95% HDI: 0.47–0.84) using visual surveys and 0.40 (95% HDI: 0.19–0.65) using eDNA surveys. Cave crayfish and cavefish detection decreased sharply with increasing water volume using visual surveys. Cavefish detection decreased significantly when using visual surveys in sampling units classified by coarse rather than fine substrates. In contrast, cave crayfish detection decreased in when using eDNA surveys in fine compared to coarse substrates. Lastly, detection probability of cavefishes using visual surveys decreased significantly when water was flowing (i.e., water velocity > 0). R̂ was < 1.1 for all coefficients. The calculated Bayesian p-value was 0.36.

The number of surveys needed to be confident the taxa were detected if present depended on sampling method and underlying environmental conditions. At mean levels of all predictor variables, approximately four eDNA surveys and nine visual surveys would be necessary to achieve a cumulative detection probability near one for cavefishes (i.e., confident the taxon was truly absent if undetected, Figure 3a). Alternatively, it would take approximately 5 visual surveys versus 10 eDNA surveys to achieve a cumulative detection probability near 1 for cave crayfishes when sampling under reference conditions (Figure 3b). When sampling in higher water volumes, greater than 10 visual surveys would be needed to confidently detect both cave crayfishes and cavefishes compared to less than 6 surveys in lower volume. Visually sampling for cavefishes at sites with fine substrates would require only four surveys to be confident of detection, whereas 10 surveys would be needed if the substrate was coarse. Seven surveys would be needed to confidently detect cavefish via eDNA surveys in both coarse and fine substrates. If we used eDNA sampling for cave crayfishes, then we would need 10 surveys in coarse substrate to be confident of detection versus more than 10 surveys in fine substrates. If we used visual surveys for cave crayfishes, then only five surveys

Table 2. Detection probability estimates from the final model for cavefishes and cave crayfishes using environmental DNA (eDNA) and visual surveys. Estimates for each parameter included in the detection model are reported on the logit scale as the mean ± standard deviation (SD) with a 95% high density interval (HDI). Mean values are reported as detection probabilities (Prob) by completing a logit transformation. The reference categories for categorical variables were visual surveys, water not flowing, cave crayfishes, and coarse substrate.

Parameter	Mean ± SD	95% HDI	Prob
Intercept	0.73 ± 0.45	-0.15, 1.62	0.67
Taxa	-1.42 ± 0.48	-2.36, -0.50	0.19
Method	-1.22 ± 0.74	-2.65, 0.22	0.23
Velocity	-0.67 ± 0.41	-1.47, 0.13	0.34
Substrate	-0.17 ± 0.58	-1.30, 0.96	0.46
Volume	-1.43 ± 0.37	-2.19, -0.74	0.19
Taxa X method	1.87 ± 0.75	0.42, 3.36	0.87
Method X velocity	1.55 ± 0.60	0.38, 2.72	0.82
Taxa X substrate	-0.77 ± 0.77	-2.26, 0.75	0.32
Method X substrate	-0.98 ± 1.03	-3.01, 1.03	0.27
Taxa X volume	0.83 ± 0.45	-0.04, 1.73	0.69
Method X volume	1.62 ± 0.55	0.52, 2.69	0.83
Taxa X method X substrate	2.89 ± 1.21	0.52, 5.30	0.95
Taxa X method X volume	-1.16 ± 0.64	-2.45, 0.09	0.24
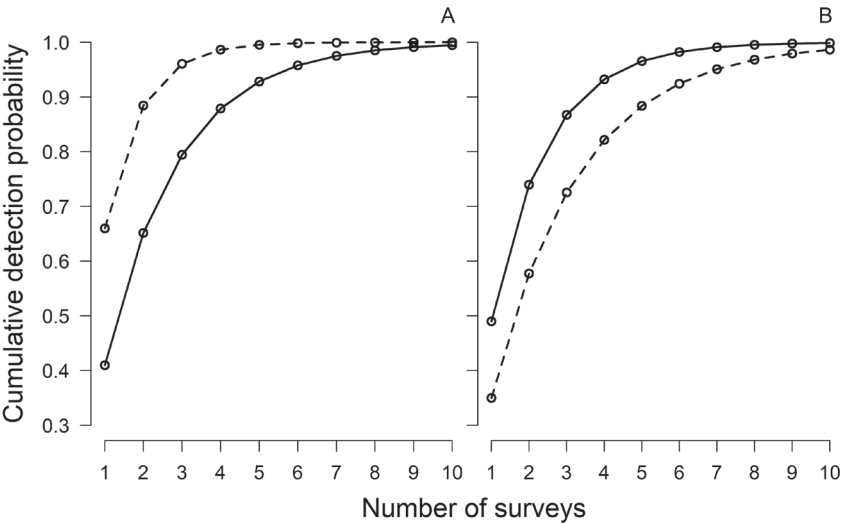
Figure 3. Cumulative detection probability as a function of the number of surveys for cave crayfishes (panel A) and cavefishes (panel B). Solid lines eDNA surveys and dashed lines are visual surveys. Cumulative detection probability (p_c) was calculated as: $p_c = (1 - (1 - p)^k$, where p is detection probability at mean and reference levels (i.e., visual surveys, water not flowing, cave crayfishes, and coarse substrate) of predictor variables and k is the number of surveys.

would be needed in both coarse and fine substrates to achieve a detection probability near one. When water is flowing, it would take 5 visual surveys for cave crayfishes and > 10 surveys for cavefishes to achieve a cumulative detection probability near 1, whereas it would only take 4 surveys to detect both taxa using eDNA.

Discussion

We show detection probabilities for both cavefishes and cave crayfishes depend on both the sampling environment and method. Several studies have demonstrated that detection can be extremely low (< 0.01–0.18) for cave organisms when using visual surveys (Culver et al. 2004; Krejca and Weckerly 2008). We found that detection can be low for stygobionts and that it would take at least nine visual surveys to ensure cavefish detection with traditional visual survey methods. With relatively low detection probabilities for cavefishes, it would be possible to conclude that a cave is unoccupied when caves are often surveyed less than once per year in the Ozark Highlands (e.g., Graening et al. 2010). Because of the relatively low detection, managers would benefit from considering study designs that account for detection (i.e., accounting for the sampling bias). If repeat surveys are not possible, another option is to use the most efficient survey method for the target taxa under the prevailing environmental conditions realizing that underestimating occupancy would be likely.

Detection probability via eDNA surveys can depend on the target species and its associated density. We observed that detection using eDNA surveys was typically higher for cavefishes than for cave crayfishes. Although some of the discrepancy in detection between cavefishes and cave crayfishes can be explained by the availability of genetic data, physiological differences may also play a role. For example, fish have a slime coat and release more DNA in the environment than crayfish that have a hard exoskeleton (Tréguier et al. 2014), thus making it easier to detect fishes. The abundance or biomass of the target organism also relates to how much DNA will be released into the environment (Takahara et al. 2012) and can influence detection (Dougherty et al. 2016; Baldigo et al. 2017). For example, we were unable to detect *T. eigenmanni* at sampling units where only one fish was observed across all surveys, but we detected them at sampling units where multiple individuals were observed. Other studies, however, have observed little relationship between target organism and detection (Rice et al. 2018). Water volume may interact with species abundance to further influence detection because eDNA may be diluted when there is more water. For example, we observed decreased detection with increased water volume.

The movement and persistence of eDNA in the environment can further complicate detection of aquatic organisms. In surface waters, eDNA flows downstream (up to 12.3 km; Deiner and Altermatt 2014) and can settle vertically (Turner et al. 2015). For example, Asian carp DNA was detected upstream of a fish barrier near the Great Lakes (Jerde et al. 2011), but flow reversals, not presence, were provided as the explanation (Song et al. 2017). In karst environments, water can flow in many directions due to gravity and topography (Aley and Kirkland 2012), which makes it difficult to understand the movement of eDNA in those environments. For example, we detected *O. stygocaneyi* DNA in sampling unit 20.1 which is approximately 100 m upslope from sampling unit 10.1 (i.e., the only location where it has been recorded), suggesting those locations may share water during flooding. We hypothesized *O. stygocaneyi* may not occur in that cave, but its DNA is present due to groundwater shared among systems during particularly wet periods. Environmental DNA can persist for up to 25 d in ex-

perimental ponds (Dejean et al. 2011), in terrestrial soil for at least 6 y (Andersen et al. 2012), and in cave soils for thousands of years (Hofreiter et al. 2003). In relatively stable underground aquifers, eDNA may persist for months or even years resulting in detections that are not indicative of the current population status. Alternatively, large floods can quickly move sediment and organisms out of caves (Van Gundy and White 2009; Graening et al. 2010) resulting in quick expulsion of DNA. Our model results indicated flowing water increased detection for cavefishes and cave crayfishes via eDNA surveys, which would be expected because some flow would mix and transport eDNA that had been held in the soil or deeper groundwater, but the retention time of DNA is unknown.

Substrate and water velocity also influenced detection probability of cavefishes and cavefishes via visual surveys. Visual counts of stream fishes have been used for a variety of species in clear coldwater and warmwater streams (e.g., Lambert and Hansom 1989; Heggenes et al. 1991; Brewer and Ellersieck 2011). Sampling bias via visual surveys has been associated with water velocity (Heggenes et al. 1991), water depth (Brewer and Ellersieck 2011), surface glare, turbidity, and fish behavior (Bozek and Rahel 1991). Similarly, we found that coarse substrate and flowing water were negatively associated with our ability to detect cavefishes using visual observations. Both variables were represented as binary in our model, which does not provide a measure of the magnitude of the relationship (i.e., it is a shift in the intercept, rather than a slope). Nevertheless, our findings do suggest that substrate and water velocity are factors to consider when conducting traditional visual surveys.

We found false negative samples associated with cave crayfishes were often related to the presence of pseudogenes in some species' DNA. Pseudogenes are mitochondrial genes that have moved into the nucleus, become nonfunctional, and then acquire mutations (Buhay 2009). Pseudogenes can be identified by the presence of stop codons in the sequence and "messy" chromatograms (i.e., the presence of many PCR products; Buhay 2009). Therefore, the presence of pseudogenes can make it difficult or impossible to determine the species (Buhay 2009). We found pseudogenes in the DNA of *O. stygocaneyi* and *C. setosus*, which resulted in non-specific binding of the primers and probes and lower detection probability. Future efforts might attempt use of other genetic techniques to isolate the actual mitochondrial gene (e.g., cloning, RT-PCR, long PCR, mtDNA enrichment, sequencing mitochondrial rich tissues) or target different genes; however, all of these techniques have associated difficulties to overcome (e.g., expense and technicality; Song et al. 2008; Buhay 2009).

Our data suggest increasing the number and spatial distribution of cave crayfish DNA sequences would allow researchers to design better assays that might improve detection. Knowing the genetic variation of the population is critical when designing assays to successfully amplify the DNA of the target species while avoiding amplification of any non-target taxa (Furlan et al. 2015). We had access to 23 sequences for *T. rosae*, 21 sequences for *T. eigenmanni*, and 8 for *C. tartarus* to represent the genetic variation across the known distribution of these species. Consequently, the assays we developed for the aforementioned species worked well. Alternatively, we only had seven *C. setosus* DNA sequences to represent genetic variation for a species that is more broadly distributed than

other cave taxa in this study (Suppl. material 1: Table S3). More samples of genetic material across the range of more broadly distributed species would be necessary to adequately capture the species' genetic variation (Niemiller et al. 2018). We also do not have a comprehensive understanding of the genetic variation and species designations among cave crayfish populations. For example, *C. setosus* individuals that were collected from opposite ends of their range were genetically different by almost 6% as reflected by their CO1 gene (Suppl. material 1: Table S3, accession numbers JX514464 and MN984899). We suggest future efforts focus on understanding the genetic variation among these species.

Conclusion

Environmental DNA is a useful tool; however, the limitations we identified indicate eDNA surveys for these taxa are currently not adequate to replace traditional surveys in subterranean environments. Environmental DNA is a viable option for sampling cavefishes from locations that provide access to groundwater but cannot be physically accessed easily (i.e., springs, wells, and flooded caves). In fact, we detected cavefishes' DNA in locations where they have not been previously identified (i.e., McDonald and Ozark counties, Missouri). Further, we show that fewer surveys using eDNA would be needed for cavefishes when compared to traditional visual surveys. Environmental DNA may serve as a useful initial surveillance method when followed up by focused, on-the-ground surveys or dye tracing to identify possible sources of DNA beyond the cave. Lastly, the life history and ecological data gained from traditional surveys provide important information necessary for developing conservation strategies though increasing survey effort to adequately capture species presence should be considered if that is the sampling goal. If eDNA surveys are to be used to supplement visual sampling in subterranean environments, it would be beneficial for future efforts to 1) examine DNA movement through karst environments, 2) evaluate the genetic diversity among the Ozark Highland cave crayfishes, and 3) attempt to isolate the actual CO1 (or other) gene of cave crayfishes to improve use of eDNA in these systems.

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Supplementary material 1

Table S1–S3

Authors: Joshua B. Mouser, Shannon K. Brewer, Matthew L. Niemiller, Robert Mollenhauer, Ronald A. Van Den Bussche

Data type: tables

Explanation note: **Table S1.** Environmental covariates were measured or estimated at each sampling unit to model detection probability of cavefishes and cave crayfishes. Sampling units (SU) are referenced by the site number, then the sequential number of units within the site (e.g., 1.2 refers to the second sampling unit within site 1). Sampling units 3.1–3.5 and 7.1 were also sampled on 01 April 2017. Using eDNA surveys only, sampling unit 10.1 was sampled a fourth time on 15 May 2017 and a fifth time on 17 May 2017. Due to high water, sampling unit 18.1 was surveyed only with eDNA on 24 April and 19 May 2017. Values for continuous environmental variables (turbidity = turb, velocity = Vel, and volume = Vol) are reported as the average across survey dates ± standard deviation; however, values were not averaged for the analysis. Values for the categorical variables light and substrate (Sub) are reported as ambient light visible (Yes) or not (No) and fine or coarse substrate, respectively. Sampling unit 10.1 did not have ambient light on the first survey, but did on later surveys due to cave flooding. The species of cavefish (*Troglichthys rosae* = ros, *Typhlichthys eigenmanni* = eig) or cave crayfish (*Cambarus aculabrum* = acu, *C. setosus* = set, *C. subterraneus* = sub, *C. tartarus* = tar, *Orconectes stygocaneyi* = sty, unknown = unk) known or thought to occur at each sampling unit are also reported. **Table S2.** Results of the environmental DNA (eDNA) and visual surveys (Vis) for each sampling unit (SU). Yes indicates that the species was detected, no indicates that the species was not detected, and NA indicates that a survey was not completed for that sampling unit. A dash indicates water samples were collected, but we did not complete the DNA analysis. **Table S3.** DNA sequences were obtained for each of our study species from various sources to design species-specific Taqman˚ assays. The number of sequences derived from each source are listed in parentheses. The accession number can be used to locate the sequence on GenBank. MDC = Missouri Department of Conservation, USFWS = United States Fish and Wildlife Service.

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RESEARCH ARTICLE

Variability in macrozoobenthic assemblages along a gradient of environmental conditions in the stream water of karst caves (Lower Shakuranskaya Cave, western Caucasus)

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Abstract

The fauna of the stream water in the Lower Shakuranskaya Cave in central Abkhazia, western Caucasus, was studied. This cave has a large inlet and an extended entrance ecotone area of approximately 60 m, which makes it a convenient area for studying macrozoobenthic assemblages across a gradient of environmental factors. The cave has 13 species of stygobionts, 10 species of stygophiles and 18 species of stygoxenes. The number of species and the abundance and biomass of stygobionts per station were the highest near the boundary of the photic zone, at a distance of 50–60 m from the entrance to the cave, and gradually decreased toward both the remote parts of the cavity and the cave exit. The most abundant stygobionts were gastropod mollusks of the Hydrobiidae family, and *Xiphocaridinella* shrimp comprised the main part of the biomass. It has been shown that the main environmental factors determining the distribution of macrozoobenthos are luminosity and distance from the entrance to a cave. According to the differences in their reactions to these environmental factors, several groups of species were identified. In addition, three main assemblages of macrozoobenthic species were described: (1) an assemblage of epigean species near the cave entrance area; (2) stygobionts in remote parts of the cave outside the photic zone; and (3) a mixed assemblage in the cave ecotone, where a faint light penetrates. The specific details related to the faunal structure in the ecotone of the cave are discussed, as well as active and passive methods by which stygoxenes invade underground cavities.

Introduction

Natural communities are usually not discrete but gradually change each other under the influence of environmental factors (Riesch et al. 2018). The values of environmental factors at both local and geographical scales often have a pronounced gradient. Appreciating that ecological gradients prevail in nature allows us to look at the distribution of organisms from the standpoint of continuality. A question that has a broad interest is how fauna and community structures change along ecological gradients.

Caves can serve as a model system for studying community variation along an environmental gradient at a local spatial scale. The entrances of caves are transition zones where epigeic and endogeic organisms can encounter each other. These ecotones are rich in food due to primary producers and accumulated debris from epigeic ecosystems, especially in comparison to the food in deeper parts of the same systems (Pentecost and Zhaohui 2001; Culver and Pipan 2009). However, the variability in the community structures in streams on a gradient of epigeic-endogeic conditions has received little attention. Presumably, communities with intermediate epigean and underground characteristics can live in a cave ecotone. It has been noted that near cave entrances, an invertebrate community may be characterized by higher species richness than that in neighboring epigeal communities or communities deeper in a cave (Prous et al. 2004, 2015). However, the general patterns of changes in aquatic communities along the gradient of environmental factors in caves remain undescribed.

