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First record of a cavernicolous Kinnaridae from the Old World (Hemiptera, Auchenorrhyncha, Fulgoromorpha, Kinnaridae, Adolendini) provides testimony of an ancient fauna

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Abstract

A new obligately cavernicolous species in the planthopper family Kinnaridae is described from Spain. This is the first record of a cavernicolous kinnarid from the Old World, and the first record of a troglobitic fulgoromorphan hemipteran from mainland Spain, and also the 7th cavernicolous kinnarid species worldwide. Epigean Kinnaridae are not known from the present-day fauna of the Iberian Peninsula nor from Western Europe at large. The new species is regarded as a relict from an ancient fauna which is now extinct. The new cavernicolous species could not be assigned to any of the existing genera, thus a new genus is established. Molecular data (COI barcode sequence) for the new species are presented. For the first time, a detailed description of the nymphal morphology of a kinnarid is provided. Information on its ecology, behaviour, distribution and conservation status is given, and biogeographic implications are discussed.

Keywords

Caves, conservation, Iberian Peninsula, morphology, taxonomy, troglobite, troglomorphy

Introduction

During several years of biospeleological research in the cave ecosystems of the Valencian Community (Spain), the former team of biologists from the Museu Valencià d'Historia Natural (Torres Sala Foundation), collected several specimens of a highly troglomorphic species of the planthopper (Fulgoromorpha) taxon Kinnaridae, previously unknown in the Iberian Peninsula. Its discovery received considerable attention in the local media, as the tiny creature is a) morphologically stunning with glassy wings characterized by a shining blue rim in males, evoking images of a "fairy", and b) its existence in the dark subterranean spaces of the last remains of Mediterranean forests in Valencia. Thus, this remarkable taxon was named the „fairy of the forests" (see below, etymology). At the moment, the new species has been studied from two caves, and has been observed in seven other caves, all in the most eastern reliefs of the Iberian Mountain Range in the east of the Iberian Peninsula. The species is characterized by several troglomorphies, e.g., absence of compound eyes and ocelli, very pale body pigmentation and reduced tegmina and wings, and is accordingly assumed to be obligately cavernicolous (troglobitic). This species represents the first record of a troglobitic planthopper species in mainland Spain, the first record of the taxon Kinnaridae in mainland Spain, the 7th cavernicolous kinnarid species worldwide, and the 3rd record of an obligately cavernicolous planthopper in the Mediterranean.

With currently 115 species in 24 genera (Bourgoin 2019), Kinnaridae is one of the smallest families of the Fulgoromorpha. Kinnarids are distributed throughout the world (Bourgoin 2019), predominantly in the tropics and subtropics (Wilson 2010). From the Palearctic region, several genera are documented, with species known from Iran and Tadzhikistan (Emeljanov 1984), Afghanistan (Dlabola 1957), India (Himalaya, Simla) (Distant 1916), the United Arabian Emirates (Wilson 2010) and from the Canary Islands (Remane 1985).

The majority of Kinnaridae species are epigean, with well-developed compound eyes, vivid body coloration, fully developed tegmina and wings, enabling flight. Several lineages, however, are known to have colonized caves in various regions of the New World: the Caribbean (Jamaica: Fennah 1980), Central America (Mexico: Fennah 1973) and South America (Brazil: Hoch and Ferreira 2013, 2016, Xing et al. 2013) and accordingly, display varying degrees of troglomorphies, such as the reduction of eyes, wings and body pigmentation (see information in Hoch and Ferreira 2016).

Knowledge of the biology and ecology of Kinnaridae in general is scarce. Most species are apparently associated with dicots (Asteridae and Dillenidae: Fennah 1948), but there are also records from ferns, gymnosperms (Ephedraceae) and monocots (Agavaceae) (see information in Ai-Ping 2001). Adults of epigean species feed on the exposed parts of plants, while nymphs are subterranean, feeding on roots (Fennah 1948, 1980).

The current state of Kinnaridae classification has been summarized by Hoch and Ferreira (2016). No hypothesis to explain phylogenetic relationships within Kinnaridae has yet been provided. The characters on which the current tribal classification is based are of important diagnostic value; they have not been evaluated as potential

synapomorphies yet. This also applies to the established genera, rendering the accommodation of the new species into any of the supraspecific taxa problematic. According to the key given by Emeljanov (2006) the new cavernicolous kinnarid from Spain can be classified as a member of the subfamily Prosotropinae Fennah, 1945, and – with some *caveat* – of the tribe Adolendini (see Discussion: suprageneric classification). In characters of the male genitalia, it does not share similarities with any species of the known kinnarid genera (in or outside the Adolendini) which could be interpreted as synapomorphies. Thus, a new genus is established to accommodate this new species described below. We also provide a description of the 5th instar nymph as the nymphal morphology of Kinnaridae in general is largely unknown.

Material and methods

Collecting, preservation, permanent storage

The specimens were discovered by visual search, collected by hand, and transferred into vials containing 70% and 96% ethanol. For permanent storage, after dissection and examination, genitalia were transferred to polyethylene vials, and individually associated with the specimen vial.

Morphological examination techniques, visualization

Measurements and examinations of external body features were made from the specimen in ethanol. To prepare male genitalia for dissection, the genital capsule was removed from the specimen, macerated for 24 h in 10% KOH at room temperature, washed in water, transferred to glycerine for storage or to glycerine-jelly for drawings. Examinations and drawings were made using a Leitz stereomicroscope with a *camera lucida* attachment.

Scanning electron microscopy

Specimens preserved in 96% Ethanol were critical point dried with a Leica EM CPD 300, and gold coated for 2, and 4 minutes, respectively. Morphological investigations were conducted with a Zeiss EVO LS 10 electron microscope.

Molecular data

DNA was purified individually from two whole nymphs with a Qiagen Blood & Tissue kit using the manufacturer's protocol. Polymerase chain reaction (PCR) was used to amplify a mitochondrial gene fragment, a 710 bp fragment of the Cytochrome Oxidase subunit I gene (COI) using primers LCO1490 and HCO2198 (Folmer et al. 1994), the so-called DNA Barcoding fragment.

PCR was performed in 25 µl volumes containing 1× Taq buffer, 1.5 mM MgCl2, 200 µM each dNTP, 1 U Taq polymerase, ca. 50–100 ng DNA and ddH2O. After an initial denaturation step of 3 min at 94 °C, cycling conditions were 35 cycles at 94 °C for 35 s, 45 °C (COI) for 60 s, and 72 °C for 1 min, with a final elongation step of 5 min at 72 °C. The same primers were used in PCR and sequencing. PCR products were sent to Macrogen Europe for purification and cycle sequencing of both strands. The sequences were processed and corrected using CodonCode Aligner v. 5.1.5 (CodonCode Corporation), both nymphs had identical sequences (sharing a single haplotype). The haplotype sequence has been deposited in GenBank, access number MW323405.

Results

Taxonomy

Kinnaridae Muir, 1925: 158
Prosotropinae Fennah, 1945: 449
Adolendini Emeljanov, 1984: 470 (51)

***Valenciolenda* Hoch & Sendra, gen. nov.**
http://zoobank.org/87FFCB90-92D3-4CED-AAA2-DEDDD769C90E

Type species. *Valenciolenda fadaforesta* sp. nov. (type locality: Spain, València, Murciélagos cave).

Diagnosis. Small kinnarid (ca. 3–4 mm body length), strongly troglomorphic: compound eyes and ocelli absent, tegmina reduced in length but surpassing lateral body margins, wings vestigial, body whitish, pigmentation largely reduced (Fig. 1a). *Valenciolenda* gen. nov. can be distinguished from all other genera of the Kinnaridae by the unique combination of the following characters: narrow and short vertex; short and wide tegmina, in life held nearly horizontally over the body and, in dorsal view, forming a near circle; male genitalia with genital segment in caudal aspect longish ovate, not constricted; anal segment short, ventrocaudally with two arm-like processes, genital styles slender at base, apically strongly enlarged, medially concave; aedeagus tubular stout, distally widening, ventrocaudal margin with a short, acute tip.

Description. ***Head*** (Fig. 2). Vertex short, with a very obtuse median carina, area of vertex slightly tilted against area of frons, slightly wider posteriorly than anteriorly, anterior margin slightly concave or nearly straight, posterior margin shallowly concave. Frons narrow, ca. 1.45 × longer than maximally wide (widest between level of antennae and frontoclypeal suture); ca. 2.1 × longer than post- and anteclypeus combined; surface medially smooth, devoid of a median carina; lateral margins distinctly ridged and directed laterally. Frontoclypeal suture shallowly arched, in a furrow between frons and postclypeus. Post- and anteclypeus with a distinct median carina, carina gradually

Figure 1. *Valenciolenda fadaforesta* sp. nov., habitus, dorsal view **a** adult male, dorsal view **b** nymph (IV. instar) from 'Murciélagos' cave (Vilamarxant, València) (photos by: Sergio Montagud Alario) **c** morphological analogy in the troglobitic *Solonaima baylissa* Hoch & Howarth, 1989 (Cixiidae) from Australia: habitus, adult male, dorsal view. Body length 4.5 mm (photo by H. Reimer, Marburg, used with permission).

vanishing towards frontoclypeal suture. Rostrum elongate; in repose well surpassing caudal margin of hind coxae; third joint shorter than second. Compound eyes and ocelli absent. Antennae with short scape, subcylindrical; pedicel subcylindrical, ca. 1.8 × as long as wide, with distinct sensory plate organs; sensory plate organs of the „flattened star-shaped plate" as reported for Kinnaridae (partim) by Bourgoin and Deiss (1994); arista ca. 3.5 × as long as pedicel.

Thorax. Pronotum tricarinate, ca. 2.3 × wider than head at level below antennae, short, ca. 5 × wider than medially long, posterior margin shallowly concave; carinae distinct, median carina reaching, but not surpassing anterior margin of pronotum; lateral carinae very shallowly S-shaped, joining hind margin laterally; mesonotum distinctly tricarinate, slightly wider than medially long; tegulae vestigial. Hind tibiae laterally unarmed, distally with 6–7 slender, terete spines, indistinctly grouped (3+4), and forming a shallow arc. First metatarsal joint with 4–5, 2nd metatarsal joint with 3–5 sturdy denticles (bilaterally and individually variable). Pretarsal claws and arolia small, inconspicuous. 2nd and 3rd metatarsal joints together slightly shorter than 1st metatarsal joint. Tegmina (Fig. 3) in males short and wide, ca. 1.6 × longer than wide, with

terminal margin obliquely transversely truncate; in life held nearly flat over the body and forming a near circle, in the female narrower: ca. 2 × longer than wide, laterally considerably exceeding the lateral margins of thorax and abdomen. Costal vein strong, in life covered with conspicuous filamentous waxy exudations (so far only observed in males). Venation in proximal part as in epigean Kinnaridae with a large and wide subcostal cell, clavus cixioid (*sensu* Emeljanov 1984), i.e., common claval vein (Pcu and A1) reaching hind margin of clavus (vein A2); basal cell of forewing open, i.e., not closed by anastomosis of M and CuA, no arculus developed; tegmen distally of nodal line distinctly reduced, with 6–7 marginal cells (Fig. 3: vein terminology according to Bourgoin et al. 2015). Hindwings vestigial, ca. 1/6 the total length of tegmen; venation strongly reduced.

