Subterranean Biology 38: 1–18 (2021)
doi: 10.3897/subtbiol.38.61733
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Three new subterranean species of *Baezia* (Curculionidae, Molytinae) for the Canary Islands

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Academic editor: P. Hlavac | Received 7 December 2020 | Accepted 24 January 2021 | Published 2 March 2021

http://zoobank.org/CBC7B4A6-4CEE-41D1-A667-9094CE8ACB3F

Citation: García R, Andújar C, Oromí P, Emerson B, López H (2021) Three new subterranean species of *Baezia* (Curculionidae, Molytinae) for the Canary Islands. Subterranean Biology 38: 1–18. https://doi.org/10.3897/subtbiol.38.61733

Abstract

The genus *Baezia* Alonso-Zarazaga & García, 1999 is endemic to the Canary Islands, where four species were known to date. Based on morphological evidence, three new species of *Baezia* are described in this study: *Baezia aranfaybo* García & López, **sp. nov.** from El Hierro island, and *Baezia madai* García & Oromí **sp. nov.** and *Baezia tizziri* García & Andújar, **sp. nov.** from La Palma island. Notes on their biology, habitat, and distribution are presented. The number of taxa in this endemic Canarian genus increases to seven eyeless species. One species has been reported from the soil (endogean environment), with the other six associated with caves and the mesovoid shallow substratum (hypogean or subterranean environment). Frequent association with the presence of roots suggests that species of *Baezia* may inhabit the continuum represented by the endogean and hypogean environments. Identification key to the seven species are provided.

Keywords

Canary Islands, Coleoptera, Curculionidae, identification key, lava tubes, mesovoid shallow substratum, new species

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Introduction

The volcanic terrains of the Canary Islands harbour a wide variety of subterranean environments (= hypogean *sensu* Giachino and Vailati 2010), most of them suitable for the establishment of fauna adapted to an underground lifestyle (Oromí 2004b). The small area of the Canary archipelago as a whole and its inherent fragmentation into islands had been considered a limiting factor for the establishment of a rich subterranean fauna (Leleup and Leleup 1970). However, these islands are rated as the richest volcanic region in troglobiont invertebrates worldwide, with more than 160 described species, followed by the Hawaiian Islands (80), Undara Cave in Australia (23), Azores (20), and Galapagos (14) (Peck and Finston 1993; Borges et al. 2012; Naranjo et al. 2020, and own unpublished data). Intensive systematic surveys of Canarian volcanic caves since the 1980's have greatly improved knowledge of the hypogean fauna of these islands (Oromí 2004a). In addition, in the last few years the number of known subterranean species has further increased in this archipelago due to studies on other non-cave subterranean environments, such as the **m**esovoid **s**hallow **s**ubstratum (henceforth referred as MSS; see Juberthie et al. 1980; Medina and Oromí 1990; Culver and Pipan 2009) and pyroclastic deposits (Oromí et al. 2018).

The richest group of Canarian troglobionts is Coleoptera, with 97 described and yet-undescribed species. Most of the subterranean beetle species are Curculionidae Latreille, 1802 (38% according to Oromí et al., in press). All Canarian weevils belonging to the tribe Typoderini Voss, 1965 in the subfamily Molytinae Schoenherr, 1823 have a subterranean lifestyle, and are classified into three genera: the non-endemic genus *Styphloderes* Wollaston, 1873, and the Canarian endemic genera *Oromia* Alonso-Zarazaga, 1987 and *Baezia* Alonso-Zarazaga & García, 1999. The genus *Styphloderes* is widely distributed in the Mediterranean and Macaronesian regions and is represented in the Canary Islands by the microphthalmic and endogean species *S. lindbergi* Roudier, 1963. *Baezia* and *Oromia* are Canarian endemic genera, with eyeless species living in lava tubes and the MSS, except for *Baezia litoralis* Alonso-Zarazaga & García, 1999, found under stones partly embedded in the soil in Tenerife (García et al. 2007). The genus *Baezia* is closely related to *Oromia* (Alonso-Zarazaga & García, 1999), however they are easy to be distinguished mainly because *Baezia* has: a smaller body size (2.5–3.8 mm) (4–6.4 in *Oromia*); a shorter and more robust rostrum (larger and narrower in *Oromia*); a rostrum dorsally strongly striated (dorsally strongly punctated in *Oromia*); a pronotum without longitudinal keels or with only a slight median keel (with obvious median and/or lateral longitudinal keels in *Oromia*); abdominal sternites I and II (hidden under metacoxae) not united to the metaventrite and the elytra (they are united to the metaventrite and the elytra in *Oromia*); metapleurosternal suture absent (present in *Oromia*).

The genus *Baezia* includes four species to date: *B. bimbache* García & López, 2007 (Fig. 1A) from El Hierro, *B. martini* García, 2002 (Fig. 1B) and *B. vulcania* Alonso-Zarazaga & García, 2002 (Fig. 1C) from La Palma, and *B. litoralis* (Fig. 1D) from Tenerife. All species of *Baezia* are probably rhizophagous, since in lava tubes they have been usually found on or inside roots hanging from the roof or lying on the ground.

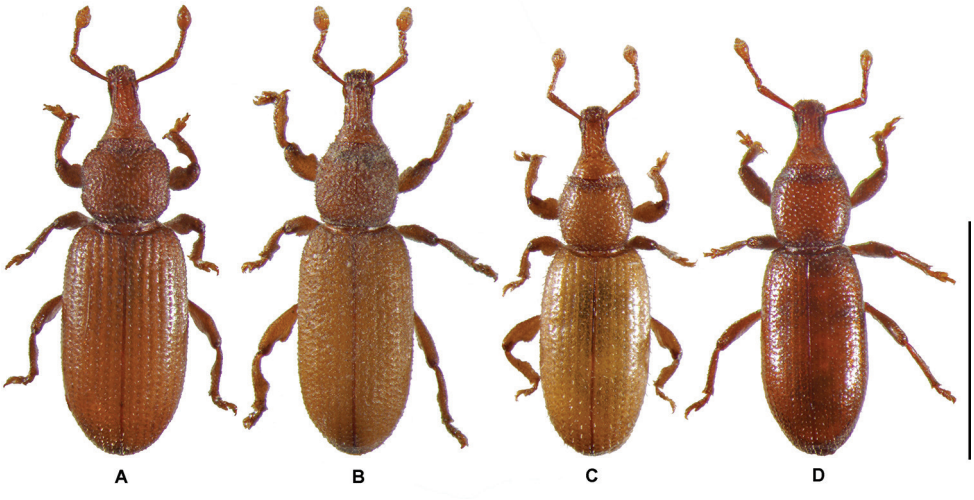

Figure 1. Dorsal habitus of **A** *Baezia bimbache* **B** *B. martini* **C** *B. vulcania* and **D** *B. litoralis*. Scale bar: 2 mm.

The presence of *Baezia* is frequently associated with the occurrence of roots in subterranean environments, suggesting that species of *Baezia* may inhabit in the continuum represented by the endogean and hypogean environments.

Our prospections since 2006 in lava tubes and MSS on El Hierro and La Palma have provided several individuals of two unknown *Baezia* species. Furthermore, on revising the Coleoptera collected in 1986 in a cave on La Palma, we detected one specimen of another unknown *Baezia* species. The purpose of the present paper is to describe these three new species and provide morphological identification keys to all seven known species of the genus.

Material and methods

Sampling and imaging

The specimens of these new species were collected using different methods. Specimens of *Baezia aranfaybo* sp. nov. from El Hierro emerged in the laboratory from dead roots collected in a cave during two different visits, and remains of six individuals were recently obtained by washing soil samples from inside the same cave. The single specimen of *Baezia madai* sp. nov. from La Palma was collected in a volcanic cave with pitfall traps baited with blue cheese, whereas those of *Baezia tizziri* sp. nov. derive from the MSS (mesovoid shallow substratum) on La Palma using subterranean traps similar to those designed by López and Oromí (2010). Despite of the intensification of samplings throughout the last decade to obtain a larger number of specimens, the results have been negative. The low number of available specimens does not compromise the

taxonomic validity of the new species, as there are clear morphological differences between these new species themselves, and with their already known congeneric species.

Soil residues adhering to the individuals were removed with a fine paintbrush and warm water with a little dish-washing liquid. Type specimens of *B. bimbache*, *B. vulcania*, *B. martini* and *B. litoralis* from the authors' collections were used for comparative morphological analysis. Examination, dissection, measurements, and drawings were completed with a Carl Zeiss Citoval 2 stereomicroscope fitted with an ocular micrometre. Photographs were taken under magnification using a Canon Powershot A650 attached to a Zeiss Stemi 2000 stereomicroscope or a Canon EOS 6D digital camera equipped with macro-lens MPE65. Photographs were processed with the program Zerene Stacker (V. 1.04, Zerene Systems, LLC., Richland, WA), combining them into a single image using pmax and dmap methods. The software Photoshop was used for final retouching.

Depositories

The material examined is deposited in the following collections:

DZUL Entomological collection of the Department of Animal Biology (Zoology), University of La Laguna, Tenerife, Canary Islands, Spain;

IPNA-CSIC Invertebrates collection of the Institute of Natural Products and Agrobiology (IPNA-CSIC), Tenerife, Canary Islands, Spain;

RGB Personal collection of Rafael García Becerra, La Palma, Canary Islands, Spain.

Results

Taxonomic acts

Class Insecta Linnaeus, 1758
Order Coleoptera Linnaeus, 1758
Superfamily Curculionoidea Latreille, 1802
Family Curculionidae Latreille, 1802
Subfamily Molytinae Schoenherr, 1823
Tribe Typoderini Voss, 1965
Genus *Baezia* Alonso-Zarazaga & García, 1999

***Baezia aranfaybo* García & López, sp. nov.**
http://zoobank.org/F66E1C68-E6F8-4A5D-A5CB-CA2FB98838C8
Figs 2A–H, 5I, J

Type locality. Spain, Canary Islands, El Hierro, Frontera: Cueva de Longueras (27°44'46.03"N, 18°1'32.04"W, 470 m a.s.l.).

Type material. ***Holotype*:** 1♂, El Hierro, Frontera, Cueva de Longueras (27°44'46.03"N, 18°1'32.04"W, 470 m a.s.l.), emerged from roots, 9 February 2011, code H680, H. López leg. (DZUL). ***Paratypes*:** same locality as for the holotype, 1♀, emerged from roots, 20 October 2006, P. Oromí leg. (IPNA-CSIC); 1♀, 20 August 2007, code H681, H. López leg. (RGB).

Other material. same locality as the holotype, remains of six individuals, washing samples of soil from inside the cave, 4 February 2020, H. López leg. (IPNA-CSIC).

Description. Male. Total length (including rostrum) 3.5 mm, 2.7 mm without rostrum and head, and maximum width 1.1 mm. Body bright reddish-brown (Fig. 2A); apex of rostrum, antennae and legs covered with scattered yellow-testaceous erect setae, and pronotum and elytra with short fine testaceous pilosity denser and aligned on whole surface. Apterous.

Head partially retracted into pronotum, microreticulated with abundant irregular punctation, lacking eyes.

Rostrum robust, similar in both sexes, widest at antennal insertion, 2.04× as long as wide at scrobes level, 0.75× as long as pronotum. In lateral view lower margin concave, and upper margin slightly convex, more declivous near apex; apex smooth, shiny, with testaceous erect setae. Scrobes deep, their apical third visible from above. Mandibles smooth and black. Rostrum slightly more depressed than forehead, with dorsal surface irregular with longitudinal sulci separated by fine keels; ventral surface smooth.

***Antennae*.** Scapes straight, increasingly widened towards ¼ of apex, 6.5× as long as its maximum width and 1.4× as long as funicule, covered with small erect setae. First funicular antennomere conical, 2.25× as long as wide, as long as next four antennomeres together; 2nd to 7th funicular antennomeres obconical, transverse. Club oval, 1.78× as long as wide and 1.3× as long as the last six funicular antennomeres.

Pronotum isodiametric with slight median keel, sides somewhat convex, constricted behind apex, with a slight sinuation at middle, anterior margin 0.93× as wide as posterior (Fig. 5J). Surface smooth and shiny, with traces of microreticulation around well-defined punctures; setae decumbent and scattered, little more erect towards edges.

Scutellum small, triangular.

Pterothorax with elytra elongate, lacking humeral calli; 2.6× as long as pronotum and 1.86× as long as wide, base wider than base of pronotum; maximum width at middle, basal margin 0.64× that width. Surface smooth and shiny; interstriae smooth; striae very fine, slightly defined by aligned punctures coinciding with small, erect setae. In lateral view, apical declivity somewhat pronounced, slightly projecting in peak.

Abdomen with integument surface shiny, slightly microreticulate; with fine, short setose pilosity; well-defined punctures separated by a distance of 0.6× to 1.0× of their diameter. First and second ventrites with wide median depression (as in all Typoderini); remaining ones slightly convex, a little but increasingly elevated towards apex, suddenly cut down at end, giving stair-like appearance. Ventrite 5 2.6× as wide as long, with strong punctation.

Legs moderately elongate, with shiny surface, microreticulate with abundant semierect setae. Procoxae separated by distance of 0.25× of their diameter. Mesocoxae separated by distance of 0.75× of their diameter. Femora slightly dilated at middle,

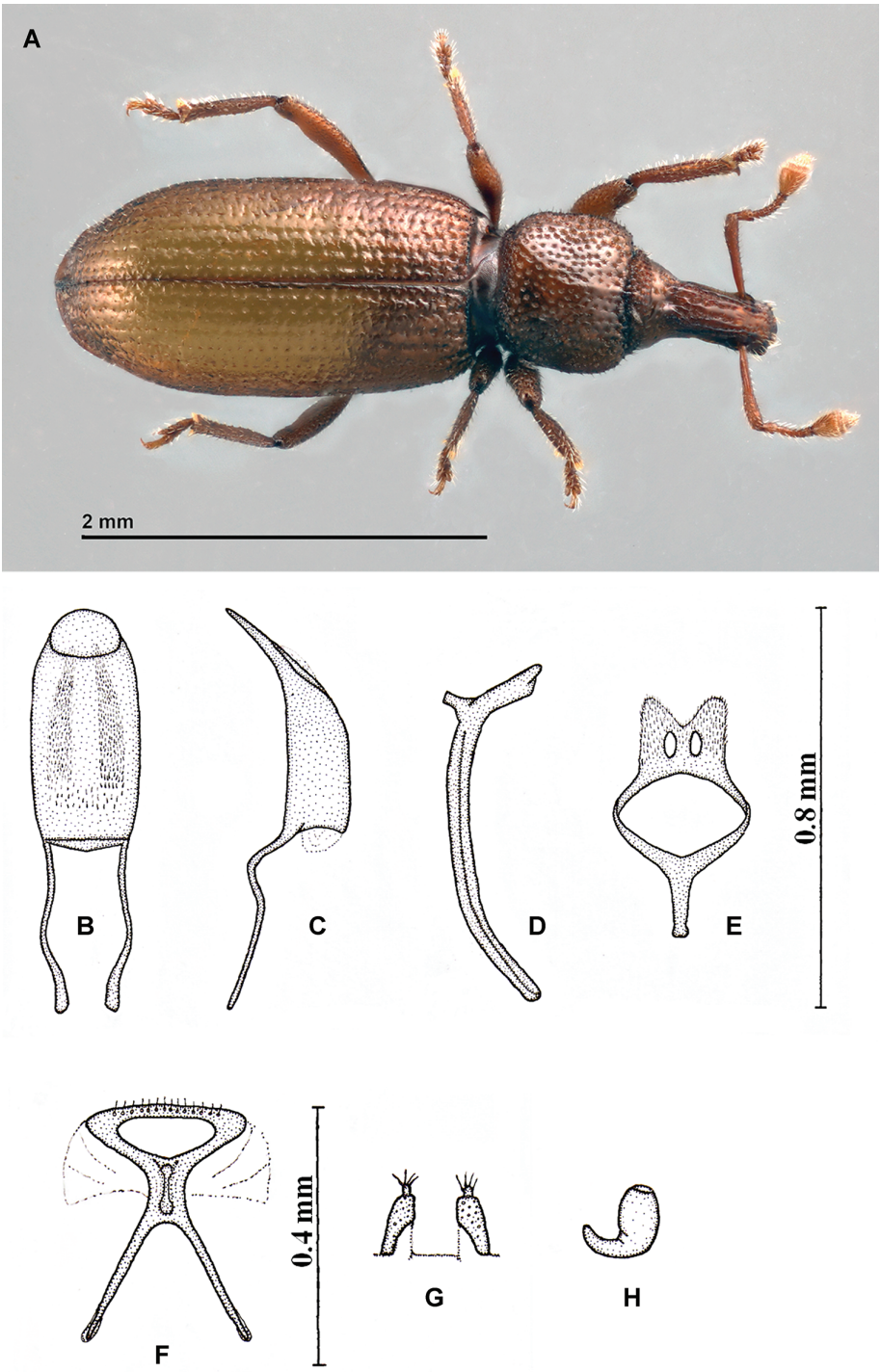

Figure 2. *Baezia aranfaybo* sp. nov. **A** dorsal habitus **B, C** aedeagus in dorsal and lateral view **D** *Spiculum gastrale* **E** tegmen **F** *Spiculum ventrale* **G** ovipositor **H** spermatheca.

strongly narrowed towards apex (Fig. 5I); pro-, meso- and metafemora 3.1×, 3.3× and 4.9× respectively as long as their maximum width. Pro- and mesotibiae straight, external edge slightly convex, with weak internal apical sinuation; metatibiae slightly concave on external edge (Fig. 5I); tibiae uncinate, apex with spiny short comb; pro-, meso- and metatibiae 5.37×, 5.7× and 6.25× respectively as long as their maximum width (excluding uncus). Protarsi with tarsomeres I 1.5×, II 0.8×, III 0.6× and V 3.3× respectively as long as wide, tarsomeres III clearly bilobed, onychium bearing two free simple claws; tarsal brushes with long sparse hyaline hairs.

Aedeagus. Median lobe dorsally almost symmetrical, with slightly convex sides and rounded apex (Fig. 2B); clearly curved in lateral view, with acute apex (Fig. 2C). Internal sac with abundant, densely arranged teeth and spicules in two elongated groups. *Spiculum gastrale* robust and bowed with highly asymmetric arms (Fig. 2D). Tegmen with short manubrium; quite wide, with two small transparent ovals and hairy parameroid lobes separated by a notch (Fig. 2E).

Female. Similar to male with slight sexual dimorphism. Total length 3.1 mm, maximum width 0.9 mm. Elytra 2.45× as long as pronotum, 1.74× longer than wide. 5th ventrite 1.9× as wide as long. Pro-, meso- and metafemora respectively 3.4×, 3.1× and 3.8× as long as wide. Pro-, meso- and metatibiae respectively 5.9×, 6.3× and 6.8× as long as wide.

Spiculum ventrale bearing about 16 macrosetae (Fig. 2F); manubrium with short median arm forking into two longer arms forming an acute angle. Ovipositor with free conical apical styles, bearing 7–8 apical macrochaetae; coxite with numerous sensilia (Fig. 2G); spermatheca with ramus and collum not developed, and hook shaped cornu (Fig. 2H).

Differential diagnosis. This new species is morphologically close to its allopatric species *Baezia bimbache* García & López, 2007, also from El Hierro. However, *B. aranfaybo* can be differentiated by its larger size and brighter body surface, proportionally longer antennae, longer scapes increasingly widened towards a ¼ of the apex, and the isodiametric pronotum (slightly transverse in *B. bimbache*). In addition, its elytra are proportionally longer with a pronounced apical declivity slightly projecting in peak in lateral view. The femora and tibiae are proportionally longer with less pronounced dilations on inner sides. Median lobe of the aedeagus with dorsally slightly convex sides (slightly diverging in *B. bimbache*) and rounded apex (slightly acute in *B. bimbache*), and less concave in lateral profile. In females, the *spiculum ventrale* has a larger manubrium and arms, these latter forming an acute angle (obtuse in *B. bimbache*).

Etymology. Specific name in apposition of Aranfaybo, considered by the Bimbaches (aboriginal people of El Hierro) as a sacred animal that lived in the cave Asteheyta (in the locality of Tacuytunta). This animal, with a pig-like appearance, was invoked as a magical intermediary to attract rains (Abreu 1848).

Habitat and distribution. This new species lives in Cueva de Longueras, a lava tube discovered in the 1980's on the northern slope of El Hierro island. It is located in a moderately old lava flow covered by thermo-sclerophyllous vegetation, which is partially degraded by long-abandoned agricultural activity in the locality. Despite its relatively short length (300 m), it offers good conditions for the subterranean fauna

due to its high humidity and stable low temperature during the whole year, as well as roots hanging from the ceiling at several cave parts (Oromí et al. 2001). The cave-adapted fauna found in this cave is composed of the pseudoscorpion *Paraliochthonius martini* Mahnert, 1989, an undescribed spider species probably of the genus *Robertus* (C. Ribera, pers. comm.), an undescribed species of the planthopper genus *Cixius*, the thread-legged bug *Collartida anophthalma* Español & Ribes, 1983, the cockroach *Loboptera ombriosa* Martín & Izquierdo, 1987, the rove beetle *Alevonota hierroensis* Assing & Wunderle, 2008, and the ground beetle *Trechus minioculatus* Machado, 1987. In the last 15 years, the cave has been visited and sampled with pitfall traps several times but no additional specimens of *B. aranfaybo* were collected. The three so far known specimens emerged from dead roots collected on three occasions, and stored in glass recipients in dark conditions. In 2020, we found the remains of six individuals by washing soil collected from the vicinity of roots inside the cave. Therefore, within the cave, this subterranean weevil seems to live associated with patches of roots that penetrate downwards from above-ground vegetation.

Baezia madai **García & Oromí, sp. nov.**
http://zoobank.org/C1BDDC6F-6737-407B-88FC-D76E45875C59
Figs 3A–E, 5E, F

Type locality. Spain, Canary Islands, La Palma, El Paso: Cueva de Las Tijaraferas, also named Cueva de Barros (28°39'43.89"N, 17°53'23.97"W, 536 m a.s.l.).

Type material. ***Holotype*:** 1♂, La Palma, El Paso, Cueva de Las Tijaraferas, also named Cueva de Barros (28°39'43.89"N, 17°53'23.97"W, 536 m a.s.l.), 10 July 1986, J.L. Martín leg. (DZUL).

Description. Male. Total length (including rostrum) 2.5 mm, 1.9 mm without rostrum and head, and maximum width 0.95 mm. Body matte reddish-brown (Fig. 3A); apex of rostrum, antennae and legs covered with scattered yellow-testaceous erect setae, pronotum and elytra with short fine testaceous and claviform pilosity denser and aligned on whole surface. Apterous.

Head partially retracted into pronotum, microreticulated with abundant irregular punctation, lacking eyes.

Rostrum robust, widest at antennal insertion, 2× as long as wide at scrobes level, 0.63× as long as pronotum. In lateral view lower margin concave, upper margin slightly convex, more declivous near apex; apex punctated, shiny, with erect setae. Scrobes deep, their apical third visible from above. Mandibles smooth and black. Rostrum slightly more depressed than forehead, with dorsal surface irregular with longitudinal sulci separated by five fine broken keels; ventral surface rough.

***Antennae*.** Scapes straight, increasingly widened from middle, 5.6× as long as its maximum width. The specimen lacks the rest of the antennae.

Pronotum slightly elongated with fine median keel, maximum width towards middle and sides slightly convex, constricted behind apex, with a slight sinuation at

middle; anterior margin as wide as posterior one (Fig. 5F). Surface matte chagrinated with microreticulation; punctures obvious, almost coalescent, setae lying down and scattered, little more erect towards margins.

Scutellum very small, triangular.

Pterothorax with elytra elongate, lacking humeral calli; 2.6× as long as pronotum, 1.84× as long as wide, base wider than base of pronotum; maximum width towards middle, basal margin 0.68× that width. Surface matte, chagrinated, strongly microreticulated; interstriae smooth; striae very fine, slightly defined by aligned punctures coinciding with small, erect setae.

Abdomen with integument surface slightly shiny, microreticulated; with fine, short setose pilosity; well-defined punctation. First and second ventrites with wide median depression; remaining ones slightly convex, a little but increasingly elevated towards apex, suddenly cut down at end, giving stair-like appearance. Ventrite 5 2.4 × as wide as long, strongly chagrinated, with deep punctation.

Legs elongate, with matte surface, microreticulate with abundant semierect setae. Procoxae separated by distance of 0.16× of their diameter. Mesocoxae separated by distance of 0.71× of their diameter, and metacoxae 1.63× of their diameter. Femora slightly dilated at middle, from middle they gradually narrow until they strangle near apex (Fig. 5E); pro-, meso- and metafemora 3.6×, 4× and 4.8× respectively as long as their maximum width. Tibiae straight, external edge slightly convex; internally with weak apical sinuosity and small bump towards middle (Fig. 5E); tibiae uncinate, with uncus provided of a sharp tip; pro-, meso- and metatibiae 4.75×, 4.75× and 6.66× respectively as long as their maximum width (excluding uncus). Protarsi with tarsomeres I 1.67×, II 0.76×, III 0.87× and V 2.5× as long as wide respectively, third one clearly bilobed, fifth bearing two free simple acute claws; tarsal sole brushes with long sparse hyaline hairs.

***Aedeagus*.** Median lobe almost symmetrical in dorsal view, sides slightly convex, apex rounded (Fig. 3B); clearly curved in lateral view, with acute apex (Fig. 3C). Internal sac with abundant teeth and spicules arranged in two elongated groups, with large acute teeth mixed with others smaller and with asperities; three basal sclerotized pieces. *Spiculum gastrale* robust and bowed with highly asymmetric arms (Fig. 3D). Tegmen with short manubrium; wide, hairy parameroids forming lobes, separated by a deep wide notch slightly more than half its length (Fig. 3E).

Female. Unknown.

Differential diagnosis. This new species is morphologically close to *B. vulcania.* However, *B. madai* can be differentiated by its smaller size, matte body surface and lesser and shorter pilosity; scape longer and increasingly widened towards middle; elongated pronotum, with sides almost straight (slightly convex in *B. vulcania*) and with a weak median keel (absent in *B. vulcania*). In addition, its elytra are proportionally longer, the femora and tibiae less dilated on inner side, the tibiae proportionally longer; the median lobe dorsally parallel-sided (sides gently rounded in *B. vulcania*) and acute apex (rounded and slightly prominent at middle in *B. vulcania*), with straighter profile, and the temones proportionally longer.

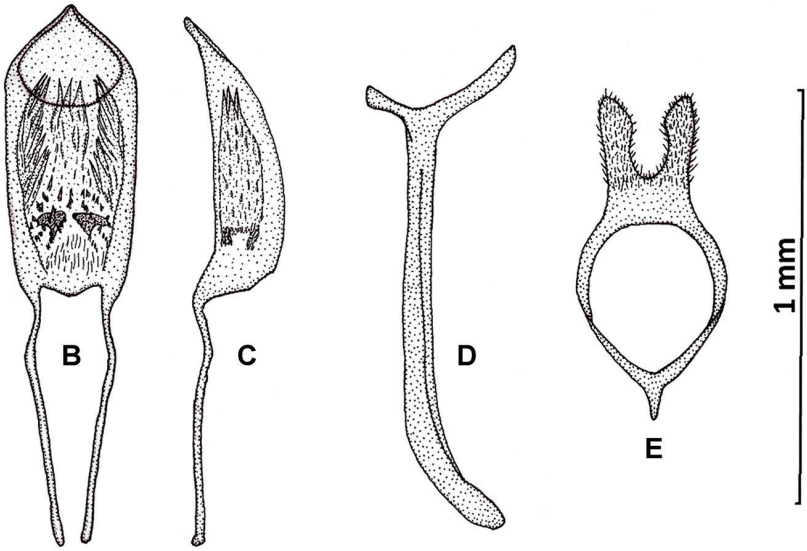

Figure 3. *Baezia madai* sp. nov. **A** dorsal habitus **B, C** aedeagus in dorsal and lateral view **D** *Spiculum gastrale* **E** tegmen.

Etymology. Specific name in apposition of Madai, a Guanche (Tenerife aboriginal) word meaning "deep" (Álvarez 1991), alluding to the habitat of this species.

Habitat and distribution. *Baezia madai* has only been collected in Cueva de Las Tijaraferas lava tube, despite systematic biospeleological surveys conducted in other

caves of the same area for many years (e.g. Fernández et al. 2007). The cave is located in the wall of a small ravine, in a place where the potential vegetation is thermo-sclerophyllous, but partially degraded and nowadays mainly replaced by *Amygdalus communis*, *Opuntia* sp., *Euphorbia lamarckii* and *Rumex lunaria*. In addition to its biological interest, this cave (only 63 m in length) holds many archaeological remains (pottery, bones, shells, etc.) that attest to its use as home by the prehispanic inhabitants of the island, the Auaritas. Inside this cave, there are several sections with high environmental humidity, roots hanging from the ceiling and walls, and soil covered with fine sediments. These points in the cave are the most suitable for underground fauna. Besides *Baezia madai* sp. nov., the presence of other troglobitic species has been confirmed, such as the sandhopper *Palmorchestia hypogaea* Stock & Martín, 1988, the cockroach *Loboptera teneguia* Izquierdo & Martín, 1999, and the ground beetles *Licinopsis angustula* Machado, 1987 and *Thalassophilus subterraneus* Machado, 1990. During the last decade, the authors have sampled the cave several times with pitfall traps and collected dead roots to remove weevil individuals from them, but without obtaining additional material beyond the only known specimen.

Baezia tizziri **García & Andújar sp. nov.**
http://zoobank.org/2FFAF945-071B-4EA5-BB0F-824BBB9C7FE4
Figs 4A–E, 5G, H

Type locality. Spain, Canary Islands, La Palma, Garafía: MSS Barranco de los Hombres (28°49'33.57"N, 17°52'07.95"W, 249 m a.s.l.).

Type material. ***Holotype*****:** 1♂, La Palma, Garafía: MSS Barranco de los Hombres (28°49'33.57"N, 17°52'07.95"W, 249 m a.s.l.), 20 September 2017, R. García leg. (DZUL). ***Paratypes*****:** same locality as the holotype, 1♂, 18 November 2020, R. García leg. (IPNA-CSIC).

Other material. Spain, Canary Islands, La Palma, Garafía, Cueva de La Fajana de Franceses (28°49'57.07"N, 17°51'56.89"W, 120 m s.n.m.), 23 January 2002, remains of elytra, R. García leg. (RGB).

Description. Male. Total length (including rostrum) 3.7 mm, 3 mm without rostrum and head, and maximum width 1.1 mm. Body matte reddish-brown (Fig. 4A); apex of rostrum, antennae and legs covered with scattered yellow-testaceous erect setae, and pronotum and elytra with short fine testaceous pilosity denser and aligned on the whole surface. Apterous.

Head partially retracted into pronotum, microreticulated with abundant irregular punctation, lacking eyes.

Rostrum robust, widest at antennal insertion, 2.08× as long as wide at scrobes level, 0.73× as long as pronotum. In lateral view lower margin concave, and upper margin slightly convex, more declivous near apex; apex smooth, shiny, with erect setae. Scrobes deep, their apical third visible from above. Mandibles smooth and black. Rostrum slightly more depressed than forehead, with dorsal surface irregular with longitudinal sulci separated by five fine keels; ventral surface smooth.

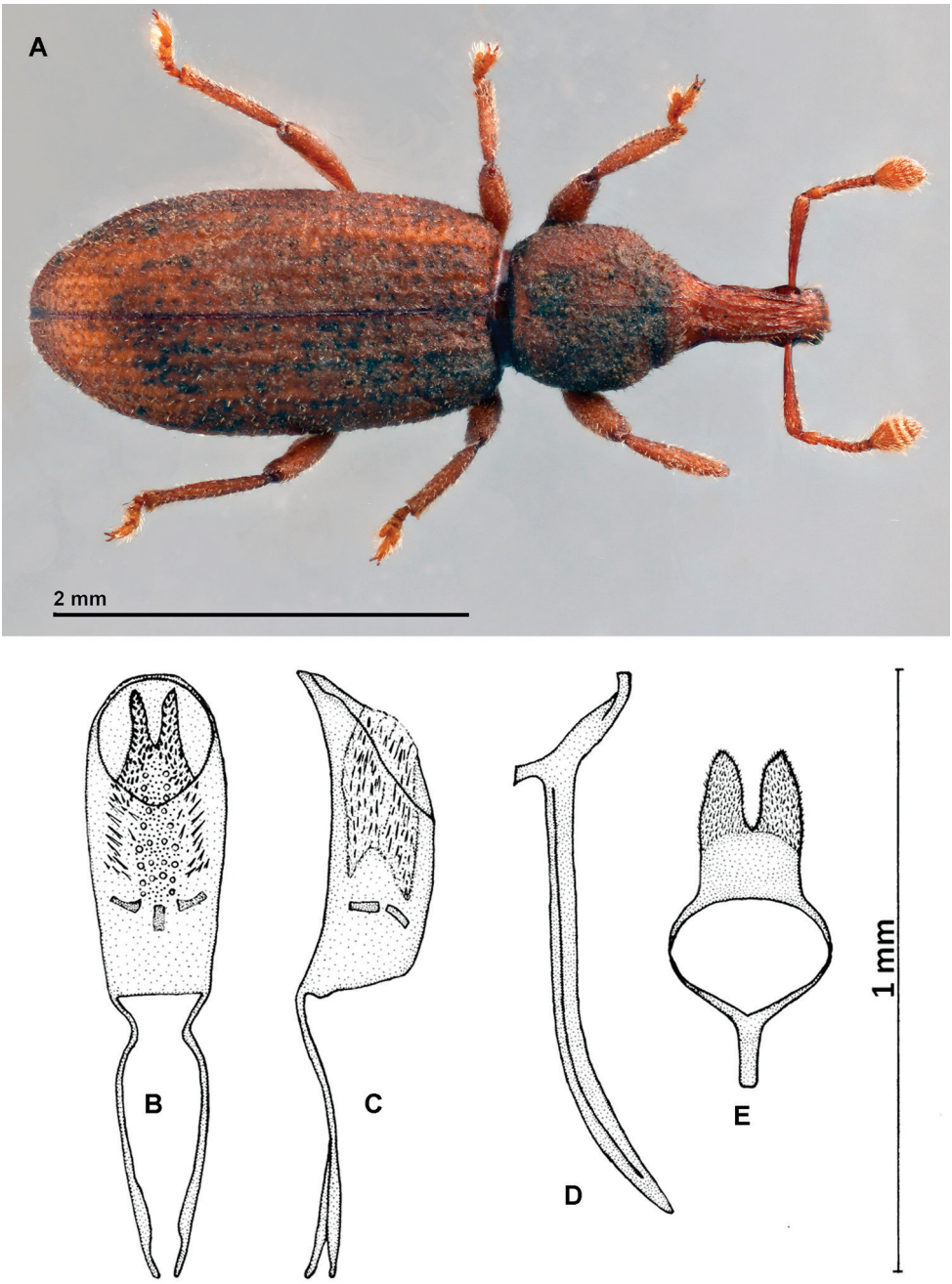

Figure 4. *Baezia tizziri* sp. nov. **A** dorsal habitus **B, C** aedeagus in dorsal and lateral view **D** *Spiculum gastrale* **E** tegmen.

Antennae. Scapes straight, increasingly widened towards ¼ of apex, 9.7× as long as its maximum width and 1.3× as long as funicule, covered with small erect setae. First funicular antennomere conical, 2× as long as wide, as long as next three antennomeres

together; 2nd to 7th funicular antennomeres obconical, transverse. Club oval, 1.6× as long as wide and 1.3× as long as the last six funicular antennomeres.

Pronotum slightly elongated with weak median keel, maximum width in the middle and sides somewhat convex, constricted behind apex, anterior margin 0.96× as wide as posterior (Fig. 5H). Surface matte chagrinated with microreticulation; punctures obvious, setae lying down and scattered, little more erect towards margins.

Scutellum very small, triangular.

Pterothorax with elytra elongate, lacking humeral calli; 2.4× as long as pronotum, 1.98× as long as wide, base wider than base of pronotum; maximum width in the middle, basal margin 0.63× that width. Surface matte, chagrinated, strongly microreticulated; interstriae smooth; striae very fine, weakly defined by aligned punctures coinciding with small, erect setae.