The aquatic invertebrate fauna of caves from the western Caucasus is rich (nearly 110 species) and highly taxonomically specific, with endemics accounting for more than 90% of the species (Kniss 2001; Shumeyev 2008; Sidorov 2014; Vinarski et al. 2014; Barjadze et al. 2015; Sidorov et al. 2015a, b; Turbanov et al. 2016). The large number of caves and, at first glance, the accessibility to explorers could make this region convenient for studying community changes on the gradient from epigean to underground conditions. However, integrated studies comparing the invertebrate assemblage structures in the different parts of an entire cave are rare for the western Caucasus (Chertoprud et al. 2016, 2020). Studies of the fauna in underground watercourses in the region face a number of problems: the presence of substrates with monolithic slabs, which complicates the collection of data; the inaccessibility of a large part of underground watercourses; and different technical difficulties related to underground research work. Incidentally, the success of such studies is largely due to the choice of a suitable cave system.

The work in this study was devoted to analyzing the structure and spatial distribution of macrozoobenthos assemblages in the watercourse of the Lower Shakuranskaya Cave (Abkhazia, western Caucasus). Here, we tested the hypothesis that the macroinvertebrate assemblages in the cave ecotone may significantly differ from the assemblag-

es in the remote parts of the cave, both in terms of species composition and structural dominance. We attempted to identify the main factors determining the penetration of epigeic species into the underground cavities.

Materials and methods

Explored area

The research was carried out in the Lower Shakuranskaya Cave, located in the Gulripshi district of Abkhazia, on the orographically right shore of the Jampal River, 1.5 km south of the village of Amtkel. The configuration of this cave allowed us to conduct research on a 650 m long transect, with a focus on the ecotone zone of the cave. The substrate of the Lower Shakuranskaya Cave consists of Late Cretaceous limestones and belongs to the speleological area of the southern slope (speleological province of the Greater Caucasus) of the Gumishkhinsko-Panavsky speleological district (Dublyansky et al. 1987). The total length of the galleries of the accessible part of the cave is approximately 1300 m (Maksimovich 1965; Dublyansky et al. 1987). The water inflow in the cave has a condensation-infiltration origin (Amelichev et al. 2007). The water flow is represented by a stream originating at the deepest part of the cave from a small waterfall (Fig. 1). The cave is characterized by a large number of rimstone dams and pools. Due to the presence of rimstone dams, shallow water areas with a fast current alternate with deep (1–1.5 m) areas with a slow drift. The stream occupies the entire width of the main cave gallery over a considerable length of the cave. The height of the Lower Shakuranskaya Cave entrance is 13 m, and the width is 10 m that, at a distance of 60 meters, decrease to 7 m and 3.5 m, respectively. Illumination penetrates the cave at a distance of 36 m from the entrance (ecotone zone).

Sampling strategy

Sampling stations were set in a transect along the stream course in the main cave gallery. The transect had a length of approximately 650 m and included eight stations located from the deepest halls to the entrance area (Fig. 1). The transect stations were located in areas of the stream with an apparent flow. The studies were carried out at three time points: February 2018 and May and October 2019. In October, three more stations were added from the ecotone zone to the main transect, with eight stations (Fig. 1). In total, 27 quantitative and complex samples of macrozoobenthos were obtained.

The high heterogeneity of the biotopes and low values of faunal abundance and species richness often make it difficult to carry out ecological studies in caves to a full extent. To compose a complete picture of the structure of species assemblages, quantitative complex samples of hydrobionts were obtained at each station (one complex sample per station). Each complex sample included organisms from three sites 3 m away from each other at a given station. At each station, the samples covered both the areas
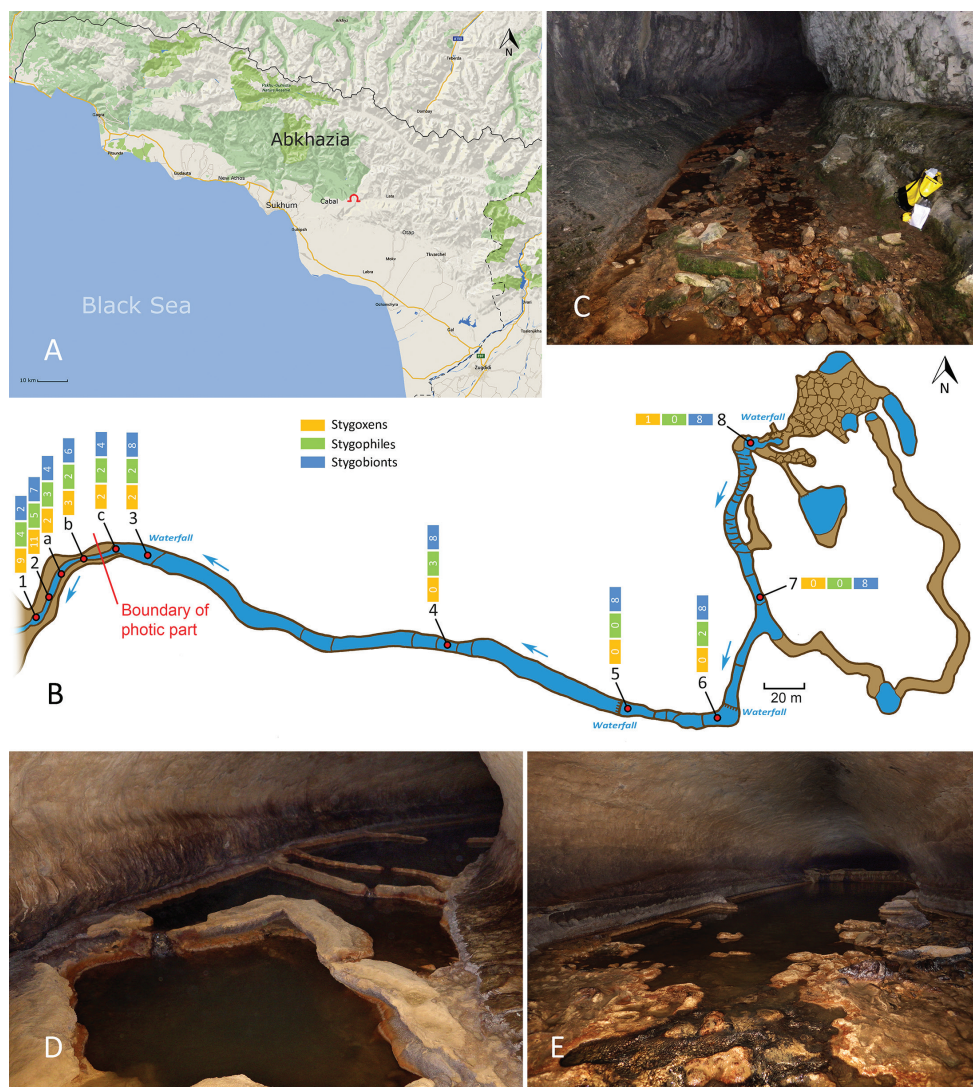
Figure 1. A Location of the Lower Shakuranskaya Cave on the map of Abkhazia **B** sampling stations location scheme in the Lower Shakuranskaya Cave in 2018–2019. In numbers – stations, sampled in February 2018, May and October, 2019; in letters – additional stations, sampled in October, 2019 (for each station indicated numbers of stygobionts, stygophiles and stygoxenes). Views on the cave stream **C** at station 2 (ecotone zone) **D** at station 4 **E** at station 5.

with the maximum depths and those at the water edge. The main substrate types at the studied stations were stones and clay sand as well as calcified rimstone walls. Collecting aquatic invertebrates was conducted with a hemispherical sampler (diameter 11 cm) with a mesh size of 0.5 mm. The total area of one complex sample at each station was 0.5 m². All the collected organisms were fixed with 90% ethanol. The species composition, abundance and fresh biomass were determined. The biomass was measured with Acculab ALC-210d4 electronic scales (Germany) with an accuracy of 0.001 mg.

Table 1. The main characteristics of the studied stations in Lower Shakuranskaya Cave. (Temperature and hydrochemical characteristics of water are given for October 2019).

Characteristic	Stations										
	1	2	a	b	c	3	4	5	6	7	8
Substrates*	1	1	1	1	1	2	2	2	2	2	2
Distance from the cave entrance, m	0	12	24	36	48	60	280	380	460	520	650
Illuminance, lx	555	13.17	2.70	0.07	0	0	0	0	0	0	0
Maximum stream depth, m	0.25	0.20	0.20	0. 03	0.30	0.40	0.50	0.40	0.30	0.50	0.3
Maximum stream width, m	1.0	1.0	1.0	1.0	2.5	3.0	3.0	3.0	3.0	5.0	4.0
Maximum flow rate, m/s	0.47	0.35	0.30	0.20	0.20	0.30	0.30	0.30	0.30	0.20	0.20
Temperature, °C (October 2019)	12.8	12.8	12.8	12.8	12.8	12.5	12.5	12.5	12.7	12.6	12.9
Mineralization, ppm (October 2019)	176	178	175	170	170	188	211	226	250	250	250
pH (October 2019)	7.7	7.8	7.8	7.8	7.8	7.9	7.9	7.8	7.85	7.9	7.7

*Types of substrates: 1 – stones and clay sand; 2 – stones, clay sand and rimstone walls.

At each station, the main hydrological characteristics of the water inflow (width, depth, water discharge, and type of sediments) and illumination (at midday) were measured (Table 1). In 2019, the water temperature, total mineralization (ppm) and pH were additionally determined (Table 1). The measurements were performed using a Hanna portable water analyzer (HI 98129) and Peak Meter MS6612 luxmeter.

Measurements were obtained by the same person at all stations of a transect. The sampling protocol followed the classic scheme used to study freshwater invertebrates (for example, Walseng et al. 2018).

Ecological groups

In this research, the term "stygon" is used, which is suggested for aquatic underground communities, and the terms "stygobionts", "stygophiles", and "stygoxenes" are used for classifying such organisms (Husmann 1966, 1967). The species were classified into three ecological groups on the basis of published data (Kniss 2001; Shumeyev 2008; Sidorov 2014; Vinarski et al. 2014; Barjadze et al. 2015; Sidorov et al. 2015a, b; Turbanov et al. 2016; Chertoprud et al. 2020). According to the scheme by which these ecological groups are differentiated, stygobionts can be distinguished from stygophiles by morphological adaptations to cave habitats. Specific morphological adaptations of stygobionts limit their penetration into epigean communities, rendering them vulnerable to predators that can see and negative effects of ultraviolet radiation (correct for some groups) (Fišer et al. 2014). Stygophiles, in turn, differ from stygoxenes by possessing ecological adaptations to life in underground cavities, such as the ability to survive and complete their full life cycle in oligotrophic cave environments. Stygoxenes are epigeic organisms trapped in caves for random reasons.

Statistical analysis

To evaluate the effects of environmental factors on the community structure, we used distance-based linear modeling (DistLM) and redundancy analysis (RDA). The analysis was performed twice, for the whole massive of data and separately for the data of 2019

year. Our environmental data contained four variables for the whole dataset (year, season, distance, and luminosity), and six additional variables were included for the set of samples collected in 2019 (maximum depth of the stream, maximum width, flow rate, water temperature, total mineralization (total dissolved solids (TDS) and pH). All the available factors were included to each DistLM test. First, marginal tests were performed to determine the effect of each variable on the variation in species assemblage structure. Then, the best-fitting model was selected using the Akaike information criterion (AICc). This criterion is used to select significant factors in a model and take into account sample size by increasing the relative penalty for model complexity with small data sets. Sequential tests are provided for each variable that is added to the model.

A dbRDA (distance-based redundancy analysis) analysis was used to ordinate the fitted values from a given model. Additionally, the original data were analyzed using the MDS (nonmetric multidimensional scaling) factored with luminosity. The analysis was performed in Primer and Permanova+ PRIMER-E, Plymouth, UK (Clarke and Gorley 2001). The ordination of the samples was performed on the basis of the rank matrix of Bray-Curtis similarities.

Regression analysis was performed to indicate the variation in the number of species along the gradient effect of the environmental factors. We used linear regression analysis in Microsoft Excel (Microsoft, Redmond, WA, USA) for the dataset including number of species at each station and four explanatory factors – season, distance, luminosity and year. The Shannon diversity index was calculated for the samples using Excel too. We also applied the constrained ordination technique canonical correspondence analysis (CCA) to determine the impact of the environmental variables on the invertebrate community and show the variations in the species assemblages in accordance with the observed environmental factors in PAST (Hammer et al. 2001).

Results

Species richness

In total, 42 species of aquatic invertebrates were found in the stream of the Lower Shakuranskaya Cave in 2018–2019: Turbellaria – 2; Oligochaeta – 4; Hirudinea – 1; Gastropoda – 6; Bivalvia – 1; Amphipoda – 5; Decapoda – 2; Ephemeroptera – 3; Plecoptera – 2; Coleoptera – 5; Trichoptera – 7; and Diptera – 4. Among them, 14 species were categorized as stygobionts, 10 as stygophiles, and 18 as stygoxenes based on the available literature data (Table 2). Of the 28 species of stygophiles and stygoxenes, most (21 species) were insects. In the illuminated ecotone zone, 33 species were found; outside the photic zone, 26 species were found. Moreover, only 17 species of aquatic invertebrates were recorded at stations more than 60 m from the cave entrance. The highest species richness (23) was observed at station 2 (Table 2), located 12 m away from the cave entrance. The species richness at stations more than 60 m away from the cave entrance varied from 8 to 11 species per sample. The Shannon diversity index varied from 1.58 to 2.98 and generally decreased from the cave entrance to the deepest parts of the cave (Fig. 2).

Table 2. Distribution of aquatic invertebrates on the transect stations in the stream of the Lower Shakuranskaya Cave in 2018–2019.

Species	Stations										
	1	2	a†	b†	c†	3	4	5	6	7	8
Turbellaria											
[2]*Dugesia taurocaucasica* (Livanov, 1951)	***	**	***	**	*	*					
[3]*Dendrocoelum* sp.						*					
Oligochaeta											
[2]*Haplotaxis gordioides* (Hartmann, 1821)							*				
[2]*Rhynchelmis* sp.							*				
[3]*Stylodrilus* sp.						***	**	***	***	**	*
[2]*Eisenia* sp.									*		
Hirudinea											
[1]*Haemopis sanguisuga* (Linnaeus, 1758)		*			*						
Gastropoda											
[2]*Tschernomorica caucasica* (Starobogatov, 1962)	**	***	***	***	*						
[3]*Caucasogeyeria horatieformis* (Starobogatov, 1962)						**	**	**	**		
[3]*Pontohoratia birsteini* (Starobogatov, 1962)		***	**	***	***	***	***	***	**	*	*
[3]*Caucasopsis schakuranica* (Starobogatov, 1962)						*	**	*	**	**	*
[3]*Caucasopsis shadini* (Starobogatov, 1962)											**
[3]*Caucasopsis* sp.		*								*	*
Bivalvia											
[3]*Euglesa* cf. *ljovuschkini* (Starobogatov, 1962)		**	**	**	**						
Amphipoda											
[3]*Niphargus magnus* Birstein, 1940	*			*							
[3]*Niphargus inermis* Birstein, 1940		**	*	**	**	**	*	**	*	**	*
[3]*Niphargus* cf. *ablaskiri* Birstein, 1940								*			
[3]*Zenkevitchia yakovi* Sidorov, 2015		*				**	**	**	**	**	**
[2]*Gammarus* cf. *komareki* (Schaferna, 1922)	****		*								
Decapoda											
[3]*Xiphocaridinella falcirostris* Marin, 2020		*		*			**	***	*	**	
[3]*Xiphocaridinella osterloffi* (Juzbaš'jan, 1941)	*	***	***	***	***	****	***	**	*	**	*
Insecta											
Ephemeroptera											
[1]*Electrogena zimmermanni* (Sowa, 1984)				*							*
[1]*Baetis* (*Rhodobaetis*) cf. *gemellus* Eaton, 1885	****	**		*							
Leptophlebiidae											
[1]*Paraleptophlebia werneri* Ulmer, 1920	*	*									
Plecoptera											
[1]*Nemoura martynovia* Claasen, 1936		*									
[2]*Leuctra* sp.		*							*		
Coleoptera											
[1]*Agabus* (*Gaurodytes*) *guttatus* (Paykull, 1798)	*										
[2]*Limnius colchicus* Delève, 1963		*									
[1]*Riolus somcheticus* (Kolenati, 1846)		*									
[1]*Elmis* sp.	*										
[2]*Odeles* sp.	*						*				
Trichoptera											
[1]*Tinodes valvatus* Martynov, 1913	*										
[2]*Plectrocnemia latissima* Martynov, 1913		*				*					
[1]*Chaetopterygella abchazica* Martynov, 1916	*	*		*							
[1]*Stenophylax clavatus* (Martynov, 1916)	*										
[1]*Lithax incanus* (Hagen, 1859)	**			*							
[1]*Ernodes palpatus* (Martynov, 1909)							*				
[1]*Schizopelex cachetica* Martynov, 1913		*		*	**						
Diptera											
[1]*Macropelopia* sp.		*									
[1]*Parametriocnemus* sp.		*				*					
[1]*Cnetha* sp.	*	*									
[1]*Dixa submaculata* Edwards, 1920		*									
Total number of species	15	23	9	11	8	12	11	9	10	8	9