Male genitalia (Figs 4, 5). Genital segment, anal segment, genital styles and aedeagus bilaterally symmetrical. Genital segment in caudal aspect longish ovate, not constricted medially, anal segment short, ventrocaudally with two arm-like processes, and ventrobasally with a median blunt process; genital styles slender at base, apically strongly enlarged, medially concave, aedeagus tubular, stout, distally widening.

Females (Fig. 6). Females with abdominal tergites VI, VII, and VIII bearing wax fields. Female genitalia as in other Kinnaridae of the non-piercing type; sternite VII in ventral view trapezoidal, with lateral margins diverging caudally, caudal margin straight; gonocoxae VIII bilobate; anal segment tubular.

Etymology. The genus name is a combination of Valencia, the capital city of the Valencian Community, an autonomous region of Spain in the east of the Iberian Peninsula where the type locality is located, and the tribe of Kinnaridae, Adolendini, to which the type species is assigned. The gender is feminine.

Valenciolenda fadaforesta Hoch & Sendra, sp. nov.

http://zoobank.org/571CB15C-1A95-40F9-AB0C-FD438C342218
Figs 1–9, 11d

Diagnosis. Habitus (Fig. 1a). Strongly troglomorphic species, predominantly whitish colouration, compound eyes and ocelli absent, body dorsoventrally compressed, tegmina short and wide, in repose very shallowly tectiform, almost flat, caudally reaching or slightly surpassing tip of abdomen, laterally surpassing external body margin with about half of their width, together creating a nearly circular shape; with a light blue wax fringe – which in life is quite eye-catching – accompanying costal vein; hind wings vestigial.

Description. ***Body length*.** Measurements refer to distance between anterior margin of head to tip of abdomen (= caudal margin of genital styles in males, and tip of anal segment in the female).

Males. 2.8 (in a specimen with contracted abdominal segments) – 3.8 mm (in the holotype which displays fully extended abdominal segments) (n = 4). Females. 4.1 mm (n = 1).

***Colouration*.** Head, thorax and abdominal segments largely unpigmented, whitish except lateral carinae of frons and rostrum, legs and genitalia in both sexes (genital

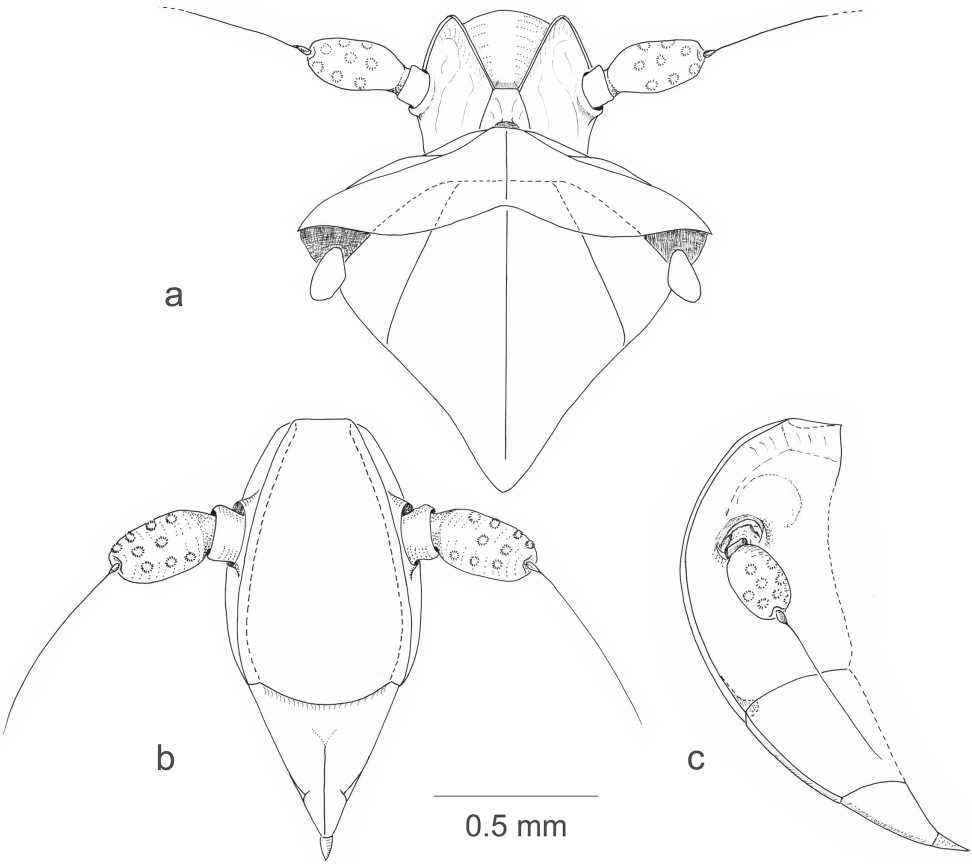

Figure 2. *Valenciolenda fadaforesta* sp. nov. **a** head and thorax, dorsal view **b** head, ventral view **c** same, left lateral view. Scale bar: 0.5 mm.

segment, genital styles, gonocoxae VIII) which are light yellowish. Distal spines on hind tibiae, 1st and 2nd metatarsal joints sordid brown. Tegmina translucent, unpigmented, veins whitish.

Configuration, shape and proportions of head and thorax as described for the genus.

***Male genitalia*.** Genital segment bilaterally symmetrical, in lateral aspect short, narrow in dorsal half, gradually widening caudoventrally; ventrally ca. 3.2 × longer than dorsally. Anterior margin of genital segment smooth, devoid of median apodemes. Genital segment in caudal aspect highly ovate, lateral margins in ventral portion more or less parallel, slightly diverging in dorsal third, dorsally gently arched medially, without conspicuous transverse bridge; caudal margin of genital segment ventrally smooth. Anal segment bilaterally symmetrical, short, stout, caudally on each side with a short, apically rounded, arm-like process directed laterocaudally. Paraproct short, stout, mushroom-shaped; epiproct broadly disc-shaped, laterally wider than paraproct, caudally not exceeding paraproct. Genital styles bilaterally symmetrical, slender at base, apically considerably enlarged; enlarged portion medially deeply concave, bearing 3

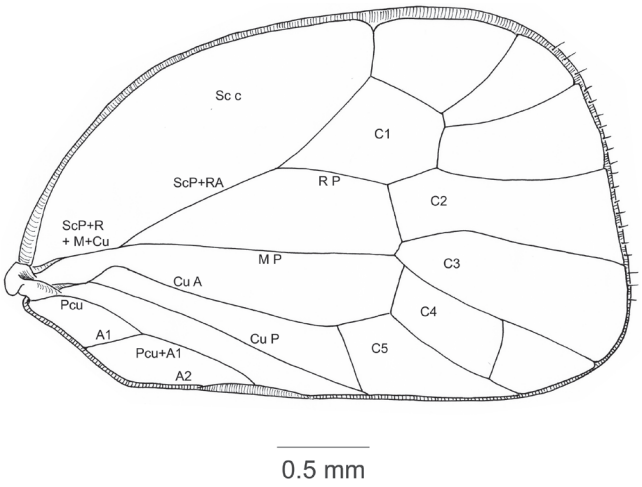

Figure 3. *Valenciolenda fadaforesta* sp. nov. Right tegmen, male. Scale bar: 0.5 mm. Abbreviations: ScP+R+M+Cu, subcosta posterior + radius + media + cubitus; scc, subcostal cell; ScP+RA, subcosta posterior + radius anterior; RP, radius posterior; PCu, cubitus posterior; A1, first anal vein; A2, second anal vein; CuA, cubitus anterior; PCu+A1, cubitus posterior + first anal vein; CuP, cubitus posterior; MP, media posterior; C1-C5, nodal cells.

apically rounded processes: one arising from ventral margin, directed mediodorsally, the others arising from dorsal margin and directed dorsally and laterodorsally, respectively. Genital styles densely covered with strong setae; setae predominantly on dorsal processes. Connective straight, and narrow. Aedeagus bilaterally symmetrical, short, stout, tubular, ventrally narrow and slightly compressed, distally widening and with ventral margin of strongly sclerotized part produced into a median tip which in repose is pointing ventrocaudally. Periandrium without any spinose or lobate processes; near base laterally on each side with a short, wing-like process, which is connected to the genital segment; apically with a wide membranous portion exposed caudally. Phallotreme not visible. Proximal apodeme of aedeagus („tectiform structure“ *sensu* Bourgoin (1997), term coined for Meenoplidae) shorter than periandrium, with dorsal and ventral margins almost parallel, proximal margin truncate.

Females with abdominal tergites VI–VIII bearing wax fields. Genitalia with sternite VII in ventral view trapezoidal, with lateral margins diverging caudally, caudal margin smooth, straight; gonocoxae VIII wide at base, distally bilobate with dorsal lobe larger than ventral one, lobes medially converging, both lobes apically with setae; tergite IX in dorsal aspect short, expanding ventrally and forming a continuous sclerotized bridge surrounding the anal segment. Anal segment (segment X) tubular, in dorsal aspect with lateral margins more or less parallel; anal style (segment XI) with paraproct prominent.

Immatures (Figs 1b, 7–9).

V. instar nymph. Body length 2.5 mm (specimen with contracted abdomen) – 3.1, resp. 3.2 mm. (specimens with extended abdomen) (n = 3).