Abdomen with integument surface shiny, slightly chagrinated, microreticulated; with fine, short setose pilosity; no apparent punctation. First and second visible ventrites 1 and 2 with wide median depression; remaining ones slightly convex, elevated towards apex, giving stair-like appearance. Ventrite 5 2.1× as wide as long, strongly chagrinated.

Legs elongate, with matte surface, microreticulate with abundant semierect setae. Procoxae separated by distance of 0.11× of their diameter. Mesocoxae separated by distance of 0.62× of their diameter, and metacoxae 1.9× of their diameter. Femora not specially dilated at middle (Fig. 5G); pro-, meso- and metafemora respectively 3.6×, 3.5× and 5× as long as their maximum width. Tibiae straight (Fig. 5G); pro- and mesotibiae with external edge slightly convex, internally with a slight apical sinuation, and internal edge sinuate towards middle; metatibiae slightly concave on apical external edge, apical internal edge slightly denticulated; tibiae uncinate, uncus with wide blunt tip; pro-, meso- and metatibiae respectively 5×, 5.3× and 6.65× as long as their maximum width (excluding uncus). Protarsi with tarsomeres I 1.2×, II 0.66×, III 0.66× and V 2.5× as long as wide respectively, third clearly bilobed, fifth bearing two free simple blunt claws; tarsal sole brushes with long sparse hyaline hairs.

Aedeagus. Median lobe dorsally almost symmetrical, with sides slightly convex, apex acute (Fig. 4B); clearly curved in lateral view, with acute apex (Fig. 4C). Internal sac with abundant teeth and spicules arranged in three elongated groups, with big acute teeth mixed with others smaller and with asperities; two basal sclerotized pieces. *Spiculum gastrale* robust and bowed with highly asymmetric arms (Fig. 4D). Tegmen with short manubrium; wide, hairy parameroid lobes, separated by a deep fine notch almost than half its length (Fig. 4E).

Female. Unknown.

Differential diagnosis. This new species is morphologically close to *B. martini*. However, *B. tizziri* can be differentiated by its larger size and matter body surface (shinier and with larger and more abundant scales in *B. martini*); antennae proportionally longer, scapes longer and thickening to 1/4 of apex (thickening uniformly towards apex in *B. martini*); pronotum moderately elongated with slight median keel (transverse and without median keel in *B. martini*); elytra proportionally longer;

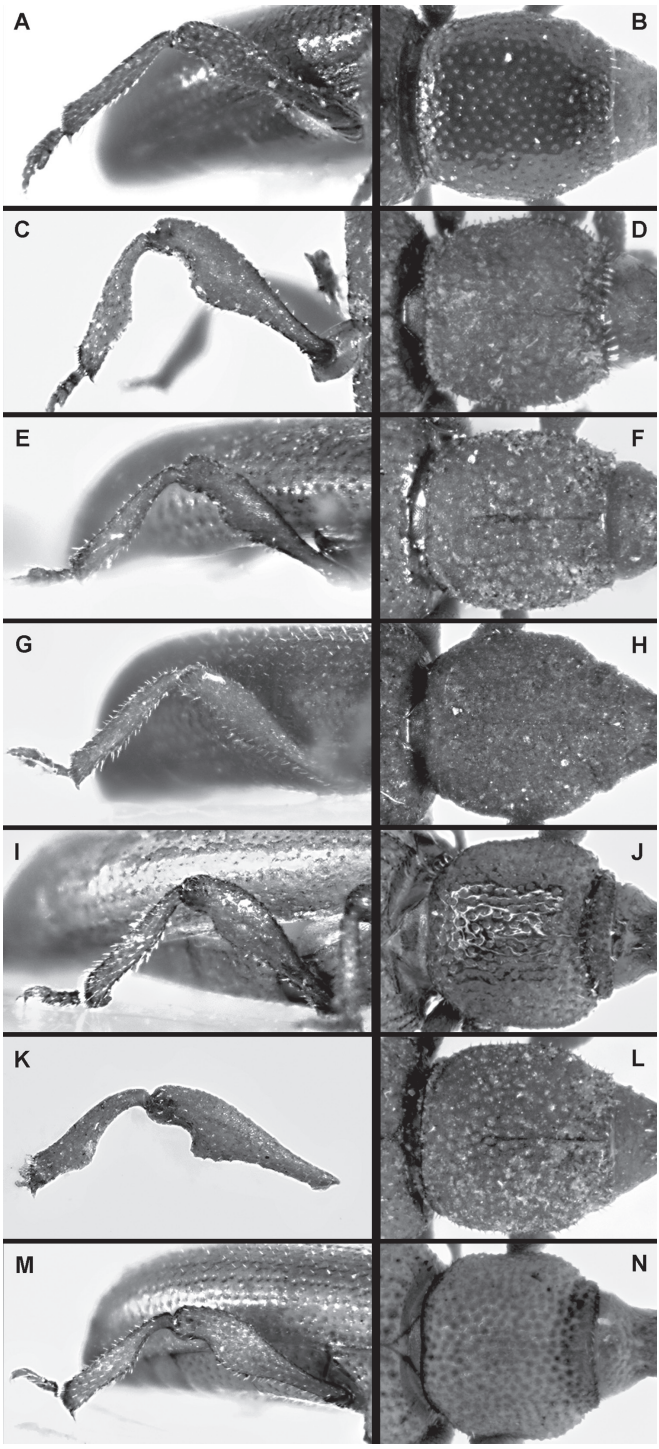

Figure 5. Hind leg and pronotum of **A, B** *Baezia litoralis* **C, D** *B. vulcania* **E, F** *B. madai* sp. nov. **G, H** *B. tizziri* sp. nov. **I, J** *B. aranfaybo* sp. nov. **K, L** *B. martini* **M, N** *B. bimbache*.

femora and tibiae proportionally longer and barely dilated on inner side (more dilated on inner side in *B. martini*); the penis, dorsally with slightly convex sides (almost straight in *B. martini*) and more rounded apex, more curved profile, and temones proportionally longer.

Note. the known specimens have blunt, thickened and blackened claws as well as uncus. These characteristics are not present in any other *Baezia* species.

Etymology. Specific name in apposition of Tizziri, Guanche (Tenerife aboriginal) word meaning "ray of light", related to the belief in your surroundings making you feel good and sleepy.

Habitat and distribution. The existence of this new species has been known since 2002 from the remains of elytra found in La Fajana de Franceses Cave. During the last 15 years, this and other caves in the same area have been actively studied with pitfall traps and by collecting dead roots, failing to obtain new individuals of this species. However, the two fresh specimens known of *B. tizziri* were collected in a close locality of the North of the island, in the MSS of the ravine Barranco de los Hombres, using subterranean traps similar to those designed by López and Oromí (2010). The ravine has well-preserved thermo-sclerophyllous vegetation, dominated by species including *Hypericum canariense*, *Apollonias barbujana* and *Bosea yervamora*, with some sparse exotic trees like *Persea americana*. In these traps, other subterranean species have been collected besides *B. tizziri*: the ground beetle *Licinopsis angustula*, the rove beetle *Domene benahoarensis* Oromí & Martín, 1990, and unidentified specimens of the cockroach genus *Loboptera* and the rove beetle genus *Medon*.

Key to the species of *Baezia*

1 Inner side of metafemora and metatibiae not dilated at middle (Fig. 5A, G)....**2**
– Inner side of metafemora and metatibiae dilated at middle (Fig. 5C, E, I, K, M) **3**
2 Pronotum not constricted at apex (Fig. 5B) .....***B. litoralis*** **(Tenerife)**
– Pronotum constricted at apex (Fig. 5H)..... ***B. tizziri*** **sp. nov. (La Palma)**
3 Setae of pronotum with clavate or flaming apex....**4**
– Setae of pronotum with acute apex **5**
4 Pronotum transverse (Fig. 5D), shiny and with punctures separated by the distance of their diameter. Pilosity length 0.05 mm .....***B. vulcania*** **(La Palma)**
– Pronotum elongated (Fig. 5F), matte and with larger and almost coalescent punctation. Pilosity length 0.025 mm, less abundant.....
.....***B. madai*** **sp. nov. (La Palma)**
5 Pronotum subquadrate, sides converging to the posterior margin (not clearly parallel-sided) (Fig. 5J). Metafemora and metatibiae slightly dilated (Fig. 5I)....
.....***B. aranfaybo*** **sp. nov. (El Hierro)**
– Pronotum subquadrate, straight sides and almost parallel-sided. Metafemora and metatibiae strongly dilated **6**

6 Inner side of metafemora strongly dilated and very suddenly narrowed at apical third (Fig. 5K). Pronotum as long as wide (Fig. 5L). Integuments very microreticulated and chagrinated. *Spiculum ventrale* of females with a large manubrium, forking at the end with inverted T-shaped arms..... .....***B. martini*** **(La Palma)**

– Inner side of metafemora dilated but not suddenly narrowed (Fig. 5M). Pronotum transverse (Fig. 5N). Integuments shiny. *Spiculum ventrale* of females with a manubrium forking directly from the plate into two arms with inverted V–shaped .....***B. bimbache*** **(El Hierro)**

Concluding remarks

The differences suggested by Alonso-Zarazaga and García (1999) to diferenciate *Baezia* and *Oromia* have been clear until now to assign them to two independient genera. However, the new species described during last years (Machado and López 2015; García et al. 2020; present work) show that some morphological characters have a wider variability than initially observed. The female spiculum ventrale is an example of this morphological variability, since the shape of manubrium and its arms show different morphologies which are present in both genera. The *Baezia* species from El Hierro have a manubrium forking into two arms directly from the plate, or after a short median arm, being these morphologies shared with the different *Oromia* species. Similar situations are present in other morphological structures, but their taxonomical importance is not clear and difficult to evaluate at the moment. Further ongoing studies, including molecular genetic data, will help to clarify the nature of this variation, testing whether the biological entities diagnosable by fixed genetic differences are coincident with the current taxonomy, and exploring phylogenetic relationships to understand the patterns of morphological differenciation.

Acknowledgements

We are indebted to Besay Franchy Gil and Javier García Pérez for their help in the fieldwork on La Palma for several years in trying to collect specimens of *Baezia*. Antonio Machado kindly provided the photographs of the specimens in Figures 2–4. The English of this manuscript was edited by Guido Jones, currently funded by the Cabildo de Tenerife under the TFinnova Programme supported by MEDI and FDCAN funds. Suggestions of the reviewers Roman Borovec and Jiří Skuhrovec, and of the editor Peter Hlaváč have improved and clarified several points of the article. We thank the Cabildo de La Palma and Cabildo de El Hierro for permits for several years to study the invertebrate fauna. This study was partly supported by the Spanish Ministry of Science (MINECO) (CGL2015-74178-JIN and CGL2017-85718-P).

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Subterranean Biology 38: 19–45 (2021)
doi: 10.3897/subtbiol.38.61787
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Stygobromus bakeri, a new species of groundwater amphipod (Amphipoda, Crangonyctidae) associated with the Trinity and Edwards aquifers of central Texas, USA

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Academic editor: Thomas Sawicki | Received 8 December 2020 | Accepted 2 March 2021 | Published 12 April 2021

http://zoobank.org/695318A5-5399-48D9-BC92-9C846F7D397E

Citation: Gibson R, Hutchins BT, Krejca JK, Diaz PH, Sprouse PS (2021) *Stygobromus bakeri*, a new species of groundwater amphipod (Amphipoda, Crangonyctidae) associated with the Trinity and Edwards aquifers of central Texas, USA. Subterranean Biology 38: 19–45. https://doi.org/10.3897/subtbiol.38.61787

Abstract

A new stygobitic groundwater amphipod species, *Stygobromus bakeri* **sp. nov.**, is described from 4 central Texas limestone karst springs; John Knox Spring (Comal County), Jacob's Well (Hays County), Mormon Spring (Travis County) and Salado Springs (Bell County). This species belongs to the predominately western Nearctic *hubbsi* species group of *Stygobromus* and differs from other species in the group by gnathopod 1 with row of 4 to 5 setae posterior to the defining angle, pereopods 6 and 7 with broadly expanded bases and distinct distoposterior lobes, gnathopods 1 and 2 with 2 rows of 3 singly inserted setae on the inner palm, and uropod 3 with single, slightly distal peduncular seta. Habitat, sympatric groundwater species, and conservation issues are discussed.

Keywords

cave, crenobiont, interstitial crustacean, karst, spring, stygobiont, stygobite, subterranean, sympatric, troglomorphy

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Introduction

The Nearctic amphipod genus *Stygobromus* Cope, 1872 (Amphipoda, Crangonyctidae) currently includes 137 described species and 4 subspecies in the United States and Canada (Hay 1882; Hubricht 1943; Derzhavin 1945; Holsinger 1967; Holsinger 1969; Holsinger 1974; Holsinger 1978; Wang and Holsinger 2001; Holsinger 2009; Sidorov et al. 2010; Holsinger et al. 2011; Taylor and Holsinger 2011; Holsinger and Ansell 2014; Holsinger and Sawicki 2016; Cannizzaro et al. 2019; Palatov and Marin 2020). The genus is entirely stygomorphic, and, as with most groundwater-obligate taxa, low detection probability (a function of inaccessible habitat and low population densities), coupled with pervasive convergent evolution (Eberhard et al. 2009; Culver and Pipan 2015; Devitt 2019) results in an incomplete understanding of diversity and systematics within the genus. Many species, cryptic and otherwise, await description. Holsinger (1974) and Wang and Holsinger (2001) provisionally assigned 45 of 54 currently recognized taxa in the western United States and southwestern Canada to the *hubbsi* group. Although molecular data supporting systematic relationships are lacking for most taxa (but see Ethridge et al. 2013; Aunins et al. 2016; Cannizzaro et al. 2019), the *hubbsi* group is supported morphologically by lack of sternal gills on the pereonites and a combination of additional characters summarized by Wang and Holsinger (2001). The distribution of *Stygobromus* in Texas is mapped in Figure 1. In the Edwards Plateau and Balcones Escarpment of central Texas, USA, 9 *Stygobromus* species are documented alongside diverse assemblages of stygobiontic crustaceans (Holsinger 1967; Gibson et al. 2008; Hutchins 2018; Nissen et al. 2018). Four central Texas *Stygobromus* species belong to the *tenuis* group: *S. balconis* (Hulbricht, 1943), *S. reddelli* (Holsinger, 1966), *S. russelli* (Holsinger, 1967), and *S. bifurcatus* (Holsinger, 1967). Four comprise the central Texas endemic *flagellatus* group: *S. flagellatus* (Benedict, 1896), *S. longipes* (Holsinger, 1966), *S. dejectus* (Holsinger, 1967), and *S. pecki* (Holsinger, 1967). *Stygobromus hadenoecus* (Holsinger, 1966) is the sole described member of the *hadenoecus* group found in the western Edwards Plateau. The nearest *hubbsi* group species, *S. limbus* Wang & Holsinger, 2001, occurs more than 300 km away from the Edwards Plateau taxa in a southeastern extension of the Basin and Range Province of the western United States in far west Texas. Huston and Gibson (2018) reported an undescribed species in the *hubbsi* group from a single spring site in the Chisos Basin of Big Bend National Park, Texas, also within the Basin and Range Province. Only a single *hubbsi* group species, *S. putealis* (Holmes, 1909) from wells in Wisconsin, is currently known from east of North America's western Cordillera (Wang and Holsinger 2001).

Stygobromus species of the *hubbsi* group are recorded in a variety of groundwater habitats including caves, wells, the hyporheic zone of surface streams, karstic and non-karstic springs, and hypotelminorheic seeps. Two species live in the depths (5–495 m) of Lake Tahoe (California and Nevada) although Wang and Holsinger (2001) hypothesized that their preferred habitat may actually be adjacent groundwater.

Many groundwater habitats and groundwater-obligate organisms are at risk from a myriad of threats, including groundwater contamination, physical habitat destruction

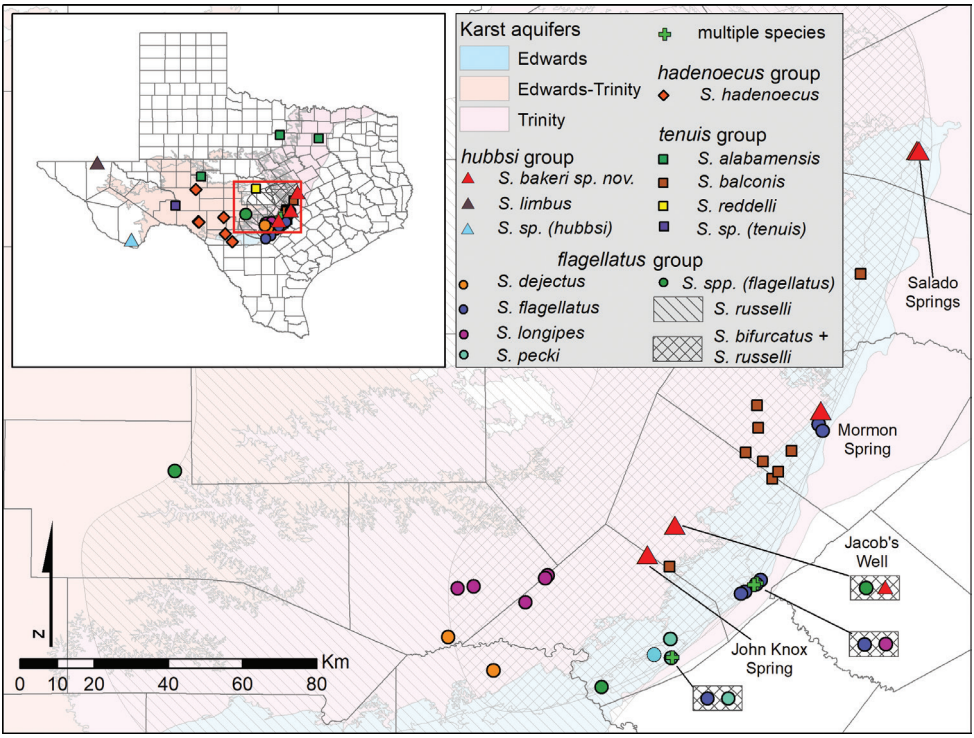

Figure 1. *Stygobromus* distribution in Texas. Red square expanded to view central Texas species. Individual undescribed species represented by *S.* sp. and *S.* spp. (*flagellatus*) refers to several undescribed site endemic species in the *flagellatus* group (Hutchins 2018).

(e.g. gravel mining), and groundwater extraction (Burri et al. 2019; de Graaf et al. 2019), lending urgency to continued documentation of groundwater biodiversity. Here, we describe the 12th species of *Stygobromus* from Texas, USA and the first species belonging to the *hubbsi* group in the Edwards Plateau/Balcones Escarpment region, an area where groundwater extraction to meet the demand of a rapidly growing human population along the Interstate 35 highway corridor increasingly threatens groundwater resources.

Materials and methods

Specimens were collected from karst springs using 150–250 µm mesh drift nets and using multiple techniques at an underwater cave, Jacob's Well (Fig. 2A) accessed via the resurgence entrance using SCUBA (Fig. 2B, C). Divers performed surveys at Jacob's Well, Hays County on 20 occasions from May 2009 to June 2011, using visual searches, a drift net placed at a vertical constriction at -25 m depth and 42 m penetration, cloth lures secured to the side of the cave shaft near the entrance, and baited bot-

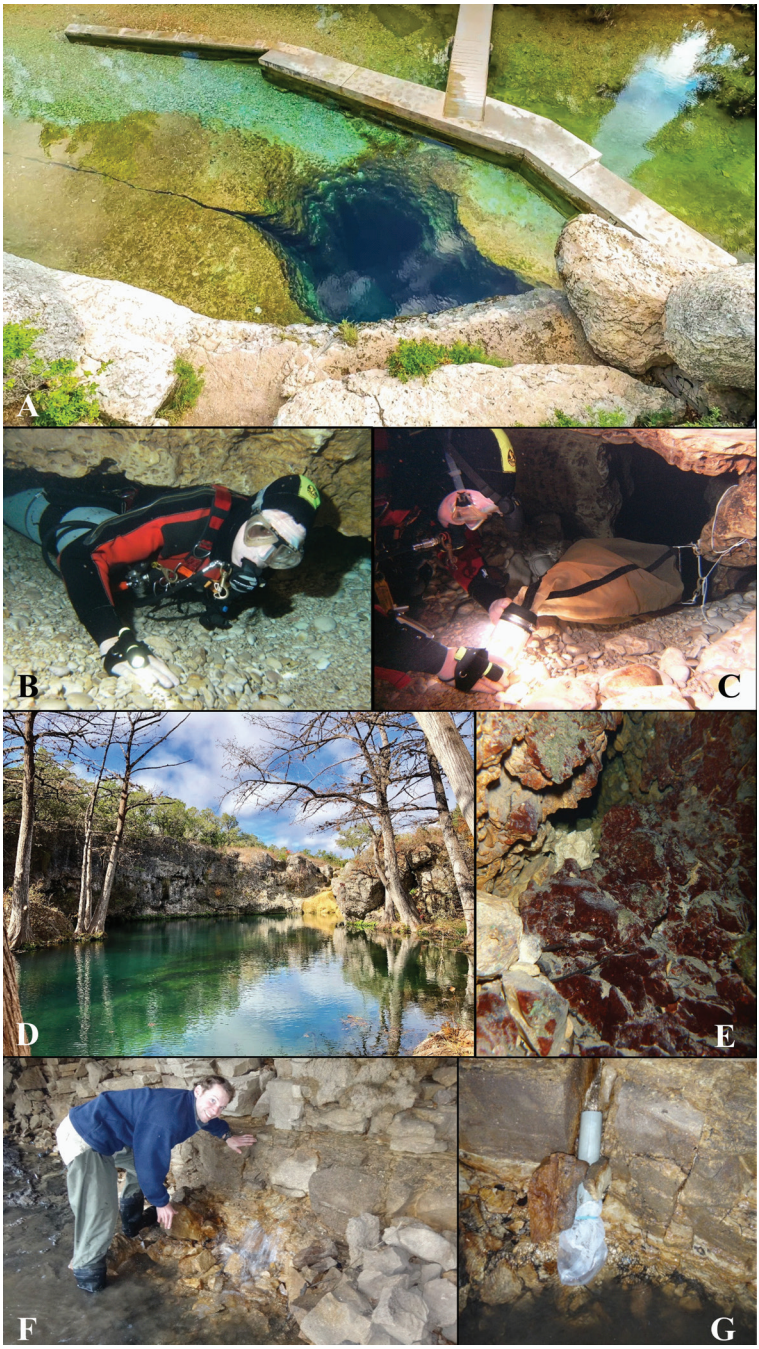

Figure 2. Comal, Hays, and Travis counties, Texas spring sites for *Stygobromus bakeri* sp. nov. Jacob's Well **A** cave entrance **B** second constriction point (30 m water depth), *Stygobromus bakeri* sp. nov. were found deeper than this point **C** drift net at first constriction point (23 m water depth). John Knox Ranch **D** Blue Hole, perennial headwaters of Carper's Creek **E** John Knox Spring issuing within Blue Hole. Mormon Spring #3 **F** Spring exposed under boat dock during drought **G** drift net on spring site. Photograph credit: Jean Krejca (**A–C**); courtesy of John Knox Ranch (**D**); Randy Gibson (**E**); Peter Sprouse (**F, G**).

Figure 3. Salado, Bell County, Texas spring sites for *Stygobromus bakeri* sp. nov. Robertson Spring Complex **A** Beetle Spring **B** Middle Spring **C** Ludwigia Spring **D** Creek Spring upwelling below water surface. Downtown Spring Complex **E** Big Boiling Spring **F** Anderson Spring. Photographs by Pete Diaz.

tle traps placed in a broad, low room at -30 m depth and 70 m penetration. The visual search area within the underwater passage began at the shaft below the spring opening and up to a maximum penetration of 330 m. Hand sampling was performed using basters, fine aquarium dipnets, and centrifuge vials. Külköylüoğlu and Gibson (2018) and Külköylüoğlu et al. (2017) described collection details at John Knox Springs, Comal County. Surveys using 250 µm drift nets at springs in the city of Salado, Bell County (Fig. 3) began in 2015 and have continued seasonally as part of a monitoring project for the endangered Salado salamander, *Eurycea chisholmensis* Chippindale, Price, Wiens & Hillis, 2000 at 10 spring orifices at Robertson Springs and 2 spring orifices at the Downtown Spring Complex (Diaz and Warren 2019). Mormon Spring #3, in Travis County was sampled using 150 µm drift nets from 20 January to 23 February 2017. All samples were stored in 95% ethanol.

Specimens were dissected, and mouthparts and appendages were mounted in glycerin. Images and measurements were acquired at multiple magnifications and focal points using an Olympus BX-45 compound stereomicroscope and Nikon DS-5M digital camera. Images were imported into Abode Illustrator Creative Suite 5 software and line drawing illustrations were produced using a Wacom Cintiq 22HD Interactive Pen Display. Nomenclature for setal arrangement on the mandibular palps follows Cole (1980) for segment 2 and Stock (1974) for segment 3. The "defining angle" of the gnathopod propodus is the area where the tip of the dactylus rests on the posterior margin of the palm. "Robust setae" and "setae" were used in place of the traditional "spine teeth" and "spines" following Watling (1989) and Cannizzaro et al. (2019).

Results

Systematics

Class Crustacea Brünnich, 1772
Order Amphipoda Latreille, 1816
Suborder Senticaudata Lowery & Myers, 2013
Infraorder Gammarida Latreille, 1802
Parvorder Crangonyctidira Bousfield, 1973
Superfamily Crangonyctoidea Bousfield, 1973
Family Crangonyctidae Bousfield, 1963; emended by Holsinger 1977
Genus *Stygobromus* Cope, 1872

***Stygobromus bakeri* Gibson & Hutchins, sp. nov.**
http://zoobank.org/AF7212A6-35E0-481C-ADCC-29A809611191
Fig. 4

Type locality. USA, Texas, Comal County, John Knox Ranch, John Knox Spring (29.9642, -98.1956).

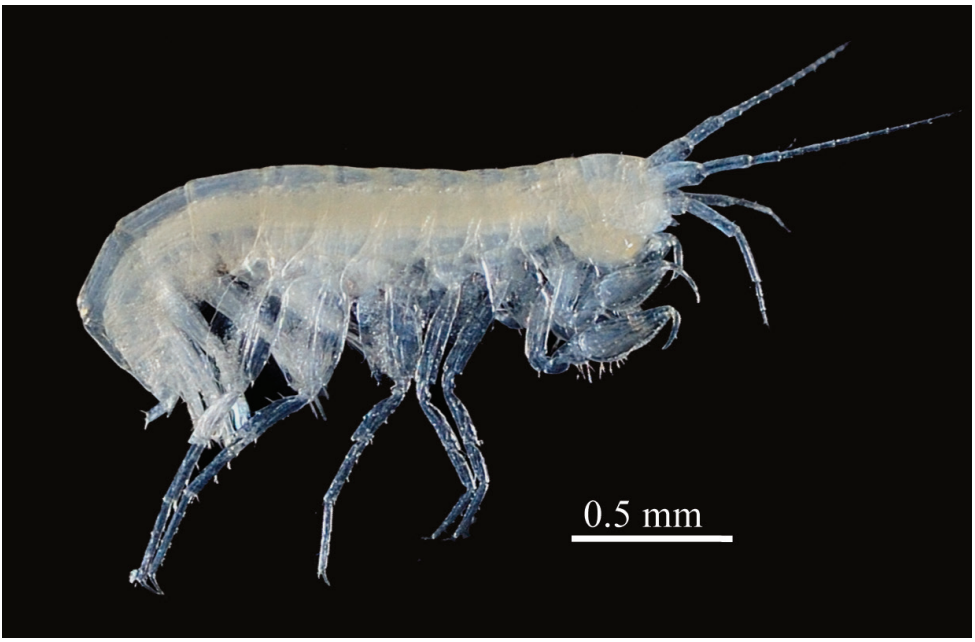

Figure 4. *Stygobromus bakeri* sp. nov. collected live from Jacob's Well. Photograph by Jean Krejca.

Material examined. All sites are in Texas, USA. ***Holotype*** **female** (4.6 mm) – Comal County, John Knox Ranch, John Knox Spring: collected by Randy Gibson (RG), 6 May 2010, dissected slide mounts Texas State University Aquifer Biodiversity Collection. ***Allotype*** **male** (3.1 mm) – Comal County, John Knox Ranch, John Knox Spring: collected by RG, 16 April 2010. ***Paratypes*** – John Knox Spring: juvenile (1.7 mm) collected by RG, 6 January 2010; 2 males (3.5 and 3.6 mm) and juvenile (2.4 mm) collected by RG, 6 April 2010; 2 males (2.5 and 3.4 mm) collected by RG, 2 May 2010; juvenile (2.4 mm) collected by RG, 6 May 2010; damaged female (4.5 mm) collected by RG, 10 May 2010; 2 males (3.1 mm and 4.1 mm) collected by RG, 13 May 2010; male (3.5 mm) and juvenile (2.3 mm) collected by RG, 21 May 2010. – Hays County, Jacob's Well (30.0345, -98.1261): male (4.2 mm) collected by Jean Krejca (JK), 21 October 2009; female (4.5 mm) collected by JK, 6 May 2010; female (3.5 mm) collected (≈ 43 m depth) by JK and James Brown, 4 August 2010; brooding female (4.4 mm), male (4.1 mm) and 2 juveniles collected by JK 31 May 2011; 2 males (4.4 and 2.9 mm) collected by JK, 16 June 2013; male (3.2 mm) collected by Matt Turner, 8 September 2013.

Additional material examined. – Travis County, Mormon Spring no. 3 (30.3132, -97.7747): 1 damaged female (4.4 mm) collected by P. Sprouse, 30 January 2017 (Zara # 9450, TMM # 91,955). – Bell County, Middle Robertson Spring (Robertson Springs Complex) (30.9444, -97.5410): female (4.2 mm) collected by Andy Gluesenkamp, 12 Dec 2009; juvenile (1.7 mm) collected by RG and Pete Diaz (PD), 30 July 2015; male (2.7 mm) collected by PD, 26 April 2017. Sam Bass Spring

(Robertson Springs Complex) (30.9444, -97.5410): male (4.5 mm) collected (drift net) by PD and RG, 1 Mar 2016. Upper Ludwigia Spring (Robertson Springs Complex) (30.9444, -97.5411): male (3.1 mm), collected (drift net) by PD, 20 January – 6 June 2017. Creek Spring (Robertson Springs Complex) (30.9445, -97.5414): male (3.0 mm) collected by PD, 12 April 2018; 2 males (4.1 and 3.2 mm) collected by PD, 27 November 2018. Big Boiling Spring (Downtown Springs Complex) (30.9437, -97.5366): male (3.2 mm) collected by RG and PD, 25 September 2015. Anderson Spring (Downtown Springs Complex (30.9441, -97.5346): damaged specimen (2.9 mm) collected (drift net) by PD, 1 November 2016–20 January 2017. Whole paratypes are deposited in the National Museum of Natural History, Smithsonian Institution (USNM accession # 1638682–1638690) and Texas State University Aquifer Biodiversity Collection (ABC accession # 0021059–002171).

Diagnosis. Small stygobitic species similar to most members of the *hubbsi* group with mature females larger than males, gnathopod 2 larger than gnathopod 1, posterior margin of gnathopod 1 typically shorter than palm, and telson as long as broad with shallow notch in apical margin. Distinguished from other *hubbsi* group species by gnathopods 1 and 2 with 2 rows of 3 singly inserted setae on the inner palm, and combination of following characters: gnathopod 1 with row of 4 to 5 setae posterior to the defining angle (compare to *S. quatsinensis* Holsinger & Shaw, 1986 with 2 or 3 postmarginal setae, 3 *hubbsi* group species have 1 or 2 postmarginal setae and all others are lacking); pereopods 6 and 7 with broadly expanded bases and distinct distoposterior lobes (similar to *S. salturus* Wang & Holsinger, 2001); and uropod 3 with single, slightly distal peduncular seta (similar to *S. lanensis* Wang & Holsinger, 2001).

Description. Female (4.6 mm). ***Antenna 1*** (Fig. 5, A1): 39% length of body, ca. 1.7× longer than antenna 2; primary flagellum with 10 segments, aesthetacs on segment 3–10; accessory flagellum 2 segmented reaching up to flagellar segment 2. ***Antenna 2*** (Fig. 5, A2): flagellum with 3 segments. ***Mandibles*** (Fig. 5, lMdb, rMdb): left mandible incisor 5-dentate, lacinia mobilis 5-dentate, with 5 robust, plumose accessory setae, molar protruding and non-triturative with tuft of setae on anterior margin, blade-like teeth and single long rastellate seta. Right mandible incisor 5-dentate, lacinia mobilis 3-dentate, 1 serrate, 1 forked apically, and last with row of denticles and long setae at base; accessory setae row with 2 robust serrate setae and tufts of long setae basally; molar and palp similar to left mandible. Palp 3-segmented, segment 2 and 3 subequal; segment 2 with 2 alpha and 1 beta setae, fringe of small hair-like setae lining inner margin and fringe of pubescence lining outer distal margin; segment 3 with 1 B seta, 5 E setae, 6 C setae, small hair-like D setae, A setae absent, and fringe of pubescence lining outer distal margin. ***Lower lip*** (Fig. 5, Li): covered in fine hairs with inner lobes poorly developed. ***Maxilla 1***(Fig. 5, Mx1) inner plate with 4 plumose setae; outer plate with 7 comb-spines (1 with inner medial margin lined with setae, 2 serrate, and 4 gaffate); palp 2-segmented, with 3–4 setae (2 apical, 1–2 subapical) and ca. 10 small hair like setae. ***Maxilla 2*** (Fig. 5, Mx2) inner plate with 13 setae, 7 longer plumose setae along inner margin and 6 shorter apical setae; outer plate with 9 setae

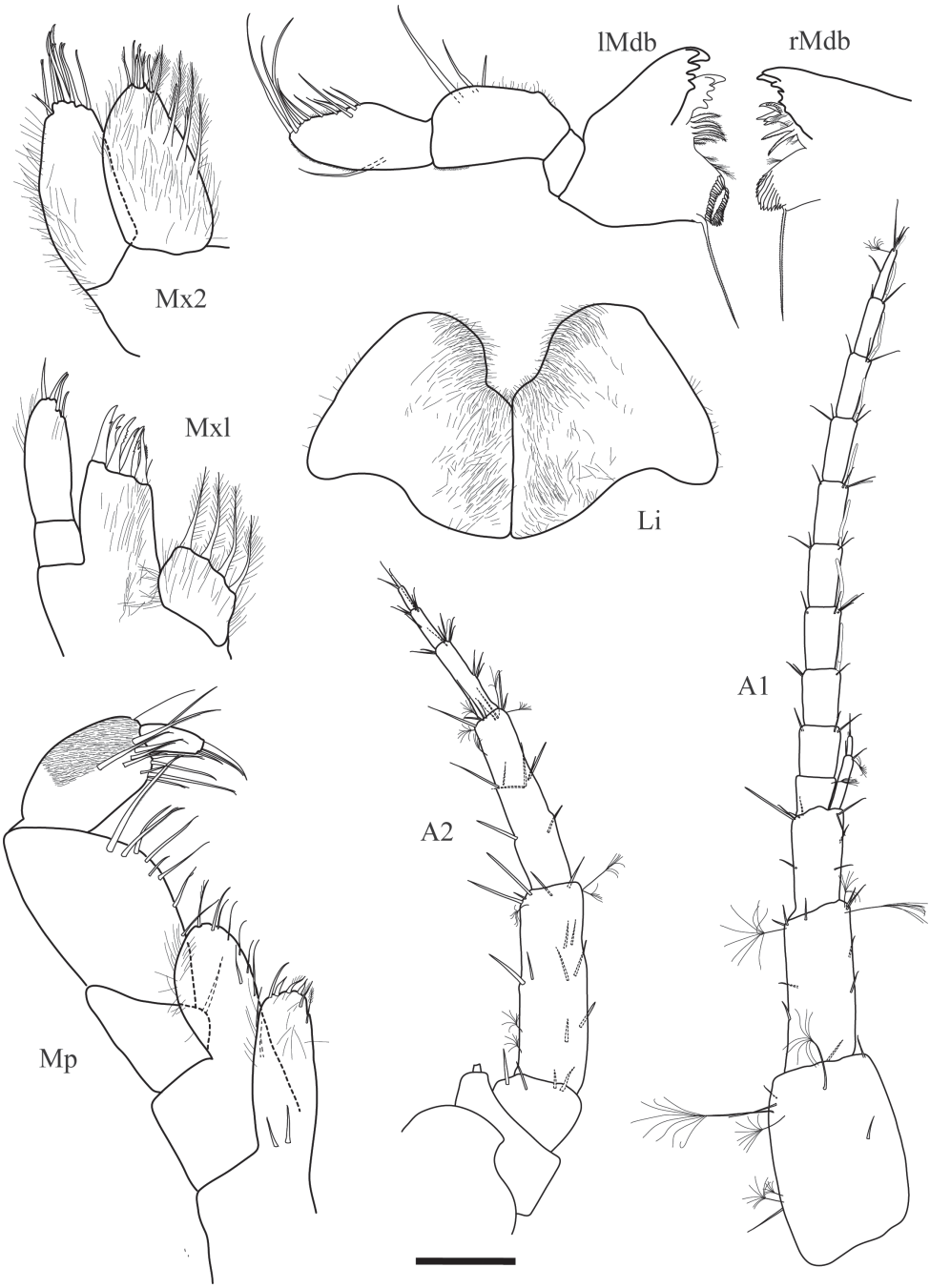

Figure 5. *Stygobromus bakeri* sp. nov., Holotype ♀ (4.6 mm), John Knox Spring, Comal Co., Texas **A1** antenna 1 **A2** antenna 2 **Li** lower lip **Mp** maxilliped **Mx1** maxilla 1 **Mx2** maxilla 2. Paratype ♂ (3.1 mm), John Knox Spring, Comal Co., TX. **lMdb** left mandible and mandibular palp **rMdb** right mandible. Scale bars: 0.05 mm (**Li, Mp, Mx1, Mx2, lMdb, rMdb**); 0.1 mm (**A1, A2**).