[1] Stygoxenes, [2] Stygophiles, [3] Stygobionts. Occurrence: * – single (1–2 specimens per sample); ** – rarely (3–8 specimens per sample); *** – often (9–26 specimens per sample); **** – frequent (27–80 specimens per sample); † – addition sampling stations taken in October 2019.
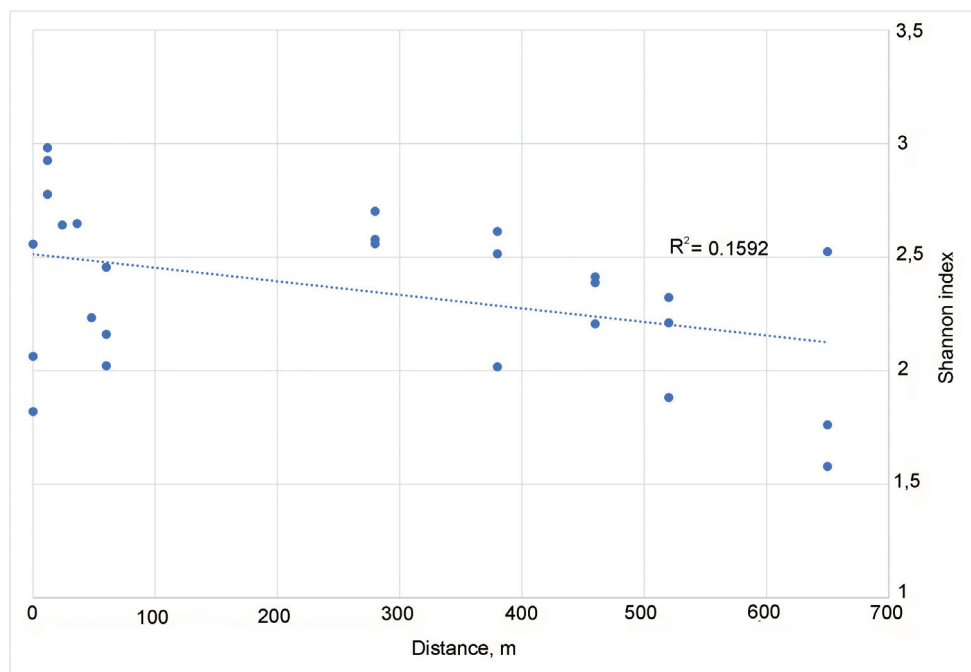
Figure 2. The variation in the values of Shannon diversity index along the distance from the cave entrance (2018–2019).

Number and biomass

The highest abundance values (up to 250 ind/m^2) were recorded at the stations in the ecotone zone (Fig. 3). With increasing distance from the cave entrance, a tendency towards decreasing aquatic invertebrate abundance was observed (up to 31 ind/m^2 at the furthest station of the transect). The most numerous stygobionts in all years of this research were shrimp (*Xiphocaridinella*) and gastropods (*Pontohoratia birsteini*) (Starobogatov, 1962) (both up to 68 ind/m^2) (Fig. 4). These species have been recorded throughout the main cave gallery. Another species recorded at all the stations, except for the first station, was *Niphargus inermis* Birstein, 1940. However, its abundance did not exceed 14 ind/m^2. In addition, oligochaetes (*Stylodrilus* sp.), amphipods (*Zenkevitchia yakovi* Sidorov, 2015), and snails (*Caucasopsis schakuranica* (Starobogatov, 1962) and *Caucasogeyeria horatieformis* (Starobogatov, 1962)) were common outside the photic zone. At station 1, which was outside the cave and had the highest illuminance (555 lx), specimens of stygobiont fauna were found only occasionally. However, at the station located 12 m from the cave entrance (illumination 13 lx), the average proportion of stygobionts was 55% of the total number of invertebrates (Fig. 3). At the ecotone stations, which had slight or no illuminance (0.07 lx), the proportion of stygobionts in the samples increased. At a distance of 36 m from the entrance, stygobionts accounted for 73% of the total number of organisms; at 48 m, 90%; and at 60 meters, 97%.
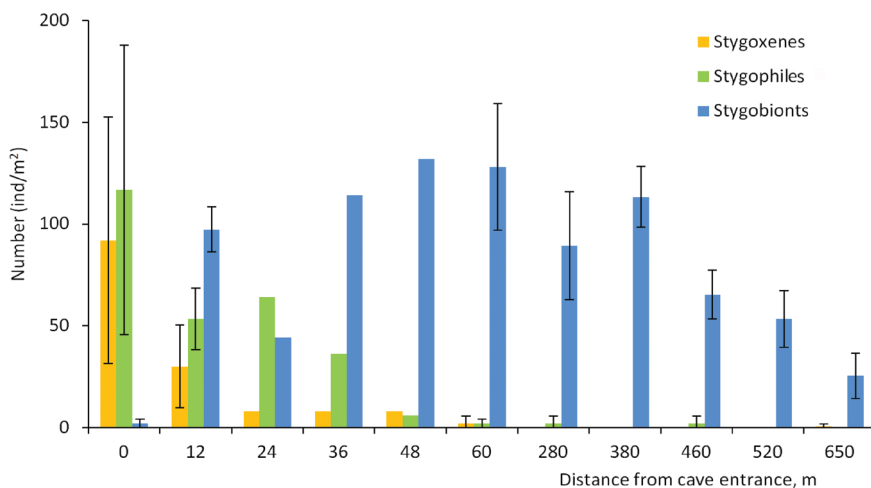
Figure 3. Changes in the number of stygoxenes, stygophiles and stygobionts according to the distance from the cave entrance (2018–2019).

Figure 4. The most numerous stygobionts in the Lower Shakuranskaya Cave **A** *Xiphocaridinella osterloffi* (Juzbaš'jan, 1941) **B** *Pontohoratia birsteini* (Starobogatov, 1962).

Among the stygophiles, the most abundant were a flatworm (*Dugesia taurocaucasica* (Livanov, 1951)) (up to 54 ind/m²), snail (*Tschernomorica caucasica* (Starobogatov, 1962)) (up to 52 ind/m²) and amphipod (*Gammarus* cf. *komareki* (Schaferna, 1922)) (up to 52 ind/m²). These species were associated mainly with the slightly illuminated part of the ecotone zone.

Mayfly larvae *Baetis* cf. *gemellus* Eaton, 1885 (up to 142 ind/m²), and caddisfly larvae *Lithax incanus* (Hagen, 1859) (up to 20 ind./m²) were the most numerous among the stygoxenes. These species were recorded in the ecotone part, and their maximum abundance was observed at the most illuminated station 1.
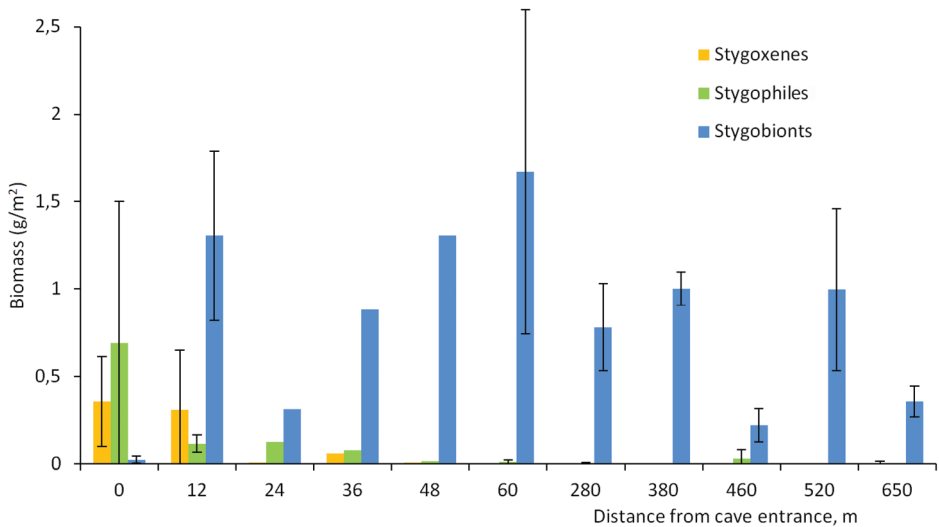
Figure 5. Changes in the biomass of stygoxenes, stygophiles and stygobionts according to the distance from the cave entrance (2018–2019).

The highest biomass values were recorded in the ecotone zone at stations 2 and 3 (Fig. 5). The main contribution to biomass at these stations was from stygobionts. The predominance of stygoxenes and stygophiles over stygobionts in the biomass was noted only outside of station 1. The biomass values recorded for stations located in the ecotone (at a distance less than 100 m from the cave entrance) were higher than those in the more distant parts of the cave (Fig. 3). The lowest biomass values were noted at stations 6 and 8, located at distances of 460 and 650 m from the cave entrance, respectively. It should be noted that *Xiphocaridinella* shrimp accounted for the main part of the biomass at most of the transect stations, including all the stations in the ecotone zone, except for station 1 near the cave entrance (Fig. 6).

Community structure across a gradient of environmental factors

Of the four environmental variables we measured for the whole dataset (year, season, distance, and luminosity), the DistLM analysis identified luminosity and distance as explaining the highest amount (31.7% and 29%, respectively) of the variation in species assemblage structure (Table 3). The set of sequential tests shows whether adding every particular variable contributes significantly to the explained variation. The column labeled "Cumul." provides a running cumulative total. Thus, these variables explained 55.6% of the variation in the species composition at the observed sampling stations (Fig. 7). Of the variables, only distance and luminosity were statistically significant (P = 0.001).

A significant proportion of the species assemblage variations remains unexplained, which is due to the high heterogeneity of the other environmental conditions in the biotopes studied. By taking into account a greater variety of environmental factors, we attempted to conduct a separate, more detailed analysis for the third sampling event
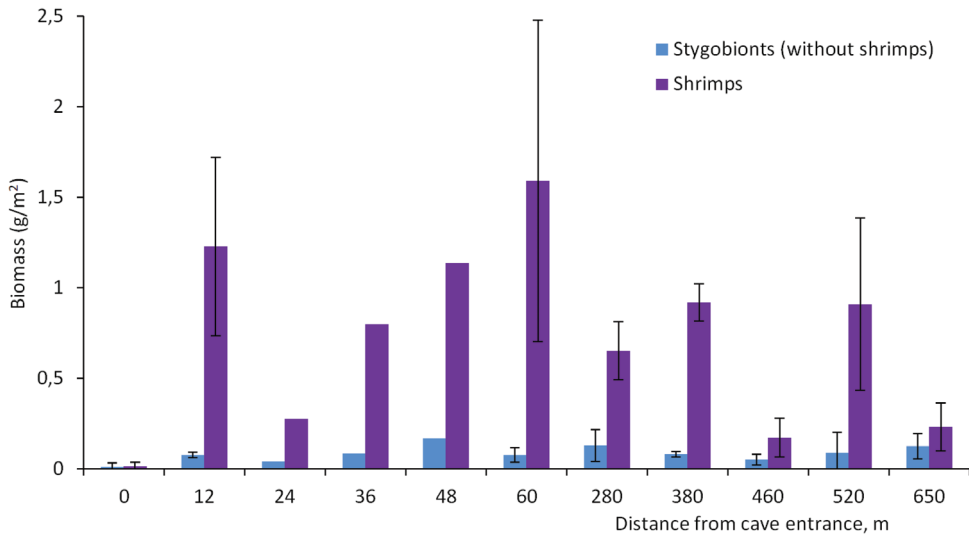
Figure 6. Changes in the biomass of stygobionts according to the distance from the cave entrance (2018–2019).

Table 3. The results from DistLM test, including marginal and sequential tests.

Variable	AICc	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
MARGINAL TESTS							
Distance		16257	10.209	**0.001**	**0.29**		
Luminosity		17783	11.612	**0.001**	**0.317**		
Year		3256.3	1.5414	0.152	0.051		
Season		2506.7	1.17	0.301	0.045		
SEQUENTIAL TESTS							
+ Season	209.51	2506.7	1.17	0.298	0.045	0.045	25
+ Distance	202.74	15615	9.875	**0.001**	**0.278**	**0.323**	24
+ Luminosity	195.43	11833	10.42	**0.001**	**0.211**	**0.534**	23
+ Year	197.19	1203.5	1.063	0.408	0.0215	0.556	22

Factors with p < 0.005 are in bold. AICc – modified Akaike information criterion by which only significant factors in model are selected; SS (trace) – the total sum of squares of the deviations explained with this; Pseudo-F – the multivariate analogue of Fisher's ratio, estimates by how much the sum of squares deviates from; random P – probability of random influence of a factor; Prop. – the proportion of variability which explains each factor (in the marginal tests – without coactions of factors); Cumul. – running cumulative total (percent of the variability explained by the model); res.df – number of degrees of freedom (number of groups allocated by this factor).

in autumn 2019. For this period of research, some additional data were available. The DistLM analysis showed that of all the factors (season, distance from the cave entrance, illumination, maximum depth of the stream, maximum width, flow rate, water temperature °C, total mineralization TDS ppm and pH), only two, the flow rate and pH, were nonsignificant and therefore eliminated (Fig. 8). Among the variables, illuminance, distance and TDS explained the most variation. Overall, the model explained 85.8% of the variation in species composition at the observed sampling stations.

The observed factors affected both the species composition and species richness of organisms in the samples. Using the regression analysis, only the factor of distance was selected as significant (P-value 0.00006). Altogether, 54.4% of the variation in the number of species can be explained by the model. The obtained regression equation
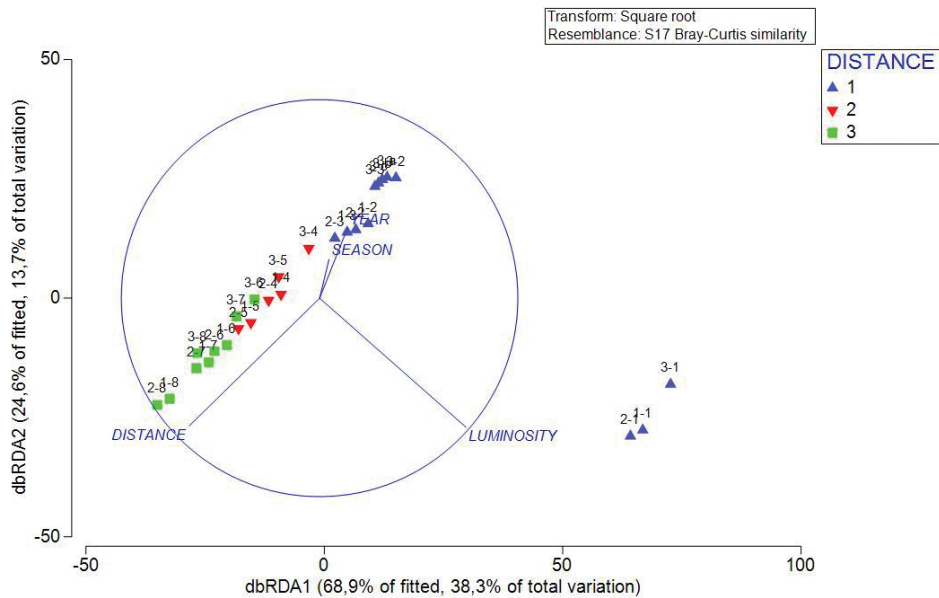
Figure 7. dbRDA ordination for the model of the investigated cave sites (based on Bray–Curtis similarity) factored with distance ranges: 1 – 0–60 m, 2 – 280–380 m, 3 – 460–650 m.

predicts a decrease in the number of species by 0.009184 with a one-meter increase in distance; in other words, a 100-meter decrease in the distance from the cave entrance leads to a one-species drop in the number of species.

Species assemblages

To further illustrate the ordination of the investigated stations according to their species compositions, we used nonmetric MDS, which revealed three groups, i.e., three species assemblages, that were clustered together on the basis of preference for luminosity (Fig. 9). There was a group of stations located at the entrance to the cave, where the illuminance was highest (555 lx, three dots on the left side of the nMDS plot), a group of stations (six dots) in semidarkness and a scatter of dots with a lux value of zero (18 dots on the right side of the nMDS plot).