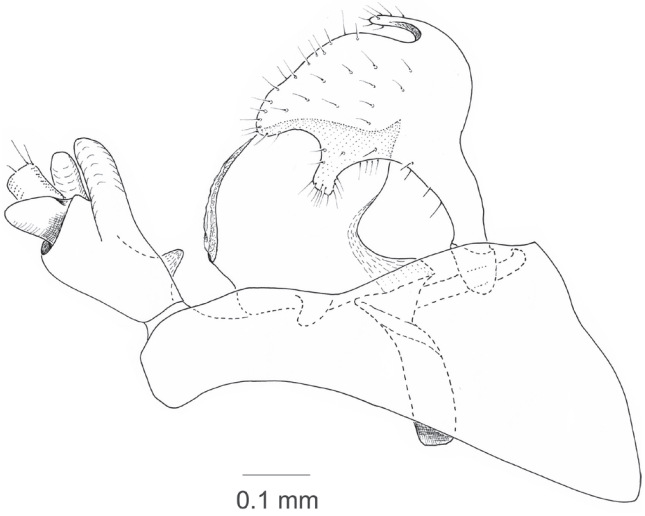

Figure 4. *Valenciolenda fadaforesta* sp. nov. Male genitalia *in situ*, left lateral view; paratype. Scale bar: 0.1 mm.

Habitus. ***Body*** ca. 1.7 × longer than maximally wide; maximum width at lateroposterior margins of forewing pads. Vertex short, compound eyes absent; rostrum elongate, well surpassing hind coxae, with ca. half its total length; frons, thoracic nota and abdominal tergites IV–VIII with numerous sensory pits.

***Colouration*.** Vertex, frons, thoracic and abdominal nota as well as distal parts of legs (tibiae, tarsi) light yellowish; head laterally, thorax ventrally as well as proximal parts of legs (coxae, femora) and abdomen ventrally white. Carinae of head and lateral carinae of pronotum yellowish brown; distal teeth of metatibia and metatarsomeres dark brown.

***Head*.** Vertex short, ca. 4 × wider than medially long, medially divided by a narrow longitudinal membraneous furrow, separated from frons by a distinct ridged transverse carina which medially slightly arches anteriorly. Frons smooth, without median carina, but with submedian carinae present, arising from frontoclypeal suture, parallel to lateral carinae of frons, converging towards apex and uniting into a short common stem which connects to anterior margin of vertex. Lateral as well submedian carinae of frons and their common stem distinctly ridged; frontoclypeal suture nearly straight, only slightly arching towards frons. Frons ca. 1.2 × as long as maximally wide (widest at level of antennae), apically straight; frons ca. 2.4 × longer than post- and anteclypeus together. Frons between lateral and submedian carinae with two parallel rows of sensory pits, in upper portion with complementary sensory pits between the two rows. Setae of pits directed towards adjacent carinae and those of complementary pits directed towards lateral carina. Post- and anteclypeus smooth, without median carina. Tip of rostrum with dorsal and ventral sensory fields convex, as described by Brozek and Bourgoin (2013), covered with numerous sensilla; type, number, and arrangement of sensilla very similar to those of the Kinnaridae species studied by Brozek and Bourgoin (2013), *Southia capnorhina* Fennah, 1980, *Atopocixius major* Fennah,

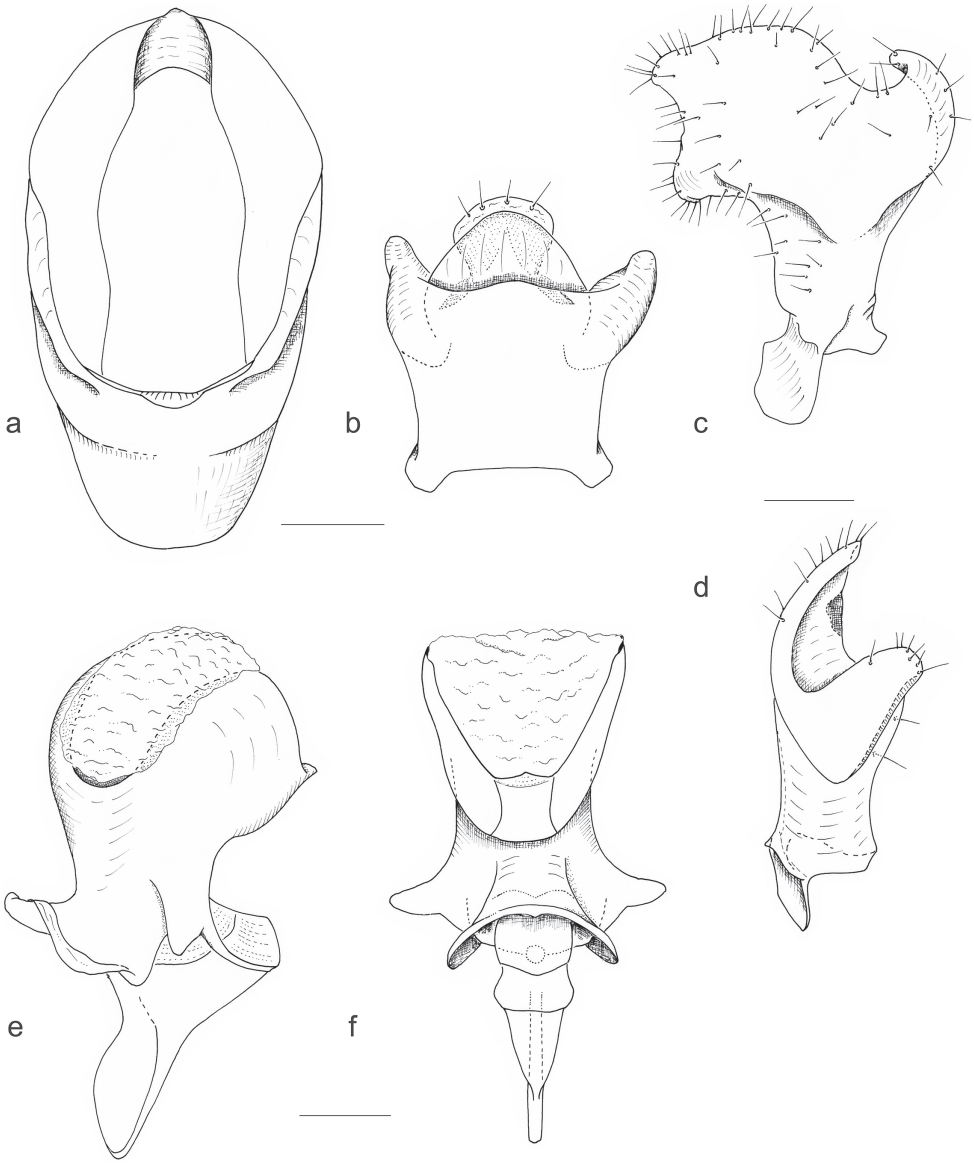

Figure 5. *Valenciolenda fadaforesta* sp. nov. Male genitalia **a** genital segment, caudal view **b** anal segment, dorsal view **c** left genital style, left lateral view **d** same, ventral view **e** aedeagus, left latero-dorsal view **f** same, ventral view. Scale bars: 0.1 mm.

1945, and *Nesomicrixia insularis* Synave, 1958. Compound eyes absent, their former position recognizable as a slightly vaulted area dorsally of antennae. Antennae with scape short and ring-like, pedicel nearly cylindrical, ca. 1.5 × longer than wide; arista ca. 3.6 × longer than pedicel.

Thorax. Pronotum short, in dorsal aspect medially ca. 2 × as long as vertex, and ca. 2 × as wide as maximum width of head; lateral carinae of pronotum nearly straight,

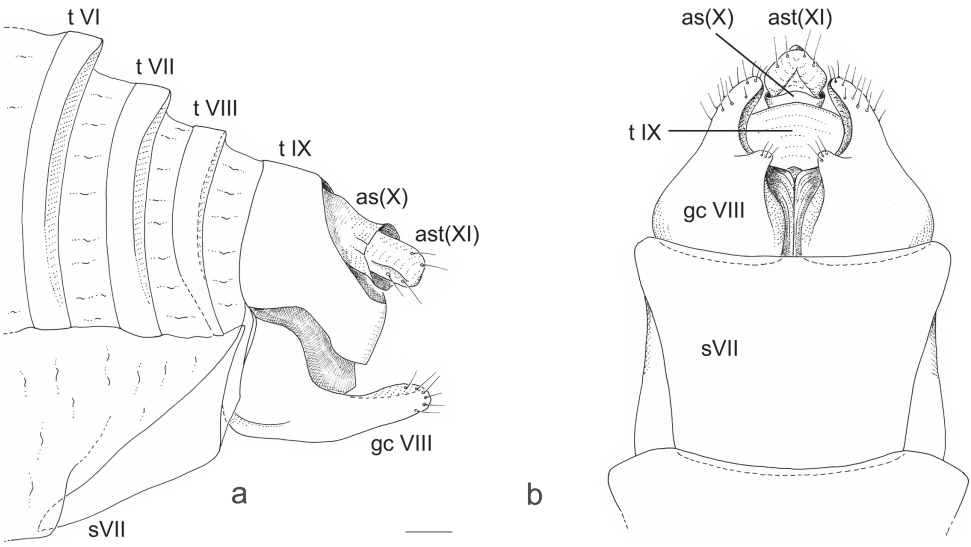

Figure 6. *Valenciolenda fadaforesta* sp. nov. Female genitalia **a** left lateral view **b** same ventral view. Scale bar: 0.1 mm.

strongly diverging posteriorly; „additional carina" of pronotum (*sensu* Yang and Yeh 1994: 3, fig. 1H), dividing anterior portion of pronotum into a smaller median, and a larger lateral area. Pronotum dorsally on each side with two rows of sensory pits: one, consisting of 5 larger sensory pits, parallel to posterior, resp. median margin, the other, consisting of 8 smaller sensory pits, adjacent to lateral carina; anterior portion of pronotum in larger, lateral area with a row of 9–11 sensory pits adjacent to lateral carina; smaller, median area devoid of sensory pits. Mesonotum medially ca. 2 × longer than pronotum, with posterior margin laterally more or less angularly curved; forewing pads well developed, ca. 2 × longer than mesonotum medially, posteriorly slightly surpassing caudal margin of hind wing pads; lateral carinae of mesonotum (separating the nota from the wing pad) distinctly ridged, slightly diverging posteriorly, attaining hind margin of mesonotum; mesonotum with a short, but distinct furrow on anterior portion of forewing pad; forewing pad with two distinct longitudinal carinae. Mesonotum with numerous sensory pits arranged on each side as follows: medially of lateral carina – a row of 6 larger sensory pits parallel to carina and 2 smaller sensory pits closer to midline, on forewing pad, between median furrow and lateral carina in anterior part of mesonotum – a group of 6 sensory pits, and three rows of sensory pits between longitudinal carinae, along exterior carina and parallel to lateral margin, respectively. Metanotum medially about as long as mesonotum, hind margin nearly straight, laterally only slightly, expanding caudally; lateral carinae of metanotum (separating the nota from the hind wing pad) distinctly ridged, straight, slightly converging posteriorly, reaching hind margin of metanotum. Hind wings pads inconspicuous, vestigial, their posterior margin shorter than posterior margin of forewing pads, covered by forewing pad. Metanotum on each side with ca. 15 small sensory pits, seemingly irregularly arranged across notum, and on hind wing pad with a group of 3 larger sensory pits adjacent to