(mostly finely serrate, and 1 with apical minute setule) and an additional shorter seta on outer margin. ***Maxilliped*** (Fig. 5, Mp) inner plate with 3 apical spine-teeth, 2 plumose submarginal setae, and 2 longer setae arising at base; outer plate with 8–9 long simple setae and 1 outer apical seta with apical marginal setules; palp 4 segmented, second segment about 2× longer and wider than third, segment 1 with seta on inner margin, segment 2 with 6–8 setae on inner margin, 2 of these finely serrate, apical seta longer than rest, segment 3 with 8–11 setae, a few finely serrate and many forked apically, a densely pubescent patch located on dorsal face from midline to outer margin of segment, segment 4 with 2 setae apically at base of nail, one seta on outer margin medially, nail subequal in length to segment 4.

Gnathopod 1 (Fig. 6, G1): propodus shorter than that of gnathopod 2; coxal plate longer than broad, with 1 ventral and 2 anterior marginal setae; basis with 5 long setae inserted along posterior margin; ischium with 2 setae and scale patch along posterior margin; merus with 9 posterodistal setae and patch of pubescence; carpus 36% length of propodus, with 9 setae; propodus 1.5× longer than widest point with 3 superior and 3 inferior medial setae, palm slightly convex with 10 inner and 11 outer bifurcate robust setae, 2 of these posterior to defining angle, defining angle rounded and sub angular, margin posterior to defining angle ca. half the length of the palm with 4–5 setae and scaly patch becoming finer at defining angle and granular along inner margin of dactylus depression; dactylus with 1 seta on outer margin and 3 on distal half of inner margin; dactylus nail ca. 21% length of dactylus.

Gnathopod 2 (Fig. 6, G2): propodus 1.3× longer than that of gnathopod 1; coxal plate quadrate, with 1 ventral and 2 anterior marginal setae; basis with 1 long seta on anterior margin, shorter seta on posterodistal margin; ischium with 1 seta and scale patch on posterior surface; merus with 2 posterodistal setae, scale patch on posterior surface; carpus 25% length of propodus, with 3 distal setae, 7 setae and scale patch on posterior surface; propodus ca. 2× longer than widest point with 3 superior and 3 inferior medial setae, palm straight to slightly convex with 11 inner and 13 outer bifurcate robust setae, 2 of these posterior to defining angle and 3 sets of paired setae, 2 of these posterior to defining angle, defining angle rounded, margin posterior to defining angle ca. half the length of the palm with scaly patch on poster margin becoming finer at defining angle and granular along inner margin of dactylus depression; dactylus with 1 seta on outer margin; dactylus nail ca. 15% length of dactylus.

Pereopod 3 (Fig. 7, P3): subequal in length to pereopod 4; coxal plate subquadrate with 2 apical setae; merus 1.2× longer than carpus; propodus 1.4× longer than carpus, 2 setae on outer distal margin; dactylus ca. 30% length of propodus, apical sensory seta on outer margin. ***Pereopod 4*** (Fig. 7, P4): coxal plate deep, quadrate with 3 ventral marginal setae, reaching more than 50% length of basis; merus subequal in length to carpus; propodus 1.3× longer than carpus, 2 setae on outer distal margin; dactylus ca. 30% length of propodus, apical sensory seta on outer margin. ***Pereopod 5*** (Fig. 7, P5): coxal plate bi-lobate with single seta on anteroventral margin; basis broad with posterior margin slightly convex; merus 75% length of carpus; propodus subequal to carpus, 4 setae on outer distal margin, 1 of these longer than dactylus; dactylus ca. 30% length of propodus, apical sensory seta on outer margin. ***Pereopod 6*** (Fig. 7, P6): subequal

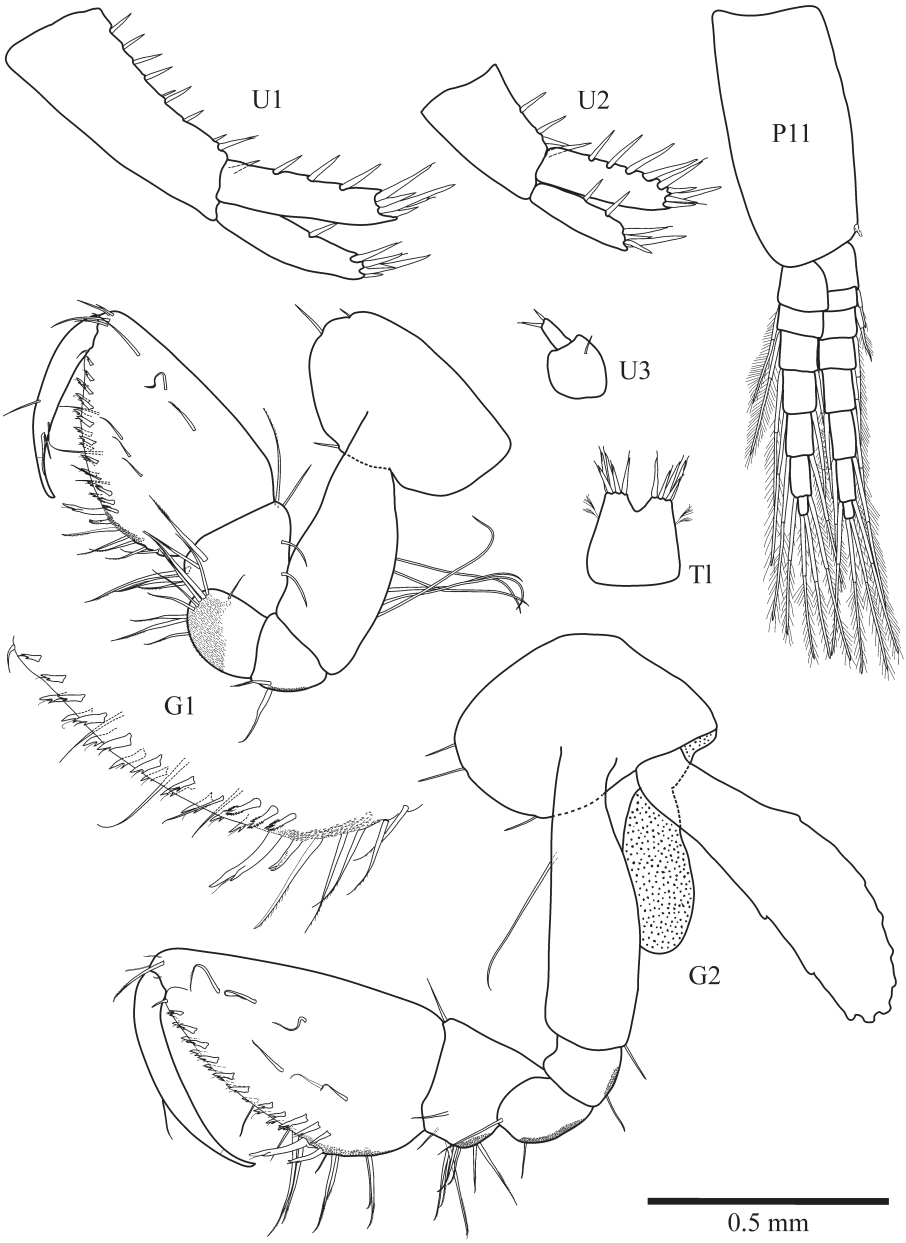

Figure 6. *Stygobromus bakeri* sp. nov., Holotype ♀ (4.6 mm), John Knox Spring, Comal Co., Texas **G1, G2** gnathopods (**G1** palmer margin enlarged 2×) 1–2 **U1–U3** uropods 1–3 **P11** pleopod 1 **T1** telson. All body parts drawn to same scale.

in length to pereopod 7 and 25% longer than pereopod 5; coxal plate bi-lobate with single seta on posteroventral margin; basis broad with posterior margin convex; merus 90% length of carpus; propodus 1.2× longer than carpus, 1 seta on outer distal margin;

dactylus ca. 30% length of propodus, apical sensory seta on outer margin. ***Pereopod 7*** (Fig. 7, P7): coxal plate ovate with single seta on ventral margin; basis broad with posterior margin convex with well developed distoposterior lobe; merus 85% length of carpus; propodus 1.2× longer than carpus, 1 long and 2 shorter setae on outer distal margin; dactylus ca. 30% length of propodus, apical sensory seta on outer margin.

Coxal gills on somites 2–7, stalked and subovate, smallest on pereopod 7 (Fig. 7, P3–P7). Brood plates present on somites 2–5 (Fig. 6, G2; Fig. 7, P3–P5) bearing long distal setae (not illustrated). Sternal gills absent but small processes sometimes visible on sternites between pereopods 3–7.

Pleonal plates (Epimera) (Fig. 7, Plp): posterior margins weakly convex with single setule; distoposterior corners weakly rounded; ventral margin weakly convex in plate 1 and 2 and nearly straight in plate 3, with single marginal seta on plate 2 and 2 setae on plate 3. Pleopods biramous, rami subequal, outer rami 7 segmented, inner rami 8 segmented, bearing many plumose setae, typical for genus (Fig. 6, Pl1). ***Uropod 1*** (Fig. 6, U1): peduncle with 9–10 robust setae; inner ramus ca. 70% length of peduncle, slightly longer than outer ramus, armed with 3 inner and 5 apical robust setae; outer ramus with 1 inner and 6 apical robust setae. ***Uropod 2*** (Fig. 6, U2): peduncle with 4 robust setae; inner ramus subequal to peduncle, ca. 30% longer than outer ramus, armed with 5 inner and 4 apical robust setae; outer ramus with 2 inner and 4 apical robust setae. ***Uropod 3*** (Fig. 6, U3): peduncle with single slightly distal seta; ramus ca. 51–55% length of peduncle, with 2 apical setae. ***Telson*** (Fig. 6, Tl): quadrate (subequal in length and width) with apical v-shaped notch with depth ca. 20% length of telson, 8 to10 apical setae and bearing tufts comprised of 2 sensory setae on each lateral side.

Male (3.1 mm). Closely resembles female but typically smaller, with fewer setae on appendages, and differing in the following characters: Antenna 1 proportionally longer, up to 46% length of body; primary flagellum with 9 segments. Uropod 2 inner and outer rami subequal in length.

Etymology. Epithet *bakeri* honors David Baker, a citizen scientist, conservationist and visionary who has worked for decades to bring together scientists, politicians, funders, regulators and stakeholders from near and far to conserve the watershed of Jacob's Well. David lived immediately over the underwater cave for some time, working hard to make his own property environmentally friendly and to educate those who visited. He formed the Wimberley Valley Watershed Association, a non-profit with the mission to protect Jacob's Well and Cypress Creek.

Distribution and ecology. To date, this small species has been collected in 2 karst springs in southwestern Hays and northwestern Comal counties, Texas, a single karst spring 50 km northeast, on the shoreline of the Colorado River (usually emerging beneath a boat dock in Lake Austin) in Austin, Travis County, Texas and 2 karst spring complexes 74 km further northeast in Salado, Bell County, Texas (Fig. 1). All 4 sites are hydrogeologically distinct.

Jacob's Well (Fig. 2A–C) is a natural spring and cave located in the bed of Cypress Creek with enterable passage typically 1–2 m in diameter, and mapped using cave SCUBA to about 42 m deep and 1.6 km in length. Water issues out of the cave from Glen Rose Limestone (Middle Trinity Aquifer) and flows down Cypress Creek: a

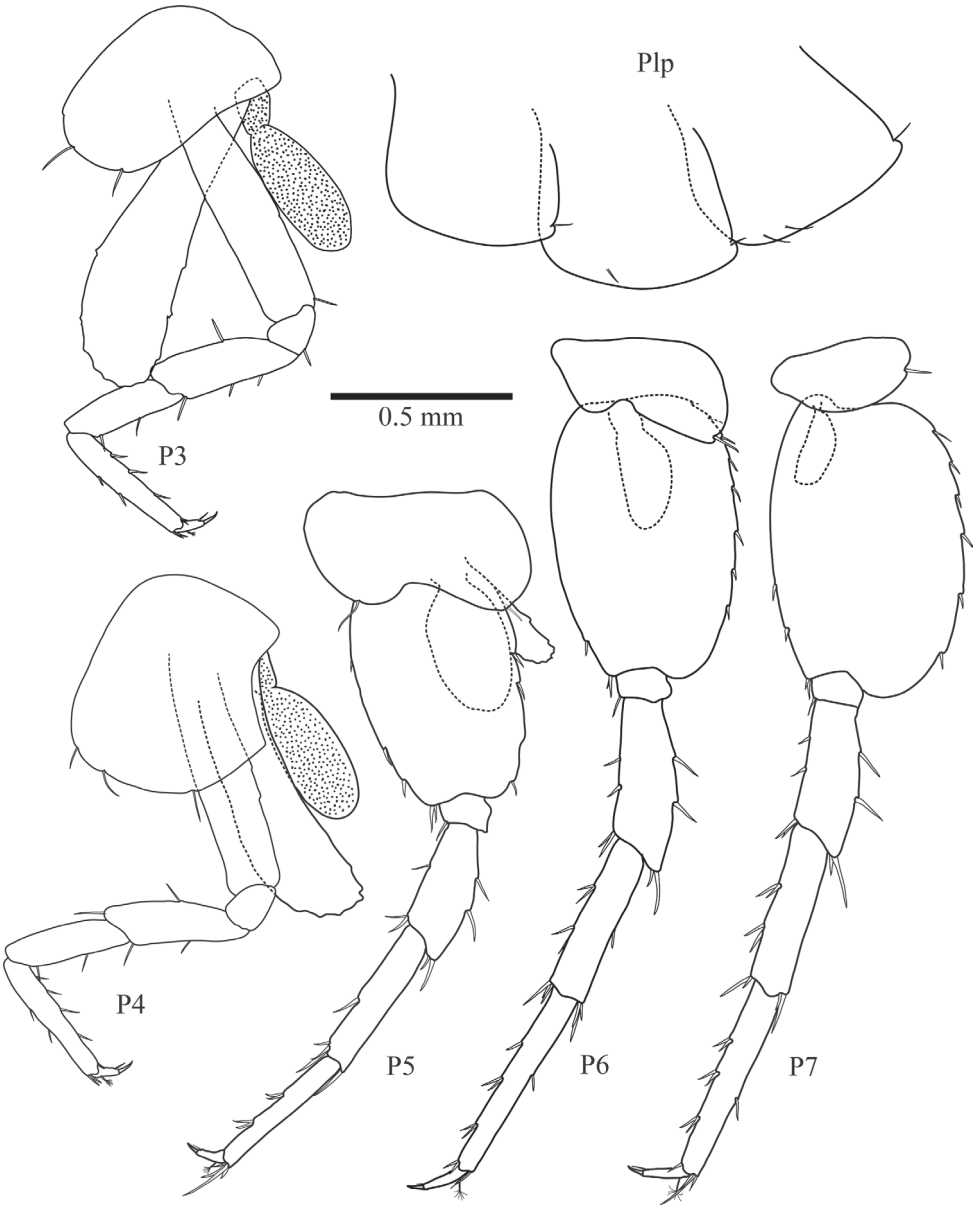

Figure 7. *Stygobromus bakeri* sp. nov., Holotype ♀ (4.6 mm), John Knox Spring, Comal Co., Texas. **P3–P7** pereopods 3–7 **Plp** pleonal plates (epimera).

tributary of the Blanco River (Gary et al. 2019). *Stygobromus bakeri* sp. nov. was only sampled successfully by hand, with divers using basters, dipnets, and centrifuge vials. Typically, the species was seen in the water column or on the floor, which consisted of silt, breakdown and cobble. Drift nets, bottle traps and cotton lures were not effective at capturing this species at Jacob's Well. It was seen only in a deeper portion of the cave between 70 m and 215 m penetration. At 70 m penetration, the cave passage descends

from -30 m to -42 m through a vertical slot called "The Knife Edge", beyond which, *S. bakeri* sp. nov. was collected. But beyond 215 m, the passage ascends back to 30 m and the species was not observed in that section. The habitat was not obviously different in the areas where the species was observed, with the exception of the entrance area (within 70 m of the surface) that has both surface influence and passage constrictions with greater flow, leading to smooth, scoured rocks and less silt.

John Knox Spring (Fig. 2D, E) emerges from Lower Glenn Rose Limestone (Middle Trinity Aquifer) through a 0.3 m vertical crevice-like orifice 1 meter below the water surface and along the bank of a limestone grotto pool forming the headwaters of Carper's Creek which discharges into the Blanco River. Interstitial surface insect fauna collected from this spring along with *Stygobromus russelli* (the most widespread *Stygobromus* species in central Texas) suggests that some portion of the flow might arise from hyporheic or vadose origin. Details of this habitat are described in Külköylüoğlu and Gibson (2018).

Robertson Springs (Fig. 3A–D) and Downtown Spring Complex (Fig. 3E, F) are 0.4 km apart, located on opposite sides of Interstate Highway 35 in the city of Salado. Both spring complexes comprise several spring orifices issuing from Edwards and Comanche Peak limestones forming spring runs that empty into Salado Creek. A diverse interstitial groundwater fauna (Table 1) including relatively widespread species has been recorded from these springs. *Stygobromus bakeri* sp. nov. was initially sampled by drift netting at Middle Robertson Springs (Norris et al. 2012). Robertson Springs has numerous spring openings (over 30 sites mapped during high flows) with variable discharge and substrate issuing from both banks and from under the 300 m long spring run. *Stygobromus bakeri* sp. nov. was collected at 4 spring sites throughout the run at different spring zones within the Robertson Springs complex (Diaz et al. 2016; Diaz and Warren 2019). The upper springs cease flowing seasonally and often produce troglofauna (terrestrial cave fauna) when flows resume after adequate rain events, while the lower springs are perennial and occasionally produce troglofauna. The Downtown Spring Complex is more discrete than Robertson Springs, with each of the 7 spring sites issuing from a single origin. Most of the springflow issues from Big Boiling Spring and flows down a short 5 meter spring run that empties into Salado Creek. Anderson Spring is 350 m downstream of Big Boiling Spring, has much less flow, issues from a fissure underneath a spring run (250 m downstream of the headwater Critchfield Spring), and flows 21 m before joining Salado Creek (Norris et al. 2012). Hydrological studies of this area in the Northern Segment of the Edwards Aquifer showed the Downtown Spring Complex to be part of an integrated fracture system with groundwater flow velocities recorded at 1.8 m/sec and confirmed mixing of groundwater and Salado Creek surface water at Big Boiling Spring (Wong and Yelderman 2017).

Mormon Spring # 3 (Fig. 2F, G), which normally lies beneath Lake Austin on the Colorado River, issues from Fredericksburg Group consolidation (limestone, dolomite, chert, and marl). Lake Austin was constructed in 1940, replacing the damaged Austin Dam, which may have also inundated the spring. From time to time, the Lower Colorado River Authority lowers the level of Lake Austin to allow for dock maintenance. During the last such lowering of 3 m in January 2017, multiple spring

Table 1. Groundwater fauna collected at each *Stygobromus bakeri* sp. nov. site. Stygobionts (S) are hypogean and colonize various groundwater habitats. Crenobionts (C) are epigean and preferentially colonize springs.

Taxon	Stygobiont/crenobiont	John Knox Spring	Jacob's Well	Salado Springs	Mormon Spring #3
Turbellarians					
Order Tricladida					
Family Kenkiidae					
Sphalloplana mohri Hyman, 1938	S	X			
Annelids					
Order Lumbriculata					
Family Lumbriculidae					
Eremidrilus **sp. 1** Fend & Rodriguez, 2003	S	X	X		
Eremidrilus **sp. 2**	S		X		
Molluscans					
Order Gastropoda					
Family Cochliopidae					
Phreatoceras taylori (Hershler & Longley, 1986)	S		X		
Phreatodrobia conica Hershler & Longley, 1986	S		X		
Phreatodrobia micra (Pilsbry & Ferriss, 1906)	S		X		
Phreatodrobia nugax (Pilsbry & Ferriss, 1906)	S	X	X		
Stygopyrgus bartonensis Hershler & Longley, 1986	S			X	
Family Hydrobiidae					
Marstonia comalensis (Pilsbry & Ferriss, 1906)	C	X	X	X	
Family Pluerocerídae					
Elimia comalensis (Pilsbry, 1890)	C		X	X	
Crustaceans					
Order Amphipoda					
Family Parabogidiellidae					
Parabogidiella americana Holsinger, 1980	S			X	
Family Crangonyctidae					
Stygobromus bifurcatus (Holsinger, 1967)	S		X	X	X
Stygobromus russelli (Holsinger, 1967)	S	X	X	X	X
Stygobromus (*flagellatus group*) sp. nov. Cope, 1872	S		X		
Order Bathynellacea					
Family Parabathynellidae					
Texanobathynella bowmani Delamare Deboutteville, Coineau & Serban, 1975	S			X	
Order Isopoda					
Family Asellidae					
Caecidotea bilineata Lewis & Bowman, 1996	S			X	
Caecidotea reddelli (Steeves, 1968)	S			X	X
Lirceolus bisetus (Steeves, 1968)				X	
Lirceolus hardeni (Lewis & Bowman, 1996)	S	X	X	X	
Lirceolus pilus (Steeves, 1968)	S		X	X	
Order Podocopida					
Family Candonidae					
Comalcandona tressleri Külköylüoğlu & Gibson, 2018	S	X			
Schornikovdona bellensis Külköylüoğlu, Yavuzatmaca, Akdemir, Diaz & Gibson, 2017	S		X		
Pseudocandona albicans (Brady, 1864)	C	X			
Pseudocandona cf *semicognita* (Schäfer, 1934)	C	X			
Physocypria cf *globula* Furtos, 1933	C	X			
Darwinula stevensoni (Brady & Robertson, 1870)	C	X			
Cypridopsis sp. (Brady, 1867)	C	X			
Physocypria denticulata (Daday, 1905)	C			X	
Chlamydotheca texasiensis (Baird, 1862)	C			X	
Cypridopsis cf *helvetica* Kaufmann, 1900	C	X			

Taxon	Stygobiont/c renobiont	John Knox Spring	Jacob's Well	Salado Springs	Mormon Spring #3
Arachnids					
Order Trobidiformes					
Family Arrenuridae					
Arrenurus **spp**. Dugès, 1834	S	X	X		
Insects					
Order Coleoptera					
Family Dytiscidae					
Sanilippodytes sp. Franciscolo, 1979	C	X	X		
Uvarus texanus (Sharp, 1882)	C		X		
Family Elmidae					
Microcyloepus pusillus (LeConte, 1852)	C	X	X	X	
Salamanders					
Order Urodela					
Family Plethodontidae					
Eurycea chisholmensis Chippindale, Price, Wiens & Hillis, 2000	C			X	
Eurycea pterophila Burger, Smith & Potter, 1950	C	X	X		

complexes were visible over a stretch of about 100 m along the east shore of the lake. North to south, Mormon Springs numbers 1 and 2 emerged from sediment, without visible bedrock apertures. Mormon Spring # 3 consists of multiple bedrock outlets in a cutbank excavated in bedrock below a boat dock. The spring appears associated with the Trinity Aquifer but is located near the Edwards Aquifer boundary and could potentially be a gravity spring from local recharge intersected by the Colorado River. Alternatively, this spring could represent flow from the north, from the Balcones Escarpment. No dye tracing has been conducted at this spring to characterize its springshed.

Stygobromus bakeri sp. nov. co-occurs with *S. russelli* (all sites), *S. bifurcatus* (Jacob's Well, Salado Springs, and Mormon Springs), and a large undescribed species of *Stygobromus* in the *flagellatus* group (Jacob's Well). The presence of 4 *Stygobromus* species at Jacob's Well is notable, and presumably all 4 species occupy different ecological niches. Due to its unusually small size and robust body, *S. bakeri* sp. nov. is able to inhabit both deep cave and shallow spring habitats. It may also occupy interstitial habitats such as the hyporheic zone of surface streams, although it was not collected in hyporheic samples from Carper's Creek, near John Knox Spring. The small adult size and general stout teardrop body shape is reminiscent of *Seborgia relicta* Holsinger, 1980 and *S. hershleri* Holsinger, 1992 found in groundwater habitats (deep aquifer caves and wells, springs, and hyporheos) throughout the Edwards Plateau and Balcones Escarpment. *Seborgia* has not been found associated with the Glen Rose formation of the Trinity Aquifer nor with the northernmost section of the Edwards Aquifer extending north of the Colorado River in Austin to Salado where *Stygobromus bakeri* sp. nov. occurs (Fig. 1). The furthest northern record for *Seborgia relicta* is from Cold Spring issuing from the Edwards Aquifer on the southern bank of the Colorado River (Ladybird Johnson Lake) only 4 km downstream of Mormon Spring #3. Groundwater fauna records for *Stygobromus bakeri* sp. nov. sites (Table 1) include interstitial and relatively widespread, ecological generalist stygobionts

and crenobionts, yet widespread cavernicolous stygobionts such as *Cirolanides* spp. isopods are absent at these sites (Holsinger 1967; Lewis 2000; Krejca 2009; Gibson et al. 2018; Hutchins 2018; Devitt et al. 2019; Schwartz et al. 2019; Alvear et al. 2020). Many of the springs have been thoroughly sampled and regularly monitored for multiple years, so the stygofauna is better characterized relative to many other Texas groundwater sites.

Key to Texas species of *Stygobromus* adapted from Holsinger (1967)

Species lengths reported from original descriptions for relative comparisons and likely vary.

1a Gnathopod 2 longer than gnathopod 1 ..... **2, *hubbsi* group**

1b Gnathopod 1 longer or subequal to gnathopod 2 ..... **3**

2a Pereopod 6 and 7 basis broadly expanded posteriorly with dorsoposterior lobe, adults < 6 mm long, central Texas .... ***S. bakeri* sp. nov. Gibson & Hutchins**

2b Pereopod 6 and 7 basis narrow, distoposterior lobe poorly developed, adults ≤ 13 mm long, rare, Culberson County, west Texas..... ..... ***S. limbus* Wang & Holsinger, 2001**

3a Gnathopod 1 often stouter but subequal in length to gnathopod 2, pereopod 6 and 7 subequal in length **4**

3b Gnathopod 1 stouter and usually longer that gnathopod 2, pereopod 7 longer than pereopod 6..... **8, *tenuis* group**

4a Pereopod 4 coxal plate extending distally > ½ length of basis, pereopod 5–7 basis broadly expanded posteriorly, well defined distoposterior lobe, midwest Texas..... ***S. hadenoecus* (Holsinger, 1966)**

4b Pereopod 4 coxal plate extending distally < ½ length of basis, pereopod 5–7 basis posterior margin not entirely expanded, usually narrow or narrows distally with poorly defined distoposterior lobes, central Texas .... **5, *flagellatus* group**

5a Uropod 3 with ≤ 3 apical setae, gnathopod 1 palmer margin straight or convex, telson with or without lateral setae, medium to large size (11–15 mm long) **6**

5b Uropod 3 typically with ≥ 4 apical setae, gnathopod 1 palmer margin convex, telson without lateral setae, medium size (8–11 mm long) **7**

6a Pereopod 5 basis subtriangular (expanded proximally), gnathopod 1 palmer margin convex with 2 rows each with many (ca. 20) robust bifurcated setae, pereopod 7 dactylus 1/4 length of propodus, telson often with multiple lateral setae (3 pair typical in females), large ≤ 15 mm long .... ***S. flagellatus* (Benedict, 1896)**

6b Pereopod 5 basis narrow (parallel sided), gnathopod 1palmer margin straight, with 2 rows each with fewer (ca. 10) relatively stouter robust bifurcated setae,

pereopod 7 dactylus 1/3 length of propodus, telson sometimes with single pair of lateral setae, medium ≤ 11 mm long.... ***S. longipes* (Holsinger, 1966)**

7a Pereopods 5–7 dactylus 1/3 length of propodus; uropod 3 with 3 or 4 apical setae, ≤ 8 mm long, rare, Kendall and northern Bexar counties..... ..... ***S. dejectus* (Holsinger, 1967)**

7b Pereopods 5–7 dactylus 1/4 length of propodus; uropod 3 with 5 apical setae, ≤ 11 mm long, rare, Comal County.....***S. pecki* (Holsinger, 1967)**

8a Gnathopod palmer margins deeply concave (pronounced in larger specimens).....**9**

8b Gnathopod palmer margins straight to convex.....**10**

9a Lateral sternal gills simple, rare, central Texas, ≤ 16 mm long .... ***S. balconis* (Hubricht, 1943)**

9b Lateral sternal gills bifurcated, widespread in northern Texas, ≤ 10 mm long ..... ***S. alabamensis* (Stout, 1911)**

10a Gnathopod 1 palmer margin convex, large (recorded >14 mm long) widespread in central Texas ..... ***S. bifurcatus* (Holsinger, 1967)**

10b Gnathopod 1 palmer margin straight, medium to large **11**

11a Pereopod 5–7 slender (pereopod 7 carpus width ca. 15% that of length), pereopod 7 ca. 25% longer than pereopod 5, pereopod 7 basis posterior margin mostly straight (parallel sided), uropod 3 with 3 apical setae, telson with ca. 14 apical setae, large (≤14 mm long) rare, San Saba County..... .....***S. reddelli* (Holsinger, 1966)**

11b Pereopod 5–7 less slender (pereopod 7 carpus width ca. 25% that of length), pereopod 7 ≥ 30% longer than pereopod 5, pereopod 5–7 basis posterior margin mostly convex, telson with ca. 11 apical setae, medium sized (≤12 mm long) widespread in central Texas..... ***S. russelli* (Holsinger, 1967)**

Discussion

The description of *S. bakeri* sp. nov. represents a southeast extension of the known range of the *hubbsi* group into the eastern Edwards Plateau and Balcones Escarpment of central Texas. *Stygobromus putealis*, described from eastern Wisconsin is the only other representative of the *hubbsi* group that occurs east of the western Cordillera of North America (Wang and Holsinger 2001). The presence of this species at Robertson Springs, over 70 km away from the other nearest known site for the species is somewhat unusual not only because of the distance, but because the sites are discharge points for 2 distinct karst aquifers, the Trinity and Edwards. However, at least 3 other *Stygobromus* species (*S. bifurcatus*, *S. longipes*, *S. russelli*) also occur in both the Trinity and Edwards aquifers, and limited, localized hydrologic connections between these aquifers have been documented (Tian et al. 2020), opening the possibility of inter-aquifer dispersal. Additionally, numerous members of the *hubbsi* group have been recorded from the hyporheic zone of surface streams (Wang and Holsinger 2001) which

could provide another potential avenue for dispersal by *S. bakeri* sp. nov. While the current range of *S. bakeri* sp. nov. is within the range reported for other *Stygobromus* species (Hutchins 2018) and certainly within the 200 km range reported for macrostygobionts by Trontelj et al. (2009), genetic analysis would still be prudent to evaluate whether southern and northern populations represent cryptic lineages.

At all sites where *S. bakeri* sp. nov. was detected, it occurs in apparently low abundance, particularly compared to co-occurring *Stygobromus* (e.g. at John Knox Spring, *S. bakeri* sp. nov. composed 10% of all *Stygobromus* (27 versus 236 *S. russelli*) collected from 6 January to 21 May 2010. In comparison, at Robertson Springs, the highest yearly abundance of *S. bakeri* sp. nov. was 5% of all *Stygobromus* (26 versus 532 *S. russelli* and *S. bifurcatus* combined) collected from 12 April to 7 November 2018. However, it is uncertain whether perceived rarity reflects actual rarity or a lack of effective sampling (Niemiller et al. 2018). Indeed, we lack much basic ecological information about the species including niche breadth and microhabitat preference. A better understanding of the species' rarity and distribution is a high priority given growing threats to water quantity and water quality in the region.

Both Jacob's Well and John Knox Springs are fed by the Trinity Aquifer, although the recharge area for John Knox Spring has not been delineated. Recharge for Jacob's Well primarily occurs in an 80 km^2 area within the Dry Cypress Creek watershed northwest of the spring (Gary et al. 2019). Increased pumping in the region has resulted in reduced spring flow at Jacob's Well, which now flows intermittently (Hunt et al. 2013), and projected rapid human population growth in the region will put additional pressures on groundwater resources. Hays County is the 3rd fastest growing county, by percent, in Texas (Texas Demographic Center 2019). Desired future conditions set by Groundwater Management Area 9 allow for an increase in average drawdown of the Trinity Aquifer in the region up to approximately 9 m by 2060, which if realized, would result in reduced spring flow or spring failure for many Trinity-fed springs. A Recharge Study Zone created in January 2020 and a proposed Jacob's Well Groundwater Management Zone afford some pumping curtailments and restrictions on new well construction in the region. Neither of these zones, however, provides conservation benefits for the John Knox Spring springshed, which probably occurs farther west in adjacent Comal County, Texas. Texas Parks and Wildlife Department recorded discharge measurements of 28–57 L/s at Carper's Creek in 2005, 2007, and 2010 (Chad Norris, pers. comm.) and the springshed proportional to this discharge is assumed to be approximately 13–18 km^2 (Marcus Gary, Edwards Aquifer Authority, pers. comm.).

The listing of several aquifer-obligate species in the adjacent Edwards Aquifer in the 1960s, 1970s, and 1980s spurred formation of the Edwards Aquifer Authority (EAA) in 1993. Charged with protecting minimum spring flows at Comal and San Marcos springs, the EAA implemented programs that helped protect groundwater recharge and reduce groundwater use (including enforcement of pumping limitations). The regulatory actions of the EAA and conservation measures established in the Edwards Aquifer Habitat Conservation Plan (HCP) have been successful in maintaining spring flow despite a rapidly growing population. Indeed, existing models predict that

HCP conservation measures are sufficient to maintain springflows during drought-of-record conditions that would otherwise result in spring failure (Votteler and Gulley 2014). However, EAA jurisdiction does not cover sites at which *S. bakeri* sp. nov. occurs. Although similar regulatory frameworks do not exist for the Jacob's Well and John Knox Springs area, the proposed management practices outlined by the Jacob's Well Groundwater Management Zone were designed explicitly to maintain springflow during periods of drought (Gary et al. 2019).

Recharge of the northern section of the Edwards Aquifer and groundwater conservation in Salado is not regulated by the EAA. In this section of the aquifer, Clearwater Underground Water Conservation District is responsible for management of groundwater in Bell County. Recharge for the springs where *S. bakeri* sp. nov. and the federally threatened salamander, *Eurycea chisholmensis* occur are thought to be in the west and southwest portion of Bell County with flow moving generally southeast (Wong and Yelderman 2017). In addition, Brune hypothesized that the recharge to Salado Creek began from fissures along the Williamson and Bell county line (Brune 1981). Bell County is the most northern extension of the Edwards Aquifer in Texas (Wong and Yelderman 2017) and the most northern location known for many Edwards Aquifer associated taxa (Alvear et al. 2020). Edwards limestones thin in Bell County relative to areas to the south, and the southeastern extent of the county was described as being devoid of substantial caves (McKenzie and Reddell 1964).