The CCA plot (Fig. 10) shows the variation in the species assemblages of aquatic invertebrates in accordance with the observed environmental factors. The first ordination axis (axis 1, eigenvalue 0.72081) positively correlated with luminosity and negatively correlated with the distance from the cave entrance. Thus, it reflected the most strongly pronounced gradients of the environmental conditions in the cave, along which stygobiont organisms are gradually replaced with epigean organisms. The species *G.* cf. *komareki*, *Agabus guttatus* (Paykull, 1798), *Elmis* sp., *Stenophylax clavatus* (Martynov, 1916), *Tinodes valvatus* Martynov, 1913, *L. incanus*, *B.* cf. *gemellus* and others, located on the right side of the CCA plot (Fig. 7), are typical of epigeic communities, while
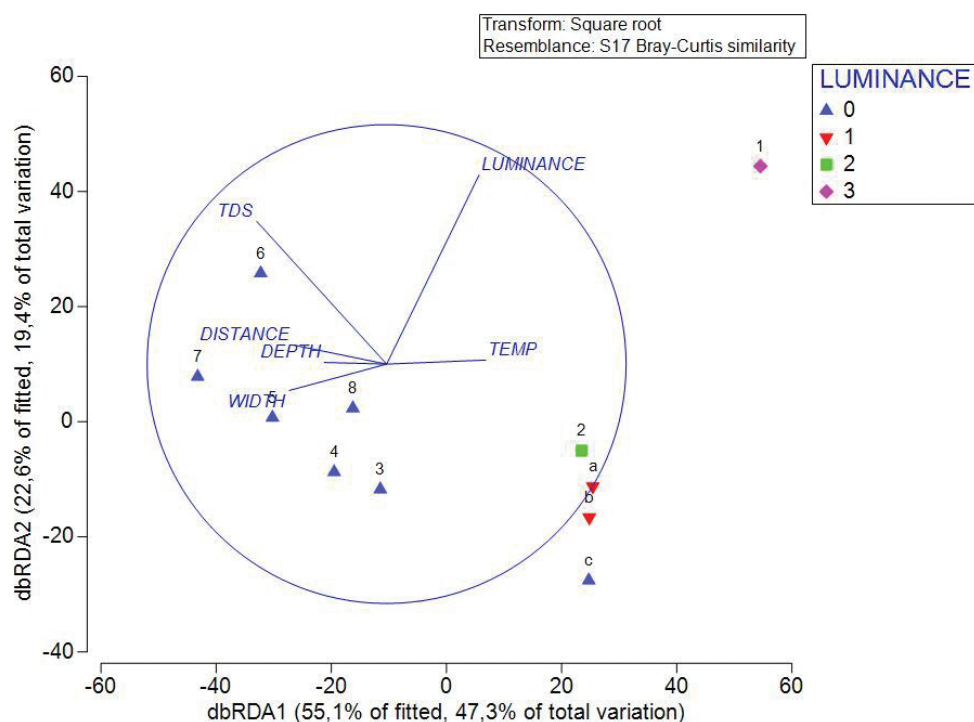
Figure 8. dbRDA ordination for the investigated cave sites during the research in autumn 2019 (based on Bray–Curtis similarity) factored with luminosity ranges: 0 – 0 lx, 1 – 0.07–2.7 lx, 2 – 13.17 lx, 3 – 555 lx.

the species *Stylodrilus* sp., *Esenia* sp., *C. horatieformis*, *C. schakuranica*, *C.* sp., *Z. yakovi*, *Xiphocaridinella falcirostris* Marin, 2020 and others on the left side are typical stygobionts. The second CCA axis, axis 2 (eigenvalue 0.26147), was positively correlated with the season of research; however, its contribution to explaining the variability in the structure of species assemblages was extremely low. Apparently, the location of species along this axis primarily characterized the rare species found in only one of the temporal surveys.

The main characteristics of the three identified species assemblages of macrozoobenthic organisms are presented below:

1. Assemblage of epigean species near the cave entrance area. This community was characterized by the predominance of epigean organisms and abundant stygophilic taxa. Larvae of amphibiotic insects (*B.* cf. *gemellus* and *L. incanus*) and epigean Amphipoda (*G.* cf. *komareki*) were dominant (66% of the total fauna). Stygophilic snails (*T. caucasica*) and flatworms (*D. taurocaucasica*) were also abundant (27%). Stygobionts (two species) were very rare, accounting for only 1% of the total number of macrozoobenthic species, and they must have been driven from the remote parts of the caves. The number of species totaled 15.
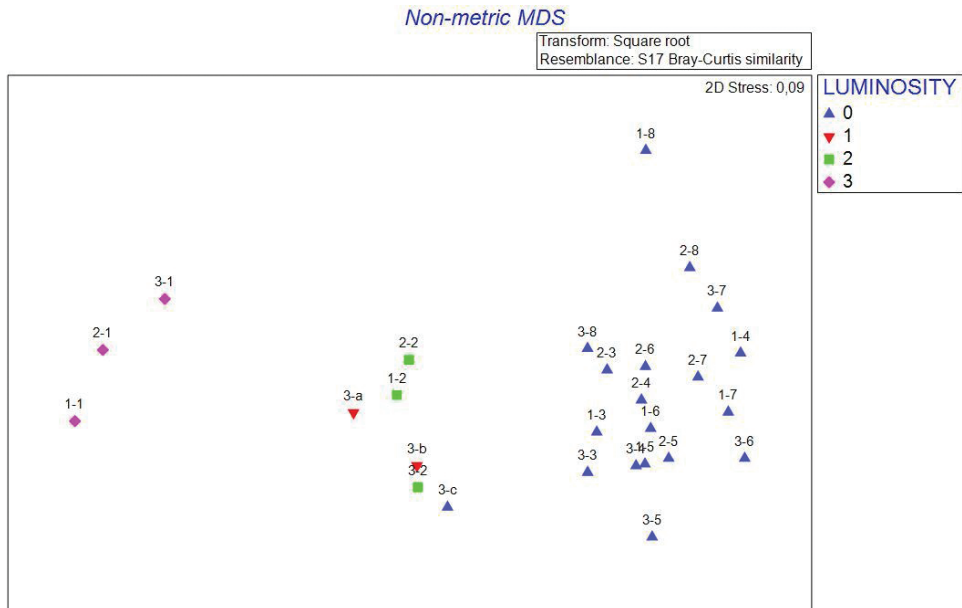
Figure 9. The two-dimensional nMDS ordination of the investigated cave sites, based on Bray–Curtis similarities (stress = 0.09) and factored with luminosity: 0 – 0 lx, 1 – 0.07–2.7 lx, 2 – 13.17 lx, 3 – 555 lx. Dots are labeled: first number – season/year of research: 1 – winter 2018, 2 – spring 2018, 3 – autumn 2019; second number – no of sampling station.

2.   Assemblage of stygobiont species in remote (> 40 m from the entrance) parts of the cave outside the photic zone. Stygobiont oligochaetes (*Stylodrilus* sp.), amphipods (*Z. yakovi*), shrimp (*Xiphocaridinella* spp.) and snails (*P. birsteini* and *C. schakuranica*) formed the bulk of the community (81% of the total abundance). Stygoxenic species were rare (1%< of total number). The number of species totaled 25.

3.   Mixed assemblage of the cave ecotone (first 40 m from the entrance to the border of the photic zone). This assemblage is transitional between the two previously described assemblages. The common species include both stygoxenes (*B.* cf. *gemellus*, 8% of the total fauna) and stygophiles (*T. caucasica* and *D. taurocaucasica*, 29%) as well as stygobionts (*P. birsteini* and *Xiphocaridinella osterloffi* (Juzbaš'jan, 1941), 41%). The total number of species was the highest here (27 species).

Discussion

Main characteristics of stygobiont fauna

A total of 14 stygobiont species were found in the Lower Shakuranskaya Cave in 2018–2019, and this number is comparable to the variety of stygophiles (10) and stygoxenes (18). Earlier (in 2012), 14 species of stygobionts were observed in this cave (Chertoprud et al. 2016). Most were found in the present study. Thus, the general list
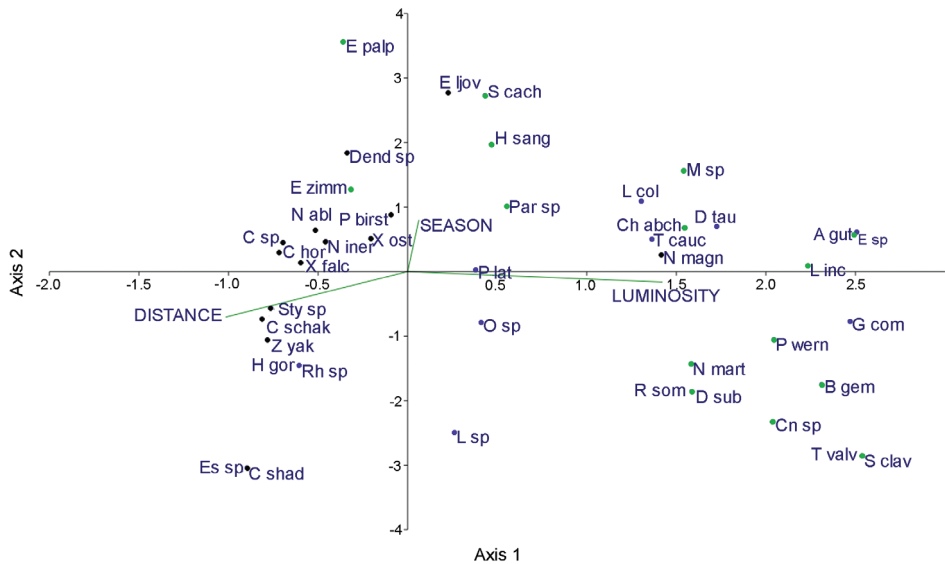
Figure 10. The CCA ordination of hydrobionts species from Nizhnyaya Shakuranskaya Cave. Black points – stygobionts, blue points – stygophiles, green points – stygoxenes. Abbreviations: A gut – *A. guttatus*, B gem – *B. gemellus*, C hor – *C. horatieformis*, C schak – *C. schakuranica*, C shad – *C. shadini*, C sp – *Caucasopsis* sp., Ch abch – *C. abchazica*, Cn sp – *Cnetha* sp., E sp – *Elmis* sp., D sub – *D. submaculata*, D tau – *D. taurocaucasica*, Dend sp – *Dendrocoelum* sp., E ljov – *E.* cf. *ljovuschkini*, Es sp – *Eisenia* sp., E palp – *E. palpatus*, E zimm – *E. zimmermanni*, G kom – *G.* cf. *komareki*, H gor – *H. gordioides*, H sang – *H. sanguisuga*, L col – *L. colchicus*, L inc – *L. incanus*, L sp – *Leuctra* sp., M sp – *Macropelopia* sp., N abl – *N.* cf. *ablaskiri*, N iner – *N. inermis*, N magn – *N. magnus*, N mart – *N. martynovia*, O sp – *Odeles* sp., P birst – *P. birsteini*, P lat – *P. latissima*, Par sp – *Parametriocnemus* sp., P wern – *P. werneri*, R som – *R. somcheticus*, Rh sp – *Rhynchelmis* sp., S cach – *S. cachetica*, S clav – *S. clavatus*, Sty sp – *Stylodrilus* sp., T cauc – *T. caucasica*, T valv – *T. valvatus*, X falc – *X. falcirostris*, X ost – *X. osterloffi*, Z yak – *Z. yakovi*.

of stygobiont fauna of the Lower Shakuranskaya Cave includes 17 species: Turbellaria (1 species), Oligochaeta (2), Gastropoda (6), Bivalvia (2), Amphipoda (4) and Decapoda (2). The temporal variability (seasonal and interannual) in the composition of the stygobiont fauna was not significant and probably reflected the probability of capturing any rare species. Overall, the Lower Shakuranskaya Cave has the highest species richness of stygobionts among the hitherto studied caves of Abkhazia (Kniss 2001; Barjadze et al. 2015; Chertoprud et al. 2016).

The two major groups in the stygobiont assemblage were gastropods belonging to *Caucasopsis*, *Caucasogeyeria*, and *Pontohoratia* (f. Hydrobiidae) and shrimp belonging to *Xiphocaridinella* (f. Atyidae) (Fig. 4). Mollusks were the most abundant group in terms of the number of individuals, while shrimp comprised the main biomass (Fig. 6).

The cave ecotone

Significant changes in the dominance structure and qualitative and quantitative characteristics along the Lower Shakuranskaya Cave gallery occur. Thus, three types of

macrozoobenthic assemblages, continually changing each other, were indicated. The ecotone consists of mixing assemblages in which stygoxenes, stygophiles and stygobionts are abundant simultaneously. The abundance and species richness of stygobionts increase from the onset of the ecotone zone, peak at 50–60 m from the cave entrance and decrease further into the cave (Table 2, Figs 3, 5).

The peak abundance in the ecotone may be related to bottom sedimentation and food availability. The bottom in the ecotone zone is covered with rocky soils with a large number of microcavities forming favorable habitats for organisms. In contrast, substrate in deeper parts comprises calcified hump dams and baths without suitable shelters. Some other researches demonstrated positive relationships between environmental heterogeneity and the diversity of aquatic organisms in cave and surface streams (Palmer et al. 2010; Pellegrini et al. 2018). Furthermore, the amount of organic matter monotonically decreases from the ecotone zone towards deeper parts of the cave. The Lower Shakuranskaya Cave is oligotrophic (Amelichev et al. 2007), whereas the ecotone zone seems to be less food deprived because of the inflow of plant detritus and filamentous algae in the presence of light. The food that may be safely accessed through the microcavities might attract stygobionts to the boundary of the aphotic zone.

Apart from the beneficial aspects of the ecotone zone, stygobionts can passively drift out from the cave with water currents. Indeed, stygobionts are occasionally found outside the caves as a result of seasonal floods. For example, *Xiphocaridinella* shrimp (Marin and Sokolova 2014) and the snail *Radomaniola curta germari* (Frauenfeld, 1863) (Perić et al. 2018) are found in epigean streams during spring and autumn high water. Although intuitively logical, these explanations need to be considered with care. It has been observed that a number of stygobionts (for example, stygobiont amphipods) that live at the border of belowground and aboveground environments can actively avoid the water current and illuminated areas, thus resisting being transported from cave biotopes (Borowsky 2011; Fišer et al. 2016).

It must be noted that the abundance and biomass of stygobionts in our study were not extremely low in the deeper and oligotrophic parts of the studied cave (more than 200 m), where species apparently feed on the microbial community containing heterotrophic and, to a lesser extent, chemoautotrophic bacteria (Kováč 2018). This food source might also explain the dominance of the cave shrimp and gastropods. The Atyidae family includes pereopods adapted to collecting bacterial biofilms due to their specific bristle armament (Page et al. 2007). Most likely, numerous gastropod mollusks can be considered consumers of biofilms, which they scrape off underwater fouled surfaces with their radula. Perhaps bacterial communities serve as one of the main food sources for stygobiont fauna in the Lower Shakuranskaya Cave. While these hypotheses need to be tested with stable isotope analysis, we acknowledge that the ecotone zone might act as a food attractant mainly to less frequent species and to a lesser extent to collectors of biofilms.

Thus, this study confirms the hypothesis about the increase in species richness and abundance of aquatic organisms in the ecotone zone (Prous et al. 2004, 2015; Culver 2005). Our hypothesis that the ecotone macroinvertebrate assemblages may significantly differ from the assemblages of the remote cave parts was confirmed.

Active and passive ways to penetrate epigeic species in cave communities

The entrance of the Lower Shakuranskaya Cave is large (approximately 70 m²). Adult amphibiotic insects were not found inside the cave, and their larvae usually do not occur further than 60 m deep. Only certain stygoxenes (*Haemopis sanguisuga* (Linnaeus, 1758), *Ernodes palpatus* (Martynov, 1909), *Schizopelex cachetica* Martynov, 1913 and *Parametriocnemus* sp.) can penetrate through the photic zone. The active penetration of stygophiles and stygoxenes further than the ecotone zone indicates their ability to actively migrate against the flow. Most likely, the intensity of these migrations is determined by the presence of an available food, as in the case of the leech *H. sanguisuga* (Linnaeus, 1758), which feeds on stygobiont oligochaetes.

The finding of stygoxenic and stygophilic insect larvae at a great distance from the cave entrance may be a consequence of drift (i.e., the movement of benthic organisms with the current). This phenomenon is widespread in watercourses and plays a significant role in the distribution of benthos in mountain regions (Brittain and Eikeland 1988; Naman et al. 2016). In the investigated cave, larvae of the Ephemeroptera *Electrogena zimmermanni* (Sowa, 1984) and Plecoptera *Leuctra* sp. were found at distances greater than 400 m from the entrance. These species have previously been noted in epigeic watercourses of the western Caucasus (Chertoprud et al. 2016, 2020). The most likely method of larval penetration in the cavities is passive drift with water through the rock cracks and karst tunnels. The greatest intensity of drift was observed during flood events (Perić et al. 2018).

In the context of global climate changes affecting organic matter flows in ecosystems, a significant transformation of cave ecosystems can be expected (Humphreys 2018). It has been suggested that ongoing warming of the climate may cause an increase in the nutrient status of cave watercourses, which can lead to more intensive penetration of epigeic species into underground cavities. It was observed previously that stygophiles and stygoxenes actively settle underground habitats in caves with organic pollution (Sousa-Silva et al. 2012; Venarsky et al. 2012, 2018). Establishment of long-term observations of aquatic fauna in model caves will enable the assessment of the value of biospeleology for monitoring global climatic processes.

Conclusion

In the Lower Shakuranskaya Cave, 42 species of aquatic invertebrates occurred: 14 – stygobionts, 10 – stygophiles, and 18 – stygoxenes. The species richness and abundance of stygobionts were the greatest near the boundary of the photic zone and gradually decreased both further into the cave cavity and up to the exit from it. In the cave, the distributions of most stygoxenes and stygophilic species were limited to the illuminated ecotone zone. The main factors regulating the spatial distributions of macrozoobenthic organisms were the distance from the cave entrance and the light intensity (illuminance). The greatest species richness and abundance of fauna were noted at sta-

tions in the shaded ecotone, where stygobionts, stygophiles and stygoxenes co-occur. The most likely reasons for this scenario are the higher abundance of food resources for aquatic invertebrates, the removal of stygobionts by the water current, and the possibility of faunal epigean elements penetrating the ecotone zone.