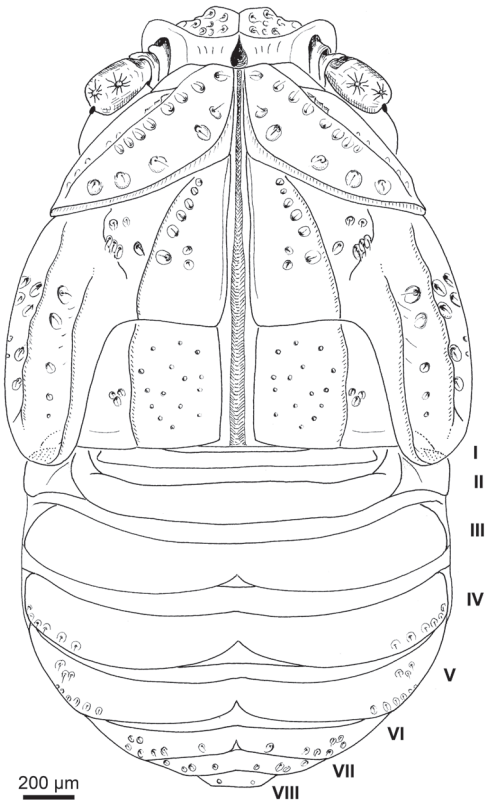

Figure 7. *Valenciolenda fadaforesta* sp. nov. V. instar nymph, habitus (distribution of sensory pits reconstructed from SEM); Cova de Murcielagos, 24.vi.2017, A. Sendra leg.

lateral carina. Legs. Hind trochanter as in most other Fulgoromorpha families (except Tettigometridae: Asche 1988) medially with cog-wheel-like opposing ledges, the „coupling apparatus“ (Emeljanov 1979) which facilitate synchronization of the hind legs during jumping (Burrows and Sutton 2013). Metacoxae with meracanthus present, inconspicuous, surface with fine tubercles. Metatibiae laterally unarmed, distally with 7–8 (bilaterally and individually variable) slender teeth, arranged in a slightly concave row. Metabasitarsus distally with 5, 2nd metatarsomere with 4 small teeth. Metabasitarsus slightly shorter than 2nd and 3rd metatarsomeres together. Pretarsus with short, slender claws, not longer than arolium.

Abdomen as in other Fulgoromorpha 9-segmented (except for Tettigometridae: Yang and Yeh 1994), ovoid, in cross section roundish; first two abdominal segments narrow, thus creating a distinct separation between thorax and abdomen. Abdomen medially about as long as head and thorax together. Abdominal tergites medially smooth, without any crest or carina; tergites I–II short, their hind margins straight; tergites I–III devoid of sensory pits; tergites III–VI with hind margin medially incised. Tergites IV–VIII with numerous sensory pits, on each side arranged as follows: IV –

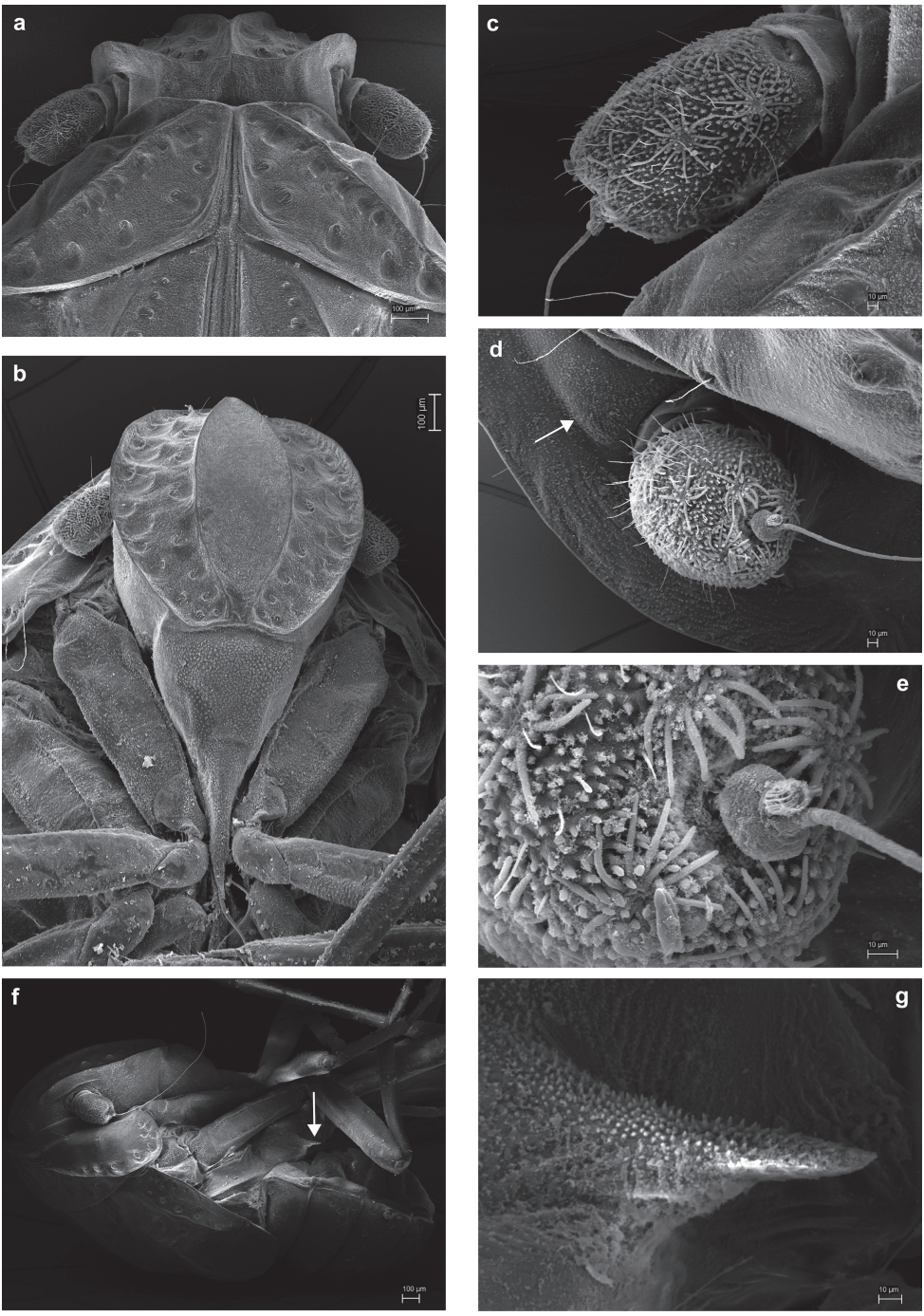

Figure 8. *Valenciolenda fadaforesta* sp. nov., V. instar nymph (SEM) **a** head and pronotum, dorsal view **b** head, ventral view **c** left antenna, dorsal view **d** head (partim) with antenna, left lateral aspect; arrow indicates the former position of the compound eye **e** tip of left antenna, as in Fig. 8e, enlarged **f** overview of body, right lateral view, arrow indicates meracanthus **g** meracanthus, as in Fig. 8f, enlarged.

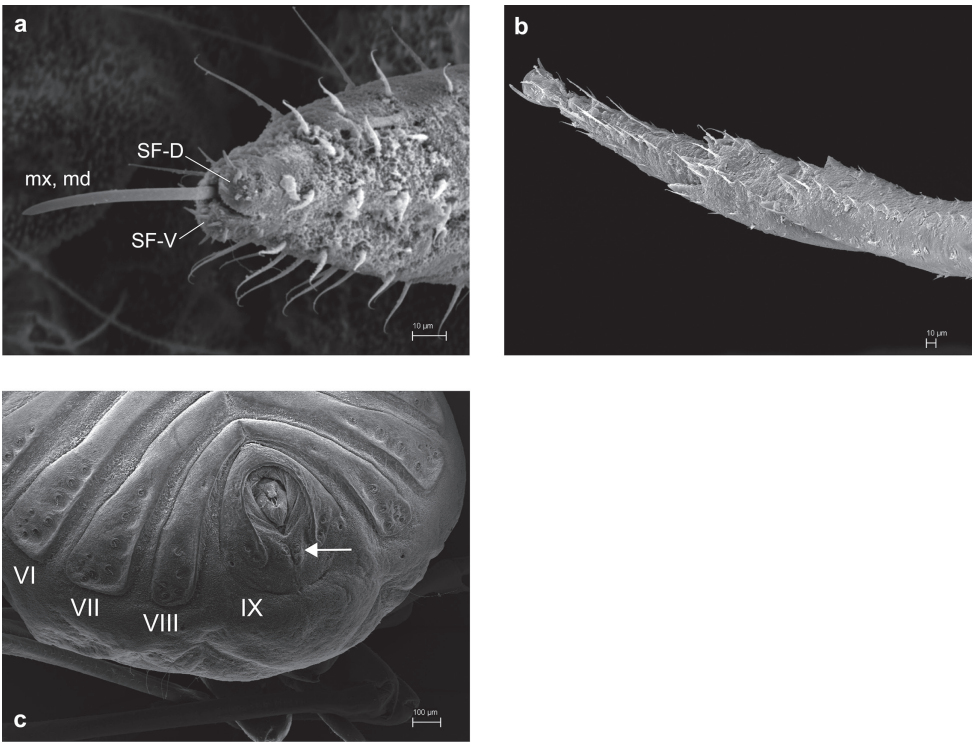

Figure 9. *Valenciolenda fadaforesta* sp. nov., V. instar nymph (SEM) **a** tip of rostrum with sensilla, ventral aspect **b** metatarsus, ventral view **c** abdominal segments VI–IX (arrow – see explanation in text). Abbreviations: mx, md, mandible, maxillae; SF-D, dorsal sensory field; SF-V, ventral sensory field.