Mormon Springs is located upstream, but near habitat for the federally endangered Barton Springs and Austin blind salamanders (*Eurcyea sosorum* Chippindale, Price & Hillis, 1993 and *E. waterlooensis* Hillis, Chamberlain, Wilcox & Chippindale, 2001), and is in the same aquifer formation as the federally endangered Jollyville Plateau salamander, *E. tonkawae* Chippindale, Price, Wiens & Hillis, 2000. Little is known about groundwater flow on the northeast side of the Colorado River on the Balcones Escarpment. Mormon Springs is not within a groundwater conservation district, and well pumping, mainly for domestic landscaping use, is unregulated.

Conclusion

The discovery of an undescribed *Stygobromus* amphipod from the Trinity and Edwards aquifers associated karst springs in Hays, Comal, Travis, and Bell counties in central Texas is unsurprising giving the high species richness and small-range endemism previously reported for the genus and previous documentation of numerous undescribed taxa (Holsinger et al. 2011). However, the assignment of *S. bakeri* sp. nov. to the predominantly western *hubbsi* group suggests that the *hubbsi* group may be more widespread than currently recognized and that additional investigation for small-bodied taxa west of the continental divide will yield additional undescribed species. *Stygobromus bakeri* sp. nov. is the 12th *Stygobromus* species described from Texas and can be distinguished from other Texas congeners by gnathopod 2 larger than gnathopod 1 and pereopods 6 and 7 with broadly expanded bases and distinct distoposterior lobes.

Stygobrumus bakeri sp. nov. is distinguished from all other *hubbsi* group species by gnathopod 1 and 2 with 2 rows of 3 singly inserted setae on the inner palm, gnathopod 1 with row of 4–5 setae posterior to the defining angle, and uropod 3 with single, slightly distal peduncular seta. Molecular analysis is recommended to assess the relatedness of southern Trinity Aquifer populations versus northern Edwards Aquifer populations. Finally, the description of small-range endemic species in the region underscores the immediate need for a comprehensive groundwater management framework that ensures adequate groundwater habitat in the face of drought exacerbated by a changing climate and growing human populations.

Acknowledgements

Thanks to the land managers at Jacob's Well for access to SCUBA and collection in the cave. James Brown and Gregg Tatum are cave divers that helped with cave diving logistics and collection of specimens and assisted author JKK with very technical SCUBA sampling, Krista McDermid helped process specimens. Special thanks to David Baker for logistical support and his decades-long dedication to the conservation of Jacob's Well. We thank John Knox Ranch for the continual access to their property and their dedication to environmental education and preservation of one of the most pristine streams in the Texas Hill Country, The Robertson family and the Bell County Adaptive Management Coalition for support of conservation research and access to springs, wells, and caves in the area. We greatly appreciate the taxonomic expertise provided by Will Coleman, Steve Fend, Robert Hershler, Okan Külköylüoğlu, Kathryn Perez, and Ian Smith, and specialized knowledge from hydrologist Marcus Gary and field biologists, Andy Gluesenkamp, Jessica Gordon, Chad Norris, James Reddell, Jennifer Bronson Warren, and staff at the San Marcos Aquatic Resources Center.

The views presented herein are those of the authors and do not necessarily represent those of the U.S. Fish and Wildlife Service.

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Subterranean Biology 38: 47–63 (2021)
doi: 10.3897/subtbiol.38.60865
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

The Pennsylvania grotto sculpin: population genetics

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Academic editor: M.L. Niemiller | Received 17 November 2020 | Accepted 16 March 2021 | Published 12 April 2021

http://zoobank.org/D7171664-2C1A-43A4-8EBC-6FD57374F654

Citation: Espinasa L, Smith DM, Lindquist JM (2021) The Pennsylvania grotto sculpin: population genetics. Subterranean Biology 38: 47–63. https://doi.org/10.3897/subtbiol.38.60865

Abstract

The Pennsylvania grotto sculpin is known from just two caves of the Nippenose Valley in central Pennsylvania, USA. They exhibit emergent troglobitic morphological traits and are the second northern-most cave adapted fish in the world. Two mitochondrial (*16S rRNA* and *D-loop* gene) and one nuclear (*S7* ribosomal protein gene intron) gene in both cave and epigean populations were sequenced. For the three markers, a large proportion of cave specimens possess unique haplotypes not found in their local surface counterparts, suggesting a vicariance in their evolutionary history. The cave population also has haplotypes from two separate lineages of surface sculpins of the *Cottus cognatus*/*bairdii* species complex. Since morphology, nuclear, and mitochondrial markers are not correlated among cave individuals, hybridization with introgression is suggested.

Keywords

Cave, Cottidae, *Cottus cognatus*, *Cottus bairdii*, hybrid, Speciation, stygobite, troglobite

Introduction

Modern biology as a science and evolutionary biology in particular have had a long history of interest in cavefishes. In evolutionary developmental biology (evo-devo), cavefishes are now viewed as a great model system (Jeffery 2001). Since the description of the first troglobitic fish, *Amblyopsis spelaea* DeKay, 1842, over two hundred species

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of blind fish or fish with some degree of eye degeneration have been found in caves around the world (Behrmann-Godel 2017; Borowsky 2018).

In 2003, Espinasa and Jeffery described a previously unreported cave population of sculpins (Cottidae: Scorpaeniformes: Actinopterygii) inhabiting Eiswert #1 Cave (Stone, 1953) in the Nippenose Valley, Lycoming County, Pennsylvania. Specimens of this cave population retain some degree of pigmentation and eye functionality. They are morphologically distinct from the surface sculpins of Antes Creek located only 445 m from the cave (Fig. 1). Espinasa and Jeffery (2003) showed that the cave population differ from the surface fish by wider and more abundant mandibular pores, wider head, longer pectoral fins, and reduced eyes (Fig. 2). This population is currently the second northern-most cave adapted fish in the world (Romero and Paulson 2001; Behrmann-Godel 2017).

Eiswert #1 Cave and Antes Creek are part of the West Branch of the Susquehanna River drainage. Two closely related species of sculpins inhabit this drainage: the mottled sculpin, *Cottus bairdi* Girard, 1850, and the slimy sculpin, *Cottus cognatus* Richardson, 1836. These cottids often exhibit overlapping meristic and mensural features (Strauss 1986). Due to the occurrence of two *Cottus* species in the area and the potential for modification in taxonomic diagnostic features as a result of inhabiting the cave environment, Espinasa and Jeffery (2003) did not assign the troglomorphic sculpins to either of the species or to a new taxon. They restricted their placement to the *Cottus bairdi-cognatus* complex until further studies could be conducted. The common name given to the cave population is the Pennsylvanian grotto sculpin.

The two local surface species of cottids are known to hybridize. For example, Strauss (1986) found that at Blockhouse Creek, located in the same county as Eiswert #1 Cave, *C. bairdi* and *C. cognatus* were hybridizing. At this locality, Strauss (1986) found F1s, but no indication of F2 progeny or backcrosses. Although genetic heterozygosity in the parental samples of Blockhouse Creek tends to refute the occurrence of introgression, morphological information may support a very limited amount of introgression (Strauss 1986). Regardless of the actual degree of introgression, it appears that either the viability of hybrids is considerably reduced, or a substantial amount of intraspecific assortative mating exists. Therefore, in the surface streams of central Pennsylvania, both *C. bairdi* and *C. cognatus* gene pools remain distinct (Strauss 1986).

The purpose of this study is to establish if the Pennsylvania grotto sculpin is genetically distinct from surface sculpins and, if such is the case, from which species it derived. For this study, we sequenced two mitochondrial and one nuclear gene. The relevance of this study is not restricted to purely academic arenas. In 2002, two corporate hog farms intended to enter the Nippenose Valley (http://old.post-gazette.com/localnews/20020922farms0922p5.asp). The proposed Concentrated Animal Feeding Operations (CAFOs) were to produce over 20,000 hogs along with 5 million gallons of antibiotic and steroid rich manure each year. This surface waste could easily enter and contaminate the underground network of groundwater through the honeycombed limestone, thereby contaminating the water supply for the inhabitants of the valley. Some residents quickly organized a group called the Concerned Citizens of Nippenose

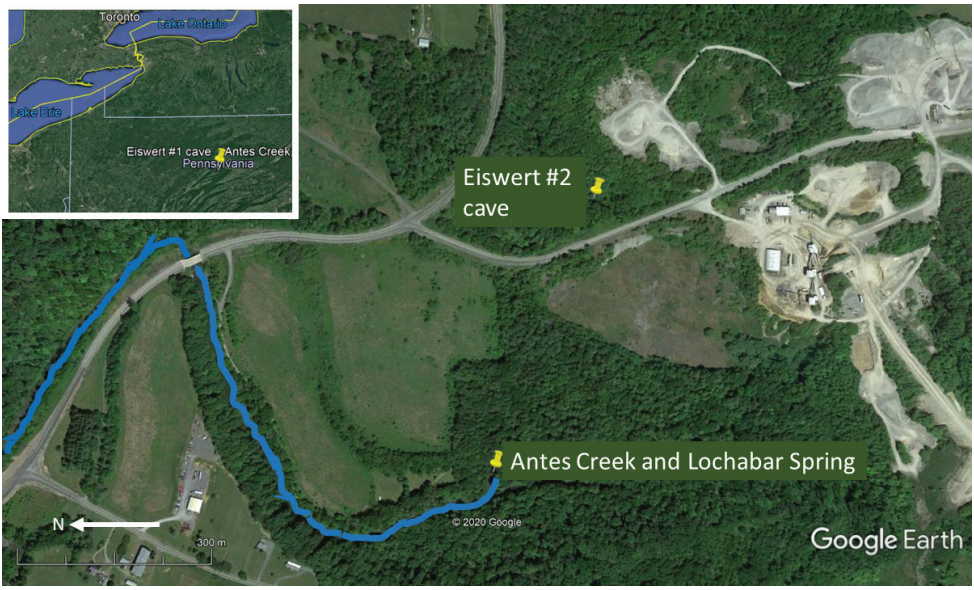


Figure 1. Nippenose Valley, showing the location of Eiswert #1 cave and Lochabar Spring, source of Antes Creek (blue). Despite being only 445 m from each other, cave and surface sculpin populations are morphologically and genetically distinct. Notice that both cave and spring are very close by to a limestone quarry, which has the potential of being a conservation threat to the Pennsylvania grotto sculpin if further developed. Inset of Pennsylvania showing location of cave. Map data 2020 Google.

Valley (CCNV). CCNV invoked a local ordinance protecting their water supply to tie up the permit applications, while mounting a comprehensive public relations campaign to build broad-based community opposition to the CAFOs. While this was occurring, the troglomorphic sculpin was found in Eiswert #1 Cave and reported in the scientific literature (Espinasa and Jeffery 2003). In particular, Espinasa and Jeffery (2003) recommended that the U.S. Fish and Wildlife Service should consider the Pennsylvania grotto sculpin to be listed under the U.S. Endangered Species Act. Afterwards, the Williamsport Sun-Gazette ran an article titled "Fish Find May Nix Pig Farms." The CCNV's efforts culminated in a victory when both CAFO applications were withdrawn. Conservation risks persist for the Pennsylvania grotto sculpins. For example, a limestone quarry is just meters away from the cave and spring (Fig. 1).

Methods

Specimens

Since the description of the Pennsylvania grotto sculpins from Eiswert #1 Cave, James C. D. Lewis (Resident Pennsylvania Fishing License number R. 703557) has identified this form in a second cave, Loose Tooth Cave, also within the Nippenose Valley,

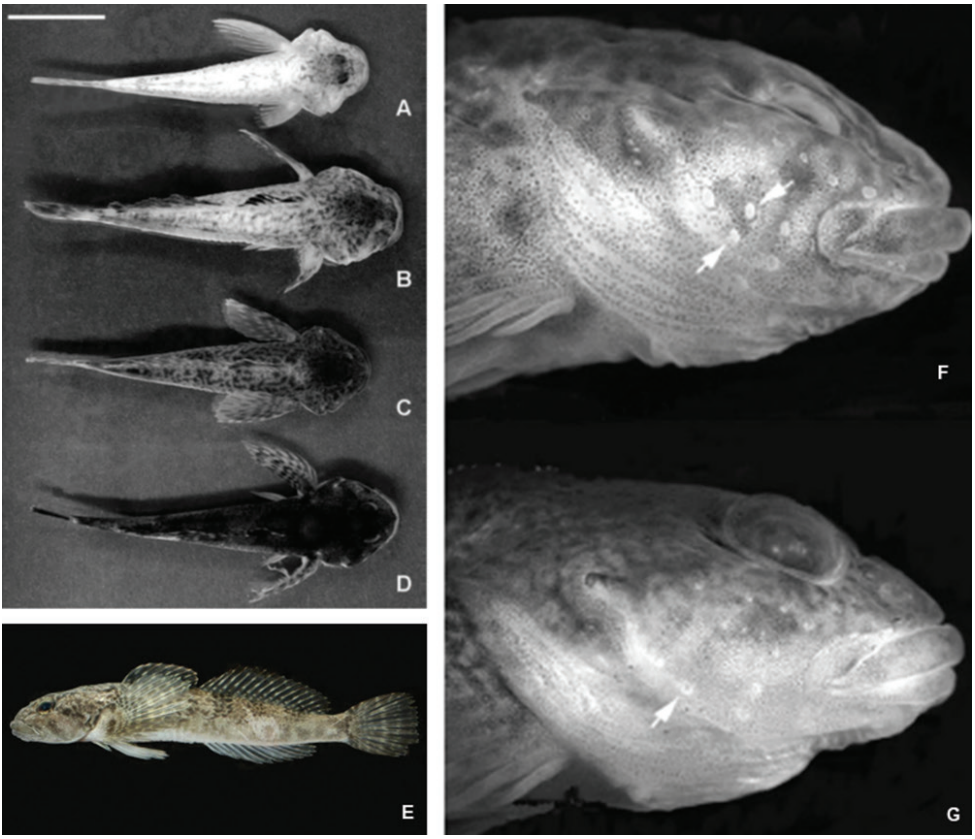


Figure 2. Nippenose Valley cave (**A–C, F**) and epigean Antes Creek (**D–E, G**) sculpins. While there is a diversity of expression of troglomorphic characters, cave fish tend to be more depigmented (**A–C** vs **D–E**), have smaller eyes (**F** vs **G**), more abundant and larger mandibular pores (**F** vs **G**, arrows point to pore III), larger heads (**B** vs **D**), and longer pectoral fins (**B** vs **D**). Scale bar: 2 cm (**A–D, F–G** modified from Espinasa and Jeffery (2003)).

2.13 km SSW of Eiswert #1. Two specimens were collected with dip nets and fin clips of fish were deposited in 100% ethanol for DNA studies. For conservation purposes, other specimens used in this study are the same as those used in Espinasa and Jeffery (2003).

Molecular data

Genomic DNA samples were obtained from:

a) Cave samples: 24 individuals from Eiswert #1 (41°9'N, 77°12'W; 8/11/02, 10/8/02, 9/16/07, 1/25/08) and two from Loose Tooth cave, Nippenose Valley, PA (41°8'N, 77°13'W; 9/16/07).

b) Surface samples: 24 individuals of presumptive *C. cognatus* from Lochabar Spring, Antes Creek, PA (41°9'28.6"N, 77°13'13.6"W; 10/8/02, 9/16/07). Lochabar

Spring is where all caves of the Nippenose Valley drain into, and it is located at a distance of 445 m WWN from the Eiswert #1 cave (Fig. 1) and 2.14 km NNE from Loose Tooth cave.

c) Surface samples: 7 individuals of *C. cognatus* from Willsey Brook, Wilsey Valley Rd. Wurtsboro, NY (41°35'N, 74°29'W; 10/7/19). According to maps provided by the New York State Department for Environmental Conservation, Willsey Brook is within a drainage inhabited by only *C. cognatus* (https://www.dec.ny.gov/animals/94615.html) and no *C. bairdii*. (Conservatiohttps://www.dec.ny.gov/animals/94617.html). Morphologic analysis of the specimens collected also showed they had three pelvic-fin rays, a diagnostic feature of *C. cognatus*, while *C. bairdii* has four pelvic-fin rays, thus confirming specimens belong to *C. cognatus*.

Standard methods for DNA purification were followed. Total DNA was extracted from tail clippings using Qiagen's DNEasy Tissue Kit. Two mitochondrial and one nuclear marker were amplified and sequenced, each as a single fragment. The primers used were *16S*ar and *16S*b primer pair for *16S rRNA* (Edgecombe et al. 2002), DL1F and DL4R primer pair for *D-loop* (Bagley and Gall 1998), and S7RPEX1F and S7RPEX2R primer pair for *S7* ribosomal protein gene first intron (Chow and Hazama 1998). Amplification was carried out in a 50 µl volume reaction, with 1.25 units of AmpliTaq DNA Polymerase (Perkin Elmer, Foster City, CA, USA), 200 µM of dNTPs, and 1 µM of each primer. The PCR program amplification cycles as in Edgecombe et al. (2002). PCR amplified samples were purified with Qiagen's QIAquick Gel Extraction Kit and directly sequenced using an automated ABI Prism 3700 DNA analyzer as in Espinasa et al. (2007). Chromatograms obtained from the automated sequencer were read and analyzed with the program Sequencher 3.0. Primers and 5' or 3' short end fragments of sequences not shared by all individuals in the analyses were trimmed. Sequences were compared against highly similar sequences available in GenBank using BLASTn. Sequences were aligned with CLUSTALW2 (Higgins et al. 1992; Thompson et al. 1994). A minimum haplotype network was obtained using PopArt program (Leigh and Bryant 2015). In addition, a maximum likelihood tree was inferred using PhyML v20160115 ran with the following model and parameters: -0 tir –pinv e –nclasses 4 –bootstrap 10 f m –alpha e, as implemented in CLUSTALW2. Branch supports were computed out of 100 bootstrapped trees. The analyses also included the sequences most similar to the Nippenose cave cottids found in GenBank through BLAST. Nei's gene diversity (h), Nei's population differentiation (*Gst*), and gene flow (Nm) were estimated with software POPGENE version 1.32 (Yeh 1999).

To determine if specimens harboring either of the mitochondrial haplotype lineages (see below) belonged to two separate species or to a single reproductive population that had undergone hybridization and introgression, morphology, nuclear, and mitochondrial markers were compared to evaluate if they were linked or correlated among individuals of the cave population. The assumption being that if they were two different species, specific nuclear haplotypes and morphologies would be found only in individuals carrying a specific mitochondrial haplotype.

Morphology

Analyses included data from the specimens described by Espinasa and Jeffery (2003), in addition to six new specimens from Eiswert #1 Cave and two from Loose Tooth Cave collected on 16 September 2007 for a total of 23 specimens. Measurements of eye and mandibular pore #3 lengths were obtained using a Motic K series stereomicroscope with a fitted eyepiece micrometer. A dial caliper was used to measure to the nearest 0.1 mm the standard length of the pectoral fin length and the head width. Eye, pore #3, pectoral fin, and head width were plotted against standard length and linear regressions were obtained using Microsoft Excel. From these plots, each of the specimens was assigned a numerical value to indicate if they had a troglobitic (0), intermediate (1), or surface-like (2) appearance based on their location with respect to the linear regression line. To do this, the seven individuals furthest above, nine closest, and the seven individuals furthest below the linear regression line were identified (Fig. 3). Overall troglomorphic appearance of an individual within the population was calculated by adding the numbers for the four characters. If an individual, when in comparison to the linear regression of the population, had small eyes and a large pore, large fin, and large head, the lowest total value it could receive would be "0". Whereas for an individual with large eyes and a small pore, fin, and head, the highest value it could receive would be "8". A non-parametric Mann-Whitney U test for ordinal data was conducted to test if specimens harboring either of the mitochondrial haplotype lineages were equally troglobitic in morphologic appearance.

Results

Molecular data

The two caves as a population had a haplotype composition completely different from the local surface population in Antes Creek. While the surface population has haplotypes corresponding to *C. cognatus*, the cave population has haplotypes shared with two sculpin lineages: *C. cognatus* and *C. bairdi*.

For the mitochondrial *16S rRNA*, analyses were performed on a 526 bp sequence fragment shared by all individuals. A total of six haplotypes were found: *Cottus* sp. 'Nippenose Valley' haplotypes 1 and 2 (GenBank accession nos. GQ280792 and GQ280792) and *Cottus* sp. 'Nippenose Valley' haplotypes A (GQ267192), B (GQ267193), C (GQ267194), and D (GQ267190). Both maximum likelihood and haplotype network analyses of the *16S rRNA* haplotypes of the cave and surface populations identified two clearly distinct clades (Fig. 4). The first clade includes only cave specimens with haplotypes 1 and 2, and the second clade includes both surface fish from Antes Creek and cave fish with haplotypes A–D. For Loose Tooth Cave specimens, one individual had haplotype A and the other haplotype 2, which were the two most common haplotypes in Eiswert #1 Cave.

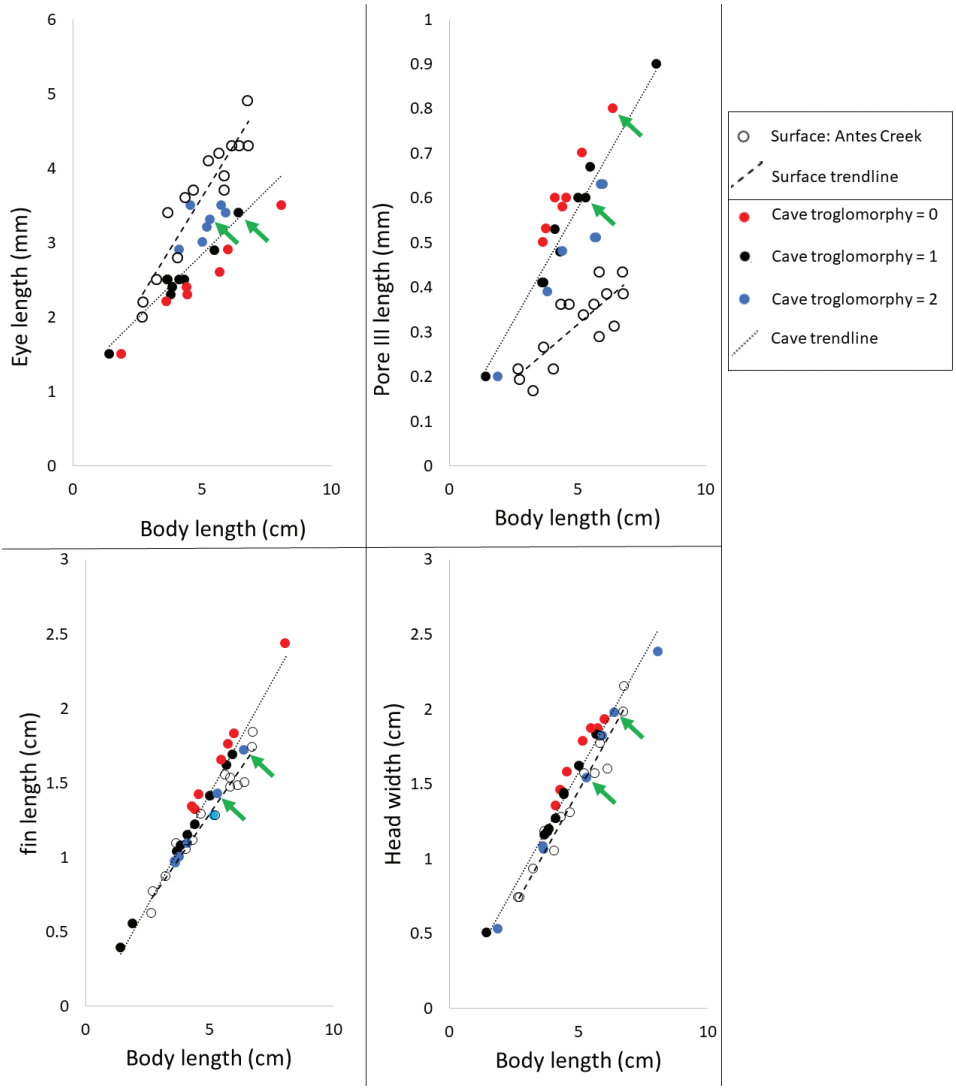


Figure 3. Morphometric comparisons between cave (closed circles) and epigean Antes Creek (open circles) sculpins (Modified from Espinasa and Jeffery 2003). Green arrows point to Lose tooth cave specimens, showing they are within the morphologically range of Eiswert #1 cave specimens. From these plots, cave specimens were further evaluated to assign their overall individual troglomorphic appearance. The seven individuals furthest above, nine closest, and the seven individuals furthest below the linear regression line were identified. Each were correspondingly assigned a numerical value to indicate they had a troglobitic (0; red), intermediate (1; black), or surface-like (2; blue) appearance.

Mean sequence divergence (*p*-distance in parenthesis) of the *16S rRNA* among the two clades was 11 (range 10–12; 1.9–2.2%) substitutions. Mean intra-clade sequence divergence within clades was of 1 (maximum of 2; 0.3% substitutions). Sequences in the first clade were most similar to *C. bairdii* (GenBank accession no. AY539018)

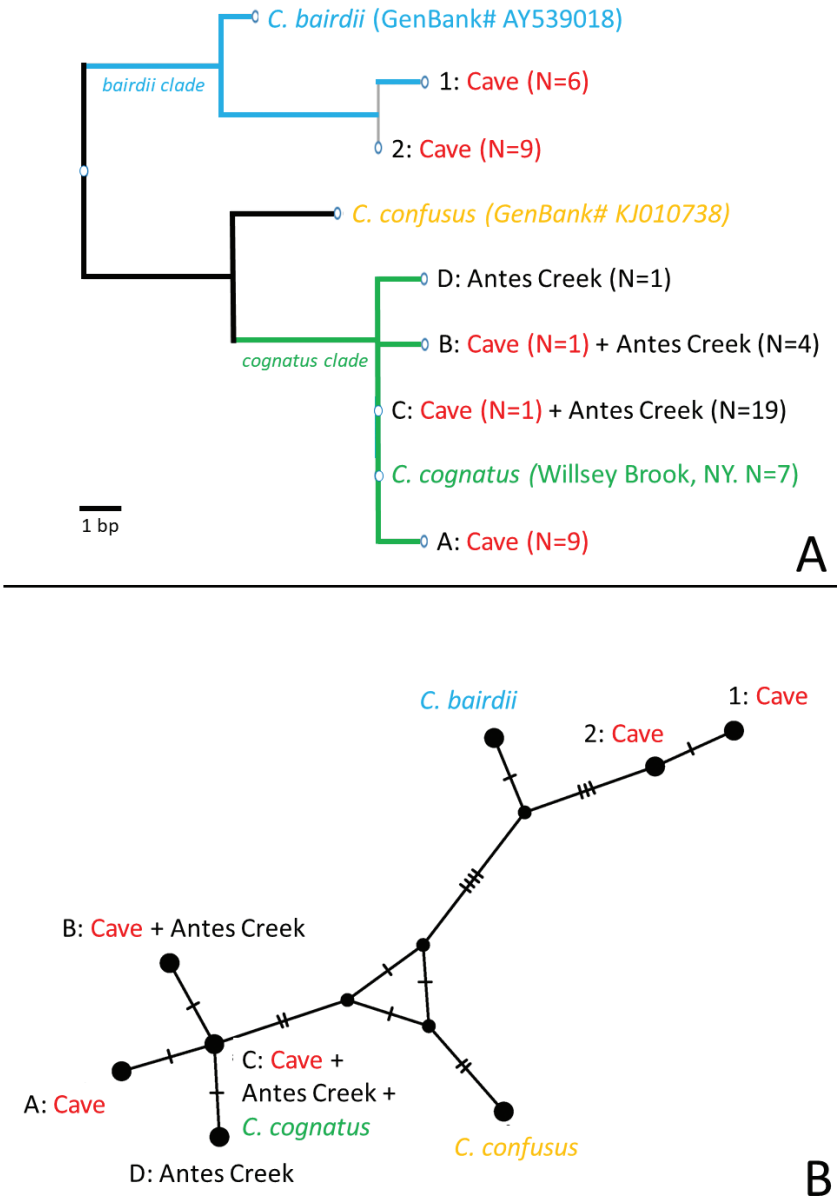


Figure 4. A maximum likelihood tree of the *16S rRNA* haplotypes found in cave and surface specimens. **B** minimum spanning haplotype network. Most similar sequences obtained in GenBank through BLAST analyses plus a sequence of *C. cognatus* from Willsey Brook, NY, are also included in the trees. Two distinct lineages were identified; one that includes *C. bairdii* specimens and one that includes *C. cognatus*. Cave haplotypes within the *C. bairdii* clade are identified by numerals (1–2) and Cave and Antes Creek haplotypes within the *C. cognatus* clade are identified by letters (**A–D**). Notice that cave individuals are found within both lineages. Despite being only 445 m apart, some haplotypes are present exclusively in cave individuals and not found in the Antes Spring population. Loose Tooth cave specimens had haplotype 2 and A.

whose sequence divergence was between 4–5 (0.7–0.9%) substitutions, and a group of three identical sequences (MT539220–MT539222). These last sequences were not included in the analyses because they were labelled by the authors as both *C. bairdii* and *C. cognatus*, despite having identical sequence, and thus are of doubtful provenance. Sequences in the second clade were most similar to seven *C. cognatus* from Willsey Brook, New York, that were sequenced in this study. Median sequence divergence between these *C. cognatus* and the Pennsylvania cave and surface members of the clade was 0 (range 0–2; 0–0.3%) substitutions. The next most similar sequence found with BLAST analyses was a *C. confusus* (KJ010738), with a sequence divergence of 5–6 (0.9–1.1%) substitutions.

16S haplotypes for all 24 surface specimens from Lochabar Spring on Antes Creek belonged to the second clade, which included *C. cognatus*. The fact that the Antes Creek population is mainly inhabited by *C. cognatus* is further supported by 90.4% of the cottids examined from Antes Creek possessing three pelvic rays (Espinasa and Jeffery 2003), which is a diagnostic character of *C. cognatus*.

Sequence data of the *16S rRNA* of the Pennsylvania grotto sculpin shows that, despite inhabiting a cave that is only 445 m from the aforementioned Lochabar Spring, it has a haplotype composition completely different from the local surface population in Antes Creek. Individuals from the cave harbor haplotypes from the first *C. bairdii* clade and from the second *C. cognatus* clade (Fig. 4). Of the 26 specimens analyzed, 15 (57.6%) had a haplotype within the *C. bairdii* clade and 11 (42.3%) within the *C. cognatus* clade. The cave population had a larger number of haplotypes (5) and a much higher gene diversity (h=0.720) than the Antes Creek population (3; h = 0.348). Haplotypes were more equitably distributed as the most common haplotype was in only 34.6% of the cave population, while in the Antes Creek, the most common haplotype was present in 79.1% of the surface population. Based on the *16S rRNA* data, there is strong population differentiation between the cave and surface populations (*Gst* = 0.361) with intermediate level of gene flow among them (Nm = 0.883).

Evidence suggests that the cave population is not simply surface *C. cognatus* from Antes Creek that happened to swim inside the cave and share the environment with local *C. bairdii*. As mentioned previously, of the 26 cave samples, 11 (42.3%) had haplotypes within the *C. cognatus* clade. Of these, 8 (30.7%) had a unique *C. cognatus* haplotype not found in any of the 24 surface Antes Creek *C. cognatus* specimens analyzed (Fig. 4). Thus, even within the individuals harboring haplotypes within the *C. cognatus* clade, the genetic structure is different between the cave and the surface populations.

Phylogenetic analysis of a second mitochondrial marker, the *D-loop* (Fig. 5), corroborated the two distinct clades identified with the *16S rRNA* data. A 786-bp fragment was analyzed in 15 cave and 11 surface individuals from Antes Creek. Twenty haplotypes were found (GenBank accession nos. GQ290440–GQ290447 in the first clade and GQ290448–GQ290459 in the second clade). Median sequence divergence between the two clades was 47 (range 46–49; 5.8%–6.2%) substitutions. Median sequence divergence within clades was of 1 (maximum 3; 0.3%) substitutions. BLAST analyses showed that the most similar sequence to the first clade in GenBank were two

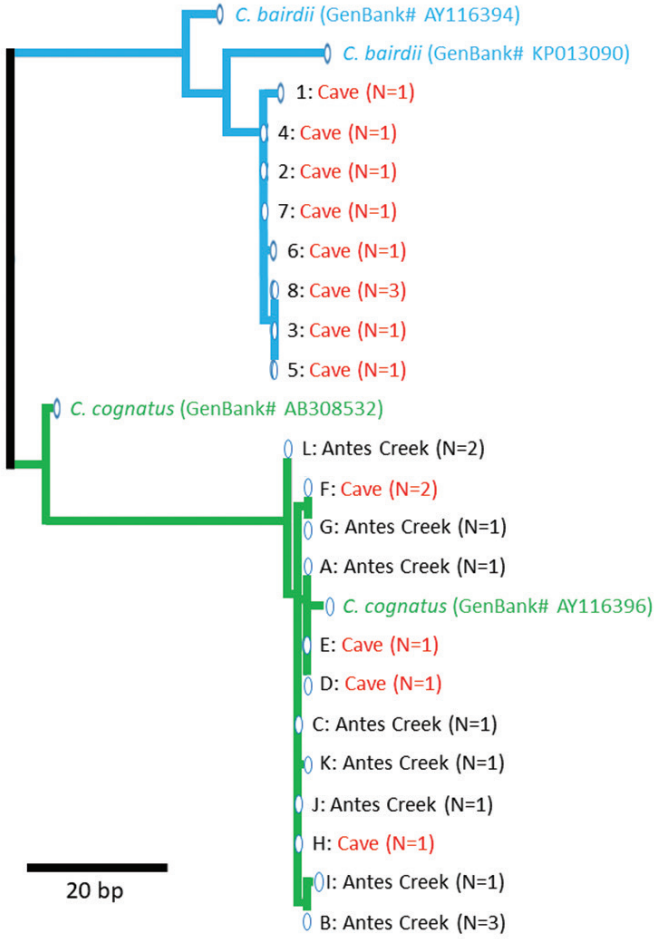


Figure 5. Maximum likelihood tree of the *D-loop* haplotypes found in cave and surface specimens. Most similar sequences obtained in GenBank through BLAST analyses are included in the tree. In agreement with the *16S rRNA* data, two distinct lineages were identified; one that includes *C. bairdii* specimens and one that includes *C. cognatus*. Cave haplotypes within the *C. bairdii* clade are identified by numerals (1–8) and Cave and Antes Creek haplotypes within the *C. cognatus* clade are identified by letters (A–L). Notice that cave individuals are found within both lineages.

sequences ascribed to *C. bairdii* (KP013090 and AY116394). Their sequence divergence against the cave specimens was between 21–22 (2.6–2.7%) substitutions. The second clade had as its most similar sequence in GenBank two sequences ascribed to *C. cognatus* (AY116396 and AB308532). Sequence divergence between these *C. cognatus* and the Pennsylvania cave and surface members of the clade ranged from 8–11 (1.0–1.3%) substitutions for AY116396 and 32–35 (4.0–4.4%) for AB308532. Just like with the *16S rRNA*, all 15 Antes Creek surface specimens analyzed harbored a haplotype within the *C. cognatus* clade, while those from the cave were mixed (Fig. 5).

Five individuals had a *D-loop* haplotype within the *C. cognatus* clade and eight within the *C. bairdii* clade. For the cave specimens harboring a mitochondrial *D-loop* haplotype within the *C. cognatus* clade, the haplotypes themselves were also distinct from the haplotypes hosted by *C. cognatus* surface fish (Fig. 5). As was expected from mitochondrial linked markers, *D-loop* results corroborate the previous findings with the *16S rRNA* that the genetic structure of the cave population is not simply the result of joining surface *C. cognatus* from Antes Creek that happened to swim inside the cave and share the environment with local *C. bairdii*.