Acknowledgements

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RESEARCH ARTICLE

A remarkable new genus and species of subterranean freshwater snail from a recently dried-up spring of Viesca, Coahuila, Northern Mexico

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Abstract

This paper describes a new genus and species of subterranean gastropod from a karstic region near Viesca, Coahuila in northern Mexico. Shells of *Phreatoviesca spinosa* **gen. nov. et sp. nov.** were found in spring-deposited sediments near the outlet of a cave that dried up in the late 20th century. The new genus can be primarily distinguished conchologically from other phreatic genera by three remarkable characteristics: (i) prominent open coiling of the last whorl, (ii) shovel-shaped spine ornamentations on the teleoconch, and (iii) a coarsely honeycomb-like pitted protoconch structure. Since only dry shells were found, the new species could already be extinct. However, in view of the relative recent drying up of the spring, we consider that *Phreatoviesca spinosa* is possibly extant in the aquifers in or adjacent to the Viesca region.

Keywords

Gastropods, interstitial habitat, new genus , North America, subterranean, systematics

Introduction

Owing to sampling difficulty associated with subterranean habitats, it is not surprising that stygobiotic (i.e., freshwater subterranean-obligate) gastropods are among the most understudied freshwater groups in the world (Prié 2019; Gladstone et al. 2021). Many stygobiotic gastropods inhabit a diversity of subterranean habitats that are near-to-completely inaccessible to humans, such as the hyporheic or phreatic zones of an aquifer system. In Mexico, the majority of the species have been discovered from these less accessible habitat types through opportunistic sampling of the groundwater saturated, interstitial spaces within the sediment of surface streams or from groundwater discharge in wells or small springs (Hershler 1985; Czaja et al. 2019a). This inaccessibility to extant populations and low probability of discovering stygobiotic gastropods *in vivo* has led to many taxonomic descriptions worldwide (whether later found extant or extinct) relying solely on empty shells (Georgiev 2013; Grego et al. 2017; Quiñonero-Salgado and Rolán 2017; Hofman et al. 2018; Czaja et al. 2019b).

Although mollusk shells generally have a high fossilization potential, there are few records of gastropod fossils that have been determined to be stygobionts worldwide. This fossil scarcity is likely owing to the usually narrow geographic distributions of stygobiotic gastropods, along with their extremely small size that does not typically exceed two millimeters (Gladstone et al. 2021). However, these small stygobiont fossils may be more likely obtained from sites that have gone through recent environmental change, such as the springs in Viesca, Mexico that dried up in the second half of the 20th century (Czaja et al. 2017, 2019a).

The aim of the present study is to describe a new subterranean genus and species from Coahuila, and to discuss unique aspects of the shell morphology compared to other stygobiotic gastropod species in North America. The new genus can be readily distinguished by three shell features: (i) prominent open coiling of the last whorl, (ii) shovel-shaped spine ornamentations on the teleoconch, and (iii) coarsely honeycomb-like pitted protoconch structure. Although the description of both genus and species based exclusively on shell morphology may appear erroneous, the shell features of the discovered specimens are so strikingly different from all known stygobiotic gastropods that we consider the erection justified. Nevertheless, in the absence of soft parts, the family designation is tentative until living specimens will be obtained for anatomical and molecular studies.

Materials and methods

The studied shells were collected during July 2015 and November 2019 in two sites within the spring "Túnel 7" (Fig. 1). Like most of the other 15 springs near Viesca, this spring began to dry up during the drought of 1958–59, but the area remained a partial wetland until the late 1990s (Czaja et al. 2015, 2019a). The shells were found in superficial spring deposits a few meters at the outlet of a cave. The area is now completely dry, but the outlines of the former water body are still clearly visible in satellite or drone
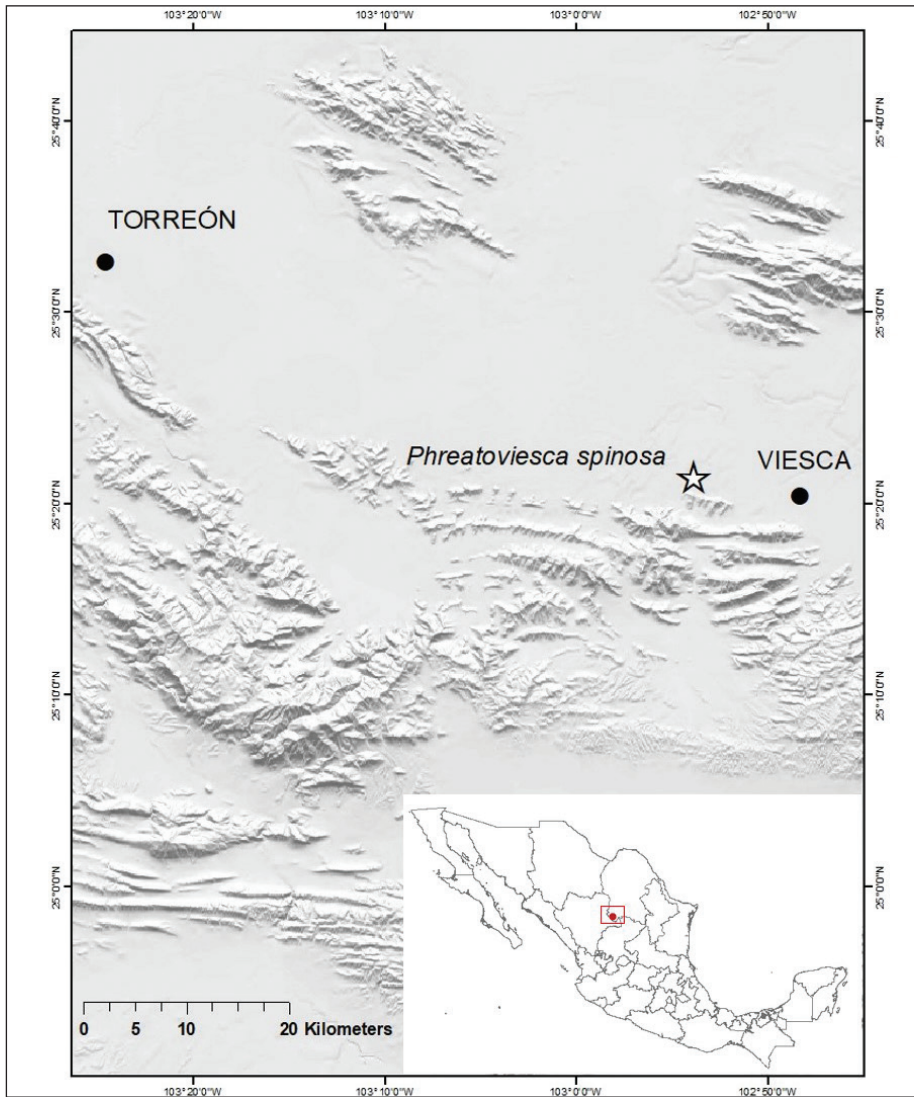
Figure 1. Map of the study area with localization of the sampling site near the town of Viesca, Coahuila, Mexico.

imagery. Moreover, the presence of remaining moisture in the subsoil is indicated by sparse vegetation within the original spring. The possible anthropogenic causes for the drying up of all 15 springs of Viesca were presented in detail by Czaja et al. (2019a).

The collected material was screened through two sieves with a mesh size of 0.5 mm and 0.3 mm. For the morphological analysis, the shells were photographed and measured with a Zeiss AxioCamERc 5s camera attached to a Zeiss Stemi 2000-C microscope. Some specimens, particularly their protoconchs, were examined in the Laboratory of Biotechnology, Universidad Autónoma de Coahuila (UAC) in Torreon, Coahuila, using a HITACHI high performance FlexSEM 1000 scanning electron microscope (SEM).

We obtained the following shell morphometrics for each specimen collected (excluding ratios): total number of whorls, shell height, shell width, aperture height, and aperture width. The mean, standard deviation and sample size are given in text (shell measurements). Shell whorls were counted according to the method of Pilsbry (1939). The studied material was deposited in the Malacological Collection of the Faculty of Biological Science of the Juarez State University of Durango.

Abbreviations used for shell morphometrics are as follows: WN, total number of whorls; SH, shell height; SW, shell width; AH, aperture height; AW, aperture width; HBW, height of body whorl; UJMC = University Juárez Malacological Collection.

Systematics

Class Gastropoda Cuvier, 1795
Subclass Caenogastropoda Cox, 1960
Superfamily Truncatelloidea Gray, 1840
Family Cochliopidae Tryon, 1866

Phreatoviesca **Czaja & Gladstone, gen. nov.**
http://zoobank.org/517E4E3B-915D-4056-A65C-312806C6DB02

Type species. *Phreatoviesca spinosa* by present designation.
Diagnosis. Shell small, conical in form, protoconch sculptured with coarsely honeycomb-like pits, teleoconch with curved ribs which are at the carina modified into regularly spaced shovel-shaped spines (Figs 14, 24), body whorl always open-coiled, some specimens with a corkscrew morphology, apertures large, ovate, rarely rounded, often trumpet-like.
Differential diagnosis. The characteristic combination of three aforementioned shell features (open coiling of the last whorl, shovel-shaped spines, and protoconch with coarsely honeycomb-like pits) separate the new genus clearly from shells of all other subterranean (and epigean) genera. Some members of *Phreatodrobia* Hershler & Longley 1986 and *Paludiscala* Taylor 1966, genera which include exclusively subterranean species, also have conical shells, but these are not uncoiled (except the slightly uncoiled *Phreatodrobia nugax* (Pilsbry & Ferriss, 1906) to this extent do not possess prominent spine ornamentations.
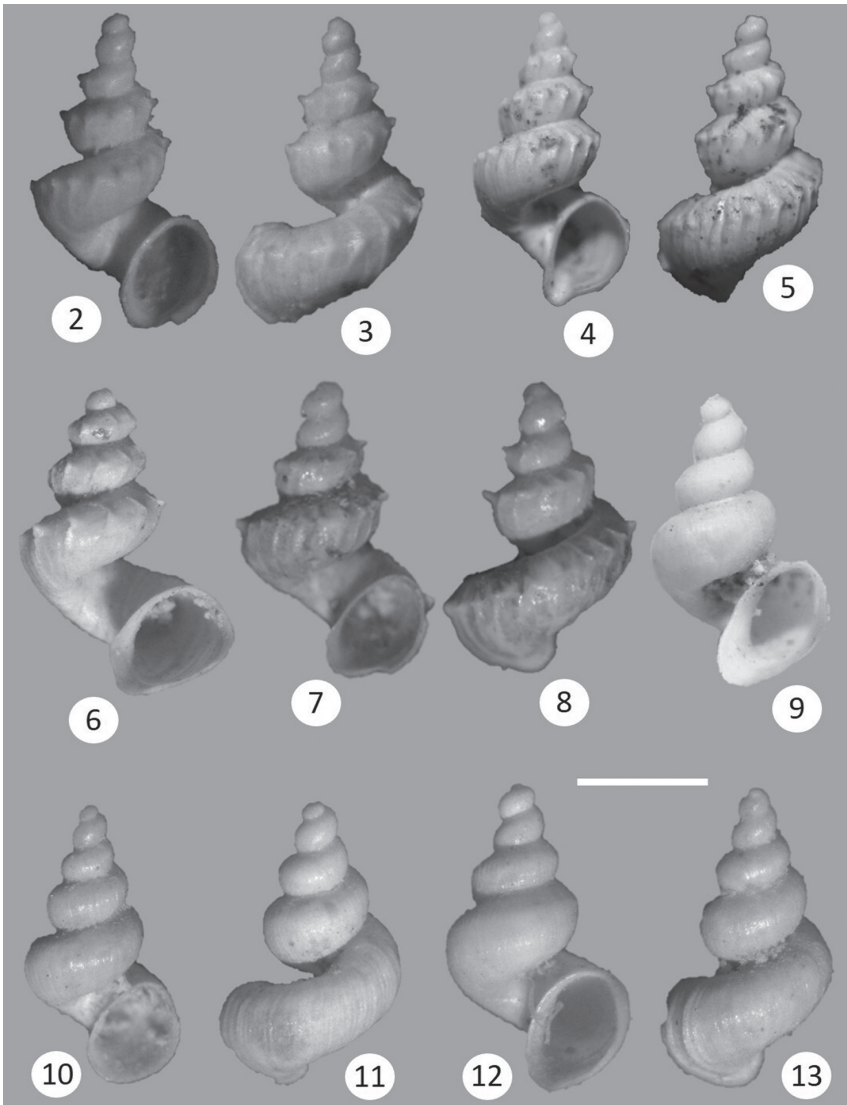**Etymology.** The name is derived from Greek *phreato* = groundwater environment, and *Viesca* (referring to the town of Viesca where the shells were found).

Phreatoviesca spinosa **Czaja & Gladstone, sp. nov.**
http://zoobank.org/C72889DC-5B7A-4366-B703-964353942786
Figs 2–24

Type locality. Mexico, Coahuila state, Viesca, spring "Túnel 7" (25°20'38"N, 102°54'19"W, 1102 m a.s.l.) (Fig. 1).
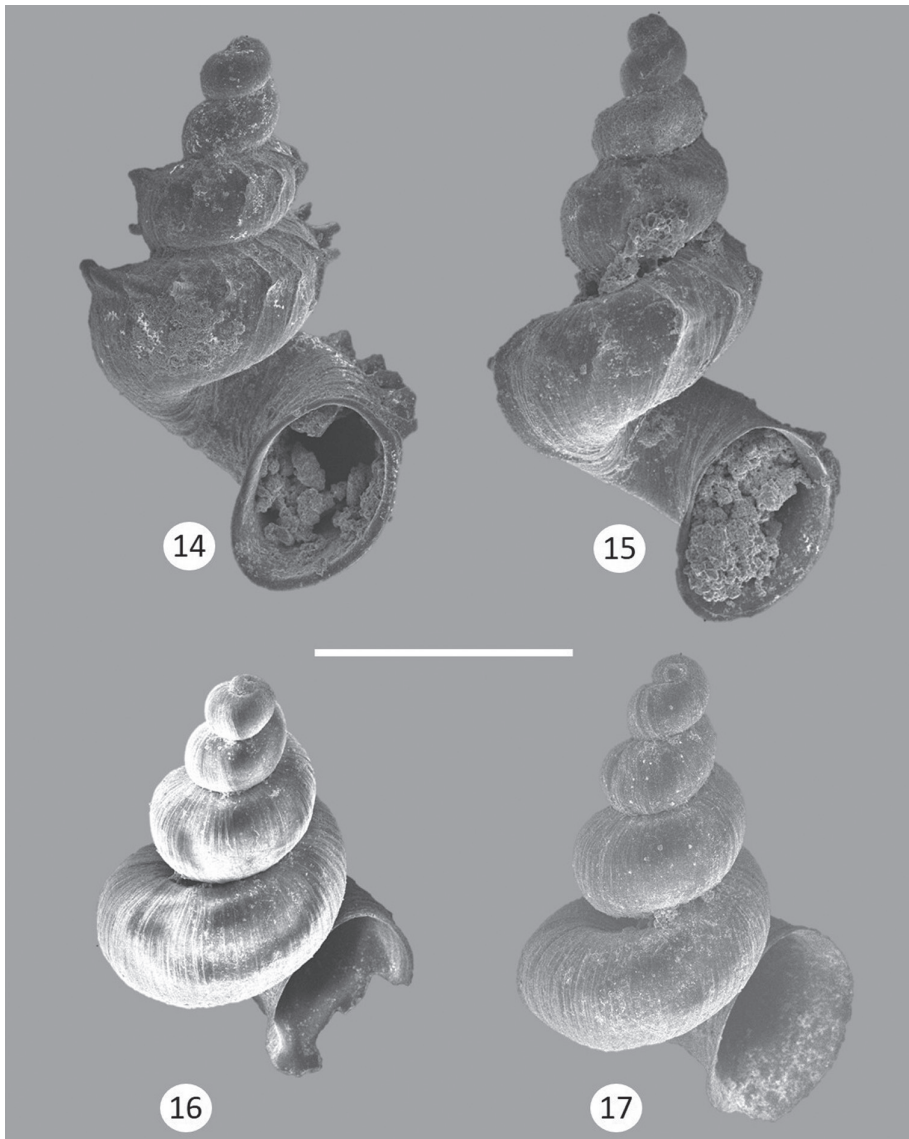
Figures 2–13. Shells of *Phreatoviesca spinosa* gen. nov. et sp. nov. **2, 3** holotype, specimen from both sides, UJMC 500 **4, 5** paratype 1, specimen from both sides, UJMC 501 **6** paratype 2, specimen with a 'corkscrew'-like morphology, UJMC 502 **7, 8** paratype 3, specimen from both sides, UJMC 503 **9** paratype 4, specimen with smooth whorls and a trumpet-like aperture, UJMC 504 **10, 11** paratype 5, specimen with smooth whorls, UJMC 505. Opercula **12, 13** paratype 5, specimen with smooth whorls, UJMC 505. Scale bar: 1 mm.

Types. Holotype (Figs 2, 3), UJMC 500, from type locality, leg. A. Czaja, 15/v/2019. Paratypes, UJMC 501-511, from same lot, >100 dry shells.