a row of 6 sensory pits laterally, parallel to hind margin; V – a row of 6 sensory pits laterally, parallel to hind margin, and a group of 3 sensory pits laterally, near anterior margin; VI – a row of 4 sensory pits parallel to hind margin, and a group of 3–4 sensory pits laterally, closer to anterior margin; VII – an irregular row of 4 large sensory pits accompanying hind and lateral margin of tergite, and 4 small sensory pits laterally, closer to anterior margin; VIII – in dorsal half with one small sensory pit laterally near caudal margin, and 4 (3 + 1) large sensory pits lateroventrally. Abdominal tergite IX (pertaining to the *anlage* of the genital segment) in dorsal view short, narrow, in caudal view bent ventrally in a horseshoe shape, with ventral margin broadly rounded, on each side with 1 sensory pit in dorsal half near caudal margin and a group of at least 3 sensory pits near lateroventral margin. *Anlagen* of abdominal segments X and XI small, triangular, tapering caudally, devoid of sensory pits. All nymphs examined display the same configuration of the pregenital abdomen and genital structures (segment IX). Abdominal tergites VI–VIII are devoid of wax-pore plates. The *anlagen* of the genitalia on the IX segment, however, are difficult to interpret. The dorsal unpaired process is likely the *anlage* of the anal style (segment XI). This structure is framed laterally and ventrally by narrow, elongated lobes (it is not recognizable whether or not they are

fused medially), which may or may not be homologues to what has been termed „anal combs“ in the other Fulgoromorpha families, except Tettigometridae (Yang and Yeh 1994). The two short conical processes located medially at the level of the posterioventral corner of tergite IX could be interpreted as *anlagen* of either the genital styles (of the male), or of the gonocoxae VIII (of the female). The high degree of reduction of the ovipositor in adults within the Meenoplidae-Kinnaridae clade apparently impedes unambiguous identification of the sex of the nymphs as already observed by Wilson (1983) for the meenoplid *Nisia nervosa* („In *Nisia nervosa* it was not possible to find obvious differences in nymphs which separate the sexes“: Wilson, 1983: 123).

Remarks. Hitherto, no information on nymphal morphology of Kinnaridae has been available – they are not covered in the seminal works on Fulgoromorphan nymphal morphology, Yang and Yeh (1994) and Emeljanov (2001), apparently due to lack of material. Thus, the description of *Valenciolenda fadaforesta* nymphs represents the first example of a kinnarid nymph. As such, it shares several characters with other Fulgoromorpha taxa which are not explicitly mentioned above. According to Yang and Yeh (1994) and Emeljanov (2001) these include: ocelli absent, second antennal segment with sensory organs, thoracic nota separated along median line by membraneous area, with numerous sensory pits on tergal parts of head, thorax and abdominal tergites III–VIII (for morphology of sensory pits, their disposition and orientation see Liebenberg 1956, Emeljanov 2001, Bräunig et al. 2012), Pro- and mesotarsi with 2 joints, metatarsi with 3 joints.

Molecular data. A blast search in GenBank and BOLD data bases for most similar COI sequence data revealed that *Valenciolenda fadaforesta* shows 15% divergence to all other Auchenorrhyncha. A molecular phylogeny of Kinnaridae, however, did not seem feasible due to insufficient taxon sampling: all other kinnarid taxa represented in Genbank and BOLD are from the New World (USA, Honduras, Mexico, Costa Rica).

Etymology. The species name is a combination of the Valencian word for „fairy“ (fada) and forest, thus meaning „fairy of the forest“. The gender is feminine.

Material examined. Type-locality: Spain, Valencia, Vilamarxant, ‚Murcielagos‘ cave, 39.537095, -0.624732, 5th April 2016, L. Beltran and A. Sendra leg.; in coll. **MfN** (Museum für Naturkunde, Berlin, Germany).

Type-specimen: ***Holotype*** male, preserved in 96% ETOH, polyethylene vial. Original label: “Spain, València, Vilamarxant, ‚Murcielagos‘ cave, (30SYJ0410579181 (UTM/MGRS Datum EUR50), 5th April 2016, L. Beltran and A. Sendra leg.”; printed label (red): “*Valenciolenda fadaforesta* Hoch & Sendra, holotype male”.

Paratypes. 1 male, same data as holotype. 1 male, 1 female, same locality as holotype, 30th April 2017, S. Teruel and A. Sendra leg; 1 male, same locality as holotype, 24.VI. 2017. 1 male, Spain, Castelló, ‚Coves de Sant Josep´cave, 4th June 2016, S. Teruel leg.

Paratypes in coll. **NAT** (Museu de Ciències Naturals de Barcelona, Spain), **MUVHN** (Museu de la Universitat de València d´Història Natural, Burjassot, València, Spain) and **ES** (Laboratório de Ecologia Subterránea/ISLA: Coleção de Invertebrados Subterráneos da UFLA – Universidade Federal de Lavras, Brazil).

Additional material. 1 nymph, V. instar, same data as holotype. 4 nymphs, V. instar, same locality as holotype, 24.VI. 2017; **MfN**.

Distribution, ecology and behaviour. Specimens of *Valenciolenda fadaforesta* have been studied in two caves (‚Murciélagos' cave and ‚Coves de Sant Josep' caves) in two karstic areas of Triassic dolomite separated by 45 kilometers and located in the eastern reliefs of the Iberian Mountain Range, from eleven to twenty-seven kilometers inland from the Mediterranean coast (Fig. 10). ‚Cueva de los Murcielagos' was excavated in a dolomitic outcrop on a small isolated mountain of the Rodanes Municipal Park, in Vilamarxant (València) (Sendra et al. 2015) and represents the best known and most abundant population of *V. fadaforesta*. In the other three caves from the same small karst rocky outcrop nymphs presumably belonging to *V. fadaforesta* have been observed. These are the caves of ‚Pedrizas, Llentiscle' and Sima del Perot' that occupy an area of less than one square kilometer in the Rodanes (Sendra et al. 2015). The other cave studied is the well-known tourist subterranean river cave ‚Coves de Sant Josep', which descends three thousands meters with explored galleries under the surface of the western slopes of the karstic region of ‚Serra Espadà' Mountains, in Vall d'Uixó (Castelló) (Garay, 2003, Sendra et al. 2017). In addition, specimens presumably belonging to *V. fadaforesta* have also been observed and also photographed in four other caves located geographically not far from the studied caves. They are the caves of ‚Soterranya', a tectonic cave with over one kilometer of narrow passages and ‚Sima Plà dels Llomes' a chasm 42 meters deep, both caves located in a large karstic area within ‚Calderona Natural Park' in Serra, València. The third one is the small ‚Cova del Cavall" cave, isolated in a limited karstic area called ‚Buitreras' hill in Llíria, València. The fourth cave colonized is „Cueva de las Raices", the southernmost locality, an epithelial cave situated in a large karstic area in the Caroig Platform in Millares, València. In summary, the presumed range of distribution of *V. fadaforesta* seems to occupy the large karstic dolomitic and limestone outcrops in the eastern reliefs of the Iberian Mountain Range, under the Mediterranean climate in the thermo-mediterranean zone characterized by scarce precipitation at low altitudes, below 570 m.asl in the case of the highest located cave, Sima Plà dels Llomes".

V. fadaforesta displays a configuration of external characters which are certainly troglomorphic traits, such as the absence of compound eyes and ocelli, and reduced body pigmentation as well as tegmina and wings. The species is known exclusively from caves, and it can be assumed that it is restricted to subterranean environments. According to the ecological classification concepts proposed by Sket (2008) and more recently, by Howarth and Moldovan (2018) it is regarded as a troglobiont. Specimens were found from the twilight to the deep zone, from a few meters up to twenty meters below the surface, however, always in humid conditions. In two of the cited caves, *V. fadaforesta* has been observed as a single male specimen probably coming from the ceiling of the gallery (‚Sant Josep' and ‚Plà dels Llomes' caves) and in the ‚Soterranya' and ‚Cavall' caves a few nymphs have been seen near to roots although juveniles were more abundant in `Raíces´cave. The only cave that has allowed ecological and behavioral observations was ‚Murciélagos' cave. Uniquely to this cave, nymphs are abundant throughout the year but male and female adults are very scarce, one or two adults were seen on four of the twelve biospeleological visits into the cave. In ‚Murciélagos' cave, *V. fadaforesta* is found after the entrance zone, very scarce in the twilight zone and more abundant in the deep humid spots of the deep zone of the cave, but only where roots are present at least

Figure 10. Distribution map of *Valenciolenda fadaforesta* gen. nov., sp. nov. Localities: **1** ‚Raíces' cave, Millares, València **2** ‚Murciélagos´cave (type locality), ‚Pedrizas' cave, , Llentiscle' cave and ‚Sima del Perot, cave in Vilamarxant, València **3** ‚Cavall' cave, Lliria, València **4** ‚Soterranya' cave, Serra, València **5** ‚Sima Plà dels Llomes', Serra, València **6** ‚Coves de Sant Josep' caves, la Vall d'Uixó, Castelló. Dark blue areas mark karst regions. (map modified after Ayala et al. 1986)

nearby. This deep zone is characterized by high humidity with slight variation of 1.8 °C in temperature from 16.5–16.6 °C during February to May up to 18.1–18.3 °C during September to November (Sendra et al. 2015). In some spots, numerous nymphs wander around on the surface, where extremely rarely adults are seen. Roots are represented by a few short sections hanging from the ceiling or the walls. In a few places, these roots produce a small pile, elevated from the soil surface three to five centimeters due to water dripping from the ceiling. Although no analysis has been made to identify the plant species to which the roots pertain, pine (*Pinus halepensis*), carob (*Ceratonia siliqua*) or mastic trees (*Pistacia lentiscus*) are likely candidates, being abundant outside the cave entrance. In ‚Cueva de las Raíces' juveniles can be seen in a few hanging roots from the ceiling, but they are not abundant (Fig. 11a–c). *V. fadaforesta* also occupies a different habitat in other spots in the cave in the interstitial spaces of the fragmented or excavated rock where roots are present too although very scarce. We assume that *V. fadaforesta* could live

among the cracks and crevices of the mesocavernous rock system, that could be considered as an epikarst of the karst wherever sufficient food resources (roots) are available.

Although there is no information on the mating behaviour of any kinnarid species, *Valenciolenda fadaforesta* may utilize the same communication system to locate potential mating partners, as has been documented for other (epigean and cavernicolous) planthoppers, i.e., surface-borne vibrations (Hoch and Howarth 1993, Hoch and Wessel 2006, Hoch et al. 2013, Soulier-Perkins et al. 2015) which have been shown to carry effectively over several meters via living plant material such as roots (Hoch and Howarth 1993).