Results from the *16S rRNA* and *D-loop* suggest that the cave population has haplotypes shared with two distinct lineages of surface sculpins. Since these two markers are mitochondrial, maternally inherited and linked, they cannot fully resolve if there has been introgression of two phyletic lines within the cave population. In order to resolve this, a nuclear marker (the *S7*) was sequenced. A 526-bp fragment of the nuclear S7 locus from 19 cave specimens showed the presence of two haplotypes differing by one bp (GenBank accession nos. MW039591 and MW039592). One of these haplotypes was identical to the sequence obtained from all 15 surface fish sequenced from Antes Creek and from the seven surface *C. cognatus* from Willsey Brook, New York. BLAST analysis of both haplotypes showed as most similar a *C. microstomus* (KY246946), from which it differs by 7–8 bp (1.3–1.5%), but it was noticed that no *S7* gene sequence for *C. bairdii* or *C. cognatus* has yet been uploaded to GenBank. Despite inhabiting a cave that is only 445 m from Antes Creek, it has an *S7* haplotype composition completely different from the local surface population in Antes Creek. While all the surface Antes Creek fish (N = 15) had the *C. cognatus* haplotype as homozygous (G base), 15 out of 19 (78.9%) cave fish had a haplotype not found in the surface fish, either as homozygous (A base) or heterozygous (A/G bases). Of the 19 cave specimens sequenced, four had the *C. cognatus* haplotype, five had the other haplotype, and ten were heterozygous for both haplotypes (Fig. 6). Loose Tooth cave specimens were homozygous, one for the A base and the other for the G base.

Of the 11 cave specimens analyzed for *S7* that harbor the *C. bairdi* clade mitochondrial haplotypes, two of them had the *S7* found in *C. cognatus*, and the rest were either heterozygous or had the other haplotype. Likewise, of the eight cave specimens that harbored the *C. cognatus* clade mitochondrial haplotypes, only two had exclusively the *C. cognatus S7* haplotype. The rest were either heterozygous or had the other haplotype (Fig. 6).

Morphology

As described by Espinasa and Jeffery (2003), the cave specimens from both caves were paler in color than the surface specimens, had smaller eyes, wider and more abundant mandibular pores, a wider head, and longer pectoral fins (Fig. 2). Some individuals after being exposed to light darken somewhat in color.

The range of troglomorphic appearance spanned from “0” when all characters analyzed in an individual were troglomorphic, to “8” when they were all epigeomorphic.

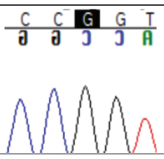
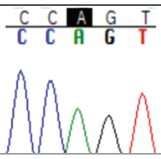
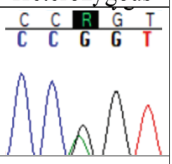
	G	A	Heterozygous
			
C. cognatus Willsey Brook, NY	N=7	-	-
C. cognatus Surface Antes Creek, PA	N=15	-	-
Cave with *C. cognatus* mitochondrial haplotypes	N=2	N=2	N=4
Cave with *C. bairdii* mitochondrial haplotypes	N=2	N=3	N=6

Figure 6. Two haplotypes were found for the *S7* ribosomal protein gene intron, which differed by a G/A base. Some individuals were heterozygous, as evidenced by a double bump in the chromatogram. *C. cognatus* individuals from Antes Creek or from New York had exclusively the *S7* haplotype with the G. Cave specimens with *C. cognatus* mitochondrial haplotypes could be homozygous or heterozygous for both *S7* haplotypes. Likewise, cave specimens with *C. bairdii* mitochondrial haplotypes could also be homozygous or heterozygous for both *S7* haplotypes. Thus, there is no correlation between nuclear haplotypes and mitochondrial clades, suggesting introgression within the cave population as a single reproductive unit. The Loose Tooth cave specimen was one of the specimens that had *C. bairdii* haplotype and an A, and the other specimen had *C. cognatus* haplotype and a G.

When a value for troglobitic appearance was assigned to each cave individual based on four distinct characters, it was found that troglobitic characteristics were spread arbitrarily among each group of mitochondrial or nuclear haplotypes, with no clear distinction between the two groups. Cave individuals harboring the *C. bairdii* clade mitochondrial haplotype had a mean troglomorphic index of 4.14 ($\tilde{x}$ = 4, stdev = 1.29, Range = 2–7, N = 15) and the cave individuals harboring the *C. cognatus* mitochondrial haplotype had a mean troglomorphic index of 3.77 ($\tilde{x}$ =4, stdev = 1.71, Range = 1–7, N = 11), which was not different from individuals belonging to the *C. bairdii* clade (P = 0.509). Furthermore, specimens from Loose tooth had morphologies within the ranges of Eiswert #1cave specimens (Fig. 3), suggesting they are part of the same population.

We also found morphological evidence of an independent assortment of morphological characters without regard to their mitochondrial haplotypes, as is expected in a reproductive population where unlinked genes/alleles segregate independently of each other. One of the clearest examples is with two specimens that have a *C. bairdii* clade mitochondrial haplotype. One specimen has a pore III width that is surface-like (Troglomorphy=2), but eye size is troglobitic-like (Troglomorphy=0). On the other hand, the other specimen has the exact opposite combination; pore III width troglobitic-like (Troglomorphy = 0) and eye size is surface-like (Troglomorphy = 2). Similar examples are also found within specimens harboring the *C. cognatus* clade mitochondrial haplotype.

Discussion

Eiswert #1 and Loose Tooth caves are both located within the same Nippenose karst valley and only 2.13 km from each other. Hydrologically, they are most likely part of the same subterranean drainage. Morphologically (Fig. 3), specimens of both populations are similar and the two Loose Tooth cave specimens have the most common *16S rRNA* and *S7* haplotypes found in Eiwert #1 cave population. The geographical proximity as well as morphological and genetic data support that specimens from both caves are part of the same underground population and it is likely that in the rainy season, when floods occur, specimens can swim from one cave to the other.

Eiswert #1 Cave is only 445 m away from the Lochabar Spring and the surface Antes Creek (Fig. 1). The cavefish have variable levels of pigmentation (Fig. 2) and at least some cave individuals have functional vision (Espinasa and Jeffery 2003). Could it be that the Pennsylvania grotto sculpin are nothing more than *C. cognatus* surface fish from Antes Creek that happened to swim upstream into the cave and became pale in the process? Our results reject this notion. A large proportion of individuals from the cave population have unique mitochondrial and nuclear haplotypes not found in the local surface counterparts, suggesting isolation in their evolutionary history. Twenty-four surface specimens were studied for the *16S rRNA* from the surface locality of Antes Creek. All individuals exhibited one of three haplotypes within a clade that includes *C. cognatus*. On the contrary, of the 26 cave specimens analyzed, only two individuals (7.6%) had the same haplotype as the surface population (Fig. 4). This result was supported with *D-loop* analyses of 12 cave and 15 surface individuals from Antes Creek, where not a single cave specimen shared a specific haplotype with the local surface population (Fig. 5). Likewise, 15 cave individuals out of 19 had a nuclear *S7* haplotype not found in the local surface population (Fig. 6).

Our results show that the cave population possesses mitochondrial *16S rRNA* and *D-loop* haplotypes shared with two distinct lineages of surface-dwelling sculpins. One line includes specimens assigned to *C. cognatus* and the other line to specimens assigned to *C. bairdi*. What could account for this? One scenario is that surface specimens from both species are simply entering the cave and the single cave population is a mixture of two surface species. Our results do not support this scenario. If two species were present, cave individuals harboring the mitochondrial haplotypes of the *C. cognatus* and the *C. bairdii* clade would have distinct and correlated nuclear and morphologic characters. Instead, we found that morphology, nuclear, and mitochondrial markers were unlinked among individuals of the cave population, suggesting a single reproductive population with introgression. Both nuclear haplotypes are found in cavefish that harbor either of the mitochondrial haplotype clades. Likewise, the nuclear *S7* shows heterozygosity in some individuals. Furthermore, there is evidence of an independent assortment of morphological characters without regard to their mitochondrial or nuclear haplotypes, as is expected in a reproductive population where unlinked gene/alleles segregate independently of each other.

If the Pennsylvania grotto sculpin as a population have a different genetic structure from the local surface *C. cognatus* and *C. bairdii*, what is their origin? One possibil-

ity is that the cave population derives from a single species who just happens to host divergent, ancient mitochondrial haplotypes. Coalescent theory shows that sometimes gene variants sampled from within a population may have originated from a common ancestor that antedates the split of its own species (Fumey et al. 2018). In essence, the presence of mitochondrial DNA haplogroups found in two different species does not necessarily imply that a population is composed of two separate species, i.e., the time of separation of the populations is not necessarily equal, not even close, to the time of coalescence of the mtDNA sequences (Fumey et al. 2018). The other scenario is that there was a hybridization event in the past between different members of the *C. cognatus*/*bairdi* species complex, whose vestiges comprise the cave population.

While the distinction may not be fully resolved until genomic studies are performed, it is our hypothesis that the hybridization scenario is the most likely. The number of rays in the pelvic fin is used as a diagnostic character to differentiate species of *Cottus* in this region. *Cottus cognatus* has three pelvic fin rays, while *C. bairdii* has four. If the Pennsylvania grotto sculpin derived exclusively from either species, it would be expected that the cavefish would conform morphologically to the species from which it derived and would either have three or four rays. Espinasa and Jeffery (2003) showed that the cave population does not fully ascribe to either. Eighty percent (80%) of the cave population has three rays, 6% has four rays, but, most significantly, 13% are asymmetric in which on one side they have three rays and on the other side they have four. Bilateral asymmetry has been employed as a criterion of developmental instability on the assumption that coordination among loci is disrupted by hybridization (Strauss 1986). In the Espinasa and Jeffery (2003) study, they showed that the percentage of asymmetric individuals for troglomorphic fish is an order of magnitude higher than in *C. bairdi* and *C. cognatus* but is comparable to the bilateral asymmetry shown in hybrid individuals found at Blockhouse Creek (Strauss 1986).

Hybridization between surface *C. bairdi* and *C. cognatus* has been reported at Blockhouse Creek (Strauss 1986), which is in the same county as Eiswert #1 Cave. Unlike the cave population, there is no indication of F2 progeny or backcrosses at this surface locality. What could account for this? A conjectural idea is that the cave habitat provided an adaptive peak where hybrids could survive that is normally not available within the adaptive landscape of surface sculpins. In other words, hybrids in surface habitats are probably eliminated by competitive exclusion of the non-hybrid surface species. The cave somehow provided a safe haven for a hybrid population, and now they may be undergoing speciation by isolation, as evidenced by the presence of unique haplotypes not found in local surface populations. While we do not discount that currently there may be surface fish that manage to enter the cave and even successfully hybridize with the cave population, the *16S rRNA* data shows there is strong population differentiation between the cave and surface populations (Gst = 0.361) with intermediate level of gene flow among them (Nm = 0.883). Another line of evidence in support of past hybridization when the cave population first evolved is the high levels of diversity. When two populations mix, hybrids acquire haplotypes from both parental populations. The cave population has a larger number of *16S rRNA*

haplotypes (n = 5) and a much higher gene diversity (h = 0.720) than the Antes Creek population (n = 3; h = 0.348).

Our results are not the first to report speciation by way of hybridization in cottids. In 2005, Nolte et al. (2005) described an invasive new lineage of sculpins in the Rhine River system of Central Europe. It was also found with mitochondrial haplotypes that the invasive sculpins are in fact hybrids between two species of the *Cottus gobio* complex. This invasive hybrid population of sculpins was determined to possess a unique ecological potential that does not occur in either of the source populations, which allowed for the colonization of new habitats that previously lacked sculpins. They concluded that hybridization led to a new, adaptationally distinct sculpin lineage.

Our results are similar to those of Nolte et al. (2005), with the difference that instead of a portion of the Rhine River, a cave was colonized. We also hypothesize that the cave population has been relatively isolated from both surface species, allowing for divergent and adaptive evolution to the cave environment. Evidence of this divergence is that most cavefish exhibit haplotypes not found in any of the surface specimens studied. If significant levels of introgression were present between the surface and cave populations, this degree of haplotype distinctiveness should not be found. Furthermore, the cave population has a unique and distinctive morphology, most likely adaptive to the cave environment, since enlarged fins and mandibular pores suggest an increased sensory capacity while in complete darkness.

Conclusions

Molecular and morphological data support the hypothesis that the Pennsylvania grotto sculpin is a distinct sculpin from *C. cognatus* and *C. bairdi*. Furthermore, data suggest that the cave population's evolutionary history may include an ancestral hybridization event between the separate members of the *C. cognatus/bairdii* species complex, but that currently there is limited gene flow from surface *Cottus* populations into the cave population. Such isolation accounts for the cave population's genetic and morphologic uniqueness. Based on these results, it is proposed that the Pennsylvania grotto sculpin deserves recognition as an independent species taxon from *C. bairdii* and *C. cognatus*. Recognition as an independent species will also help support current conservation efforts.

Acknowledgements

We thank all of the concerned citizens of the Nippenose Valley, especially David Hollick, for all of their encouragement, support, and friendship, without which this study could not have been done. We also would like to thank James C. D. Lewis for collecting the samples. Katherine D. Amodeo performed the morphological measurements. The students of the BIOL493: Molecular Biology Fall 2007 and BIOL320: Genetics Fall 2018 courses at Marist College performed some of the molecular techniques. Pa-

tricia Ornelas Garcia helped with developing the haplotype networks. We also thank Monika Espinasa, Jack Wimmershoff, Shivani Patel and Marylena Mesquita for proofreading and English language editing. Partial support for this study from the School of Science at Marist College.

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Subterranean Biology 38: 65–76 (2021)
doi: 10.3897/subtbiol.38.64762
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

First record of stygobiotic gastropod genus *Travunijana* Grego & Glöer, 2019 (Mollusca, Hydrobiidae) from Montenegro

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Academic editor: Vincent Prié | Received 21 February 2021 | Accepted 11 March 2021 | Published 15 April 2021

http://zoobank.org/6A604C23-5524-4D06-8ED1-D71344627018

Citation: Grego J, Pešić V (2021) First record of stygobiotic gastropod genus *Travunijana* Grego & Glöer, 2019 (Mollusca, Hydrobiidae) from Montenegro. Subterranean Biology 38: 65–76. https://doi.org/10.3897/subtbiol.38.64762

Abstract

The stygobiotic genus *Travunijana* Grego & Glöer, 2019 is one of the typical elements of the subterranean freshwater fauna of the Trebišnjica River Basin in Bosnia and Hercegovina. The previous records of the genus suggested that its distribution was limited to the basin of the Trebišnjica River and adjacent parts of the southwestern Neretva Basin. In this paper we describe a new species, *Travunijana djokovici* **sp. nov.** from "Vriješko Vrelo" spring in Montenegro. The recent finding of a new species in the Skadar Lake basin (The Drin River system) suggests the possibility of a subterranean route between the Trebišnjica and the Drin drainages, indicating that likely the endemic subterranean fauna in both basins has a lower level of historical isolation than has been considered so far.

Keywords

Bosnia, cave, karst-water, *Plagigeyeria*, spring

Introduction

The genus *Travunijana* Grego & Glöer, 2019 was only recently recognised based on its characteristic anatomy and shell morphology (Grego and Glöer 2019). Recent revision of another stygobiotic genus *Plagigeyeria* Tomlin, 1930 by Grego (2020) revealed that a number of species of the latter genus belongs to the genus *Travunijana*, and that the

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shell morphology with its protoconch sculpture is suitable evidence for the separation of these two genera (Grego 2020). The current distribution of ten taxa, i.e. *Travunijana vruljakensis* (Grego & Glöer, 2019), *T. ovalis* (Kuščer, 1933), *T. robusta* (Schütt, 1959), *T. robusta asculpta* (Schütt, 1972), *T. klemmi* (Schütt, 1961), *T. nitida* (Schütt, 1963), *T. angelovi* (Schütt, 1972), *T. edlaueri* (Schütt, 1961), *T. tribunicae* (Schütt, 1963) and *T. gloeri* (Grego, 2020) indicates that the Trebišnjica River Basin is the centre of endemism of the genus *Travunijana*. Outside the Trebišnjica River Basin, there are two records (a spring between Fatničko and Dabarsko Poljes and the spring of the Bunica near Mostar) from the southwestern part of the Neretva River Basin at its boundary with the Trebišnjica River Basin, with an intermittent divergence of the karst-water (Grego 2020) (Fig. 1).

The distribution pattern of the genus *Travunijana* appears to reflect well the relative isolation within the hydrogeological conditions with possible faunal dispersal pathways to adjacent basins. The genus is a typical element of the stygobiotic and crenobiotic fauna of the Trebišnjica River Basin, as a part of a remarkable and unique diversity that characterises the central part of the Dinaric Karst. The latter region is home to a number of typical stygobiotic species such as the olm *Proteus anguinus* (Laurenti, 1768), the bivalve *Mytilopsis kusceri* (Bole, 1962) and a polychaete, *Marifugia cavatica* (Absolon & Hrabě, 1930), whose southernmost distribution range is bounded by the Trebišnjica River Basin (see Pešić et al. 2018 for further discussion).

Recently, Pešić et al. (2020) moved the western border of the Southeastern Adriatic Ecoregion (which includes the Drin river system) west of the river Sutorina, located near the administrative border of Montenegro. In this concept, contrary to FEOW global freshwater ecoregion system, which places the eastern border of the Dalmatian Ecoregion near the town of Budva, the boundaries of the latter ecoregion ends or overlaps with the eastern boundary of the Trebišnjica River Basin (Lake Bileća) (Pešić et al. 2020).

The subterranean drainage boundaries between the Trebišnjica River Basin and Drin Basin together with the Kotor Bay drainage area is hydrogeologically well defined (Stevanović et al. 2014, Pešić et al. 2020) and might act as a barrier for stygobiotic and crenobiotic Mollusca dispersal. The existence of a subterranean barrier is supported by the finding of a number of typical mollusc genera at the both sides of the drainage boundaries. For the Trebišnjica Basin and the northward areas, the following stygobiotic and crenobiotic genera are typical, *viz*. *Mytilopsis* Conrad, 1958; *Travunijana* Grego & Glöer, 2019; *Emmericia* Brusina, 1870; *Belgrandia* Bourguignat, 1869; *Hadziella* Kuščer, 1932; *Kerkia* Radoman, 1978; *Narentiana* Radoman, 1973; *Belgrandiella* A. J. Wagner, 1928; *Dalmatinella* Radoman, 1973; *Sadleriana* Clessin, 1890; *Tanousia* Servain, 1881 and an undescribed genus represented by "*Pseudamnicola*" *troglobia* Bole 1961 (Kuščer 1933, Schütt 2000; Bilandžija et al. 2013; Beran et al. 2014, 2016; Rysiewska et al. 2017; Hirschfelder 2018, Falniowski et al. 2021). The above listed genera cannot be found in the Skadar Lake Basin (the Drin River system) and its associated Kotor Bay drainage basin, which have their typical freshwater malacofauna consisting of gastropod genera such as: *Bracenica* Radoman, 1973; *Anagastina* Radoman 1978; *Stygobium* Grego & Glöer, 2019; *Karucia* Glöer & Pešić, 2013; and

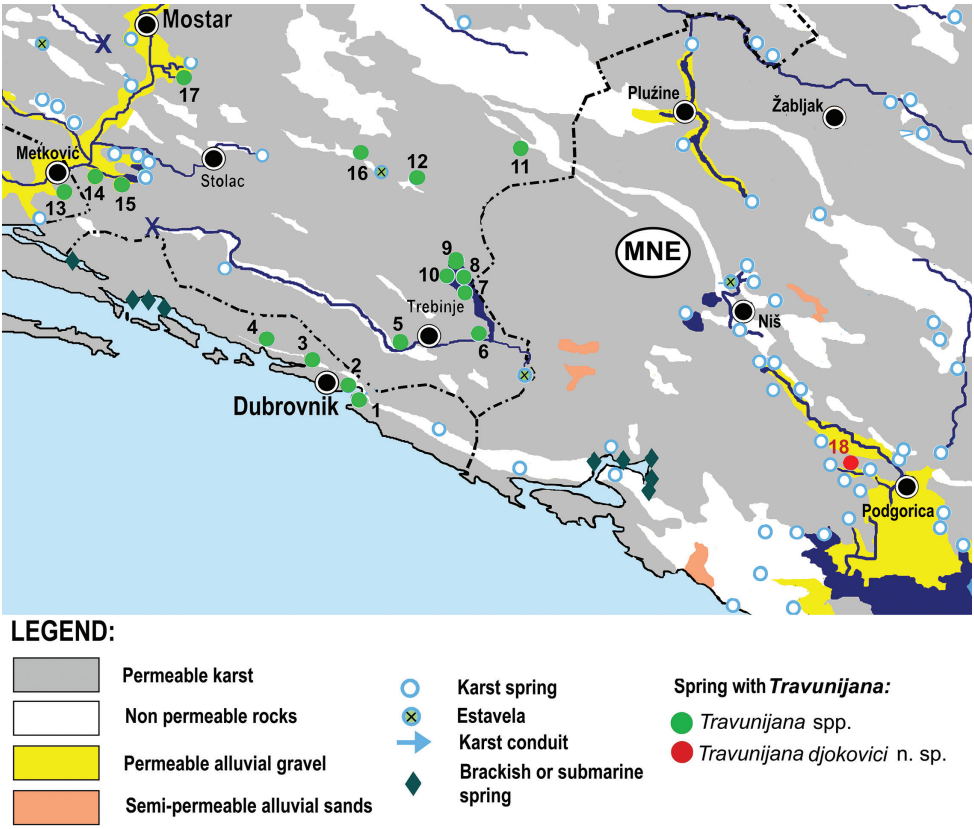

Figure 1. Distribution of the Genus *Travunijana* Grego & Glöer, 2019 (Green dots 1–17) and type locality of *Travunijana djokovici* sp. nov. (Red dot 18). Grey: Permeable karstified carbonates; White: impermeable unkarstified bedrocks; Yellow: Permeable alluvial deposits; Orange: sand deposits with limited permeability for subterranean gastropod dispersal. Light blue ring: karst springs; Blue ring with green centre and black X: estavelles; Dark green diamond: submarine or brackish karst spring.

Zeteana Glöer & Pešić, 2014 (Reischütz and Reischütz 2008; Pešić and Glöer 2012, 2013a, b; Reischütz et al. 2013, 2014, 2016; Glöer and Pešić 2014a, b; Glöer et al. 2015; Grego et al. 2017, 2019).

Several freshwater genera can be found on both sides of the drainage boundaries, but are represented in them by different species (Schütt 1961, 1963, 1972, 2000; Glöer and Grego 2015). Such stygobiotic genera include *Plagigeyeria* Tomlin, 1930; *Paladilhiopsis* Pavlović, 1913; *Iglica* A. J. Wagner, 1910; *Islamia* Radoman, 1973 and *Theodoxus* Roth, 1855 (for its subterranean species *T. subterrelictus* Schütt, 1963 in the Trebišnjica Basin) and *Adriohydrobia* Radoman, 1977 for crenobiotic fauna. Only one stygobiont hydrobiid genus and species, *Montenegrospeum bogici* Pešić & Glöer, 2013 is known to occur in both sides of the boundary over longer distances northward, suggesting that this euryvalent stygobiont is adapted to a broad range of subterranean habitats supporting its large dispersal (Falniowski et al. in press). In a similar way, the

more widespread crenobiont species such as *Radomaniola curta* (Küster, 1853) and *Litabithella chilodia* (Westerlund, 1886) can be found at both sides of the underground watershed boundaries (Radoman 1983).

Nevertheless, there is evidence for the existence of an underground hydrological connection between the Trebišnjica Basin and the neighbouring Skadar Lake Basin. A remarkable example is the existence of an intermittent karst-water connection which seasonally drains the water from the Skadar Lake Basin through the Gornjepoljski Vir Estavelle (628 m alt.) towards the "Nikšićko Vrelo" spring (468 m alt.) near Bileća in the Trebišnjica Basin. This unique subterranean route represents a distance of 38 km of aerial distance and 189 m elevational difference. The latter route is an aquatic migratory corridor for some of the stygobiotic species such as *Plagigeyeria zetatridyma* Schütt, 1960 (confirmed by empty shells; see Schütt 1970) and *Montenegrospeum bogici* (confirmed by molecular data, Falniowski et al. in press). The latter species were found at both sides of the drainage boundaries, indicating that the above-mentioned underground route plays an important role in their dispersal . There is an indication that a similar route is used by the crenobiont genus *Anagastina*, which was found at a single site in Trebinje (Grego, unpublished data). The current discovery of a new representative of genus *Travunijana* in "Vriješko Vrelo" spring belonging the Skadar Lake Basin, suggests a more complex pattern of spatial distribution of stygobiont habitats of the studied area than was hitherto known, indicating the possible existence of an underground path for the spread of subterranean species from the Trebišnjica Basin to the Drin Basin.

Material and methods

The studied material was collected during a field trip in April 2019 to both Montenegro and Bosnia and Hercegovina. The samples were collected by wet sieving (Grego et al. 2017) and sorted out from the sandy sediments, and fixed in 70–80% ethanol. The live specimens were transferred to 80% analytical ethanol. Photographs were made by a digital camera system Leica R8 (Leitz Photar 21 mm objective with Novoflex bellows), ImageJ scientific image analyzing software was used for making the measurements together with direct measurement with an eye-piece micrometer.

Abbreviations

HNHM Hungarian Natural History Museum, Budapest;
NHMW Naturhistorisches Museum, Wien;
SBMNH Santa Barbara Museum of Natural History, California, USA;
JG Collection Jozef Grego;
H Shell height;
W Shell width;
WB Width of the body whorl;
HA Aperture height;
WA Aperture width.

Systematics

Superfamily Truncatelloidea J.E. Gray, 1840
Family Hydrobiidae Stimpson, 1865
Genus *Travunijana* Grego & Glöer, 2019

***Travunijana djokovici* sp. nov.**
http://zoobank.org/93FAB9F2-2C62-46B8-92D0-8494695354E5
Figs 3A, B, 4A, B

Typelocality. Montenegro • Danilovgrad district, Bandići Village, Vriješko Vrelo spring under road to Modro Oko Lake, left tributary of Matica River, 42.481206°N, 19.145484°E (Fig. 1 and 2).

Typematerial. ***Holotype*:** Typelocality: J. Grego, G. Jakab. M. Olšavský, M. Kováčiková leg. 20.04.2019, NHMW1132628.

***Paratypes*:** same data: NHMW113629/ 2 dry specimens, HNHM105300/ 2 dry specimens and 37 dry specimens T 1293 in coll. Grego.

Measurements. ***Holotype*** H 3.23 mm; W 1.77 mm; WB 1.46 mm; HA 1.44; WA 1.14.

Paratype H 3.33 mm; W 1.86 mm; WB 1.46 mm; HA 1.49; WA 1.26.

Other material. Same locality data: 12 fragmented shells in coll. JG T 1294.

Figure 2. Photo of the type locality of *Travunijana djokovici* sp. nov. (photo M. Olšavský).

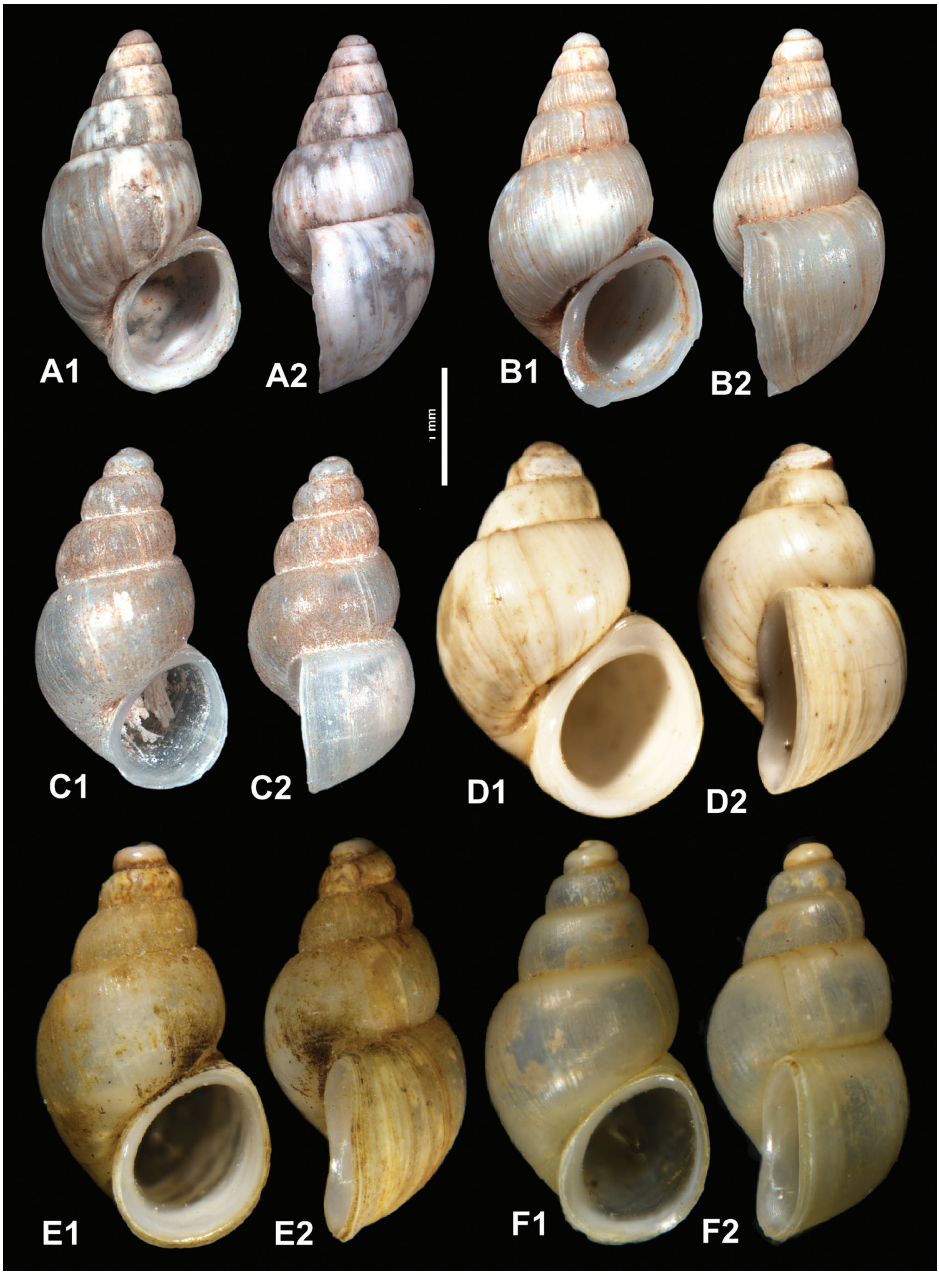

Figure 3. A, B *Travunijana djokovici* sp. nov. **A** holotype NHMW 1132628 **B** paratype, coll. JG F12393 (photo P. Glöer) **C** *T. vruljakensis* Grego & Glöer, 2019, holotype, HNHM Moll 100 174 **D** *T. ovalis* (Kuščer, 1933) **E, F** *T. gloeri* Grego, 2020 **E** holotype HNHM-Moll 104 418 **F** paratype JG F1340.

Differential diagnosis. The faintly pitted protoconch clearly distinguishes the new species from the members of the genus *Plagigeyeria* and indicates its position within the genus *Travunijana*. Moreover, the weakly sinuated columellar margin also supports its generic assignment.

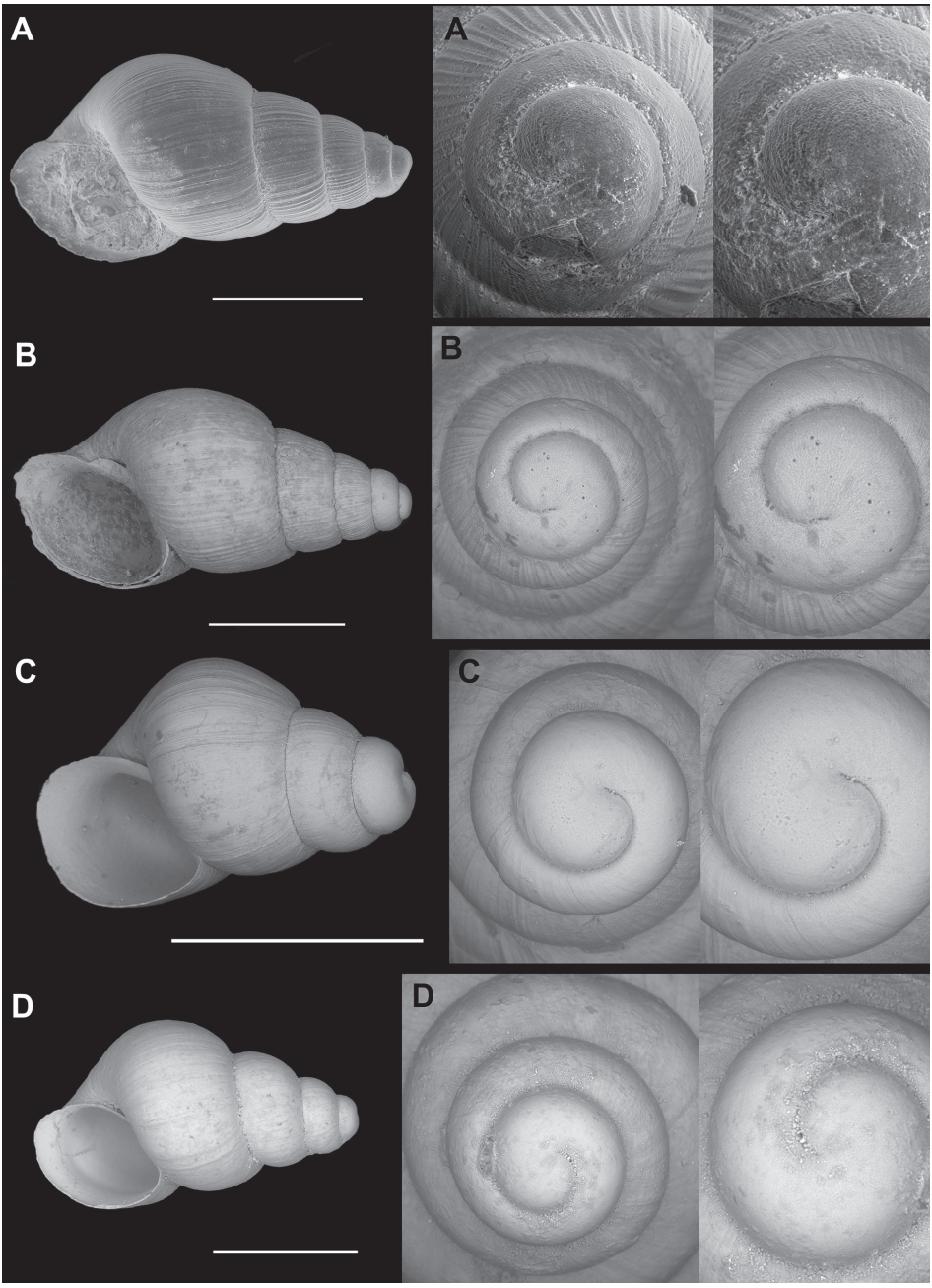

Figure 4. M protoconch SEM images of *Travunijana* **A, B** *Travunijana djokovici* sp. nov., paratype, SBMNH 632721) **C** *T. vruljakensis* Grego and Glöer, paratype SBMNH 625961 **D** *T. gloeri* Grego, 2020, paratype SBMNH 632722. Scale bars: 1 mm. (SEM SBMNH Vanessa Delnavaz).

Compared to the most closely related *Travunijana gloeri* Grego, 2020, *T. vruljakensis* Grego & Glöer, 2019 and *T. ovalis* (Kuščer, 1937), all from Trebišnjica Basin (Fig. 3C–F), the new species differs by its shell morphology, which has more elongate-

pyramidal shape, more distinct regular ribs on the shell surface and an elongate lens-like aperture, the latter with more distinct siphonal and anal grove.

Description. ***Shell:*** elongate-conical, milky whitish colour, periostracum yellowish, consisted of five moderately convex whorls with a semi-deep suture. The surface covered by dense, regular weak axial ribs. Aperture elongate, lens-like, its axis declined from the columella; the labral peristome straight in its lateral profile and slightly outward curved and callous. The columella peristome weakly sinuated at its middle part. The siphon and anal groves clearly indicated at the tips of elongated aperture. The umbilicus is tiny, almost closed and hidden behind the reflected columellar margin.

Protoconch very faintly and densely pitted, at the penultimate whorls smoothly transferring through weak malleated structures, faint regular growth lines into a regular weak ribbing covering the rest of the teleoconch.