Etymology. Name is derived from the Latin word *spinosa* = having spines.

Referred material. Coahuila. Viesca, Spring "Túnel 7", UJMC 500-511, A. Czaja, J. L. Estrada-Rodríguez 10/vi/2015 and 15/v/2019.

Figures 14–17. SEM images of *Phreatoviesca spinosa* gen. nov. et sp. nov. **14** specimen with strong spines, UJMC 506 **15** specimen with ribs, UJMC 507 **16** specimen with smooth whorls, UJMC 508 **17** specimen with smooth whorls, UJMC 509. Scale bar: 1 mm.

Diagnosis. Like for the genus.

Description. Shell small, conical, white or colorless, sometime with rests of light brown periostracum, yielding diversity in shell form, with 4–5½ rounded whorls (usually 5), whorls increasing in radius, the first three whorls never uncoiled, subsequent whorls open coiled, body whorl always uncoiled, some specimens show a 'corkscrew'-like morphology (Figs 3, 6), suture deep; teleoconch sculptured with irregular, strong

marked growth lines and with ribs (Figs 4, 5), spiny shells with whorls with a peripheral slightly pronounced carinae, ribs at the carina are modified into regularly spaced shovel-shaped spines (Figs 14, 24), transition protoconch/teleoconch distinct, marked by a change in surface texture from pitted to axial growth lines, whorls rapidly increasing in diameter, first two whorls smooth, without carina or spines, the last three whorls with increasing number spines (up to 40 on the body whorl, but usually less than 30), spiral lines beginning at the end of protoconch, a few specimens with smooth whorls without any sculpture but with thickened axial growth lines, some (smooth) specimens with a varix just behind the aperture (Fig. 13), body whorl large, apertures large, ovate to subrounded, often trumpet-like (Fig. 9). Protoconch with coarsely honeycomb-like pits, the basal and outer lip rounded and thin, some smooth specimens with trumpet-like peristome, umbiculus deep or, in corkscrew-like specimens, almost without umbiculus; Opercula not preserved. ***Shell measurements*** (mean ± standard deviation in parentheses; *n* = 17): SH 2.08 (0.31) mm, SW 1.24 (0.17) mm, AH 0.79 (0.09) mm, AW 0.61 (0.08) mm, WN 4.93 (0.44) whorls; HBW 1.23 (0.21) mm. Paratypes from the type locality.

Measurements of holotype. WN 5¼ whorls; SH 2.26 mm; SW 1.41 mm; AH 0.86 mm; AW 0.67 mm, HBW 1.46 mm.

Habitat. The new species was found exclusively in one spring near Viesca, Coahuila. The original habitat was probably the outlet of a cave, were the species likely inhabited interstitial waters.

Distribution. A microendemic species, only in spring "Túnel 7", near the town of Viesca.

Remarks. The open coiled last whorl, shovel-shaped spines and a protoconch with coarsely honeycomb-like pits are the most evident characteristics which differentiated the shells of *Phreatoviesca* gen. nov. et. sp. nov. from shells of all other described stygobiotic gastropods in North America. We considered these shell features as derived characters (apomorphy) of a new clade, most likely within the family Cochliopidae. The SEM imagines of the two different morphotypes (smooth and spinous) from Viesca show that both have identical coarsely honeycomb-like pitted protoconchs (Figs 18, 19) and also the details of the shell wall microstructure with fine growth lines are similar (Figs 22, 23). Therefore, we consider these two morphotypes as belonging to the same species. There is no significant difference in shells measurements between smooth and spiny morphotypes and therefore sexual dimorphism is unlikely. Moreover, most of the shells have strong spines and only less than 5% of the morphotypes collected are smooth. Two morphotypes (one smooth and other with lamelliform costae) not associated with sexual dimorphism, were reported also from shells of the subterranean genus *Paludiscala* Taylor, 1966, described from the neighboring Cuatro Ciénegas Basin (Hershler, 1985). Interestingly, our material is conchologically similar to members of the stygobiotic and stygophilic genus *Pyrgophorus* Ancey, 1888 in Mexico, which show similar shovel-shaped spines (Grego et al. 2019). This resemblance is surely an evolutionary convergence and result from living in subterranean habitats.
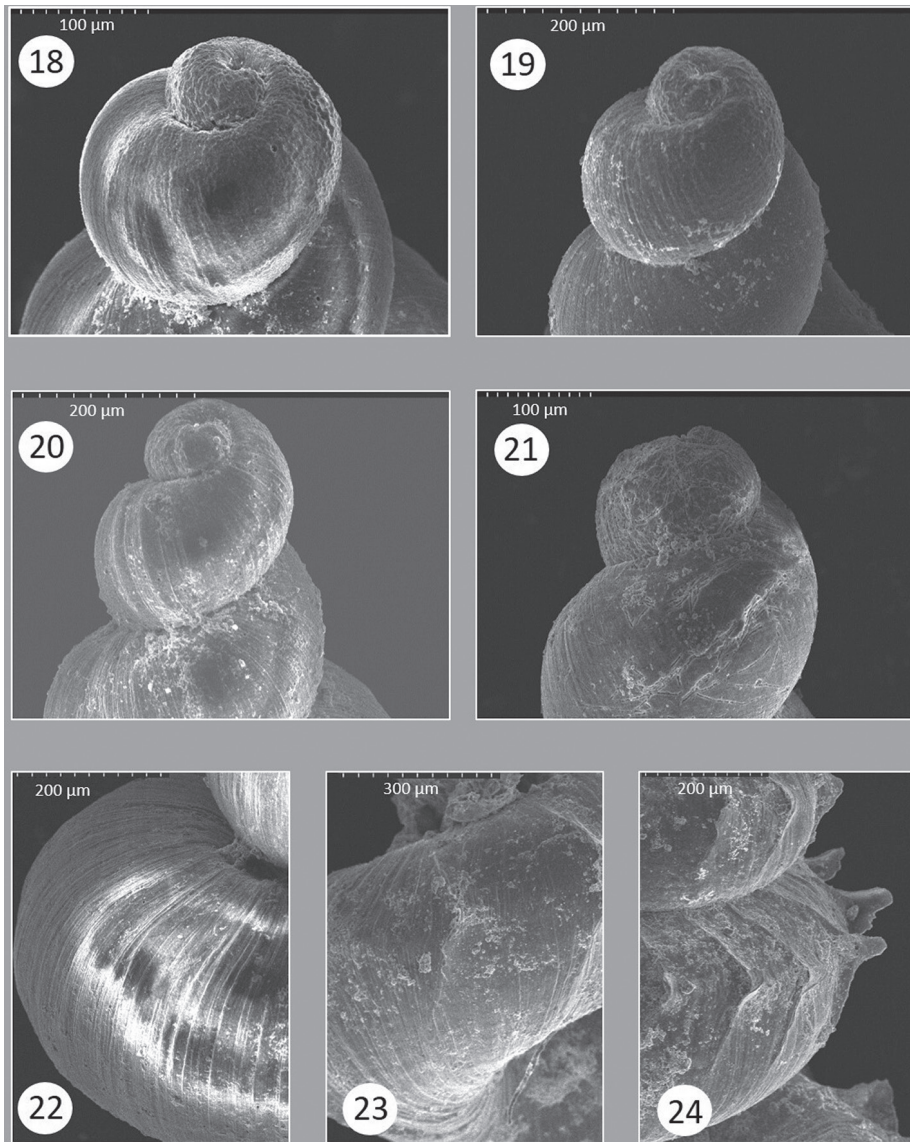
Figures 18–24. SEM images of *Phreatoviesca spinosa* gen. nov. et sp. nov. **18** shell apex with protoconch, UJMC 508 **19** paratype 3, shell apex with protoconch, UJMC 503 **20** shell apex with protoconch, UJMC 510 **21** paratype 1, apex with protoconch, UJMC 501 **22** smooth specimen with body whorl shows irregular, strong marked growth lines, UJMC 508 **23** paratype 1, body whorl shows irregular, strong marked growth lines, UJMC 501 **24** paratype 2, body whorl shows regularly spaced shovel-shaped spines, UJMC 502.

Discussion

Comparison with other North American stygobiotic gastropods

The general turriform shell shape of *Phreatoviesca spinosa* is common among other stygo-biotic cochliopids that occupy hyporheic and phreatic habitats in the Edwards Aquifer

(e.g., *Stygopyrgus bartonensis* Hershler & Longley, 1986; *Texapyrgus longleyi* Thompson & Hershler, 1991) or cave streams in the Appalachians (e.g., *Holsingeria unthanksensis* Hershler, 1989). Moreover, the large aperture and widely-reflected lip is also seen among hyporheic and phreatic taxa (e.g., *Phreatodrobia* species). However, the two primary structural differences not shared among any other stygobiotic gastropods in North America is the highly separated, uncoiled body whorl and the large spines on the teleoconch.

Regarding the open-coiling shell morphology, it seems as though *Phreatoviesca spinosa* is of an intermediate form compared to other open-coiling cochliopid stygobionts. In one case of minute open-coiling, Hershler and Longley describes the aperture of *Phreatodrobia nugax nugax* as "often free from [the] penultimate whorl", and several specimen photos from their study show *P. nugax nugax* with an open-coiled body whorl with accompanying lamelliform costae. However, *Phreatodrobia nugax nugax* shells can also appear trochoid to low conical and without costae (Hershler and Longley 1986). On the opposite side of the spectrum, *Phreatoceras taylori* (Hershler and Longley 1986) is completely uncoiled (trumpet-shaped). This suggests that an open-coiled shell morphology may be more common that previously understood for stygobiotic gastropods, and we may potentially discover more species with this feature through additional sampling efforts.

The prominent spine ornamentations of *Phreatoviesca spinosa* is not seen in any other North American stygobiotic gastropod species. The recently described species *Phreatodrobia spica* Perez & Alvear, 2020 is the only other stygobiotic gastropod species to have a 'spiny' teleoconch, but the spines on the shells of *Phreatodrobia spica* are considerably smaller and sporadically distributed across the shell (Alvear et al. 2020). The spine ornamentations of some *Pyrgophorus* species (e.g., *Pyrgophorus coronatus* (L. Pfeiffer, 1840)) show some similarities to *Phreatoviesca spinosa* regarding the structure and placement of spines along the whorls (Grego et al. 2019). However, there are still considerable differences of the spines among these two genera.

Open coiling

An openly coiled shell is a rather atypical character among gastropods, but occurs in both marine and continental (freshwater and terrestrial) groups across many independent lineages since the earlier Paleozoic (Rex and Boss 1976; Bandel & Frýda, 2004). Though far less prevalent compared to now extinct gastropod groups, open coiling is still seen among extant species (for a review of select extant species with open coiling, see Rex and Boss 1976). Many hypotheses have been generated regarding the adaptive significance of this open coiling, including that it is (but not limited to) a response to predator release (since the shell is structurally weaker and movement is more difficult; Vermeij 1987; Scholz and Glaubrecht 2010), high chemical stress (Nützel and Bandel 1993), sessility (Gould 1968), gerontic conditions (Yochelson 1971), or increased hybridization (Woodruff and Gould 1987). Notably, Rex and Boss (1976) also hypothesized that open coiling of shells that have spine ornamentations (such as the terrestrial species *Blaesospira echina* (Pfeiffer, 1864)) is a predator avoidance adaptation owing to the increased difficulty of any predator consuming the effectively larger, spiny shell. However, the predator release hypothesis is seemingly the most widely held throughout the literature (Vermeij and Covich 1978).

Several of these hypotheses were discussed in detail by Clements et al. (2008) when detailing the significance of the exaggerated open coiling of the terrestrial microgastropod *Opisthostoma vermiculum* (Architaenioglossa: Diplommatinidae), but none could be verified without further *in vivo* study. Liew and Schilthuizen (2014) performed *in vivo* predator-prey interaction studies for the terrestrial microgastropod genus *Plectostoma* (Diplommatinidae), and found their results suggested that open coiling may be an anti-predation adaptation that provides a less direct predation path when compared to a typical, tightly coiled gastropod (which counters predator release hypothesis).

Clearly the wide range of potential mechanisms that may drive open coiling makes narrowing down on any one a difficult task, and all of these hypotheses require much additional study (particularly *in vivo*) in order to be applied to a specific lineage. However, not all of these hypotheses seem plausible, and we believe that defining uncoiling or open coiling *a priori* as maladaptive (e.g., in response to chemical stress) should not be favored. It would be equally unfavorable to assume that it is a pathological phenomenon (Baynes et al. 2019) that occurs in few individuals within a population, or the sole product of ecophenotypic plasticity (e.g., Scholz and Glaubrecht 2010; Clewing et al. 2015). On the contrary, most of the uncoiled fossil and recent forms have been described as independent species with a robust number of collected specimens (Kase 1986; Scholz and Glaubrecht 2010; Alba et al. 2012).

Although we cannot validate any hypothesis with certainty for *Phreatoviesca spinosa*, we can confirm that open coiling seems to be a prevalent strategy among stygobiotic or stygophilic gastropods (Hershler 1985; Hershler and Longley 1986; Falniowski et al. 2021). Through additional sampling of the Viesca springs, we were able to successfully uncover a new openly coiled species, and we hope that these findings encourage additional sampling.

Conclusion

Phreatoviesca spinosa gen. nov. et sp. nov. is a new phreatic snail with remarkable shell characteristics such as prominent open coiling of the last whorl, shovel-shaped spine ornamentations on the teleoconch, and a coarsely honeycomb-like pitted protoconch structure. These morphological features are strikingly different compared to all known recent and fossil stygobiotic gastropods from North America. This newly described subterranean snail from Coahuila demonstrates that there continues to be great potential for discovering more stygobiont gastropods in these large unexplored karst regions in northern Mexico.

Acknowledgments

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RESEARCH ARTICLE

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Revalidation of the stygobiotic species *Haber zavreli* (Hrabě, 1942) (Clitellata, Naididae, Tubificinae) with discussion on the closely related species *Haber speciosus* (Hrabě, 1931) and *Haber monfalconensis* (Hrabě, 1966)

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Abstract

During studies of aquatic fauna in wells situated near Kraków (South Poland), many specimens of clitellates were found. The detailed description of the setal formula and genital organs of the collected individuals made it possible to distinguish *Haber zavreli* (Hrabě, 1942) from the related species: *H. speciosus* (Hrabě, 1931) including synonymized *H. simsi* and its forms known from the USA (*H. speciosus simsi* and *H. speciosus fluminialis*) and *H. monfalconensis* (Hrabě, 1966). In addition, remarks concerning the morphologically similar *Haber vetus* (Semernoy, 1982) described from Lake Baikal and the stygobiotic species *H. turquinae* (Juget & Lafont, 1979) are included.

Keywords

Oligochaete worms, southern Poland, stygobiont, wells

Introduction

The genus *Haber* Holmquist, 1978 was established by Holmquist (1978), as a result of a revision of *Peloscolex* Leidy, 1850, a species-rich genus and heterogeneous. Its definition was completed later and slightly modified by Milligan (1986). According to these authors, the genus *Haber* can be defined as follows:

- body wall without papillae and usually without adherent particles.
- modified spermathecal and penial setae of similar shape present in X and XI segments; each single seta is inserted in a conspicuous glandular sac (named also setal sac).
- smooth or hispid hairs and pectinate (or bifid) setae in dorsal bundles and bifid setae in ventral ones.
- male funnel fairly small, vas deferens long, in the majority of species with its distal part (ental – sensu Holmquist (1978, 1979) and Milligan (1986)) narrow and proximal (ectal) part 2–4 times broader; vas deferens opens apically to the tubular atrium.
- ectal region of the atrium modified into an ejaculatory duct.
- compact prostatic gland attached medially to the atrium.
- penial apparatus bulb-like, usually muscular; its internal canal lined with epithelium fitted with thick basal membrane resembling cuticular penial sheath, but true cuticular penial sheath absent.
- spermatheca with elongated ampulla and fairly short ectal duct, spermatozeugmata narrow, "worm-like".
- spermathecal pores paired, situated in different position in particular species.

Three taxa representing this genus were described by Hrabě (1931, 1942, 1966). The description of *Haber speciosus* (Hrabě, 1931) (originally *Tubifex speciosus*) was based on 12 individuals (8 mature) found in Lake Ochrida (Ohrid) at depths between 40–250 m. *Haber zavreli* (Hrabě, 1942) was described (as *Peloscolex zavreli*) from wells in the village Rajec (Slovakia) where numerous, mainly mature specimens were found. The last species, *H. monfalconensis* (Hrabě, 1966) was described as a subspecies of *H. speciosus* (originally *Tubifex speciosus monfalconensis*). The unknown number of specimens representing this taxon was collected in cave waters and in a spring in northeast Italy (Timavo region) (Hrabě 1966). Brinkhurst (1966) described very shortly *Haber simsi* (Brinkhurst, 1966) (as *Peloscolex simsi*) based on a single specimen from the diversion of the Frome River in Dorset (Great Britain). These four taxa were later synonymized by Brinkhurst and Jamieson (1971). Holmquist (1978, 1979), who re-examined the original materials of all three taxa described by Hrabě (*H. speciosus*, *H. zavreli* and *H. monfalconensis*) wrote: "there are several quite distinctive characters justifying a separation into the three species" and she redescribed them shortly (Holmquist 1979). According to her suggestion *H. monfalconensis* exclusively was assigned to the species status (Martin et al. 2017; Fauna Europaea 2021). *H. zavreli* was mentioned as an "independent" species only by Brinkhurst (1981), who indicated that its subspecific or specific rank has yet to be determined. In zoological lists it is still treated as a synonym of *H. speciosus* (Martin et al. 2017; Fauna Europaea 2021; WoRMS 2021).