An amazing similarity in behaviour has been observed in *Valenciolenda fadaforesta* and an obligate cave species of the family Cixiidae from Australia: *Solonaima baylissa* Hoch & Howarth, 1989, from Bayliss Cave, a lava tube of Undara lava flow, Queensland (Fig. 1c), which also displays a similar degree of tegmina reduction as well as the prominent blueish-white wax fringe along the costal vein (Hoch and Howarth 1989). Both species hold their tegmina nearly flat over the abdomen and – when disturbed – exercise escape jumps with the tegmina resembling tiny parachutes obviously slowing down the jumping individual (personal observation). This behavior – observed in two totally unrelated species, on two continents – is an excellent example of convergent evolution. Its context has not been studied yet, but it is conceivable that in an environment where roots may be few and far between, escape from approaching predators, such as spiders; the „parachuting“ could be a means to avoid predaters as well as ensuring the jumping planthopper is not catapulted too far from its host root. Adults and nymphs have been observed in ‚Murciélagos‘ cave jumping several centimeters to avoid predetors, such as *Dysdera* spiders and the carabid beetles (*Laemostenus terricola* (Herbst, 1784) or *Porotachys bisulcatus* (Nicolai, 1822)) with *V. fadaforesta* (Sendra et al. 2015). In addition, the tegmen morphology (strong costal vein, covered with waxy exudation, and tegmina exceeding lateral body margins) may aid to avoid predation too.

Another striking behavior has been observed in the nymphs and adults of *V. fadaforesta*. When they wander around and also when they remain in the same spot, a quick lateral movement of the abdomen has been observed. Such movement is usually followed by a change in the walking direction which is consistent with that of the lateral abdominal deflection. It could be interpreted as a signal to change the direction of movements, perhaps in response to yet unidentified stimuli.

Conservation status. In order to settle the conservation status of this remarkable endemic genus, two interesting aspects might be considered. Firstly, conservation of the general habitat of *Valenciolenda fadaforesta*. All caves in the Valencian Community are protected by law (11/94 Law of Natural Protected Spaces and Legal decree 65/2006, of 12th May) devoted to caves with special protection for 150 notable caves, among them there are all the cavities of the ‘Pedrizas’ in the Rodanes karstic area, ‘Soterranya’ and ‘Sant Josep’ caves inhabited by *V. fadaforesta*. Also, the Rodanes caves are within a natural protected park including the Turia Natural Park. Secondly, *V. fadaforesta*, according to the IUCN Red Data Book categories ought to be regarded as *vulnerable*, or even *endangered* (IUCN 2019), based on its small distribution, specialized habitat and presumed small population size. If the criteria given in Sánchez et al. (2004) are applied, which are more suitable for arthropod taxa, it can be considered as a ‘highly

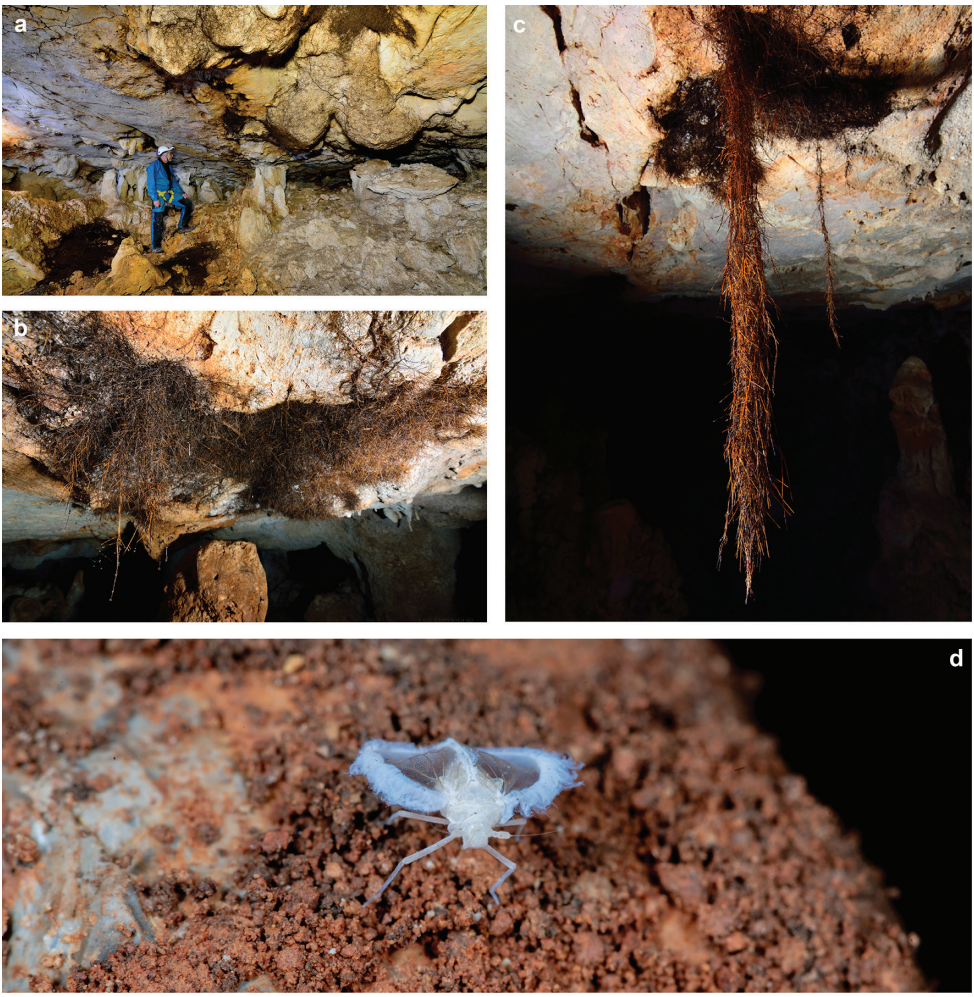

Figure 11. Root habitat in `Raices´cave (Millares, València) **a** overview main room **b** roots along cracks in the cave ceiling **c** roots dangling from the ceiling **d** *Valenciolenda fadaforesta* sp. nov., adult, on cave floor in `Murciélagos´cave (photos **a–c** by Teresa Molina Jiménez and Ricardo Giménez Mezquita, used with permission of UEE Fotogrup; photo **d** by Sergio Montagud Alario).

vulnerable taxon' according to its degree of endemicity, small distribution area, rarity and threat to its habitat.

There are real threats to the sustained occurrence of *V. fadaforesta*. Most notably, the species shows a low population density: in almost all caves where it has been reported, only one or a few specimens have been observed. Only the 'Murcielagos' cave contains a stable population all year round, but even this population apparently diminished during the extended 2013–2016 drought which affected the flora, with a particular impact on the pine trees, *Pinus halepensis*. This drought period also impacted the roots that appear inside the cave. Fortunately, after the end of the drought period at the end of 2017 there was an apparent recuperation of *V. fadaforesta* of the 'Murcielagos' cave.

Discussion

Suprageneric classification

According to the classification of Kinnaridae suggested by Emeljanov (2006), *Valenciolenda* belongs to the subfamily Prosotropinae Fennah, 1945, as it displays 1) costal vein of fore wing strong from base up to nodus, 2) branches of RP and M free, 3) a cixioid clavus, i.e., the common claval vein reaches the hind margin of the clavus.

The placement of *Valenciolenda* into Adolendini follows the concept of tribal division of Kinnaridae by Emeljanov 2006. According to this, the Adolendini are characterized by a combination of the following characters:

1) *Metope* (= frons) *without median carina, narrow, with high lateral carinae*. According to Emeljanov (personal communication, used with permission) this is an apomorphy, however, with low weight because of high potential for homoplasy.
2) *Median carina of pronotum reaching fore carina but not prolonged in front of it.*
3) *Basal cell of fore wing closed by anastomosis of M and CuA* (Emeljanov 2006: 1). According to Emeljanov (personal communication, used with permission) the anastomosis replaces the arculus, and is considered a likely apomorphy for Adolendini.

However, at least one of these characters (frons smooth, without median carina), is often observed in obligately cavernicolous planthoppers (Hoch 1994) as the reduction of the compound eyes strongly alters head morphology and may lead to a broadening of frons (and vertex), along with reduction of carinae (Hoch and Howarth 1989). It can thus not be excluded that the closest epigean relative of *Valenciolenda fadaforesta* featured or features a median frontal carina. The pronotum configuration postulated for Adolendini by Emeljanov (2006) does apply to *Valenciolenda fadaforesta*, however, the fore wing character does not: there is no anastomosis of M and CuA, and there is no distinct basal cell recognizable (Fig. 3). The configuration of the fore wing, or tegmen, observed in *V. fadaforesta* may or may not be a consequence of the troglomorphic fore wing reduction. It should be noted, though, that none of the characters mentioned have been discussed as potential synapomorphies for the pertaining taxa.

Biogeographical implications

Given the background of the current state of knowledge on the phylogeny of Kinnaridae, it is difficult to make conclusions as to which taxa may be the closest living relatives of *Valenciolenda fadaforesta*. The geographically closest Kinnaridae are species from 2 genera (*Kinnacana* Remane, 1985 and *Kinnoccia* Remane, 1985) (tribe Kinnocciini

Emeljanov 2006) recorded from the Canary Islands (Remane 1985). These display – like *V. fadaforesta* – wax fields in the female on abdominal segments VI, VII and VIII (Emeljanov 2006), but – unlike *V. fadaforesta* – the basal cell of the forewing is closed by an anastomosis of M and CuA (Emeljanov 2006).

Except for the Kinnocciini, Kinnaridae have not been documented from Western Europe. Several species are known from Iran, Tadzhikistan (Emeljanov 1984), Afghanistan (Dlabola 1957), India (Distant 1916) and the United Arabian Emirates (Wilson 2010). However, none of those taxa display character configurations which could be interpreted as synapomorphies with *Valenciolenda fadaforesta*. No close epigean relatives could thus be identified for *Valenciolenda fadaforesta* neither in Spain, nor elsewhere. Consequently, *Valenciolenda fadaforesta* must currently be regarded as a relict species which has long been isolated. This assumption is corroborated by the high divergence of its COI sequence from all other Auchenorrhyncha.

Although it cannot be determined on the basis of our current knowledge whether initial cave adaptation was driven by allopatry (extinction of closely related epigean populations: see *climatic relict hypothesis*, as postulated by e.g., Vandel 1964, or Barr 1968) or parapatry (adaptive shift of troglophilic populations in order to exploit novel food resources, as suggested by Howarth 1981, see also Howarth et al. 2019), it is clear that at some point, Kinnaridae must have been represented in the epigean fauna of Spain. The Iberian Fulgoromorpha fauna can be regarded as well investigated (Bourgoin 2019). Thus, it is unlikely that epigean Kinnaridae are still extant, but remain undiscovered. It is rather more likely that *Valenciolenda fadaforesta* provides testimony of an ancient fauna which is now extinct. The fossil record of Kinnaridae is scarce and not informative in regard to any putative epigean relatives of *Valenciolenda fadaforesta*, as there are only few species documented, exclusively from Oligocene/Miocene deposits of Dominican amber (Szwedo et al. 2004). It is conceivable that the epigean Kinnaridae which eventually gave rise to *Valenciolenda* migrated into the Western Mediterranean not from Europe, but from Asia through Northern Africa, perhaps in the context of the late Miocene Messianian salinity crisis. This southern migration route was hypothesized for other animal taxa, e.g., several genera of Muroidea (mammalia) of Eastern Spain, which show African or Asian affinities (Agustí 1989).