Etymology. The new species is named after Novak Djokovic a famous Serbian tennis player to acknowledge his inspiring enthusiasm and energy.

Distribution. Montenegro; only known from the type locality.

Ecology. The type locality is a medium sized karst spring with stable water outflow, situated at the border of a limestone massif and alluvium of the Matica River. The status of empty shells washed out of the spring-head suggests its stygobiont habitat inside the karst conduit. The new species was syntopic with *Plagigeyeria* cf. *montenegrina* Bole, 1961, *Bracenica* cf. *spiridoni* Radoman, 1973, *Zeteana* sp. and *Montenegrospeum* cf. *bogici* (Pešić & Glöer, 2012) at its type locality thanatocoenosis.

Conservation status. Number of known locations (1) fewer than 5 and AOO (area of occupancy) smaller than 20 km^2. There is no reason to suppose that AOO, EOO (extent of occurrence), number of locations, number of subpopulations or the number or mature individuals are declining or extremely fluctuating. However, due to small AOO it is assessed as Vulnerable (VU) D2 species.

Discussion

The find-site of the stygobiotic *Travunijana djokovici* sp. nov. in the Skadar lake Basin belonging to the Southeastern Adriatic Ecoregion, is located 60 km southward from the main distribution range of the genus in Hercegovina and south Dalmatia. This represents not only a significant range extension of the genus (Grego 2020), but also indicates an enigmatic stygobiotic migration dispersal route through the relatively well defined subterranean drainage boundary between the Trebišnjica and Drin basins (Pešić et al. 2020). As mentioned in the Introduction section, the existence of such a boundary is supported by finding numerous stygobiotic and even crenobiotic Mollusca genera and species that are endemic to each of the neighbouring basins. Recently, Pešić et al. (2018, 2020) stated that the south-eastern border of the distribution area of some holodinaric species such as the cave salamander *Proteus anguinus*, the polychaete *Marifugia cavatica*, the hydrozoan *Velkovrhia* and the bivalvian *Mytilopsis kusceri*

coincided with the northwest administrative border of Montenegro, which indicates the existence of "the common biotic break of holodinaric taxa in the northwest region of Montenegro" (Pešić et al. 2020).

However it is known that the drainage boundaries do not represent absolute barriers for the dispersal of subterranean animals, and as we mentioned in the Introduction, there is at least one such karst conduit intermittently cross-connecting both basins and draining water from Gornjpoljski Vir Estavelle (the Drin River Basin) to "Nikšićko Vrelo" spring in the Trebišnjica Basin (Grego 2020). This intermittent connection is active at the high water saturation during both the winter and spring time. Its possible functionality as a migration path for stygobiotic Mollusca was firstly indicated by the finding of *Plagigeyeria zetatridyma*, a species typical for stygofauna of the Zeta basin of Drin River system, in "Nikšićko Vrelo" spring (Schütt 1972), one of the three major Trebišnjica wellspring outlets that have been submerged under Bilećko Lake since 1967. However, those finds were only empty shells transported through the 38 km long karst conduit and if ever a live specimen reached the Trebišnjica basin, the species was obviously never dispersed very far from the outlet springs near Bileća (Schütt 1972).

On the other hand, the recent finding of the live specimens of *Montenegrospeum bogici*, a species that was supposed to be limited to the Skadar Lake basin (the Drin River system), in springs located far downstream and upstream of Bileća (Falniowski et al. in press) suggests the possibility of stygobiont gastropod migration from the Drin river system to the Trebišnjica River Basin (Fig. 5). It is likely that a wide habitat preference of *M. bogici*, ranging from open cave streams and cave lakes to alluvial interstices, plays an important role in the large dispersal ability of the latter species (Falniowski et al. in press).

All the above mentioned observations indicate occurrence of only one dispersal mode from the Skadar Lake Basin to the Trebišnjica Basin through the above explained connection route. On the other hand, for *Travunijana*, we can speculate a similar dispersal mode though the same route but in an opposite direction, from Trebišnjica to Zeta River Basin, which probably occurred under different historical hydrological conditions. Despite the intensive sampling of the stygobiont malacofauna of the Skadar lake basin, which began in the middle of the last century (Pešić et al. 2018), no *Travunijana* specimen has been found in the studied region. Therefore, we assume that the underground route between the Trebišnjica and Zeta River Basins is the main way for *Travunijana* dispersal to the type locality near the Matica River.

The permanent submersion of the Trebišnjica spring group at Bileća by Lake Bilećko in the 1970s (when the Trebinje 1 Hydroelectric Power Plant was completed) has made it impossible to find living specimens of stygobiont species for molecular analysis and, consequently, no molecular evidence of the gene flow between two basins which would confirm the functionality of this migration route. Therefore, the origin and dispersal route of *T. djokovici* sp. nov. still remains a mystery. We cannot exclude the existence of similar divergent channels between the basins during the geological history of the area, especially knowing that the present hydrological situation does not necessarily have to reflect the paleohydrology. Last, but not least, anthropogenic changes in the directions

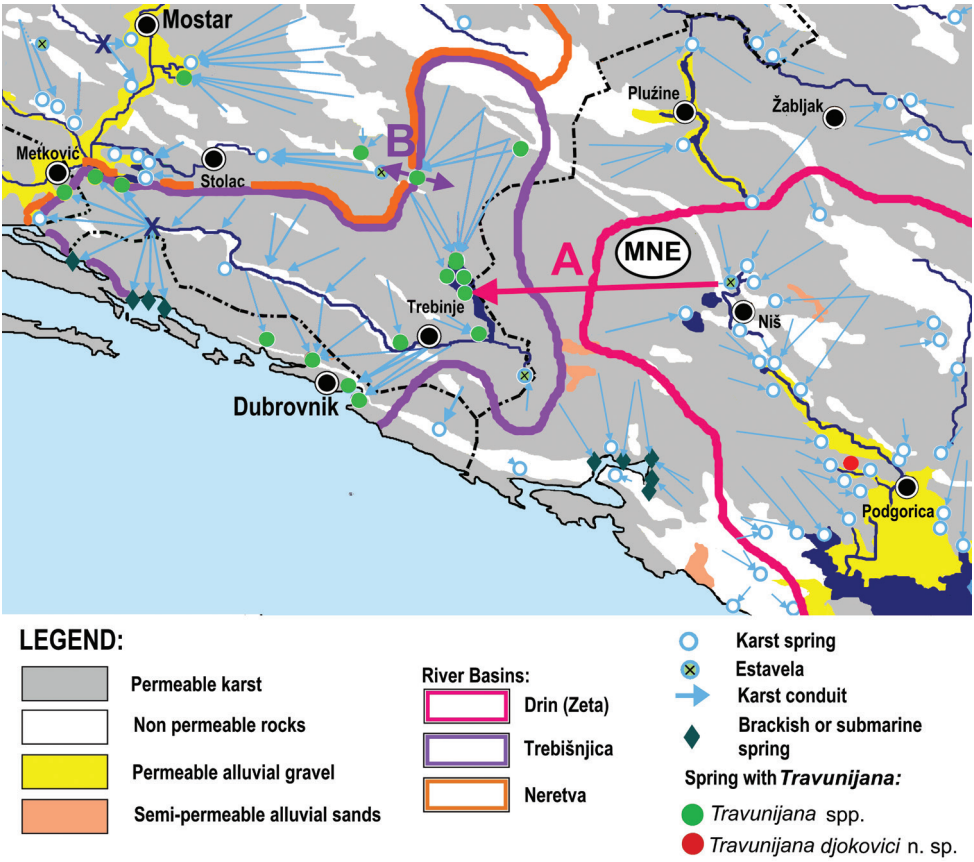

Figure 5. Distribution range of *Travunijana* and the type locality of *T. djokovici* sp. nov. superimposed by the local hydrogeological conditions.

of karst-water flows as a result of several large dams and diversion of river flows, with the aim of diverting water to power plants (on the rivers Zeta and Trebišnjica) make the understanding of possible migration routes of stygobionts even more difficult.

Acknowledgements

We would like to express our thanks to Daniel Geiger and Vanessa Delnavaz for their support with SEM imaging; to Anita Eschner and Sara Schnedl (NHMW, Wien, Austria) and Peter Glöer (Hetlingen, Germany) for their support with microphotography. We are especially grateful to Gabriel Jakab (Plešivec, Slovakia), Mário Olšavský and Miroslava Kováčiková (Banská Bystrica, Slovakia) and Milica Jovanović (Podgorica, Montenegro) for their active participation on our 2019 field trip. Special thanks to Brian Lewarne from the Devon Karst Research Society, Plymouth, UK and to the "Proteus Project in R. Bosnia & Hercegovina" for valuable discussions about the local hydrology and for the help with English editing.

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Subterranean Biology 38: 77–90 (2021)
doi: 10.3897/subtbiol.38.66254
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Collembola from Hundidero-Gato Cave in Southern Spain, with the description of a new species of *Entomobrya* Rondani, 1861 (Collembola, Entomobryidae)

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Academic editor: Ľubomír Kováč | Received 21 March 2021 | Accepted 14 April 2021 | Published 19 April 2021

http://zoobank.org/4B29C24B-8715-4620-88E9-B4D0086F98FB

Citation: Arbea JI, López VG, Soria FJ, Abellán P (2021) Collembola from Hundidero-Gato Cave in Southern Spain, with the description of a new species of *Entomobrya* Rondani, 1861 (Collembola, Entomobryidae). Subterranean Biology 38: 77–90. https://doi.org/10.3897/subtbiol.38.66254

Abstract

This paper deals with some Collembola from a cave at La Sierra de Grazalema (Málaga). In total, eight species of springtails were found. Two may represent new species but there is insufficient material available to prepare full descriptions (one species in the genus *Ceratophysella*, one in *Hypogastrura*), one species (*Folsomides* cf. *ayllonensis*) is identified to species, but differences from the nominal species suggest further studies may indicate the Grazalema populations represent a distinct form, and one other is described as new to science, *Entomobrya virginiae* Arbea, **sp. nov.** The new species is characterized by no sexual dimorphism in colour pattern, 5 central macrochaetae on Abd II and 4 macrochaetae on Abd III. For the identification and description of the new species, the set of characters proposed by Jordana and Baquero (2005) was used.

Keywords

Arthropoda, biospeleology, Entomobryidae, *Entomobrya*, taxonomy

Introduction

The Hundidero-Gato system, within the Subbaetic Mountain range (Southern Spain), is a hydrogeological system composed by more than 10 km of galleries. This cave, located between the municipalities of Montejaque and Benaoján in the province of Málaga

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(Andalusia, Spain), has two mouths: the first one is Hundidero, which acts as a sink, and the other one, which is called Gato, where the complex drains. The system harbours a wide variety of subterranean environments, including an old subterranean river course, subterranean lakes, broad chambers and galleries (Guerrero Sánchez 2015).

The information about the subterranean biology in this system is scarce and fragmented (García-López et al. 2020). The first studies were carried out in 1865 by Lucas von Heyden, a German entomologist who wanted to find blind coleopterans. Subsequently, the abbot Henri Breuil, in 1914, could see the presence of warm guano inside the cave but did not capture anyone. Years later, in the 1970s, some specific surveys were carried out by certain speleological groups, such as the GE Telefónica of Madrid, GEOS of Sevilla and the E.R.E from C.E.C. from Barcelona, among others. In 1978, a Dutch speleological group described the spider *Iberoneta nasewoa* Deeleman-Reinhold, 1984, the aquatic amphipod *Pseudoniphargus stockin* Notenboom, 1987, and the gastropod *Iberhoratia gatoa* (Boeters, 1980) (Boeters 1980; Deeleman-Reinhold 1984; Notenboom 1987). Despite the interest of many researchers throughout decades, the deepest and most extended studies did not begin until March of 2016 (Hernando and García-López 2016), which revealed a new endemic species, the troglobitic staphylinid *Phloeocharis manu* Hernando & García-López, 2016. More recently, twelve spider species have been recorded for this cave, which belong to nine families: Agelenidae, Araneidae, Dysderidae, Hahniidae, Linyphiidae, Liocranidae, Segestriiadae, Tetragnathidae and Uloboridae) (Barrientos et al. 2019).

There are few references on the cave-dwelling Collembola in the Subbaetic System. Only nine species have been recorded in some caves from Sierra de las Nieves, Sierra de Almijara and Sierra de Tejeda (Arbea and Baena 2003; Arbea et al. 2011; Arbea 2012): *Ceratophysella engadinensis* (Gisin, 1949), *Deuteraphorura cebennaria* (Gisin, 1956), *Onychiurus gevorum* Arbea, 2012, *Entomobrya pazaristei* Denis, 1937, *Lepidocyrtus flexicollis* Gisin, 1965, *Pseudosinella infrequens* Gisin & Gama, 1969, *Pseudisotoma monochaeta* (Kos, 1942), *Heteromurus nitidus* (Templeton, 1835) and *Troglopedetes absoloni* Bonet, 1931. This is the first study in which a cave from the Sierra de Grazalema have been sampled for Collembola capture. The biospeleological work has been developed in the Hundidero-Gato system. A total of 597 specimens have been captured, belonging to 8 different species. Among them there is one new species, belonging to the genus *Entomobrya*.

Material and methods

Cave description

The Hundidero-Gato system is located in the northeast extreme of Sierra de Líbar, within the mountain range of Ronda, in the northwest of the province of Málaga. In this cave we can find three characteristic types of karstification: the first floor corresponds to a first stage, while the lower floor is always flooded; the middle floor is the most relevant as it corresponds with the speleological trek and it is the most developed.

Due to the extension of the system, we have limited the study area to the most accessible part at the entrance. The coordinates of Hundidero are 36°43'39"N, 5°14'19"W, about 558 m above the sea level. The entrance of the cave has a width of 10 m and a height of approximately 60 m, and 400 m of depth accessible on foot.

Sampling

We used baited pitfall traps to collect invertebrates, which were placed in some areas of the cave that were susceptible to harbor fauna. For the pitfall traps we used clinical analysis cans (volume 150 ml), which were buried and filled with a modification of Turquin´s liquid (Domingo-Quero and Alonso-Zarazaga 2010). The traps were baited with Majorcan sausage (cured sausage from Balearic Islands made with pork, paprika, salt and other spices) as attractant (Belda-García et al. 2014). All collected specimens were preserved in 70% ethanol.

Two areas were sampled: the entrance to the cave, which covers about 400 m from the mouth to the first lake, and the area called gallery, a ledge located after about 20 m from the lake, about 20 m in the vertical of the wall, corresponding to the deepest area that was sampled. In total, sixteen pitfall traps were placed: four in semi-darkness (twilight) and four in total darkness in the entrance area; and five traps in soil with guano and three in soil without guano in gallery. The sampling was carried out in 2016 (both the entrance and gallery) and 2018 (only the entrance), and the traps were placed in the dates that the Natural Park of Sierra the Grazallema allowed us. The traps were in the cave for one week. The entry seasons established by the Park was governed by the activity of the bats, allowing us access from mid-March to mid-April and later from mid-August to mid-November, in order to not disturb the breeding and wintering periods of the bats.

Preparation and analysis

Some specimens were cleared in Nesbitt's fluid and were mounted in Hoyer's medium for compound microscope observation in phase constrast. Figures were drawn with a camera lucida.

Terminology

Chaetotaxy schemes in *Entomobrya* follow Szeptycki (1979), Jordana and Baquero (2005) and Jordana (2012). Symbols used in dorsal chaetotaxy schemes are: open circle in the figures stands for the presence of large mesochaetae or macrochaetae that could be absent in male and juvenile specimens but present in females, closed circle stands for macrochaetae that are always present, black chaetae-like drawings to sensilla, large black circles with a cross line to pseudopores, long chaetae to bothriotricha.

Abbreviations used: Abd = abdominal segment; Ant = antennal segment; as = sensilla; mc = mesochaeta(e); Mc = macrochaeta(e); psp = pseudopore; Th = thoracic segment; **MNCN** = National Museum of Natural Sciences at Madrid, Spain; **MZUN** = Museum of Zoology, University of Navarra, Spain.

Results

Family Hypogastruridae Börner, 1906

Ceratophysella sp.

Material studied. Cueva del Gato, twilight zone, 27-III-2016, 1 ♀

The single individual collected apparently represents an undescribed species similar to *C. norensis* Cassagnau, 1965, characterized by A-type of chaetotaxy (p1 chaeta shorter than p2 chaeta on Abd IV), dens with 6 chaetae, the basal chaeta more than twice as long as the others, retinaculum with 4+4 teeth, tibiotarsi with one pointed tenent hair, claw with one internal tooth and two lateral teeth, filament of empodium 2/3 as long as the inner edge of claw, Ant IV with trilobed apical bulb and 7 thin sensilla, slender anal spines 1.5 times the long of the claws and 1.8 mm length. The species is dark blue and does not show characters typically present in cave-adapted species.

Additional material is needed to confirm the identity of the species.

Hypogastrura sp.

Material studied. Cueva del Gato, twilight zone, 27-III-2016, 1 juv.

The single individual collected appears to represent a new species similar to *H. vernalis* (Carl, 1901), characterized by tibiotarsi with one slightly knobbed tenent hair, empodium with broad basal lamella, dorso-lateral parts of Th II–III with m6 chaeta, Ant IV with trilobed apical bulb and 7 sensillae, dens with 6–7 chaetae, 2.7 times as long as mucro, PAO nearly the same size as an ommatidium, anal spines minute, on high papillae, coarse body granulation (8 granules between the p1 chaetae of Abd V) and 1 mm length. The species is grey-blue and does not show characters typically present in cave adapted species.

Additional material is needed to confirm the identity of the species.

Mesogastrura ojcoviensis (Stach, 1919)

New Sierra de Grazalema (Málaga) record.

Material studied. Cueva del Gato, twilight zone, 27-III-2016, 16 ex; dark zone, 14-IV-2016, 1 ex, 13-XI-2016, 2 ex, 4-X-2018, 25 ex; cave galleries, 14-IV-2016, 1 ex, 30-IX-2016, 1 ex.

Edaphic and troglophilous, guanophilous species. This is a common European species in the caves of the Iberian Peninsula (Spain and Portugal) and the Balearic Islands (Jordana et al. 1997).

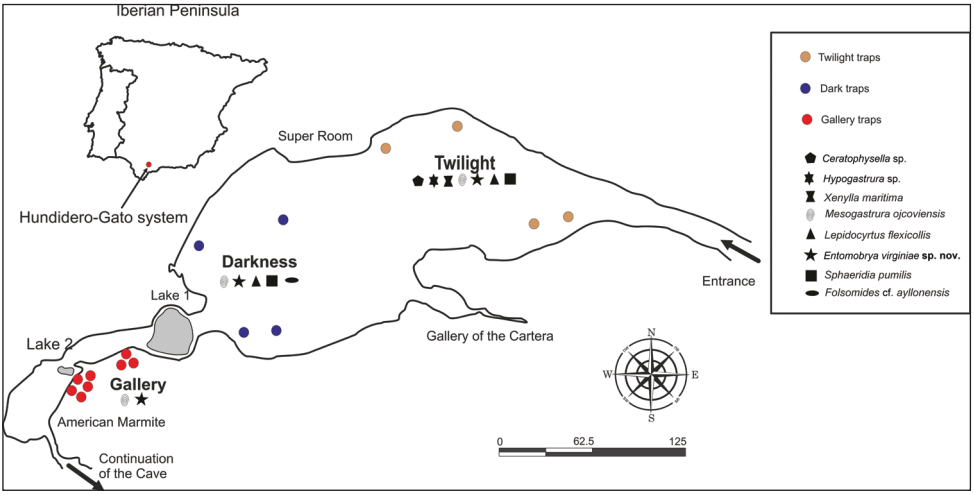


Figure 1. Topography of Hundidero-Gato system and locations where the Collembola were sampled. Modified from Guerrero Sánchez (2015). In brown, the twilight pitfall traps, in blue, the darkness pitfall traps and in red, gallery traps (five on guano and three on ground without guano). The different species that appear in each area are represented with their own symbol in the legend.

Xenylla maritima Tullberg, 1869

New Sierra de Grazalema (Málaga) record.

Material studied. Cueva del Gato, twilight zone, 27-III-2016, 3 ex, 16-VIII-2018, 1 ex.

This is a cosmopolitan species known to occur in dry habitats, surface leaf litter or lichens on rocks (Thibaud et al. 2004). May appear in the mesovoid shallow substratum or in caves accidentally.

Family Isotomidae Schäffer, 1896

Folsomides cf. *ayllonensis* Simón & Luciáñez, 1990

New Sierra de Grazalema (Málaga) record.

Material studied. Cueva del Gato, dark zone, 30-IX-2016, 1 ex.

The species lacks pigment, but has 5+5 eyes. The juvenile (0.5 mm) examined differ from *F. ayllonensis*, as original description (Simón and Luciáñez 1990) and notes made by Fjellberg (1993), in having an oval postantennal organ, 1.7–1.8 as long as omma (>2 in *F. ayllonensis*), and in having three posterior chaetae on dens (two in *F. ayllonensis*).

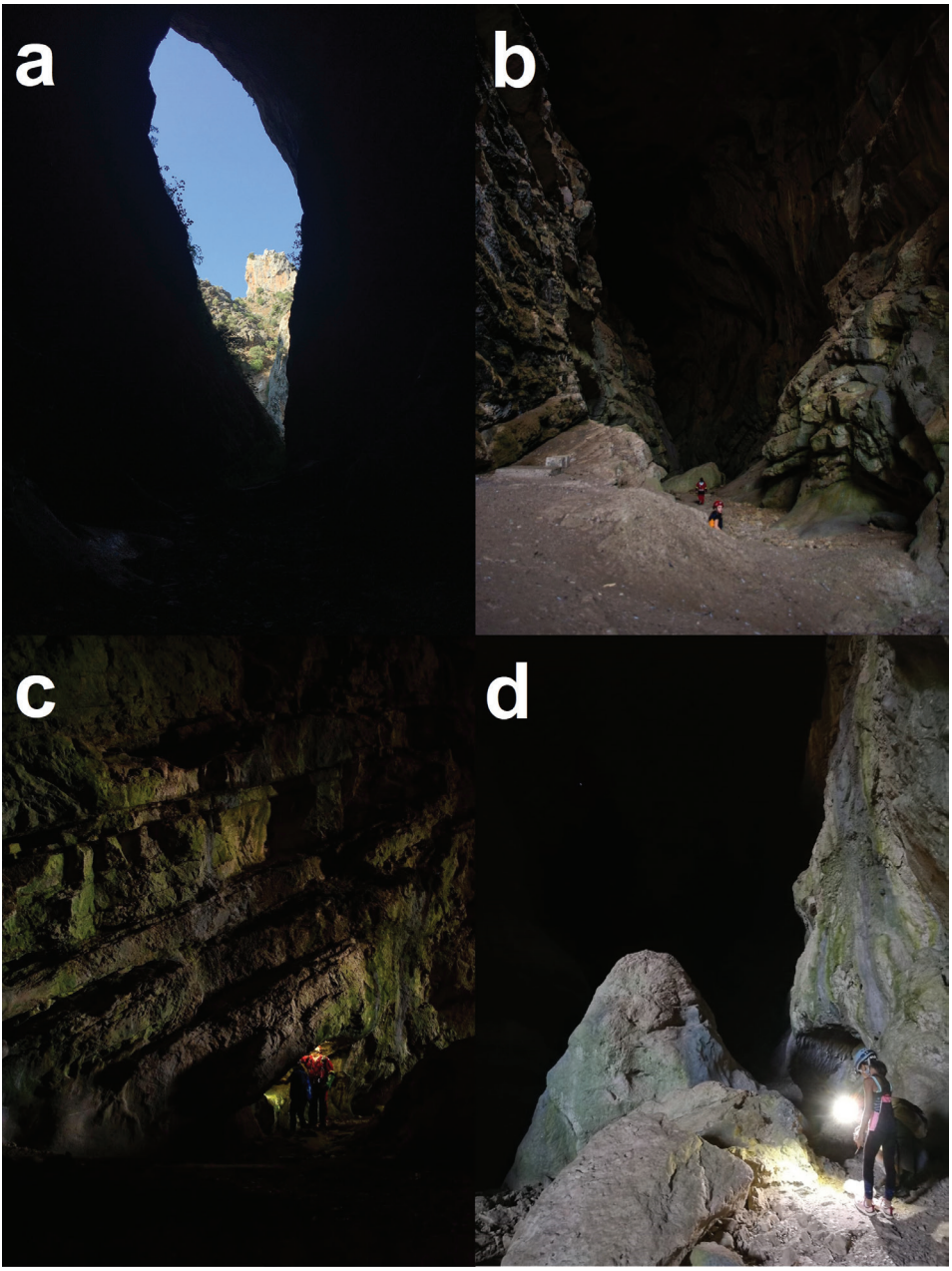


Figure 2. Hundidero Gato cave **a** exterior view from Hundidero **b** Hundidero entrance **c, d** dark zone, making direct captures (Photo by Miguel de Felipe).

The physical distance (≈690 km) and morphological differences between the Grazalema and Ayllón populations suggest they may represent distinct species, but additional material from Sierra de Grazalema will be needed to determine if the morphological differences noted here are fixed.

Family Entomobryidae Schäffer, 1896

***Entomobrya virginiae* Arbea, sp. nov.**
http://zoobank.org/0391BA36-456A-44BE-84BA-5C82C944D00A
Figs 3–15, Tables 1, 2

Type locality. Sistema Hundidero-Gato, Benaoján-Montejaque, Málaga (Sierra de Grazalema Natural Park, Spain), 36°43'39"N, 5°14'19"W, 558 m a.s.l.

Type material. ***Holotype*** female on slide, Sistema Hundidero-Gato, Benaoján-Montejaque, Málaga (Spain), dark zone, 23-VIII-2018, leg. Virginia García López; 80 paratypes, same data as holotype: 12 females, 4 males and 4 juveniles on slide (11 from twilight and 9 from dark zone) and 60 ex. in a tube with ethyl alcohol (20 from twilight and 40 from dark zone). Deposited in MNCN (holotype and paratypes in slides) and MZUN (paratypes in alcohol).

Etymology. The name is dedicated to Virginia García López, who captured the specimens.

Description. ***Body*** length 1.80 mm, excluding antennae (mean 16 ex., Table 1). Without dimorphism in colour; ground colour white or very light blue, with blue pigment on lateral body (all segments), dorsolateral segments Th II to Abd V, as two not complete stripes wider on posterior tergites, a central patch on Abd III with a truncated cone shape, other central patches on Abd I–II, and transversal bands on posterior tergites Th II–Abd III in many specimens; head with pigment on posterior lateral eyes and a dorsal V-shaped patch; on legs, pigment on distal femur and lateral tibiotarsus; antennae with pigment on Ant I tip and whole Ant II–IV (Fig. 3). Simplified Mc formula (Jordana and Baquero 2005) H1-H2-H3-H4-H5/T1-T2/A1-A2/A3-A4-A5/A6-A7-A8-A9-A10 in adult females: 3-2-0-3-2/2-4/2-3/1-2-1/0-4-3-2-2.

Head. Eight eyes, GH smaller than EF. Antennae length 1.08 mm, 3.13 times the length of the head (n = 16, Table 1); Ant IV with apical vesicle simple (Fig. 10) (slightly bilobed in some specimens); relative length of Ant I/II/III/IV = 1/2.38/2.42/2.43 (Table 1). Labrum with 4/5,5,4 chaetae; prelabral chaetae ciliated, and labral chaetae smooth. The labium has MR*ELL ciliated Mc; R* half as long as M; the remaining labial Mc are smooth. The maxillary palp has three sublobal chaetae. The papilla E has a lateral process reaching the end of the papilla (Fig. 9). In the dorsal head chaetotaxy (Fig. 4), the H1 area has three Mc: An2, An3e1 and An3, and the H2 area with two Mc: A5 and A6 (sometimes absent or as mc in male and juvenile specimens); Mc series M with M1–M4; the H3 area without Mc S'0; the H4 area with S1, S3 and S4 Mc, and the H5 area with Ps2 and Ps5 Mc. There are four chaetae on the ocular well: p, q, r and s.

Thorax (Fig. 5). Th II has two Mc: m1 and m2i, on the T1 area; the T2 area has four Mc: m4, m4i, a5 and a5' (sometimes absent or as mc in male of juvenile specimens). Th III has complete basal chaetotaxy: a1–a7, p1–p6, m6–m7.

Abdomen (Figs 6, 7). The Abd II has two Mc on area A1: a2 and a3 (sometimes absent or as mc in male or juvenile specimens) and three Mc on area A2: m3, m3ep and m3e. Abd. III has 1, 2 and 1 Mc on areas A3, A4 and A5, respectively: a1, a2, a3 (some-

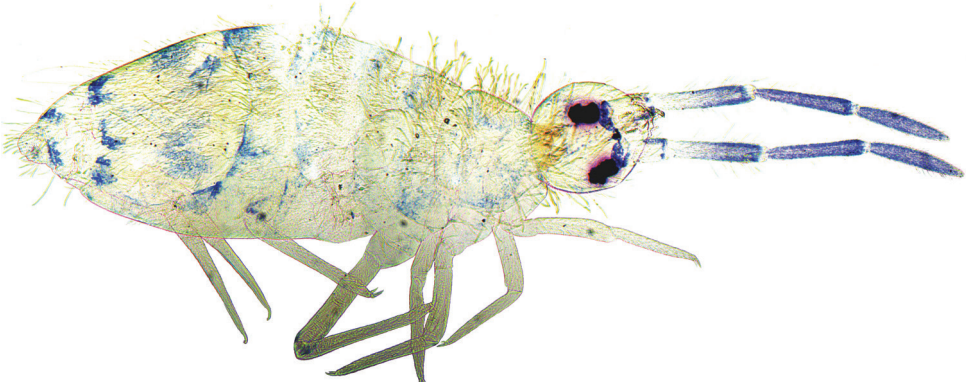
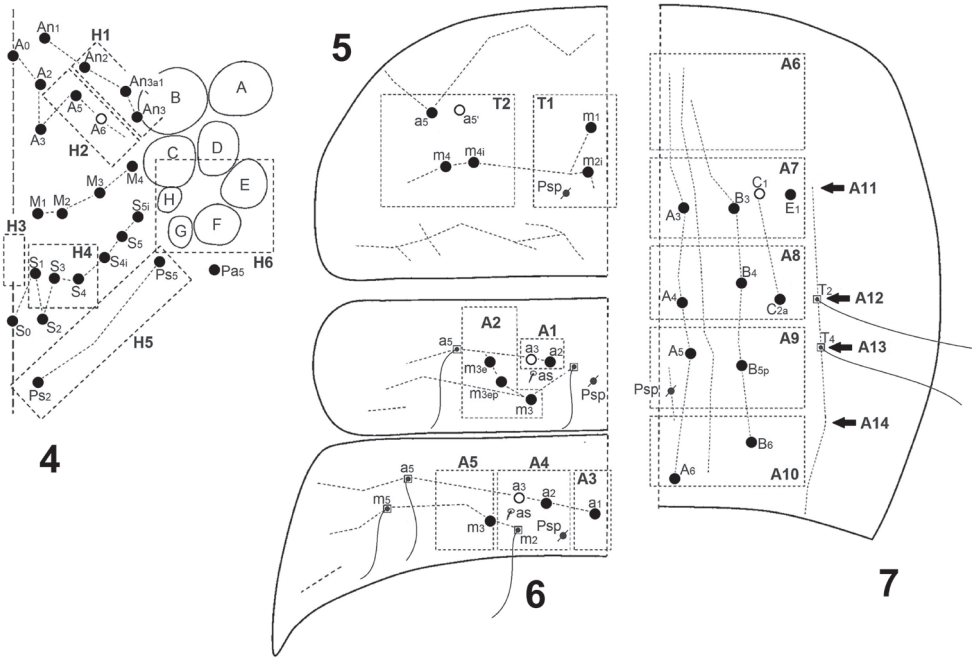
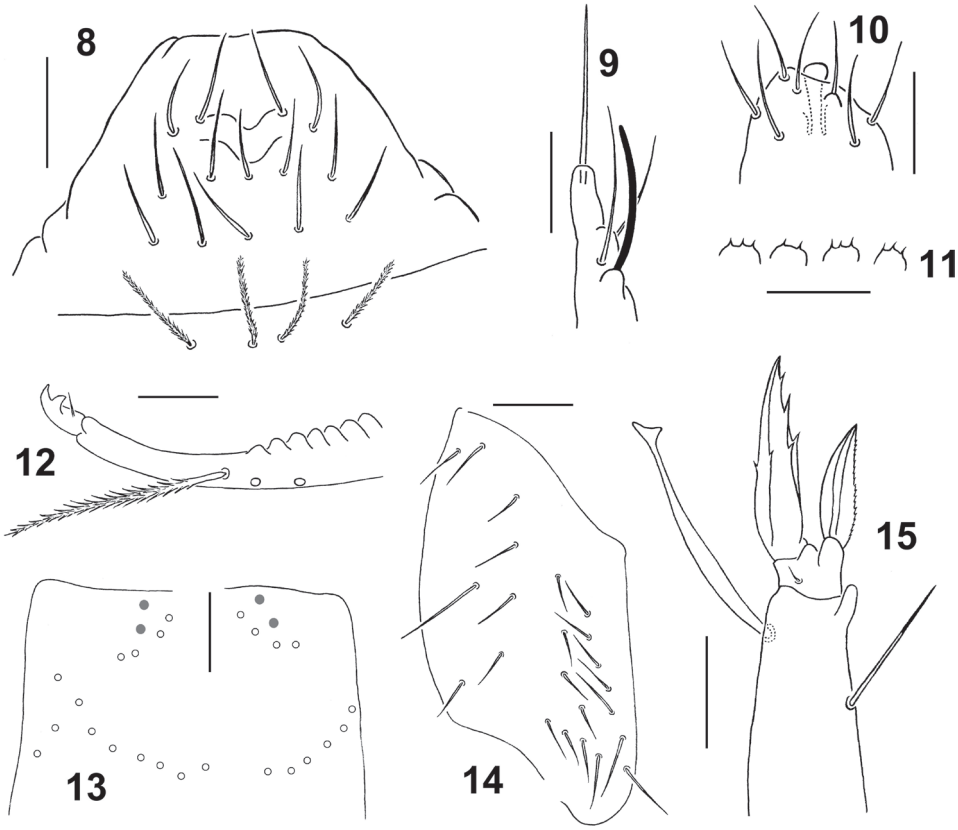


Figure 3. *Entomobrya virginiae* Arbea, sp. nov. Habitus.



Figures 4–7. *Entomobrya virginiae* Arbea, sp. nov. **4** head chaetotaxy **5** Th II chaetotaxy **6** Abd II–III chaetotaxy **7** Abd IV chaetotaxy. Symbols: open circle = Mc that could be absent in male and juvenile specimens but present in females; closed circle = Mc that are always present; black chaetae-like = sensilla; black circles with a cross line = pseudopores; long chaetae = bothriotricha.

times absent or as mc in male or juvenile specimens) and m3. Abd. IV with 11+11 central Mc: A3–A6, B3–B6, C1 (sometimes absent), C2a and E1 (Fig. 7). The trichobothria on Abd IV at the levels T2 and T4. Length ratio of Abd IV/III = 3.78 (n = 16, Table 1). Length of manubrium and dens 0.35 and 0.43 mm, respectively (n = 16, Table 1). Manubrial plate with four chaetae and two pseudopores (Fig. 13). Mucro with teeth similar in size, mucronal spine reaching tip of subapical tooth (Fig. 12).



Figures 8–15. *Entomobrya virginiae* Arbea, sp. nov. **8** labrum **9** labial papilla E **10** tip of Ant IV **11** labral papillae **12** tip of dens and mucro **13** manubrial plate (open circles = Mc; gray circles = pseudopores) **14** trochanteral organ **15** claw and empodium of leg III; Scale bars: 0.02 mm (**8, 12–15**), 0.01 mm (**9–11**).

Legs. Trochanteral organ with 15–16 smooth chaetae in V-shaped and 7–8 lateral smooth chaetae (Fig. 14). Tibiotarsus not subsegmented, without smooth chaetae, except for smooth terminal chaeta on legs III. Claw with four inner teeth: paired at 53–58% (one of them larger and forward), first unpaired at 70% from basis, and the last one closer than 90%; dorsal teeth at the level of lateral teeth (Fig. 15). Empodium lanceolate, with serrated external lamella in leg III. Tenent hair clavate, longer than claw.