This work aims to reassess the species status of *Haber zavreli* based on: 1) new material collected in a well near the Kraków city; 2) original descriptions and other literature data concerning related species: *H. speciosus* (including *H. simsi* and two forms from the USA: *H. speciosus simsi* and *H. speciosus fluminialis* (Milligan 1986) and

H. monfalconensis). In addition, taxonomical remarks on the morphologically similar epigean species *Haber vetus* (Semernoy, 1982) and the poorly described stygobiont *H. turquinae* (Juget & Lafont, 1979) will be made.

Material and methods

In 2016, studies on aquatic fauna were done in some wells dug in the cretaceous marls near Kraków (Southern Poland). Samples from the bottom of the wells were collected using an Ekman sampler, washed on 200 µm net mesh and fixed in 75% ethanol. The invertebrates were sorted under a stereoscopic microscope (magnification 10×). Whole specimens of clitellates were mounted in Canada balsam.

Biological material: 55 mature and 106 juvenile specimens were collected in one of the studied wells. 10 May 2016: 136 individuals (37 mature, 99 juv.), 2 August 2016: 25 individuals (18 mature, 7 juv.). Collected specimens were deposited in the Natural History Museum, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences in Kraków and in private Dumnicka's collection in the Institute of Nature Conservation, Polish Academy of Sciences in Kraków.

Locality: dug well in Prandocin Wysiółek village (50°15.100'N, 20°05.677'E in DDM system), 240 m asl, depth of the well – 8.1 m, bottom covered with muddy sediments. Physico-chemical parameters of water in particular dates: water temperature 10.3, 12.2 °C; pH 6.9, 7.0; conductivity 836, 826 µS; oxygen concentration 7.04, 6.72 mg O_2 L^{-1}; calcium 148.2, 167.7 mg L^{-1}; sulphates 136.3, 133.3 mg L^{-1}; nitrates 40.7, 38.6 mg L^{-1}; phosphates 0.017, 0.023 mg L^{-1}.

Results

Haber zavreli (Hrabĕ, 1942)

Peloscolex zavreli Hrabĕ 1942: 23–26 (description of species, type locality: Rajec u Žiliny, in wells);
Peloscolex speciosus (partim) (Brinkhurst and Jamieson 1971): 514–515;
*Peloscolex zavreli* (Kasprzak 1973): 421–422 (short description of two specimens found in the wells in the Beskidy Mts, Poland)
Haber zavreli (Holmquist 1979): 52–53 (redescription);
Peloscolex speciosus zavreli (Hrabĕ 1981): 87–88 (distribution, short taxonomic discussion);
Haber zavreli (Brinkhurst 1981): 1062 (citation)

Redescription. Almost all mature specimens without the posterior part of the body. Length of complete mature individuals: 10–11 mm, number of segments: 64–68. Body wall without papillae and without mucous cover, usually smooth, but in some specimens with tiny wrinkles (Fig. 1).
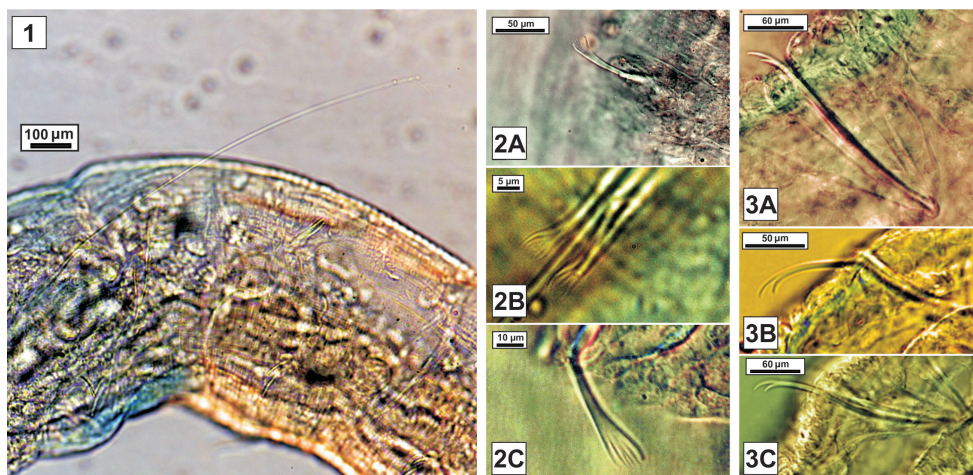
Figures 1–3. *Haber zavreli* (Hrabě, 1942) **1** fragment of the body with hair seta and tiny wrinkles seen on dorsal side **2** shape of dorsal pectinate setae: **2A** in V segment **2B** in VII segment **2C** in XV segment **3** shape of ventral setae: **3A** in IV segment **3B** in VIII segment **3C** in XV segment.
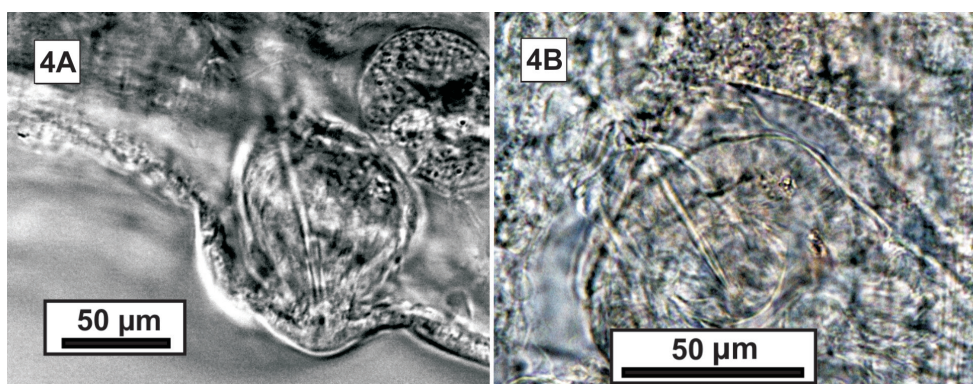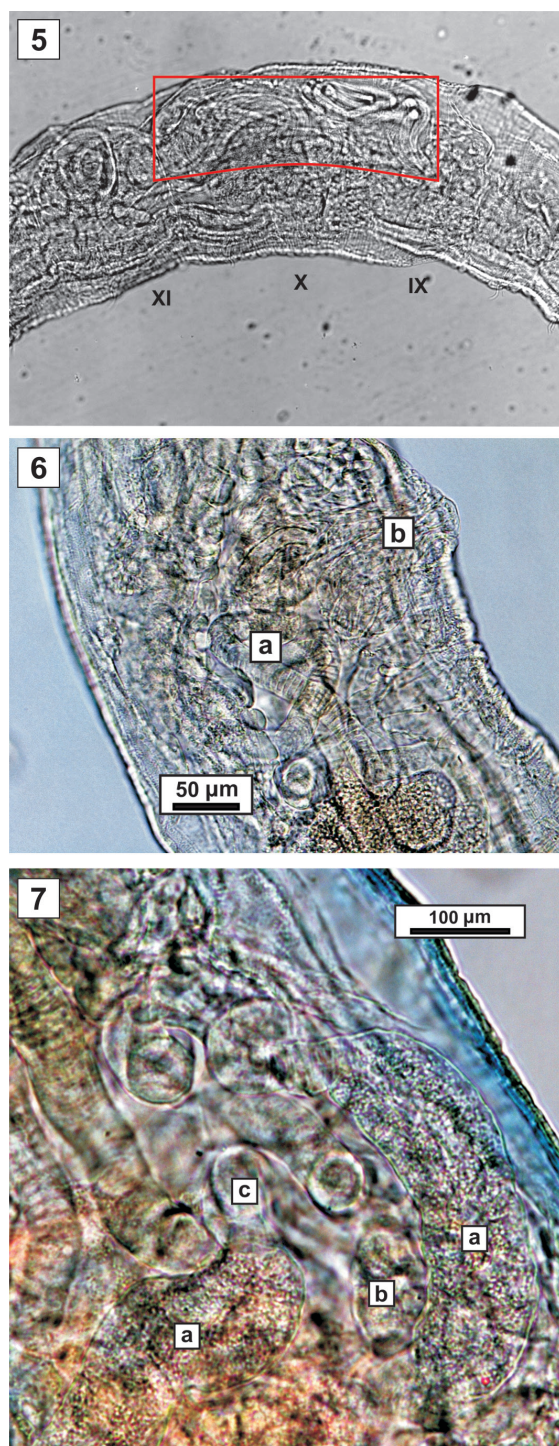

Figure 4. *Haber zavreli* (Hrabě, 1942) **A** spermathecal seta in glandular sac **B** penial seta in glandular sac.

Singular long and smooth hair seta (Fig. 1) in all dorsal bundles (exceptionally two setae in anterior segments). Pectinate setae with long teeth in all segments (Fig. 2A–C). The length of external and intermediate teeth is almost the same, but external ones are stouter. In anterior segments 5–7 intermediate teeth (Fig. 2A, B) and distal end of the setae shovel-shaped, in posterior segments it is goblet-shaped with 3–4 intermediate teeth (Fig. 2C). In the anterior dorsal bundles 1–2 setae, in the posterior segments – 1 seta. All ventral setae with the upper teeth longer than the lower (Fig. 3A–C): in anterior segments about two times longer (Fig. 3A), from segment VIII this difference is smaller (Fig. 3B, C). The singular modified spermathecal and penial seta is present respectively in segments X and XI. They are thin and sharp-ended with hollowed distal part (Fig. 4A, B) and they are inserted inside glandular sacs. The length of setae of mature individuals: hair setae up to 1000 μm long; dorsal anterior: 103–132 μm;

Figures 5–7. *Haber zavreli* (Hrabě, 1942) **5** Fragment of the body with spermathecal ampulla (marked by red frame) filled with long spermatozeugmata **6a** fragment of vas deferens **6b** penial apparatus **7** ectal part of male genital apparatus: **a** atria **b** prostate gland **c** ejaculatory duct.

posterior: 87–103 µm; ventral anterior: 230–243 µm; ventral posterior: 148–160 µm; spermathecal setae: 55–70 µm and penial setae 55–63 µm.

Long, irregular sac-like ampullae of spermathecae (Fig. 5) sometimes reach IX segment, short spermathecal duct set off suddenly from ampulla and its ectal opening occurs slightly dorsally from the line of spermathecal setae. The long, "worm-like" spermatozeugmata either fill whole ampulla or are concentrated in its ental part. Male funnel small, vas deferens very long, coiled (Fig. 6a) with distal part slightly thinner and shorter than proximal one. Proximal part of vas deferens narrower than tubular atrium (Fig. 7a) and enters to it apically. Prostate gland small and compact enters to atrium almost medially (Fig. 7b). Thin ejaculatory duct markedly sets off from the atrium (Fig. 7c). Penial apparatus elongated with two, well visible bulges – one with basal membrane (the so-called "penis sheath"), and the second one with penial seta (Fig. 6b).

Discussion

The body wall of the examined specimens is generally smooth. The fine, longitudinally arranged wrinkles (Fig. 8, left) originally described by Hrabě (1942) have not been observed. Holmquist (1979) reported the presence of fine ringlets in the post-clitellar part of the body and similar structures were present in some collected specimens, but thin cover of secretion was absent. Probably the presence or absence of fine wrinkles could be the result of different methods of material fixation.

According to original descriptions by Hrabě (1931, 1942, 1966), the shape of somatic setae is a good feature allowing to differentiate *H. zavreli* from *H. speciosus* and *H. monfalconensis* (Table 1). Of these three species, *H. zavreli* is the only one to have upper teeth of posterior ventral setae distinctly longer than lower ones and pectinate dorsal setae in all segments. The shape of ventral setae in collected specimens was almost identical to these on original Hrabě's illustrations (Fig. 8a–c) whereas anterior dorsal pectinates differed a little from that drown by Hrabě (1942) (Fig. 8d): in our specimens, the number of intermediate teeth was a little smaller than on Hrabě's picture, but the shape of the setal ectal tip was identical (shovel-shaped). For *H. speciosus* only the shape of anterior setae was shown by Hrabě (1931) (Fig. 9a, b), whereas in the original description of *H. monfalconensis*, there are no drawings of setae (Hrabě 1966). For this reason, a descriptive comparison of setal shapes is only possible for the three species mentioned above (Table 1).

Ventral setae of the *H. speciosus* forms described by Milligan (1986) from the USA (*H. speciosus simsi* and *H. speciosus fluminialis*) (Fig. 10a–d) are similar to these observed in the nominative European form, but dorsal setae (Fig. 10e–h), especially of *H. speciosus simsi* (Fig. 10e, f) differ a little: instead of bifid setae present in posterior segments of the nominative form, pectinate setae with a few thin intermediate teeth were reported (Milligan 1986). Nevertheless, the anatomy of genital organs confirms that the American forms belong to *H. speciosus*. Ventral anterior seta (Fig. 11a) of the specimen described as *H. simsi* by Brinkhurst (1966) and synonymized later with *H. speciosus* (Brinkhurst and Jamieson 1971) is typical for last-mentioned species,
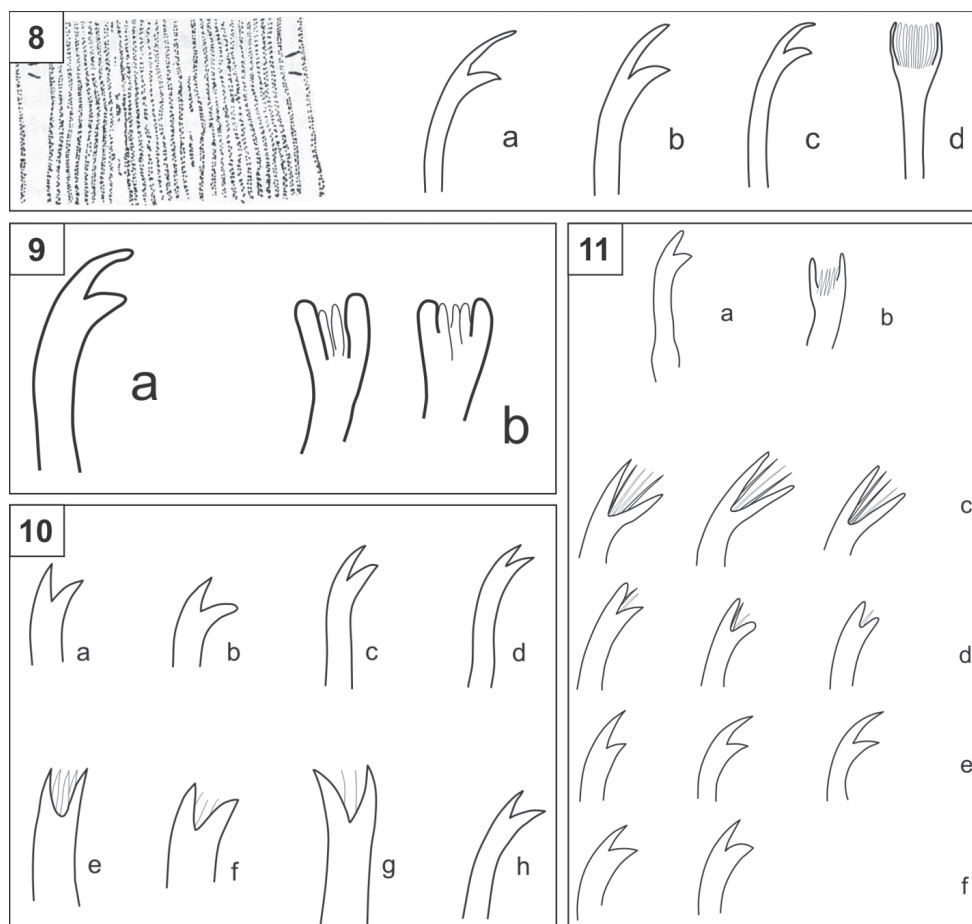
Figures 8–11. 8 *Haber zavreli* (Hrabě, 1942); Left: wrinkles on body surface; right: shape of the somatic setae **a** ectal part of ventral setae in segment III **b** in segment VIII **c** in posterior segments **d** anterior dorsal seta (after Hrabě 1942) **9** *Haber speciosus* (Hrabě, 1931); anterior somatic setae **a** ventral seta in segment II **b** dorsal setae (after Hrabě 1931) **10** somatic setae of *Haber speciosus* forms described by Milligan (1986) **a–d** ventral setae: *H. speciosus simsi* **a** anterior seta **b** posterior seta; *H. speciosus fluminialis* **c** anterior seta **d** posterior seta **e–h** dorsal setae: *H. speciosus simsi* **e** anterior seta **f** posterior seta; *H. speciosus fluminialis* **g** anterior seta **h** posterior seta (after Milligan 1986, modified) **11** *Haber simsi* (Brinkhurst, 1966) **a** anterior ventral seta **b** anterior dorsal seta (after Brinkhurst 1966) **c** anterior dorsal setae **d** posterior dorsal setae **e** anterior ventral setae **f** posterior ventral setae (after Bird and Ladle 1981).

whereas dorsal setae "seem to be intermediate to those of *P. speciosus* (…) and *P. zavreli*" (Brinkhurst 1966, p. 736) (Fig. 11b). The setae of specimens determined by Bird and Ladle (1981) as *H. simsi* (Fig. 11c–f) resemble those of *H. zavreli*, but due to the lack of full description of genital organs of these specimens, it is not possible to determine their taxonomic status. A distinctive feature of the genus *Haber* is the shape of genital setae – it is similar in all discussed species.