A similar case of a relict distribution has been documented from the Canary Islands: there, obligately cavernicolous species of the Fulgoromorpha taxon Meenoplidae are known from La Palma (Hoch and Asche 1993), El Hierro (Remane and Hoch 1988, Hoch and Asche 1993), and Gran Canaria (Hoch et al. 2012), while epigean Meenoplidae are not part of the present-day fauna of the Canary Islands.

The unexpected discovery of this planthopper of the Kinnaridae family, *Valenciolenda fadaforesta* gen. nov., sp. nov., in a relatively well-known Iberian cave highlights the importance of subterranean biodiversity, and leads us to conclude that there are still many amazing discoveries awaiting us in cave environments. The study of cave faunas may yield valuable information on evolutionary and biogeographic history, and thus provide veritable windows to the past.

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Supplementary material 1

Video sequence

Authors: Sergio Montagud Alario

Data type: Video sequence

Explanation note: Video sequence documenting *Valenciolenda fadaforesta* sp. nov. (adult male) in its natural environment (video by Sergio Montagud Alario).

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Phlebotomine sand flies (Diptera, Psychodidae) from iron ore caves in the State of Pará, Brazil

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Abstract

The present study aimed to evaluate the distribution of sand fly species in iron ore caves in the State of Pará, Brazil and to associate the richness and abundance of these insects with the capacity of leishmaniasis transmission. Entomological captures were carried out in the years 2010, 2013, 2014 and 2015, throughout active samples with brushes, along the entire caves' extension, in dry and rainy periods. A total of 9,807 sand flies were counted during the 532 samplings events, being 4,340 in the dry period and 5,467 in the rainy period. A random sample of 802 morphologically identified specimens consisted of 8 genera and 17 species, being 369 males (46%) and 433 females (54%). The predominant species was *Sciopemyia sordellii* with 60.6% of the total of sand flies collected. Differences in composition and richness were observed between caves located inside of forest and anthropized areas. The mean richness and abundance were different between the wet and rainy periods, with a greater abundance of these insects in the rainy period. The phlebotomine fauna proved to be rich and abundant in the sampled caves, however, environmental degradation seems to be the main factor determining changes in the composition and richness, reinforces the importance of these places as a shelter for sand flies in degraded areas.

Keywords

Leishmaniasis, Phlebotominae, Vector ecology

Introduction

Phlebotomines (Diptera, Psychodidae, Phlebotominae) are insects with medical importance because include vector species of *Leishmania* sp., *Bartonella bacilliformis* and arbovirus (Alvar 2001; Sherlock 2003; Lainson and Shaw 2005; Kamhawi 2006; Battisti et al. 2015). They usually take shelter in places with high moisture content and with organic matter available, shaded areas and drafts to prevent desiccation (Aguiar and Medeiros 2003).

Caves are favorable environments for the occurrence of these insects due to the stable conditions of temperature and humidity (Freitas and Littlejohn 1987; Freitas 2010; Lauritzen 2018). Vertebrates such as bats, rodents, birds, lizards and amphibians can serve as a blood source for sand flies present inside and around cavities (Auler et al. 2019).

In Brazil, the data regarding the phlebotomine fauna in caves are incomplete (Galati et al. 2003; Galati et al. 2006; Alves 2007; Barata et al. 2008; Galati 2008; Almeida et al. 2019; Campos et al. 2020) several species of sand flies have been described (Alves et al. 2008; Carvalho et al. 2010, 2011; Barata et al. 2012; Vilela et al. 2015).

In the Serra do Carajás, located in the state of Pará, the mining potential of the Curionópolis and Parauapebas municipalities has attracted enterprises that, despite contributing to the economic development of the region, have caused major environmental changes, with direct impacts on biodiversity and public health (Palheta et al. 2017). Since the 1980s, several territorial, economic, social and political conflicts have emerged or were intensified by the mining activities, such as the emergence of municipalities, population growth and increased environmental pressures on land use (Palheta et al. 2017).

Serra de Carajás has a large number of caves with economic and biological potential, but it is also considered an endemic area for cutaneous leishmaniasis (Ward et al. 1973). The expansion of municipalities and their peripheral populations living close to the caves may represent a risk of *Leishmania* transmission. In this sense, the present study aimed to evaluate the distribution of phlebotomine species in the caves and to associate the richness and abundance of these insects with the potential transmission of leishmaniasis. In addition, we sought to contribute to the knowledge of the region's biodiversity, given the economic and biological potential of the iron ore caves of the State of Pará.

Methods

Study area

The study was carried out in iron ore caves located in the regions of Morro I, Morro II (Serra Norte), within the Flona de Carajás and Serra Leste, outside a conservation unit, in the municipalities of Parauapebas and Curionópolis (Figure 1). In Parauapebas, the altitude of the caves varied from 350 to 650m asl and in Curionópolis it varied from 230 to 560m asl. Parauapebas (6°04'03"S, 49°54'08"W) is a Brazilian municipality located in the state of Pará, northern Brazil. Curionópolis (6°3'58"S, 49°33'40"W) is located around

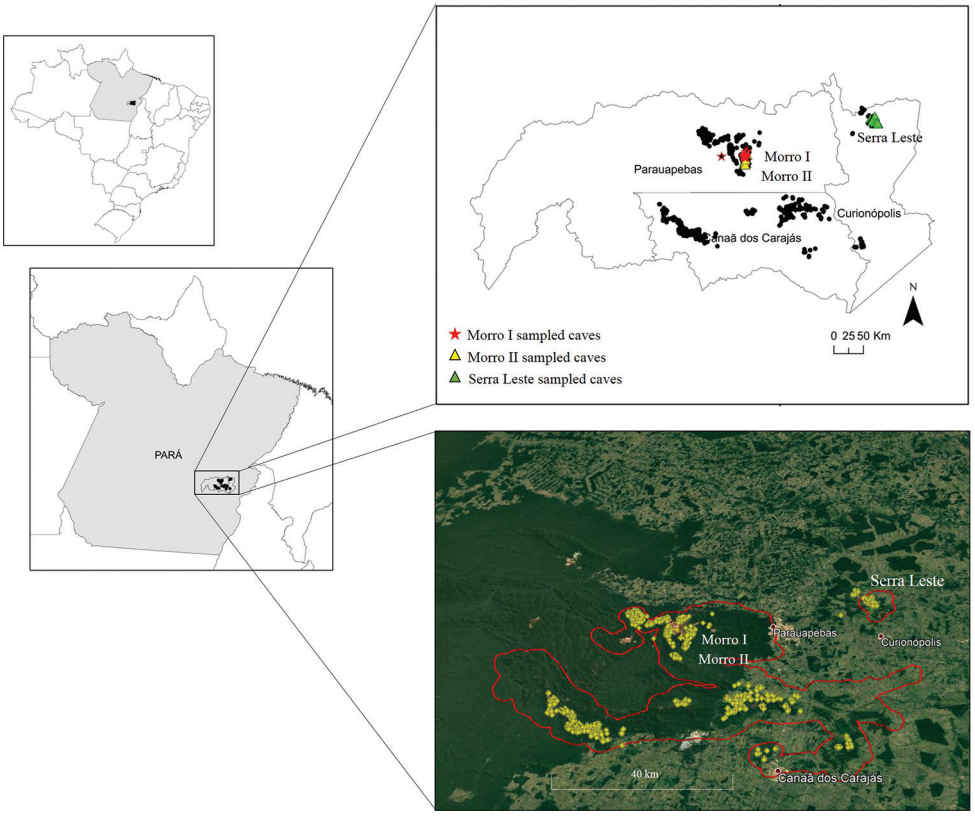

Figure 1. Ferruginous geosystem of Carajás, Pará, Brazil (limited by red lines), with iron ore caves (yellow dots) in the regions of Morro I, Morro II (Carajás National Forest) and Serra Leste. Limits of the ferruginous geosystem and the coordinates of the caves, available in https://institutopristino.org.br/atlas

40 km from Paraupebas and is a municipality known to house the Serra Pelada district, which was the world's leading area for open pit gold mining operations during 1980.

It has the largest iron ore mineral province on the planet, Serra dos Carajás, which has the highest concentration of ferruginous caves in Brazil, with approximately 20% of all caves registered in the country (Jaffé et al. 2016; Instituto Prístino 2020). The extraction of iron ore represents the main source of resources in the municipalities.

Both municipalities have a semi-humid tropical climate (Aw/As), with annual temperatures around 26 °C and high relative humidity; the rainy season occurs, from November to May, and the dry season, from June to October, with the rainfall index being around 2,000 mm annually (IBGE 2010).

Phlebotomine collections

Entomological captures were carried out in the years 2010, 2013, 2014 and 2015, through active samples with brushes, along the entire caves' extension, in dry and rainy periods from speleological activities carried out by independent consulting com-

panies, in the years, but using similar collection methods (Jaffé et al. 2016; Travellin et al. 2019). Not all sighted individuals were collected, since the authorization for collecting invertebrates in caves, issued by the Sistema de Autorização e Informação em Biodiversidade (SISBio) suggests employing a collection or capture effort that does not compromise the viability of populations in the taxonomic group of interest *in situ* condition (Normative Instruction N° 03, 01 September 2014). Not sampled sand flies were plotted on a schematic sketch of each cave and later counted from the schematic sketch and inserted in a spreadsheet of the database at Coleção de Invertebrados Subterrâneos de Lavras (ISLA). Then, the data of the total abundance of sand flies in the caves were extracted from the ISLA database. Such data refer to the counting of specimens in the field during the collections and were only used to identify greater or lesser probabilities of leishmaniasis transmission (considering the abundance as a *proxy* of transmission, probability), between areas and between seasons, especially in places with free access to residents and visitors (e.g. Serra Leste). All study material was found at the ISLA of the Center of Studies on Subterranean Biology, at the Federal University of Lavras (biologiasubterranea.com.br).