Remarks. For the identification of the species of *Entomobrya* we must consider a combination of colour pattern and dorsal macrochaetotaxy. It has been shown that species with the same colour pattern can have a different chaetotaxy (Jordana and Baquero 2005). On the other hand, species with differences in color pattern can correspond with species-level genetic divergence without any obvious differences in chaetotaxy (Katz et al. 2015b).If the macrochaetotaxy of Th II/Abd II/Abd III is considered simultaneously, twelve species of the genus share a similar simplified formula for these tergites: 2-3/2-3/1-2-1 in *E. atrocincta* Schött, 1896, *E. maroccana* Baquero & Jordana, 2008, *E. melitensis* Stach, 1963, *E. numidica* Baquero, Hamra-Kroua & Jordana, 2009, *E. vergarensis* Baquero, Arbea & Jordana,

Table 1. Measurements of *E. virginiae* sp. nov. (in micrometers), average from 16 specimens.

	Holotype	Mean	Min.	Max.
Ant I	129	131	87	179
Ant II	308	312	225	469
Ant III	326	317	230	437
Ant IV	313	319	253	437
Ant	1076	1078	795	1522
Head	377	344	267	446
Ant/Head ratio	2,85	3,13	2,54	3,81
Th II	230	216	161	322
Th III	170	144	92	184
Abd I	175	123	55	175
Abd II	170	161	92	221
Abd III	156	134	74	184
Abd IV	533	486	377	671
Abd IV/III ratio	3,42	3,78	2,80	5,60
Abd V	138	112	92	138
Abd VI	87	76	51	97
Body	2036	1795	1353	2323
Manubrium	368	347	285	483
Dens	469	430	354	561
Claw	77	71	58	86
Empodium	47	47	37	58
Tenent hair	85	79	63	86

2010, *E. fainae* Baquero & Jordana, 2018 and *E. benaventi* Rueda & Jordana, 2020; 2-4/2-3/1-2-1 in five species described by Baquero and Jordana (2018) from the Canary Islands, *E. grimanesae*, *E. achuteygai*, *E.* cf. *quinquelineata*, *E. guayarminae* and *E. gazmirae*; of these twelve species, only *E. melitensis*, *E. vergarensis*, *E. fainae* and the females of *E. atrocincta sensu* Katz et al., 2015a, and *E. benaventi* have a colour pattern similar to the new species; but the new species is the only one with Mc a5' on Th II in females. The colour pattern of the new species could also be considered similar to that of *E. multifasciata*, *E. atteneri* and females of *E. nigrocincta*, which have a different macrochaetotaxy formula for the Th II (Mc a5' present in females of the new species vs. absent in others) and Abd II tergites (2–2 in *E. multifasciata*, 2–4 in *E. atteneri*, 1–2 in *E. nigrocincta* vs. 2–3 in the new species). Table 2 shows the differences that separate these species from the *E. virginiae* sp. nov.

Lepidocyrtus flexicollis Gisin, 1965

New Sierra de Grazalema (Málaga) record.

Material studied. Cueva del Gato. 9-VIII-2018, 2 ex. in twilight and 11 ex. in dark zone; 16-VIII-2018, 4 ex. in twilight and 11 ex. in dark zone; 23-VIII-2018, 3 ex. in twilight zone; 30-VIII-2018, 2 ex. in dark zone; 16-IX-2018, 8 ex. in dark zone; 20-IX-2018, 16 ex. in twilight zone; 4-X-2018, 25 ex. in twilight and 39 ex. in dark zone.

Known species from the Canary Islands and the southeastern part of the Iberian Peninsula. This troglophilous species is relatively common in subterranean habitats (Arbea 2013).

Table 2. Differences of *E. virginiae* sp. nov. among the similar species of *Entomobrya* by colour pattern and/or macrochaetotaxy of Th II and Abd II–III tergites. The species share: Mc S'0 on H3 area of head absent; Th II with 2 Mc on T1 area; Abd III with 1/1 Mc on A3/A5 areas; Abd IV without unpair chaetae on A7 and A9 areas and the trichobothrium at the level T2 and T4. U = unknown character. Differences with *E. virginiae* sp. nov. in bold. References: 1, Christiansen and Bellinger (1998); 2, Jordana (2012); 3, Katz et al. (2015a); 4, Baquero and Jordana (2018); 5, Rueda and Jordana (2020).

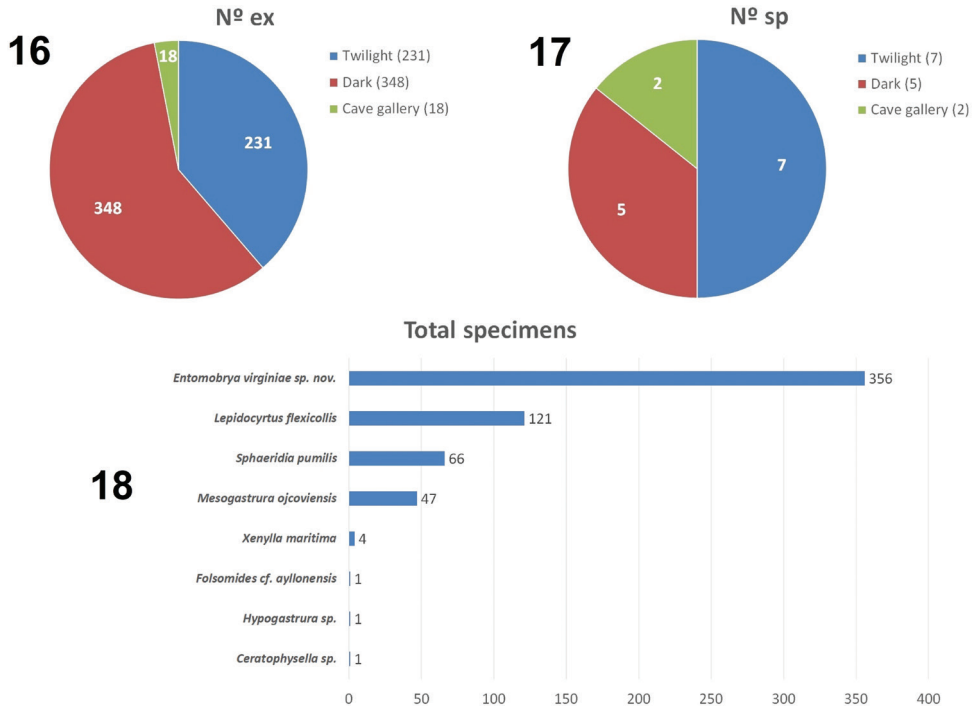
	E. acbuteygai	*E. atrocincta* Europe	*E. atrocincta* North America	*E. atteneri*	*E. benaventi*	*E. fainae*	*E. gazmiraе*	*E. grimanesae*	*E. guayarminae*	*E. marocana*	*E. melitensis*	*E. multifasciata*	*E. nigrocincta*	*E. numidica*	*E.* cf. *quinquelineata* Canary Islands	*E. vergarensis*	*E. virginiae* sp. nov.
Ch.1 H1 (Head) An2–An3	3	**2**	3	3	3	3	3	3	3	3	U	3	3	3	3	3	3
Ch.2 H2 A5–A7	2	**1**	1(2)	2–3	**1**	**1**	2	**1**	**1**	**1**	U	**1**	**1**	**1**	**1**	**3**	2
Ch.3 H4 S1–S3–S4	3	3	3	3	3	3	3	3	3	**2**	U	3(2)	**2**	3	3	3	3
Ch.4 H5 Ps2–Ps3–Ps5	2	2(3)	2	2	2	2	2	2	2	2	U	2	2	**3**	2	**3**	2
Ch.5 Labral papillae: smooth papillae (1), with some projections (2)	2	**1**	2	2	2	**1**	**1**	2	**1**	**1**	2	2	2	2	2	2	2
Ch.6 Length of lateral process subequal to labial papilla E (1) or shorter than labial papilla E (2)	**2**	U	U	1	1	1	**2**	1	1	U	U	1	U	U	**2**	U	1
Ch.7 eyes G&H size = E&F (1), < E&F (2)	2	2	2	2	2	2	2	2	2	**1**	2	2	2	2	2	2	2
Ch.8 apical antennal retractile bulb absent (0), lobe simple (1), bilobed (2)	**2**	1	1–2	**2**	**2**	**2**	1(0)	**2**	**2**	1	**2**	1–2	**2**	**2**	**2**	**2**	1
Ch.9 T2 chaetae number a5, m4–m5	4	3	3	4	3	3	4	4	4	3	3	3	3	3	4	3	4(3)
Ch.10 T2 chaeta a5'	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(0)
Ch.11 Claw internal teeth 3(3), 4(4)	4	4	4	4	4	4	4	4	4	4	**3**	4	4	4	4	4	4
Ch.12 Claw dorsal tooth absent (0), basal (1), internal teeth level (2)	**1**	2	2	**1**	**1**	2	**1**	2	2	2	**0**	**1**	2	**1**	**1**	2	2
Ch.13 A1 Abd II a2–a3	2	2	2(1)	2	2	2	2	2	2	2	2	2	**1**	2	2	2	2
Ch.14 A2 Abd II m3 series chaetae number	3	3	2(3)	**4**	3	3	3	3	3	3	3	2–3	**2**	3	3	3	3
Ch.15 A4 Abd III above m2 chaetae number	2	2	2	2	2	2	2	2	2	2	2	2	**1**	2	2	2	2
Ch.16 A6 Abd IV A1–D1 chaetae number	0	0	0	0	**1**	0	**1**	0	0	0	0	0	0	0	0	0	0
Ch.17 A7 Abd IV A2–E1 chaetae number	4	4	1(4)	4	3	3	4	**2**	**0**	**0**	**0**	2–3	**2**	**2**	**2(3)**	3	3–4
Ch.18 A8 unpair chaeta A04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0
Ch.19 A8 Abd IV A4a–C2a chaetae number	3	3	3	3	3	**4**	3	3	3	3	**4**	3	3	3	3–4	**5**	3
Ch.20 A9 Abd IV A5–B5 chaetae number	2	**3**	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Ch.21 A10 Abd IV A6–B6 chaetae number	2	**3**	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Ch.22 Manubrial plate chaetae number	**5**	3–4	3–4	4–6	U	4–6	**6–7**	4	4	U	4	4	4	4	3–4	4	4
Ch.23 Sexual dimorphism in colour present (1) or absent (0)	U	0	**1**	0	**1**	0	U	0	U	0	0	0	**1**	0	0	0	0
Number of differences in relation to *E. virginiae* sp. nov.	4	5	1	3	5	4	5	3	4	5	5	2	8	5	5	5	–
References	4	2	1,3	4	5	4	4	4	4	2	2	2,4	2,4	2	4	2	

Family Sminthurididae Börner, 1906

Sphaeridia pumilis (Krausbauer, 1898)

New Sierra de Grazalema (Málaga) record.

Material studied. Cueva del Gato. 9-VIII-2018, 21 ex. in twilight zone; 16-IX-2018, 23 ex. in dark zone; 20-IX-2018, 6 ex. in twilight zone; 4-X-2018, 5 ex. in twilight and 11 ex. in dark zone.



Figures 16–18. Diversity of Collembola in Gato cave **16** total number of Collembola specimens recorded **17** number of Collembola taxa recorded **18** abundance of eight species of Collembola collected.

This is a cosmopolitan species widely distributed in Europe and North America. Occurs on the surface and in upper layers of different moist soils and in their low vegetation. Occasionally appears in caves from Spain (Jordana and Beruete 1983) and other European caves.

Discussion

The number of springtails collected is higher in dark zone (58% of all Collembola collected), but the twilight zone proved to be the most richness – of the eight species recorded in this study, we recorded seven in the twilight and only five in the dark zone (Figs 16, 17). The troglophilous species *Ceratophysella* sp., *Hypogastrura* sp. and *Xenylla maritima* were recorded from only the twilight zone at the cave entrance (Fig. 1).

Four of the eight species were represented by only one to four specimens (Fig. 18). The high number of individuals is attributable to two species, *Entomobrya virginiae* sp. nov., for which 356 specimens (59.6% of all springtail specimens collected during this study) were collected from all zones of the cave, and *Lepidocyrtus flexicollis* with 121 specimens. The next two most abundant species were *Sphaeridia pumilis*, with 66 specimens (11% of all Collembola collected), and *Mesogastrura ojcoviensis*, with 47 specimens (7.9% of all Collembola collected).

Acknowledgements

To thank the Department of Zoology of the University of Seville for their unconditional and essential support for the surveys and the authorities responsible for the "Sierra de Grazalema Natural Park", as well as the "Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible, Junta de Andalucía" that provided the authorizations and permits for the execution of the work. Also thank our friend Toni Pérez Fernández for directing us to the underground world. The authors also would like to thank Enrique Baquero and Aron Katz who provided excellent comments and suggestions that improved this manuscript.

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Subterranean Biology 38: 91–112 (2021)
doi: 10.3897/subtbiol.31.60691
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Reproduction, development, asymmetry and late eye regression in the Brazilian cave catfish *Ituglanis passensis* (Siluriformes, Trichomycteridae): evidence contributing to the neutral mutation theory

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Academic editor: Horst Wilkens | Received 12 November 2020 | Accepted 4 March 2021 | Published 19 April 2021

http://zoobank.org/A5132226-180C-42A7-96F5-A769212D7090

Citation: Secutti S, Trajano E (2021) Reproduction, development, asymmetry and late eye regression in the Brazilian cave catfish *Ituglanis passensis* (Siluriformes, Trichomycteridae): evidence contributing to the neutral mutation theory. Subterranean Biology 38: 91–112. https://doi.org/10.3897/subtbiol.31.60691

Abstract

The troglobitic (exclusively subterranean source population) catfish *Ituglanis passensis* (Siluriformes, Trichomycteridae) is endemic to the Passa Três Cave, São Domingos karst area, Rio Tocantins basin, Central Brazil. This unique population presents variably reduced eyes and melanic pigmentation. We describe reproduction and early development in this species based on a spontaneous (non-induced) reproductive-event that occurred in the laboratory in January–February, 2009, while simultaneously comparing with data from the cave-habitat and a previous reproductive event. Egg laying was parceled. Egg-size and number were within variations observed in epigean congeners. Larvae behavior and growth is described. A single surviving specimen was monitored over two years. Eye-regression started late, one year after birth, and followed a pattern of stasis phases intercalated with slow growth and fluctuating asymmetric rates. Late eye regression, associated with asymmetry in eye development and intra-population variability of troglomorphic traits, as shown by several Brazilian subterranean fishes, provide support for the Neutral Mutation Theory.

Keywords

central Brazil, eye-regression, *Ituglanis passensis*, life-cycle, reproduction, Troglobitic fish

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Introduction

Character regression, observed in most troglobites (exclusively subterranean source populations – Trajano and Carvalho 2017), is, not surprisingly, a central theme in subterranean biology, with emphasis on visual structures and dark pigmentation. Over the last seven decades and among fishes, Mexican tetra characins of the genus *Astyanax* have been the favored source of material in genetic and developmental studies focused on the regression of eyes and pigmentation in cave derivatives (e.g., Thinès 1960; Wilkens 1971, 1987, 1988, 2007, 2010, 2011; Peters and Peters 1973; Durand 1979; Borowsky and Wilkens 2002; Jeffery 2005; Gross et al. 2009; Yamamoto et al. 2009; Bilandzija et al. 2013; Hinaux et al. 2013; McGaugh et al. 2014; Wilkens and Strecker 2017; among many others). The other troglobitic fishes studied include the cyprinids *Caecobarbus geertsi* (Thinès 1960), *Phreatichthys andruzzi* (Berti et al. 2001; Aden 2008) and *Garra barreimiae* (Aden 2010), besides the Brazilian loricariid, *Ancistrus cryptophthalmus* (Secutti and Trajano 2009). In every case, juveniles were born with apparently normally structured eyes, that degenerated throughout ontogeny. Thus, subterranean fishes provide excellent material for testing hypotheses on the evolutionary causes of character regression.

Two hypotheses have been proposed to explain regressive traits in the Mexican cave characins *Astyanax*, the so-called "cavefish", 1) the accumulation of neutral mutations, due to relaxation of selective pressure on the maintenance of eyes, dark pigmentation and other light-related structures and functions, and 2) the negative pleiotropic effect on the selection of constructive traits in these structures and functions (e.g., Wilkens 1971, 2011; Protas et al. 2007; Jeffery 2010; Yoshizawa et al. 2012; for review see Wilkens and Strecker 2017). They are not mutually exclusive. As the neutralistic model can be applied to any evolutionary regression arising from the loss of function (Wilkens 1971, among others), application is more widespread. When considering specific traits, evidence of pleiotropic effects is only available from these studies of the *Astyanax* cavefish. To date, this knowledge has not been applied to studies of other troglobitic fishes.

When compared to other countries, taxonomically and phylogenetically, Brazil harbors a high diversity of subterranean fishes, comprising troglobites (30+ differentiated lineages in seven families and three orders – Trajano 2021), and troglophiles (source populations, dwelling in epigean and subterranean habitats, genetically connected by mutually commuting individuals (Trajano and Carvalho 2017). Most troglobitic lineages are characterized by troglomophisms, i.e., autapomorphies related to the particular selective regime found in subterranean habitats, the commonest of which regression of both eyes and melanic pigmentation (Trajano et al. 2016). The degree of troglomorphism may present inter- and intrapopulation variability, passing from slightly yet significantly reduced eyes and pigmentation compared to their surface congeners, to highly variable populations, and homogeneously depigmented and anophthalmic species, without any corresponding taxonomic correlations (Trajano and Bichuete 2010). There is also high diversity in habitat type, from the epikarst and vadose tributaries to base-level streams and the phreatic zone (Trajano and Bichuette 2010).

The Trichomycteridae family contains the largest number of troglobitic derivatives in South America, with 10 described species in Brazil included in three genera, viz.,

Trichomycterus, 3 spp., *Ituglanis*, 6 spp. and *Glaphyropoma*, 1 sp., besides a few undescribed species (Gallão and Bichuette 2018)

Data on the complete reproductive cycle, from courtship (if present), egg laying, larvae growth, and development until the juvenile phase, are lacking for most of these species. Sparse information is available on *T. chaberti* from Bolivia (Pouilly and Miranda 2003), and the Brazilian *T. itacarambiensis* (Trajano 1997a) and *I. passensis*, cited as *Trichomycterus* sp. (Trajano and Souza 1994).

The reproductive cycles of relatively few among the 200+ troglobitic fishes worldwide have been described. Among these, the Mexican cave-fishes *Astyanax* (*e.g.*, Wilkens 1987, 1988; Parzefall 1993; Jeffery 2005), the USAn amblyopsids (Poulson 1963), and the Brazilian armored catfish *Ancistrus cryptophthalmus* (Secutti and Trajano 2009) based on spontaneous (non-induced) reproduction in the laboratory, the Mexican heptapterids *Rhamdia zongolicensis* and *R. reddelli* (Wilkens 2001), and *Phreatichthys andruzzi* from Somalia (Berti et al. 2001; Aden 2008) based on induced reproduction, and the Cuban bythitid *Lucifuga subterranea* (Durand 1998) based on data from the natural habitat.

The main difficulties when studying reproduction, development and eye regression in troglobitic vertebrates arise from their life-cycle tendency towards K in the r – K continuum (precocial life-cycle *sensu* Balon 1990), as an adaptation to counteract food scarcity. Infrequent reproduction, low fertility and slow development (Culver 1982; Trajano 2001a), allied to generally low population densities, the small proportion of mature individuals at any time and the secretive habit of larvae, hinders encountering juveniles in the natural habitat. Moreover, most subterranean fishes with well-defined spawning cycles reproduce during or just after the rainy season, when many caves are flooded and access is impossible or quite dangerous (Trajano op. cit.). Few species, e.g., the Mexican troglobitic *Astyanax*, reproduce regularly in the lab.

Bichuette (2003), when investigating the distribution, ecology and behavior of the four cave *Ituglanis* from the São Domingos karst area, Central Brazil, transported live specimens to a lab of the Department of Zoology, University of São Paulo, São Paulo, for observations on behavior. In the beginning of 2009, a small group of *Ituglanis passensis* reproduced spontaneously, thereby offering an excellent opportunity to describe egg laying and larval development, with a focus on growth rates and eye regression. Additional evidence bolstering the neutral mutation theory, from data on Brazilian troglobitic fishes, are herein discussed.

Materials and methods

Study area and species studied

Ituglanis passensis is endemic to the type-locality, the Passa Três Cave (13°25'S, 46°22'W), a part of the São Vicente system, São Domingos karst area, Goiás State. This cave is basically a 2,000 m long stream-conduit, part of an upper vadose tributary of the São Vicente I cave, opening onto the surface through a sinkhole. Large amounts

of organic matter are carried into the cave by flash-floods, the accumulation in the first hundreds of meters sustaining rich communities of invertebrates. Particulate and dissolved organic matter is carried on throughout the entire cave stream, thereby characterizing a mesotrophic environment that supports dense populations of *I. passensis*, as well as the syntopic armored catfish *Ancistrus cryptophthlmus*. Non-troglomorphic jejus, *Hoplerythrinus unitaeniatus* (Erythrinidae), (Bichuette and Trajano 2003) represent potential prey for troglobitic fishes.

This cave area is located in the PETER- Parque Estadual de Terra Ronca (State Park of Terra Ronca), within the Cerrado (savannah-like) domain characterized by a tropical semi-humid climate, with 4–5 dry months (May to September). The water temperature in the Passa Três Cave, measured in the dry seasons of 2000 and 2001, varied from 19 to 22 °C at 100 m from the sinkhole, thereafter stabilizing to around 21 °C at 300 m. For maps and further details on the area, as well as descriptions of the caves, see Bichuette and Trajano (2003) and Reis et al. (2006).

The São Domingos karst area presents high diversity in subterranean fishes, with seven nominal troglobitic species (three families, two orders – Bichuette and Trajano 2003). The four *Ituglanis* species inhabit separate, but parallel, micro-basins fed exogenously by high-flow rivers crossing the limestone outcrop from east to west towards the Tocantins river, in the Amazon basin. As a consequence of parallel evolution, they can be mutually distinguished by different mosaics of troglomorphic traits.

Ituglanis passensis is characterized by very reduced eyes and scarce pigmentation, with some individual variations. Color varies from yellowish-pink (most individuals) to light grey. Melanophores are scarce and limited to the dorsal region of both head and body. Eyes vary from vestigial black spots, symmetrical or not, to none visible externally (Trajano and Souza 1994; Fernandez and Bichuette 2002). In the laboratory, the largest sizes observed in females were 90 mm TL (total length) (Trajano and Souza 1994) and 73.1 mm SL (standard length) (present study), whereas in males these were, respectively, 63.0 mm SL and 64.7 SL. *I. passensis* fishes are significantly larger than individuals of the other three species from the São Domingos karst area (Bichuette and Trajano 2004).

Methods

Four adult *I. passensis* catfish were hand-netted in May, 2000, and afterwards transported to the laboratory in the basement of the Departamento de Zoologia, Instituto de Biociências da USP. They were kept in permanent darkness (except during maintenance activities) in a 55-l aquarium (1650 cm² bottom area), with biological filtering and a dolomite pebble substrate. Limestone rocks from the previous habitat provided shelter. Air and water temperatures were controlled by air conditioning at around 21 to 24 °C, thus close to the normal range in the cave-habitat.

For no apparent reason, one died in 2007. The remainder consisted of a female of 73.1 mm SL, without externally visible eyes (Fig. 1), and two individuals of undefined sex, measuring, respectively 64.7 and 63.0 mm SL, with eyes reduced to externally visible spots and diameters of approximately 0.85% of SL (Fig. 1). At least one of the latter two was a male, proven by spontaneous reproduction in January-February, 2009.

To avoid predation by the parents, free-swimming larvae were individually transferred to small (2.5-l, bottom area 200 cm²) aquaria, with aeration but devoid of a substrate, so as to facilitate observation. When collecting eggs from the substrate of the home aquarium, the female was placed in a clean aquarium without substrate for one hour. Those encountered were measured (total diameter, including the turgid hyaline membrane) and subsequently preserved in diluted formalin.

The surviving larvae were fed *ad libitum* once a week with nauplii and young *Artemia* crustaceans. When reaching 14.0–15.0 mm SL, juveniles were transferred to larger aquaria (7-l, bottom area 375 cm²), with small limestone blocks and a "mop" (a bundle of acrylic wool-yarns hanging from a floating styrofoam piece) for shelter. They were fed with young to adult *Artemia* crustaceans.

Eggs and larvae were measured and photographed under a stereomicroscope with ocular lens (millimeter scale – precision 0.1 mm) coupled to a digital camera. Once a week, living fry were placed in a Petri dish with water from their maintenance aquaria. After settling down, standard length (SL), head length (HL) measured dorsally from the tip of the snout to the beginning of spinal cord, and horizontal diameter of both eyes (40 × magnification), were measured. The study started with 12 larvae. However, in spite of every care, most died in the first few months: one individual survived for more than two years.

Results

Reproductive behavior and eggs

External sexual dimorphism was not apparent. Gender could only be distinguished in mature individuals with fully developed gonads, visible through transparency (facilitated by light coloration), as observed in the case of the female in January, 2009 (Fig. 1). These fish presented cryptobiotic habits and avoidance of light. Hence, mating could not be directly observed. Maybe it occurred inside dens and/or in the dark.

Spawning is parceled, i.e., spaced out – during the reproductive season, eggs are released several times intercalated with resting phases. Gonad maturation and egg laying in the laboratory occurred in January-February, thus corresponding to the middle of the rainy season in the natural habitat, when the minimum air temperatures are the highest.

Non-adhesive translucent eggs (Fig. 2A) were laid scattered on the substrate. There was no parental care. The diameter of newly laid eggs varied from 1.0 to 1.1 mm. Based on spawning in a clean aquarium, plus the number of eggs and larvae in the parental home-aquarium, the minimum clutch-size was calculated at ca. 120. It was impossible to establish whether all the eggs had been fertilized or not, nor whether both or only one of the males participated.

On comparing reproductive traits (number and sizes of eggs, and sizes of reproducing females) in epigean trichomycterids and the troglobitic *Trichomycterus chaberti* and *I. passensis* (Table 1), in the case of the latter, these traits fall well within the range already observed for epigean species as a whole. The troglobitic *T. chaberti* showed a

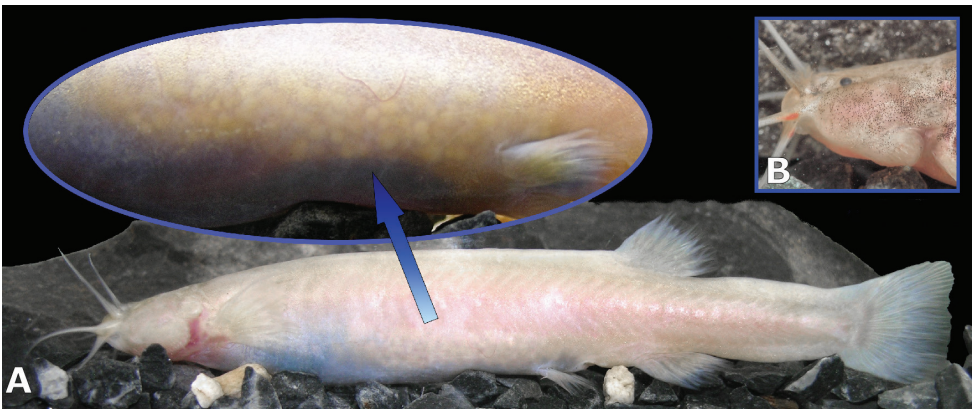


Figure 1. A *Ituglanis passensis* mature female (73.1 mm SL), eggs seen by transparency **B** detail of male head. Photos author: Alexandre Lopes Camargo.

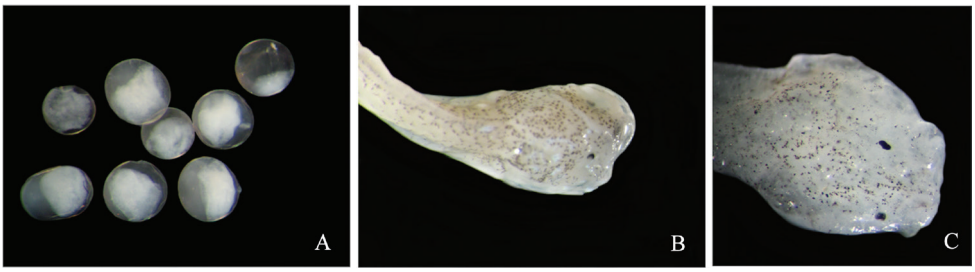


Figure 2. A *Ituglanis passensis*, newly laid eggs **B** newly hatched free-swimming larva, with visible eyes and melanophores **C** same, in zoom. Photos author: Sandro Secutti.

Table 1. Data on numbers of eggs per female, egg diameters (in mm) and respective sizes of female trichomycterids (• standard length, •• total length, in mm) from literature and the present study for *Ituglanis passensis* from Passa Três Cave; N – sample sizes; * troglobitic species.

Species	N° eggs	Egg sizes	Female sizes	N	References
Eremophilus mutisii	1820–27950	4.0–5.0	210–300••	35	González and Rosado 2005
Trichomycterus corduvense	2947	0.45–0.49	–	–	Marraro et al. 2005
Trichomycterus sp.	~73	1.83	+42•	17	Rondineli et al. 2009
T. areolatus	110–1460	1.51–2.1	55–98.5••	43	Manriquez et al. 1988
T. caliense	170–223	1.24–1.74	83•	37	Roman-Valencia 2001
T. cf. *barbouri*	79–231	0.80–1.49	37.3–61.0•	34	Pouilly and Miranda 2003
T. chaberti *	42	1.52	44–68.8•	4	Pouilly and Miranda 2003
Ituglanis passensis *	~120	1.0–1.1	73.1•	1	Present data

tendency for larger and less numerous eggs. On the other hand, compared with epigean and other troglobitic trichomycterids, *I. passensis* lays less eggs, but of the same size as other epigean relatives, whereas the epigean *Trichomycterus* sp. from the Passa Cinco stream, Corumbataí basin (SE Brazil), lays even less eggs than *I. passensis*, albeit larger than those of *T. chaberti*.

Newly hatched larvae in the parental aquarium were observed to swim actively in midwater and near the surface. There was no avoidance of illumination. When disturbed in any way, they quickly sank to the bottom, to hide under rocks or among pebbles. Larvae and juveniles kept in isolation were less active, mostly remaining stationary on the bottom, under rocks or in corners, except when feeding. When offered live food, juveniles began to forage, actively exploring the substrate and walls, and swimming up to the surface in a vertical position to forage among mob-wool yarns and floating pieces.

Development and eye regression

Measurements of standard length (SL), head length (HL) and the diameters of both eyes throughout the study are shown in Table 2. The exception is individual 12, which survived for 724 days and reached 29.9 mm SL. Newly hatched larvae (Fig. 2B, C) consistently showed 7.6 mm SL and 1.5 mm HL (Day 1 in Table 2). Nevertheless, heterogeneous growth among larvae was observed from Day 6 on. Just five days after the first measurements, SL varied from 7.65 to 8.30 mm, and HL from 1.7 to 2.0 mm. Growth rates were not consistent, varying throughout the study.

Larval eyes, black spots without distinguishable pupils and measuring ca. 0.1 mm in diameter (Fig. 3), remained visible throughout the study. Contrary to what occurred with the parental female, no visible sign of regression was noted in the longest-living fish (individual 12). Its daily growth (SL) is shown in Figure 4. The average rate was 1.24 mm month $^{-1}$. Nonetheless, the pace varied. From the 40th day on, and until the 370th there was a considerable slowdown. This was followed by an acceleration until day 600, and finally decceleration. This configures an S-shaped-growth-curve in the first 2 years, instead of a C-shaped one.

Curiously, with individual 12 and until the 370th day, no perceptible regression in eye diameter (decrease in size in relation to SL) occurred. In the second year, the increase in eye-size in relation to SL growth was slower. Furthermore, this became asymmetrical, with the right eye beginning to grow faster. However, this tendency reverted around the 550th day (Figs 4, 5). When the surviving juvenile reached ca. 15 mm SL (day 180), both eyes started presenting downward movements, as observed among fishes in general. Less than one month later, the left eye presented a small change in structure, losing the typical round-shape (Fig. 3). This would be the result of fluctuating asymmetry in eye-size, observed from the 350th on (Fig. 5), without dominance in size in either the left or the right. At 370 days, diameters were around 4.5% of SL.

Individual variability in growth rates of standard length and the left eye diameter, observed in the three longest-living catfishes, is illustrated in Figures 6, 7, respectively. A further comparison of differences as to standard lengths and diameters of not only of the left eye (Fig. 8a), but also of the right (Fig. 8b), showed variable asymmetry, but no dominance on either side (Figure 8).

The larvae skin is whitish with black melanophores all over the dorsum (Figs 2B, C, 3). The number increases with growth, with the skin gradually acquiring a yellowish coloration, as in the adults.

Table 2. Standard lengths, head sizes, diameter of left and right eyes, respectively (in mm), of 11 larvae of *Ituglanis passensis* raised in laboratory until death (data on larva 12, that survived for 724 days, are not shown).