The main features of the genital organs which allow distinguishing between species attributed to the *Haber speciosus* group were described (Table 1) and illustrated

Table 1. Comparison of the setal formula and genital organs structure of three closely related species: *Haber speciosus* (Hrabě, 1931), *H. zavreli* (Hrabě, 1942) and *H. monfalconensis* (Hrabě, 1966) (after Hrabě 1931, 1942, 1966; Holmquist 1979, modified and complemented).

Species feature	*H. speciosus* (Hrabě, 1931)	*H. zavreli* (Hrabě, 1942)	*H. monfalconensis* (Hrabě, 1966)
No of hair setae: in anterior segments in posterior segments	2–3 1 (0)	1 (2) 1	1–2 1
Dorsal setae in anterior segments	1–3; lateral teeth obtuse, almost parallel, short, 2–3 intermediate teeth	1 (2); lateral teeth long acute, inter-mediate teeth slightly shorter, up to 8	1–2 (3); lateral teeth long, acute, upper tooth slightly longer, intermediate teeth fine
Dorsal setae in posterior segments	1; bifid	1 (2); pectinate, 3–4 intermediate teeth	1; bifid, teeth short, equal, upper thinner
Ventral setae in anterior segments	3–4; upper teeth slightly longer	2–3; upper teeth two times longer	3–4; upper teeth longer, from VIII teeth equal
Ventral setae in posterior segments	1–2; teeth equal (?)	2; upper teeth longer	3–2; teeth short, equal or upper slightly shorter
Localization of spermathecal pores	between the line of ventral and dorsal setae	near the line of ventral setae	in line of ventral setae
Spermathecal duct	comparatively long, set off gradually from ampulla	short, set off abruptly from ampulla	short, set off gradually from ampulla
Spermathecal ampulla	comparatively small, sac-like	well developed, long and irregular sac	comparatively small, sac-like
Male funnel	small	small	small
Vas deferens	long	long	long
Distal part of vas deferens	distinctly thinner and slightly shorter than proximal part	moderately thinner; length of both parts similar or proximal part a little longer	moderately thinner and markedly shorter than proximal part
Proximal part of vas deferens	broad, even wider than atrium	narrower than atrium	broad, about the same width as atrium
Ejaculatory duct	gradually set off from atrium	abruptly set off from atrium	gradually set off from atrium

(Fig. 12A–C) by Holmquist (1978, 1979). The most characteristic features for *H. za-vreli* are: (1) shape and dimension of spermathecal ampulla which is distinctly bigger than in two other species; (2) localisation of spermathecal pores near the line of ventral setae, but not in this line; (3) the ectal part of vas deferens narrower than atrium whereas in remaining species it is broader or has the same width as atrium and (4) non-gradual transition between atrium and ejaculatory duct.

According to Holmquist (1978) the construction of the penial apparatus is very specific in the *Haber* genus. The basal membrane lying in the internal canal of the penial bulb resembles cuticular penial sheath and this name was used in species descriptions by Hrabě (1931; 1942; 1966), Brinkhurst (1966), Juget and Lafont (1979) and the others.

According to original species descriptions (Hrabě 1931; 1942; 1966), cylindrical "penis sheath" is about 50 μm long in *H. speciosus*, about 67 μm in *H. zavreli* and it reaches up to 80 μm in *H. monfalconensis*. For *H. speciosus* the proportion between length and width of this structure differs in various papers (Fig. 13a–e): in the original description (Hrabě 1931) this proportion is about 2 : 1 (Fig. 13a) whereas in another paper by Hrabě (1966) it was reported to be about 4 : 1 (Fig. 13b). For *H. speciosus* described by Brinkhurst (1966) as *H. simsi* and American forms (*H. s. fluminialis* and *H. s. simsi*), these proportions fluctuate from 1.5 : 1 to 2 : 1 (Fig. 13c–e). Thickened basal membranes in *H. zavreli* (Hrabě 1942) and *H. monfalconensis* (Hrabě 1966) are elongated. The proportions between length and width reach 4 : 1 for *H. zavreli*
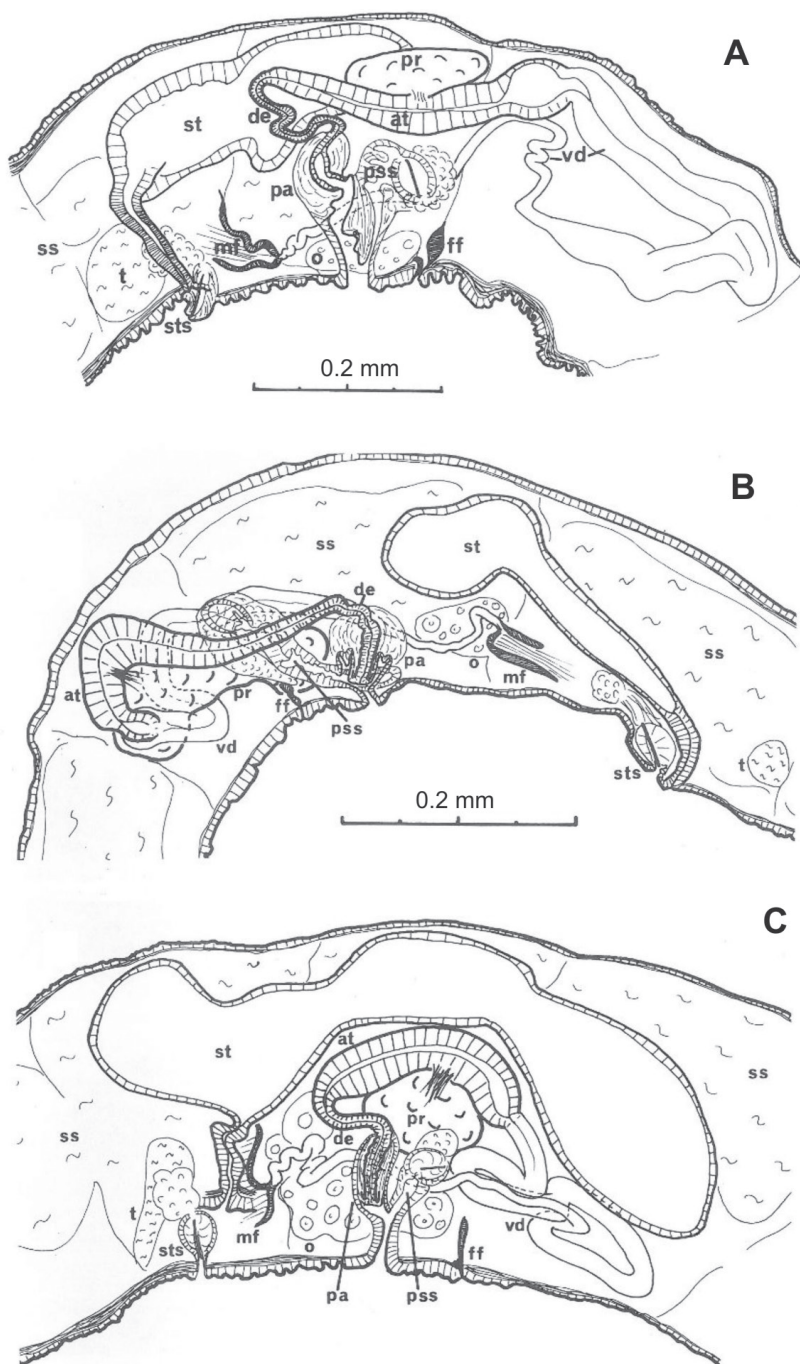
Figure 12. Reconstruction of the genital organs (from sagittal sections) **A** *Haber speciosus* (Hrabě, 1931) **B** *Haber monfalconensis* (Hrabě, 1966) **C** *Haber zavreli* (Hrabě, 1942). Abbreviations on the figure: at – atrium; de – ductus ejaculatorius; ff – femal funnel; mf – male funnel; o – ovary; pa – penial apparatus; pr – prostate gland; pss – penial setal sac; ss – sperm sac; st – spermatheca; sts – spermathecal seta; t – testis; vd – vas deferens. In original paper figure **C** without scale bar. (**A** after Holmquist 1978 **B, C** after Holmquist 1979).
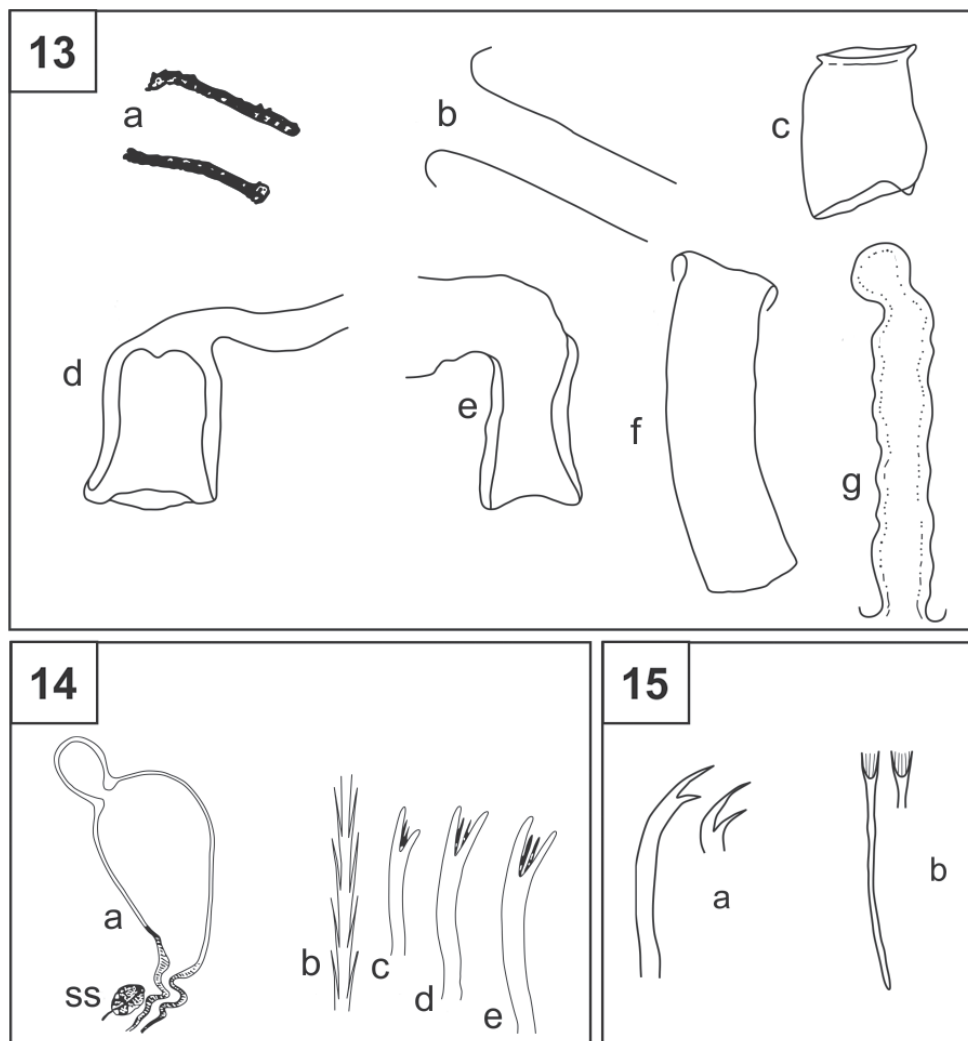
Figures 13–15. 13 Shape of thickened basal membrane **a** *H. speciosus* (after Hrabě 1931) **b** *H. speciosus speciosus* (after Hrabě 1966) **c** *H. speciosus* sensu Brinkhurst (1966) **d** *H.speciosus fluminialis* from USA (after Milligan 1986) **e** *H. speciosus simsi* (after Milligan 1986) **f** *H. zavreli* (after Hrabě 1942) **g** *H. monfalconensis* (after Hrabě 1966) **14** *Haber vetus* (Semernoy, 1982) **a** spermatheca **b** serrated hair seta **c–e** anterior dorsal setae **c** in segment II **d** in segment III **e** in segment V. Abbreviation on figure: ss – spermathecal seta (after Semernoy 1982, modified) **15** *Haber turquinae* (Juget & Lafont, 1979) shape of setae **a** anterior ventral setae **b** anterior dorsal setae (after Juget and Lafont 1979).

(Fig. 13f) and 6 : 1 for *H. monfalconensis* (Fig. 13g). On Holmquist's figures showing reconstructions of genital organs of species representing *H. speciosus* group the shape of the thickened basal membrane is not visible for nominative species (Fig. 12A) whereas their shapes and dimensions are very similar for *H. monfalconensis* and *H. zavreli* (Fig. 12B, C). All mature specimens collected by the authors have almost identical

shape and length of the basal membrane (see Fig. 6) as in the original figure (Fig. 13f) drawn by Hrabě (1942). It is possible that some differences of shape and length of thickened basal membrane depend on the method of material's preservation, which results in various degrees of its shrinking.

In accordance with Martin et al. (2017) and WoRMS the genus *Haber* comprises nine species. The majority of them could be easily distinguished from *H. zavreli* even by the shape of their setae, as illustrated by Milligan (1986) (table 2 in Milligan's paper). It seems that *Haber vetus* (Semernoy, 1982) – described as *Tubifex speciosus vetus* from Lake Baikal (Semernoy 1982) shares some morphological and anatomical features with *H. zavreli*. Both these species have ventral setae with longer upper tooth, the same localisation of spermathecal pores and irregular shape of spermathecal ampullae (Fig. 14a). In addition, both species have the ectal part of vas deferens narrower than atrium. Nevertheless, other features such as: (1) – serrated hair setae (Fig. 14b), (2) – dorsal setae with distinctly longer upper tooth in segment II (Fig. 14c) and with only two intermediate teeth (Fig. 14c–e), (3) – comparatively long ejaculatory duct and ectal duct of spermatheca (both set off gradually from atrium and ampulla, respectively) and (4) – long vas deferens having the same width along its whole length allow distinguishing sexually mature specimens of *H. vetus* from those of *H. zavreli*.

The morphology of anterior setae of the stygobiotic species *H. turquinae* (Fig. 15a, b) resembles that of *H. zavreli*. Although the genital organs of *H. turquinae* were not fully described, different shape of spermatheca, as well as different localisation of spermathecal openings, allow to distinguish these species. Moreover *H. turquinae* is significantly smaller (length 1.3–2.6 mm) than *H. zavreli* (10–12 mm) (Hrabě 1942 and our measurements).

A great part of species belonging to the genus *Haber* is known from restricted areas: *H. amurensis* (Sokolskaya & Hrabě, 1969) – from Far East (Hrabě 1969), *H. dojranensis* (Hrabě, 1958) – Greece and Macedonia, *H. hubsugulensis* (Semernoy, 1972) – Lake Hubsugul (Khuvsgul) in Mongolia (Semernoy and Tomilov 1972) and Lake Baikal (Snimščikova 1985), *H. pyrenaicus* (Juget & Giani, 1974) – France/Spain border, *H. svirenkoi* (Lastočkin, 1937) (or *H. swirenkoi* in WoRMS) – lower course of the Dnieper River (Ukraine), including its mouth (Finogenova 1972) and Black Sea (Hrabě 1973), *H. turquinae* (Juget & Lafont, 1979) – cave waters in the department Ain (France) and *H. vetus* (Semernoy, 1982) – Lake Baikal. All these species were rarely caught.

According to the literature, *H. speciosus* seems to be the only species with a wide distribution. In Europe, this species is mainly known from oligotrophic or mesotrophic water bodies of many countries, from Scandinavian Peninsula (Sloreid 1995; Erséus et al. 2005) to Turkey (Balik et al. 2004; Arslan et al. 2007). It was also found in running waters and tidal freshwater marsh in the eastern part of North America (Milligan 1986). Some data concerning the occurrence of *H. speciosus*, for example in Czech Republic (Schenkova et al. 2010) deal with *H. zavreli* (according to an earlier paper by Hrabě (1981)). Furthermore, up to now, specimens identified as *H. zavreli* have been found only in subterranean waters, either in Slovakia (Hrabě 1942; Šporka 2003), Italy (Dumnicka 1990) or in the Dinaric region (Giani et al. 2011; Martinez-Ansemil et al.

2016), which suggests that it is a stygobiotic species. The species was also mentioned in the checklist of Italian oligochaetes (beside *H. speciosus* and *H. monfalconensis*) (Paoletti and Sambugar 1996) and in the list of subterranean aquatic oligochaetes (des Châtelliers et al. 2009).

Two specimens, probably representing *H. zavreli* were previously found in Poland by Kasprzak (1973) in wells, but these specimens seem to be not fully mature – instead of typical modified penial seta, they had "in XI segment the seta similar to normal bifid somatic seta placed in big glandular sac". Moreover, the main features of genital organs were not observed, except for the "penial sheath".

On the basis of all the elements discussed above (including the detailed description of the setal formula and genital organs), *Haber zavreli* can be clearly distinguished from related species. We, therefore, feel justified to revalidate the species.

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