Larvae / Days	1	2	3	4	5	6	7	8	9	10	11
1	7.60/1.5/0.45/0.45	7.60/1.5/0.45/0.45	7.60/1.5/0.40/0.25	7.60/1.5/0.40/0.30	7.60/1.5/0.4/0.40	7.60/1.5/0.45/0.45	7.60/1.5/0.35/0.40	7.60/1.4/0.40/0.40	7.60/1.65/0.45/0.45	7.60/1.5/0.45/0.35	7.60/1.5/0.45/0.45
6	7.65/2.0/0.45/0.45	7.90/2.0/0.45/0.45	8.15/1.9/0.40/0.25	8.15/2.0/0.40/0.30	7.65/1.7/0.40/0.40	7.65/1.7/0.45/0.45	8.30/2.0/0.35/0.40	7.70/1.8/0.40/0.40	7.90/1.7/0.40/0.40	8.30/2.0/0.45/0.35	8.15/1.7/0.45/0.45
7	7.65/2.0/0.45/0.45	7.90/2.0/0.45/0.45	8.17/1.9/0.40/0.25	8.16/2.0/0.40/0.30	7.80/2.0/0.40/0.40	7.80/1.7/0.45/0.45	8.31/2.0/0.35/0.40	7.75/1.8/0.40/0.40	8.00/1.8/0.40/0.40	8.30/2.0/0.45/0.35	8.15/1.8/0.45/0.45
9	7.70/2.0/0.45/0.45	7.90/2.0/0.45/0.45	8.18/1.9/0.40/0.25	8.17/2.0/0.40/0.30	7.90/2.0/0.40/0.40	7.90/1.85/0.45/0.45	8.38/2.0/0.35/0.40	7.80/1.8/0.40/0.40	8.00/1.8/0.40/0.40	8.35/2.0/0.45/0.35	8.20/1.8/0.45/0.45
12		7.95/2.0/0.45/0.45	8.18/1.9/0.40/0.25	8.17/2.0/0.40/0.30	8.20/2.0/0.40/0.40	8.20/1.85/0.45/0.45	8.40/2.0/0.35/0.40	8.00/1.8/0.40/0.40	8.12/1.8/0.40/0.40	8.40/2.0/0.45/0.35	8.20/2.0/0.45/0.45
13			8.19/2.0/0.40/0.25	8.18/2.0/0.40/0.30	8.30/2.0/0.40/0.40	8.30/1.85/0.45/0.45	8.50/2.0/0.35/0.40	8.00/1.8/0.40/0.40	8.14/1.8/0.40/0.40	8.50/2.0/0.45/0.35	8.25/2.0/0.45/0.45
14			8.20/2.0/0.40/0.25	8.20/2.0/0.40/0.30	8.40/2.0/0.40/0.40	8.40/1.85/0.45/0.45	8.50/2.0/0.35/0.40	8.00/1.8/0.40/0.40	8.17/1.8/0.40/0.40	8.50/2.0/0.45/0.35	8.25/2.0/0.45/0.45
15					8.45/2.0/0.40/0.40	8.45/1.85/0.45/0.45	8.55/2.0/0.35/0.40	8.00/1.8/0.40/0.40	8.20/1.8/0.40/0.40	8.55/2.0/0.45/0.35	8.3/2.0/0.50/0.50
16					8.50/2.1/0.35/0.40	8.50/1.85/0.45/0.45	8.60/2.0/0.35/0.40	8.00/1.8/0.40/0.45	8.23/1.8/0.40/0.40	8.60/2.1/0.45/0.40	8.3/2.0/0.50/0.50
21							8.80/2.0/0.35/0.40	8.20/1.8/0.40/0.45	8.24/1.8/0.40/0.40	8.90/2.1/0.45/0.40	8.35/2.5/0.55/0.55
22								8.30/1.8/0.40/0.40	8.25/1.8/0.40/0.40	8.90/2.1/0.45/0.40	8.4/2.5/0.55/0.55
23								8.40/2.0/0.40/0.35	8.30/1.8/0.40/0.40	9.00/2.2/0.45/0.45	8.5/2.5/0.55/0.55
27									8.80/1.8/0.45/0.40	9.73/2.2/0.45/0.45	8.8/2.5/0.60/0.60
29									9.00/2.0/0.40/0.35	9.75/2.2/0.45/0.45	9.00/3.0/0.60/0.60
35										9.90/2.2/0.45/0.45	11.00/3.0/0.65/0.65
43										10.23/2.2/0.45/0.45	15.20/3.0/0.65/0.65
46										10.24/2.2/0.45/0.45	15.23/3.0/0.70/0.70
52										10.40/2.2/0.45/0.45	15.21/3.5/0.70/0.70
57										10.72/2.2/0.45/0.45	15.25/3.5/0.75/0.75
59											15.32/3.5/0.70/0.65
61											15.32/3.5/0.70/0.65
65											15.32/3.5/0.70/0.65
68											15.32/3.5/0.65/0.65
73											15.32/3.5/0.65/0.65
74											15.32/3.5/0.65/0.65
80											15.32/3.5/0.65/0.65
82											15.32/3.5/0.65/0.65
87											15.32/3.5/0.65/0.65
89											15.32/3.5/0.65/0.65
94											15.32/3.5/0.65/0.65

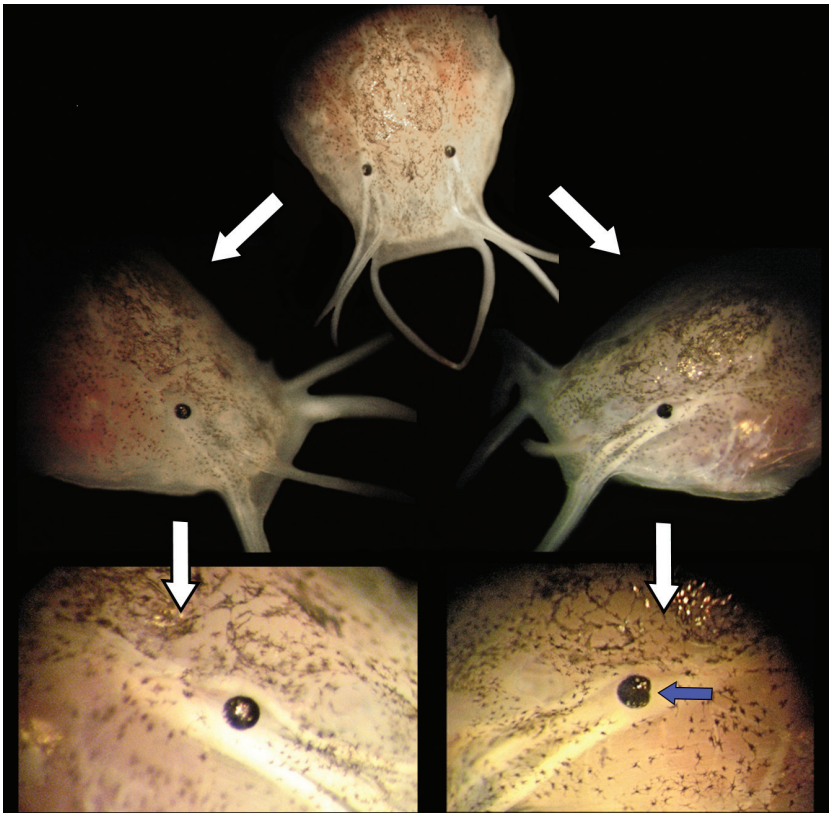


Figure 3. Juvenile *Ituglanis passensis* (200 days, 15.4 mm SL), detail of head and eyes showing melanophores and asymmetry in eyes shape. Arrow…. degeneration of round shape in left eye. Photo author: Sandro Secutti.

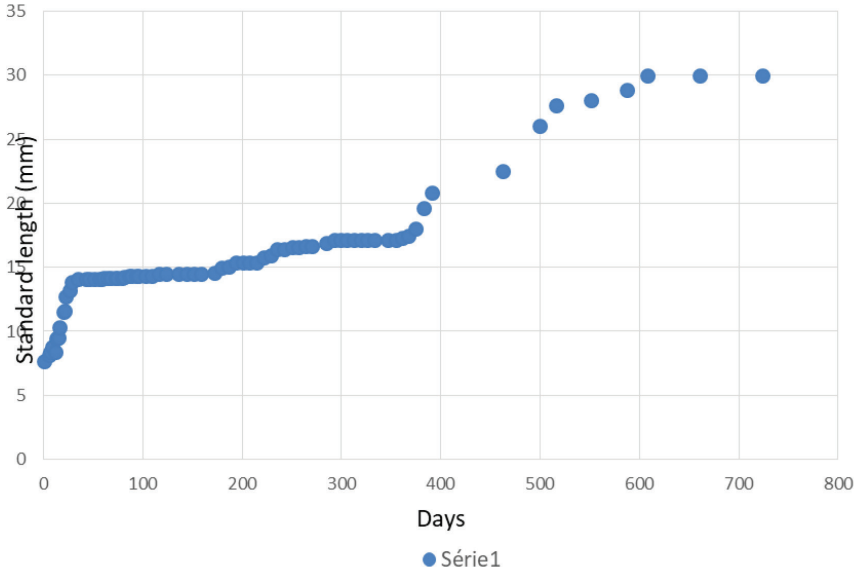


Figure 4. Size (standard length, in mm) *versus* age (in days) in specimen 12.

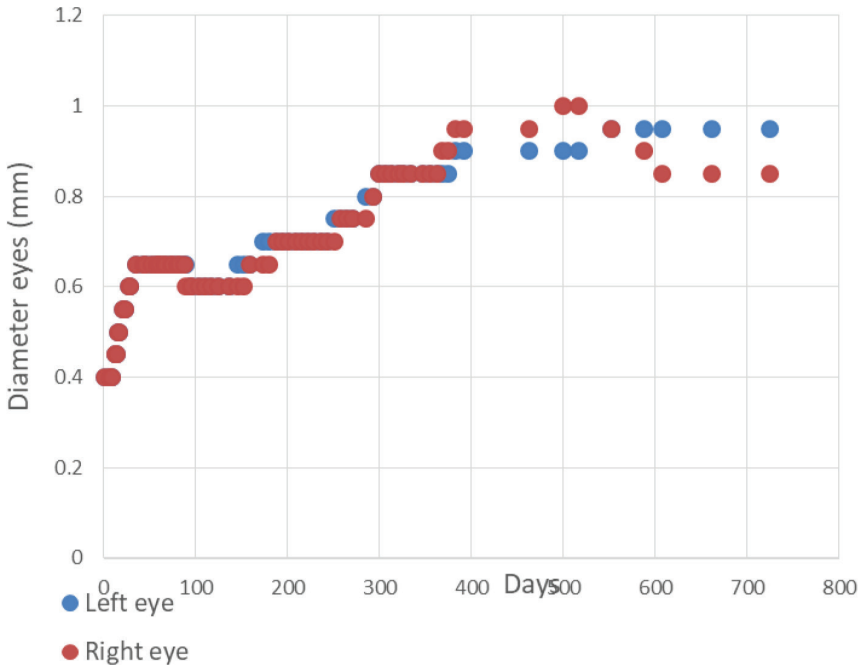


Figure 5. Eyes size (diameter, in mm) *versus* age (in days) in specimen 12.

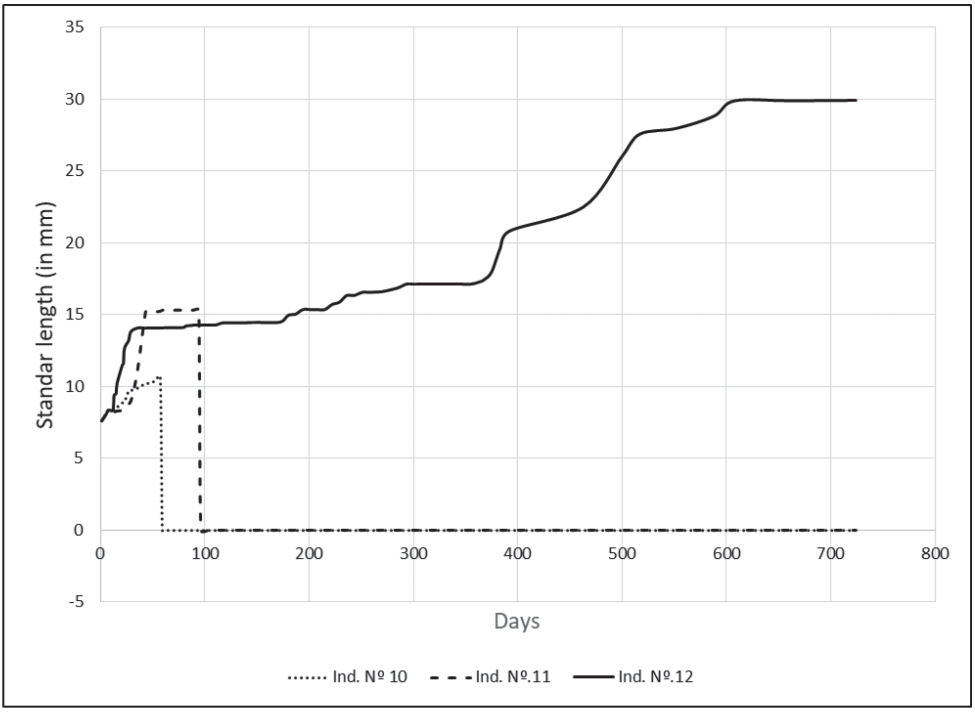


Figure 6. Standard length growth along the study period in individuals 10, 11 and 12.

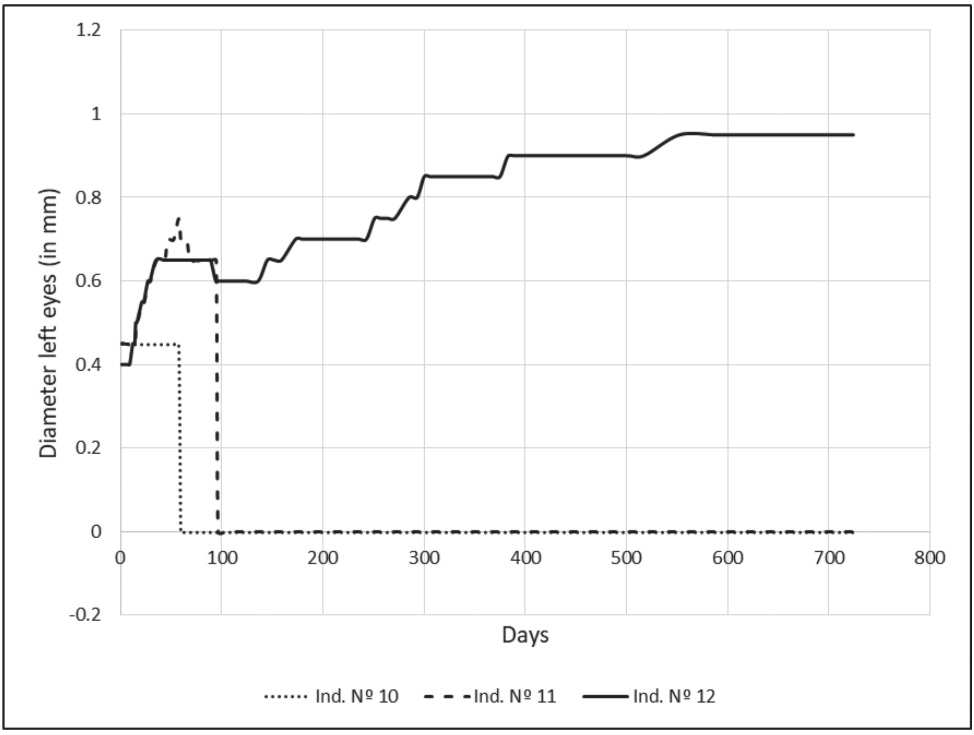


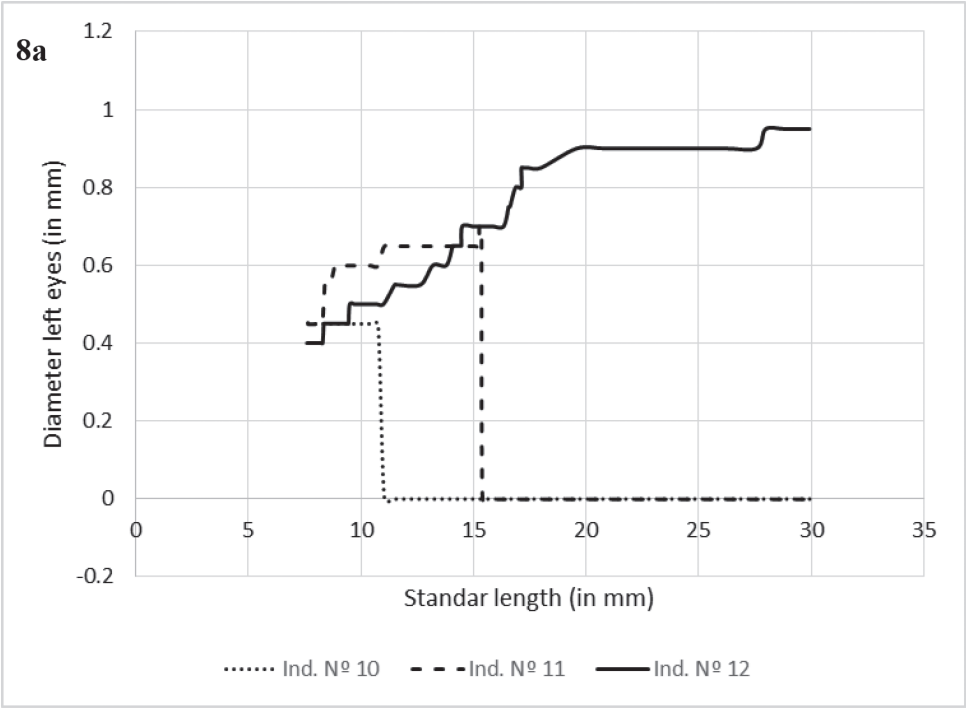
Figure 7. Diameter of left eye *versus* time in individuals 10, 11 and 12.

Discussion

Reproduction and life span

Seasonal reproduction has been recorded in several troglobitic fishes, usually during the high wet season, as is the case of the Brazilian catfishes, *Pimelodella spelaea* and *Trichomycterus itacarambiensis*, the USAn amblyopsids *Amblyopsis spelaea* and *A. rosae*, and Mexican and Cuban bythitids (*Ogilbia pearse*, *Lucifuga dentatus*, *L. subterraneus*). Counterwise, the Somalian phreatobic *Uegitglanis zammaranoi* reproduces during the warm dry season (Trajano 2001a).

Seasonal reproduction in troglobitic trichomycterids is based on differences in the proportion of mature females, which is higher in the dry season than in the beginning of the rainy, the case of *T. chaberti* (Pouilly and Miranda 2003). In *T. itacarambiensis*, the higher proportion in the beginning than at the end of the dry season, is accompanied by a parallel decrease in the mean size of oocytes, probably due to a progressive depletion in food resources (Trajano 1997b). A reproductive peak at the end of the rainy season was reported for *Rhamdiopsis krugi*, from Chapada Diamantina (BA), characterized by well-defined rain cycles (Mendes 1995; cited as *Imparfinis* sp.). Data on *I. passensis* show reproductive peaks during the rainy season, indicated in the laboratory by slight increases in water temperature (up to 2 °C).



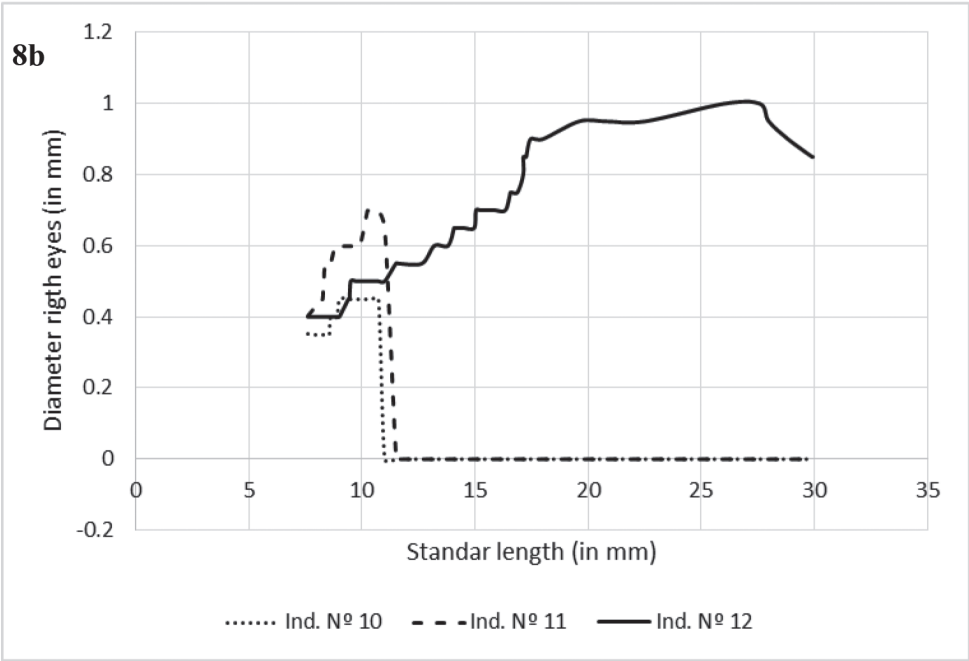


Figure 8. Diameter of left (**8a**) and right (**8b**) eyes *versus* standard length in individuals 10, 11 and 12.

The *I. passensis* female under study presented intercalated spawning, like the epigean *Trichomycteus corduvense* (Marraro 2005) and *Trichomycterus* sp. from the Passa Cinco stream (Rondileni et al. 2009), but not *T. areolatus* (Manriquez et al. 1988). Parceled spawning and a prolonged reproductive period are frequent in fishes from tropical and subtropical zones, where seasonality is not so accentuated as in the temperate (Nikolsky 1963). Hence, it is probably a plesiomorphic trait of *I. passensis*, not clearly correlated with a subterranean life.

Studies based on large samples (N > 30) reveal profuse individual variability in reproductive characters, the case of *T. areolatus*, *T. caliense* and *T. cf. barbouri* (Table 1). Further studies are required to clarify life-cycle patterns in trichomycterids in general, and troglobitic species in particular.

When caught in May, 2000, the studied individuals were at least 40 mm long. Based on growth-rates observed in the laboratory, at that time they were probably at least 3–4 years old. Subsequently, they survived for almost another 10 years in aquaria, thus indicating a longevity of more than 10 years, a high life-span for such a relatively small fish. Based on mark-recapture techniques, the estimated average longevity of the same sized troglobitic *T. itacarambiensis* was seven years (Trajano 1997a), and of the larger troglobitic heptapterid *Pimelodella kronei* (mean maximum size = 154 mm) 10–15 (Trajano 1991). When compared to epigean relatives, these life-spans, as part of K-selected life cycles, are higher (Trajano 2001a). Even considering that life expectancy may be higher under protected laboratory conditions (absence of predators, plus regular and predictable feeding), longevity of at least seven years in the natural habitat, as in the case of *T. itacarambiensis*, is a reasonable hypothesis.

Our results are consistent with previous observations based on specimens collected in 1988, and kept under conditions similar to those of the present study. Adult *I. passensis* catfish are shy and cryptobiotic, hiding most of the time under rocks or buried in the substrate, whether in the cave habitat or in aquaria. Agonistic interactions were rare, consisting of rapid pursuing and twisting movements. In the summer of 1989/1990, a couple kept in a 40-l aquarium with external filter and two rocky shelters, initiated reproductive behavior. Over several weeks, the male (83 mm SL) already in the same den as the female, nervously pursued her away from the shelter, sometimes touching her anal region with its snout. This behavior continued even after the first juveniles appeared (February, 1990), whereupon both adults were transferred to another aquarium. The eight surviving juveniles (5 to 12 mm TL) had well-developed and apparently normally structured eyes (Trajano and Souza 1994: fig. 1).

Growth and eye regression

The presence of eyes in newly hatched larvae, as reported here for *I. passensis* and apparently a general trait in troglomorphic vertebrates, has been observed in organisms as diverse as the European salamander *Proteus anguinus* (*e.g.*, Durand 1973), the USAn

amblyopsids (Niemiller and Poulson 2010), the African cyprinid *Phreatichthys andruzzii* (Aden 2008; Soares and Niemiller 2020), the characid *Astyanax* (Wilkens and Strecker 2017) and the heptapterid catfish *Rhamdia zongolicensis* (Wilkens 2001), the latter two from Mexico. Among Brazilian troglobitic fishes, eyed alevins of *Glaphyropoma spinosum* (Trichomycteridae) were collected in the cave habitat (Rizzato and Bichuette 2013: fig. 6). Incidently, ontogenetic regression in the laboratory was described for *Ancistrus cryptophthalmus* (Secutti and Trajano 2009),

Two non-mutually exclusive explanations have been proposed for the precocious presence of eyes in the larvae of troglomorphic vertebrates. 1) Since the eye anlage plays an essential role in inducing craniofacial formation in fishes, eyes undergo accelerated development during the early stages (Langecker 2000). 2) Cells contributing to the retina, as well as the rest of the secondary prosencephalon (hypothalamus plus telencephalon), are intermingled in the early developmental stages. At one point there is a strong and overall constraint in the process, which antecedes eye-development alone, to so facilitate reaching the ideal point for playing their essential role in the shaping of a correct forebrain (Pottin et al. 2011).

Heterogeneous growth, i.e., the significant variation in growth rates among individuals of the same age and living in the same habitat, seems to be a common phenomenon among fishes. It was first noticed in fish farming, and recorded for adults in mark-recapture studies of Brazilian subterranean fishes, including occasions of negative growth (*Pimelodella kronei*, Trajano 1991; *Trichomycteus itacarambiensis*, Trajano 1997a; *A.cryptophthalmus*, Trajano and Bichuette 2007; *Rhamdia enfurnada* and *P. spelaea*, Trajano unpubl. data; and the amblyopsid *Amblyopsis rosae*, Brown and Johnson 2001). Heterogeneous growth may also take place among larvae and juveniles, as herein reported for *I. passensis*. This was also observed in the laboratory in the trichomycterid *Trichomycterus dali* from the Serra da Bodoquena karst area. However, the absence of individual variations in growth rates between isolated juveniles and those kept collectively indicates that neither genetics nor social interactions can unambiguously account for this (Cordeiro and Trajano, unpubl data).

Fluctuating asymmetry in eye-size, as herein reported for *I. passensis* from the 350th day on, was also observed for *A. cryptophthalmus* from the 180th (Secutti and Trajano 2009). No left-right asymmetry was noted in other Brazilian subterranean fishes characterized by intrapopulation variability, viz., the heptapterids *Pimelodella kronei*, *Rhamdia infurnada* and *Rhamdia* undescribed sp. from the Serra da Bodoquena karst area (Trajano and Bichuette 2010). On the contrary, troglobitic Amblyopsids present this asymmetry (Niemiller and Poulson 2010), and in the Mexican catfish, *R. zongolicensis*, it is extensively common among adults (Wilkens and Strecker 2017).

Although the degeneration process had started by the end of the first year (beginning of asymmetry, change of shape in the left eye), the last surviving *I. passensis* juvenile still had external eyes, visible as black spots, after around 720 days (ca. 30 mm SL). The eyes (diameters around 3% of standard length) presented fluctuating asymmetry. In adults, externally visible eyes are, at the maximum, 1% of SL (Bichuette 2003). Thus, apparently the process of eye regression was still proceeding after two years (for

an estimated lifespan of 7+ years in the laboratory), with body-sizes already having reached 40–50% of the maximum recorded for the species (60–70 mm). Thus, in comparison with other studied troglobitic fishes, this may be considered as late regression.

Timing of eye-regression among species is extremely variable, and apparently taxonomically uncorrelated. Definitive eye regression in the Mexican troglobitic *Astyanax* from the Sabinos Cave starts when fishes are ca. 15 mm long (less than one third of the average maximum size in this population). They present temporary stops, or even a decline, in eye-growth during the development phase (Wilkens and Strecker 2017), as described here for *I. passensis*.

Late ontogenetic regression, as observed for *I. passensis*, has also been recorded for *Ancistrus cryptophthalmus.* In this case, regression started in 480/500-day-old juveniles, 40–45 mm SL (Secutti and Trajano 2009). On the other hand, early eye-regression was described for the highly troglomorphic fishes *Phreatichthys andruzzi*, from Somalia (maximum eye development was reached in 36 hours, followed by very fast regression – Berti et al. 2001) and the Brazilian *Trichomycterus dali* (Cordeiro and Trajano pers. obs.). Further studies involving a larger number of species belonging to various taxonomic groups are required, to test possible correlations between the duration of eye-regression and the degree of troglomorphism, as a means of measuring time of isolation in subterranean habitats (Wilkens 1982, but also see Trajano 2007).

Evidence for the Neutral Mutation Theory: data on Brazilian subterranean fishes

The main mechanisms supposedly involved in eye-regression are natural selection (energy economy, pleiotropy) and the accumulation of neutral mutations (for review see Wilkens and Strecker 2017).

Generality, as applied to the energy-economy-model, has been refuted, when considering troglomorphic populations dwelling in habitats where food is plentiful, viz.,troglobitic plant-hoppers and internal parasites. The presence of troglomorphic species in tropical caves, generally assumed to be food-rich, is frequently cited as an argument against this model. However, our experience in Brazil shows that the food-rich-tropical-caves paradigm is a myth. Bat caves are uncommon and mostly restricted to areas with scarce rocky shelters, such as sandstone and small karst areas (Trajano 1985). Moreover, eutrophy is a present-day ecological condition, whereas troglomorphy is the result of past historical processes (Trajano 2001b), which may have occurred under very distinct ecological conditions. The opening of large entrances favoring the input of great amounts of nutrients is a late speleogenetic event in karst areas (Ford and Williams 2007). This would be the case of the Passa Três Cave, which opens to the surface through an upper sinkhole. The large amount of organic matter, mostly vegetal debris, carried into the cave during flash floods would explain the high population densities of *I. passensis* and the syntopic *Ancistrus cryptophthalmus* (Trajano and Bichuette 2007), although this factor is probably unrelated to the higher degree of eye-regression in the later, when compared to other high density cave populations found in base-level streams.

Pleiotropic hypotheses on eye development, based on a selection of unrelated traits, are mostly the outcome of molecular studies of Mexican tetra characids, *Astyanax* spp. By cross analysis and quantitative trait loci mapping, Wilkens and Strecker (2017) presented compelling evidence against these hypotheses. Moreover, the mosaic distribution of molecular and behavioral character states in these fishes is inconsistent with this model.

Molecular studies of the cyprinids *Sinocyclocheilus anophthalmus* from China, and of *Phreatichthys andruzzii* from Somalia, point to eye-regression mechanisms different from those affecting the Mexican tetras, thereby indicating that independent evolution of eye degeneration in cave fishes could occur through other genetic mechanisms (Meng et al. 2013; Soares and Niemiller 2020).

The Neutral Mutation Theory on regression in structures, functions and behaviors that are no longer subject to selective pressures (i.e., that cease being adaptive), was proposed for troglomorphic fishes 85 years ago by the German geneticist C. Kosswig (Wilkens and Strecker 2017). A growing body of evidence has been accumulated ever since. Contrary to pleiotropic hypotheses, mostly *ad hoc*, this is a general theory, as foreseen by Darwin to explain the regression of characters that lose their functions in various and unrelated taxa.

A generally observable feature of functionless regressive traits in troglobites is their long-lasting genetic and phenotypic variability, higher than in epigean relatives and not expected for those under selection. This was reported for many troglobitic species, including the Mexican tetras, and involved molecular, morphological and behavioral aspects (Wilkens and Strecker 2017). The mosaic distribution of troglomorphic character states, including morphological such as eyes and melanic pigmentation, and behavioral traits, intra- e inter-populations, is also a common phenomenon among Brazilian subterranean fishes, thereby providing evidence of independent evolution (Trajano and Bichuette 2010).

The late eye regression, the fluctuating asymmetry and the mosaic intrapopulation variability in adult *I. passensis* catfish provide further evidence for the neutral mutation theory as a general model to explain character regression in troglobites.

Fishes from the São Domingos karst area – a comparison

The São Domingos karst area is distinguished worldwide by its rich subterranean ichthyofauna, comprising troglophilic populations and several troglobitic lineages in seven described species (Trajano 2021). However, compared to fishes from other Brazilian karst areas, troglomorphism varies from low (slightly, yet significantly reduced eyes and little depigmentation – *Pimelodella spelaea* and *Eigenmannia vicentespelaea*) to medium (*Ancistrus cryptophthalmus* and four *Ituglanis* spp.). This is in contrast to karst areas in the semiarid Bahia State, Serra da Bodoquena (MS) and Jaiba (MG), where the most troglomorphic, homogeneously depigmented and anophthalmic populations occur.

On the other hand, the relative paucity of troglobitic invertebrates, plus location in the Cerrado core area, subject to less accentuated paleoclimatic fluctuations than in sev-

eral other Brazilian karst areas, indicate that the paleoclimatic model alone would not account for the troglobitic-fish taxonomic diversity found in São Domingos. Presumedly, topographic isolation due to alluvial downcutting by fast-flowing rivers resulted in a regional lowering of the water table and the presence of waterfalls, dolines and karst canyons (Bichuette and Trajano 2004). Hence, it is not surprising that lineages from higher, older habitats, such as the epikarst and vadose water courses, e.g. *A. cryptophthalmus* from the Passa três Cave and *Ituglanis* spp., including *I. passensis*, are more troglomorphic than those living in base-level streams (*E. vicentespelaea* and *A. cryptophthalmus* from the Angélica, Bezerra and São Vicente caves), due to a longer time in isolation.

Highly troglomorphic subterranean fishes tend to become arrhythmic, loosing photophobic reactions and cryptobiotic habits (Parzefall and Trajano 2010), besides laying fewer and larger eggs than close epigean relatives (Culver 1982; Trajano 2001a). None of this was noted in *I. passensis*.

Acknowledgements

We are most grateful to Lívia Medeiros Cordeiro and Rodrigo Borghezan for assistance in maintaining the specimens and for biometric data of larvae in the first weeks of study, and to Ramon A. Clark, for revision of the English style. We are greatly indebted to the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP, for the research grant (# 03/00794-5) granted to the first author. Permission for collections was given by IBAMA.

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Subterranean Biology 38: 113–115 (2021)
doi: 10.3897/subtbiol.38.68597
https://subtbiol.pensoft.net

CORRIGENDA

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Corrigenda: *Stygobromus bakeri*, a new species of groundwater amphipod (Amphipoda, Crangonyctidae) associated with the Trinity and Edwards aquifers of central Texas, USA. Subterranean Biology 38: 19–45. https://doi:10.3897/subtbiol.38.61787

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Academic editor: O.T. Moldovan | Received 12 May 2021 | Accepted 14 May 2021 | Published 21 May 2021

http://zoobank.org/11BDC2F7-406D-408D-A12A-BB01C836C4D6

Citation: Gibson R, Hutchins BT, Krejca JK, Diaz PH, Sprouse PS (2021) Corrigenda: *Stygobromus bakeri*, a new species of groundwater amphipod (Amphipoda, Crangonyctidae) associated with the Trinity and Edwards aquifers of central Texas, USA. Subterranean Biology 38: 19–45. https://doi:10.3897/subtbiol.38.61787. Subterranean Biology 38: 113–115. https://doi.org/10.3897/subtbiol.38.68597

Keywords
subterranean, karst, stygobiont, stygobite, troglomorphy, crenobiont, interstitial crustacean, spring, cave, sympatric

Introduction

It has come to our attention that many of the records reported in Table 1 were shifted or missing and therefore incorrect.

The CORRECT Table is as follows:

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Table 1. Groundwater fauna collected at each *Stygobromus bakeri* sp. nov. site. Stygobionts (S) are hypogean and colonize various groundwater habitats. Crenobionts (C) are epigean and preferentially colonize springs.

Taxon	Stygobiont/ Crenobiont	John Knox Spring	Jacob's Well	Salado Springs	Mormon Spring #3
Turbellarians					
Order Tricladida					
Family Kenkiidae					
Sphalloplana mohri Hyman, 1938	S	X		X	
Annelids					
Order Lumbriculata					
Family Lumbriculidae					
Eremidrilus **sp. 1** Fend & Rodriguez, 2003	S	X		X	
Eremidrilus **sp. 2**	S			X	
Molluscans					
Order Gastropoda					
Family Cochliopidae					
Phreatoceras taylori (Hershler & Longley, 1986a)	S			X	
Phreatodrobia conica Hershler & Longley, 1986b	S			X	
Phreatodrobia micra (Pilsbry & Ferriss, 1906)	S			X	
Phreatodrobia nugax (Pilsbry & Ferriss, 1906)	S	X		X	
Stygopyrgus bartonensis Hershler & Longley, 1986b	S				X
Family Hydrobiidae					
Marstonia comalensis (Pilsbry & Ferriss, 1906)	C	X	X	X	
Family Pluerocerídae					
Elimia comalensis (Pilsbry, 1890)	C		X	X	
Crustaceans					
Order Amphipoda					
Family Bogidiellidae					
Parabogidiella americana Holsinger, 1980	S			X	
Family Crangonyctidae					
Stygobromus bifurcatus (Holsinger, 1967)	S		X	X	X
Stygobromus russelli (Holsinger, 1967)	S	X	X	X	X
Stygobromus (*flagellatus group*) sp. nov. Cope, 1872	S		X		
Order Bathynellacea					
Family Parabathynellidae					
Texanobathynella bowmani Delamare Deboutteville, Coineau & Serban, 1975	S			X	
Order Isopoda					
Family Asellidae					
Caecidotea bilineata Lewis & Bowman, 1996	S			X	
Caecidotea reddelli (Steeves, 1968)	S			X	X
Lirceolus bisetus (Steeves, 1968)				X	
Lirceolus hardeni (Lewis & Bowman, 1996)	S	X	X	X	
Lirceolus pilus (Steeves, 1968)	S		X	X	
Order Podocopida					
Family Candonidae					
Comalcandona tressleri Külköylüoğlu & Gibson, 2018	S	X			
Schornikovdona bellensis Külköylüoğlu, Yavuzatmaca, Akdemir, Diaz & Gibson, 2017	S			X	
Pseudocandona albicans (Brady, 1864)	C	X			
Pseudocandona cf. *semicognita* (Schäfer, 1934)	C	X			
Physocypria cf. *globula* Furtos, 1933	C	X			
Darwinula stevensoni (Brady & Robertson, 1870)	C	X			
Cypridopsis sp. (Brady, 1867)	C	X			
Physocypria denticulata (Daday, 1905)	C				X
Chlamydotheca texasiensis (Baird, 1862)	C				X
Cypridopsis cf. *helvetica* Kaufmann, 1900	C	X			

Taxon	Stygobiont/ Crenobiont	John Knox Spring	Jacob's Well	Salado Springs	Mormon Spring #3
Arachnids					
Order Trobidiformes					
Family Arrenuridae					
Arrenurus spp. Dugès, 1834	S	X		X	
Insects					
Order Coleoptera					
Family Dytiscidae					
Sanilippodytes sp. Franciscolo, 1979	C	X		X	
Uvarus texanus (Sharp, 1882)	C			X	
Family Elmidae					
Microcyloepus pusillus (LeConte, 1852)	C	X	X	X	X
Salamanders					
Order Urodela					
Family Plethodontidae					
Eurycea chisholmensis Chippindale, Price, Wiens & Hillis, 2000	C			X	
Eurycea pterophila Burger, Smith & Potter, 1950	C	X	X		