Preparation, assembly and identification of specimens

The collected specimens were sent to the Parasitology Laboratory of the Department of Biological Sciences at the Federal University of Jequitinhonha and Mucuri Valleys. The sandflies were prepared and assembled between a slide and coverslip, according to the Langeron technique (1949), modified, using Berlese's liquid to mount both sexes. The identification of the specimens was carried out according to the classification proposed by Galati (2003) and the abbreviation of generic names followed the work of Marcondes (2007). Some specimens with missing or damaged characters that impaired the identification at the specific level were considered only at the generic level (i.e., *Micropygomyia* spp.) or not identified.

Data analysis

The qualitative similarity was obtained by the Jaccard index and contrasted in a metric multidimensional scaling (MDS) with resampling using the Bootstrap method. Furthermore, the significant separation of species groups between the different sampling areas (Morro I, Morro II and Serra Leste) was evaluated by similarity analysis – ANOSIM (Clarke et al. 2014). To assess the mean dissimilarity between the sampling regions, SIMPER analysis (similarity percentage) was used. In addition, the percentage of species contribution to dissimilarity was evaluated (Clarke et al. 2014).

Differences in mean richness and abundance between areas and between dry and rainy periods were assessed using the Kruskal-Wallis test (Sprent and Smeeton 2000). To assess the eventual relationship between the sand fly's richness and the linear extension of the caves, a simple linear regression analysis was used. The normality of the data was tested using the Shapiro-Wilk test (Sokal and Rohlf 1995).

Results

In total, 306 iron ore caves (44 caves in Morro I, 115 in Morro II and 147 in Serra Leste) were inspected, and the phlebotomine fauna captured in 276 of these caves in 532 samplings (dry and rainy seasons) consisted of 8 genera and 17 species, namely: *Evandromyia carmelinoi* (Ryan, Fraiha, Lainson & Shaw, 1986), *Evandromyia monstruosa* (Floch & Abonnenc, 1944), *Evandromyia saulensis* (Floch & Abonnenc, 1944), *Evandromyia termitophila* (Martins, Falcão & Silva, 1964), *Lutzomyia longipalpis* (Lutz & Neiva, 1912), *Micropygomyia goiana* (Martins, Falcão & Silva), *Micropygomyia oswaldoi* (Mangabeira, 1942), *Micropygomyia peresi* (Mangabeira, 1942), *Micropygomyia pilosa* (Damasceno & Causey, 1944), *Nyssomyia umbratilis* (Ward & Fraiha, 1977), *Pintomyia* série *chagasi*, *Pintomyia gruta* (Ryan, 1986), *Pintomyia serrana* (Damasceno & Arouck, 1949), *Psathyromyia lutziana* (Costa Lima, 1932), *Sciopemyia sordellii* (Shannon & Del Ponte, 1927), *Trichophoromyia brachipyga* (Mangabeira, 1942), *Trichopygomyia dasypodogeton* (Castro, 1939) and *Micropygomyia* spp., totaling 802 specimens, being 369 males (46%) and 433 females (54%). The predominant species was *Sciopemyia sordellii* with 60.6% of the total of sand flies identified (Table 1). Specimens that had lost structures and could not be identified represented 15.21% of the total captured.

Similarity analysis (ANOSIM) showed a significant difference between the species composition of the Parauapebas and Curionópolis caves ($R = 0.06$ and $P < 0.05$) (Figure 2). The shade plot shows the species distribution in the Morro I, Morro II and

Table 1. Distribution of sand flies collected in iron ore caves in the State of Pará, Brazil, in the years 2010, 2013, 2014 and 2015.

Species	Curionópolis		Parauapebas		N	%
	♂	♀	♂	♀		
Evandromyia carmelinoi	2	1	0	0	3	0.38
Ev. monstruosa	0	1	0	1	2	0.24
Ev. saulensis	0	1	13	10	24	3.00
Ev. termitophila	0	2	0	0	2	0.24
Lutzomyia longipalpis	13	13	1	2	29	3.62
Micropygomyia goiana	0	0	0	3	3	0.38
Mi. oswaldoi	0	3	0	0	3	0.38
Mi. peresi	11	2	2	2	17	2.12
Mi. pilosa	2	1	1	1	5	0.63
Micropygomyia spp.	17	0	11	3	31	3.87
Nyssomyia umbratilis	0	0	1	1	2	0.24
Pintomyia série *chagasi*	0	0	0	6	6	0.75
Pi. gruta	7	9	25	22	63	7.86
Pi. serrana	0	0	0	1	1	0.12
Psathyromyia lutziana	0	0	0	1	1	0.12
Sciopemyia sordellii	46	64	184	192	486	60.60
Trichophoromyia brachipyga	0	0	1	0	1	0.12
Trichopygomyia dasypodogeton	0	0	1	0	1	0.12
Not identified	2	8	29	83	122	15.21
Sub-total	100	105	269	328	802	100
Total	205 (25.5%)		597 (74.5%)			

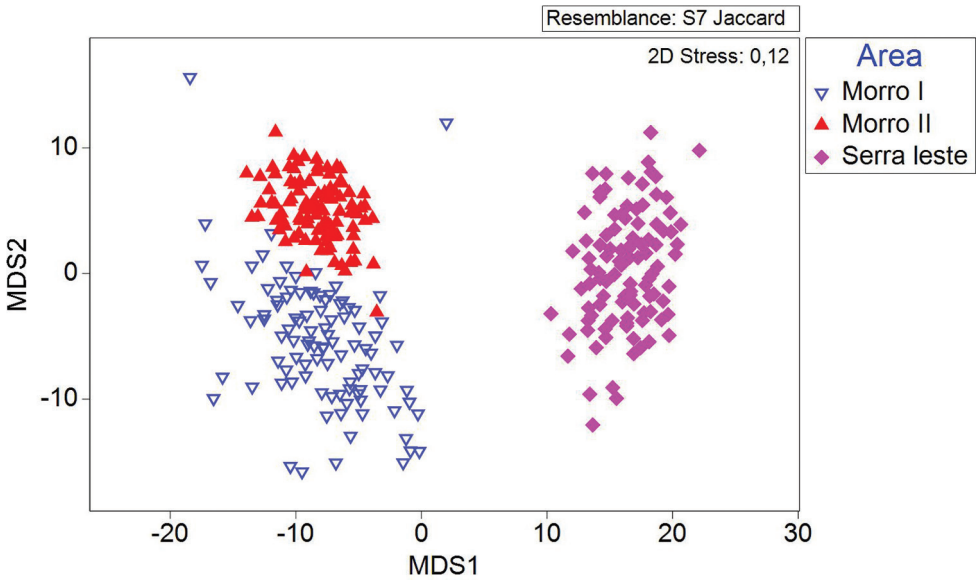

Figure 2. Metric multidimensional scaling (MDS) using bootstrap to show variations on sand flies species composition in iron ore caves at Parauapebas (Morro I, Morro II) and Curionópolis (Serra Leste) municipalities, Pará, Brazil.

Serra Leste caves, with the metric multidimensional scaling (MDS) showing the dispersion in the similarity of the fauna in the three sampled areas (Figures 2, 3).

The mean dissimilarity between the caves of Parauapebas and Curionópolis corresponded to 72.29, and the species responsible for the dissimilarity were *Sciopemyia sordellii* (77.26% contribution), *Pintomyia gruta* (12.01% contribution) and *Lutzomyia longipalpis* (7.92% contribution).

The mean richness was different between the two sampled areas (KW-H (1; 206) = 20.34; p <0.01), with a higher mean richness in the Parauapebas caves (mean = 2 spp., sd = 1) in relation to Curionópolis (mean = 1.44 spp., sd = 0.7). The mean richness was different between the regions of Morro II and Serra Leste (KW-H (2; 206) = 20.36; p <0.01), with a higher mean richness in the Morro I caves (mean = 2 spp., sd = 1.1) in relation to Morro II (mean = 1.98 spp., sd = 0.94) and Serra Leste (mean = 1.44 spp., sd = 0.7). The richness did not show any significant relation with the linear extension of the caves.

A total of 9,807 individuals were counted during the 532 samplings, 4,340 in the dry period and 5,467 in the rainy period. In the Morro I and II caves, 6,791 specimens (295 samples) were counted and 3,016 in Serra Leste (238 samples) (Suppl. material 1).

The mean abundance was different between the two sampled areas (KW-H (1; 532) = 28.37; p <0.01), with a higher mean abundance in the caves of Parauapebas (mean = 23.10 individuals, sd = 31.73) in relation to Curionópolis (mean = 13.60 individuals, sd = 29.40) (Figure 4A). The mean total abundance was different between the regions of Morro I, Morro II and Serra Leste (KW-H (2; 532) = 37.87; p <0.01), with a higher mean abundance in the Morro I caves (mean = 24.8 individuals, sd = 43.76)

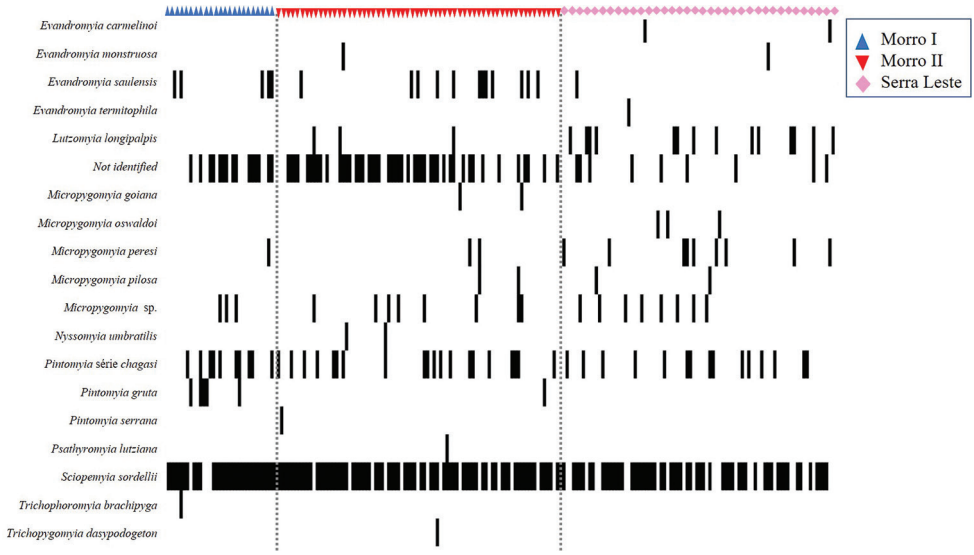

Figure 3. Distribution of species of sand flies collected in caves of Parauapebas (Morro I, Morro II) and Curionópolis (Serra Leste) municipalities, Pará, Brazil.

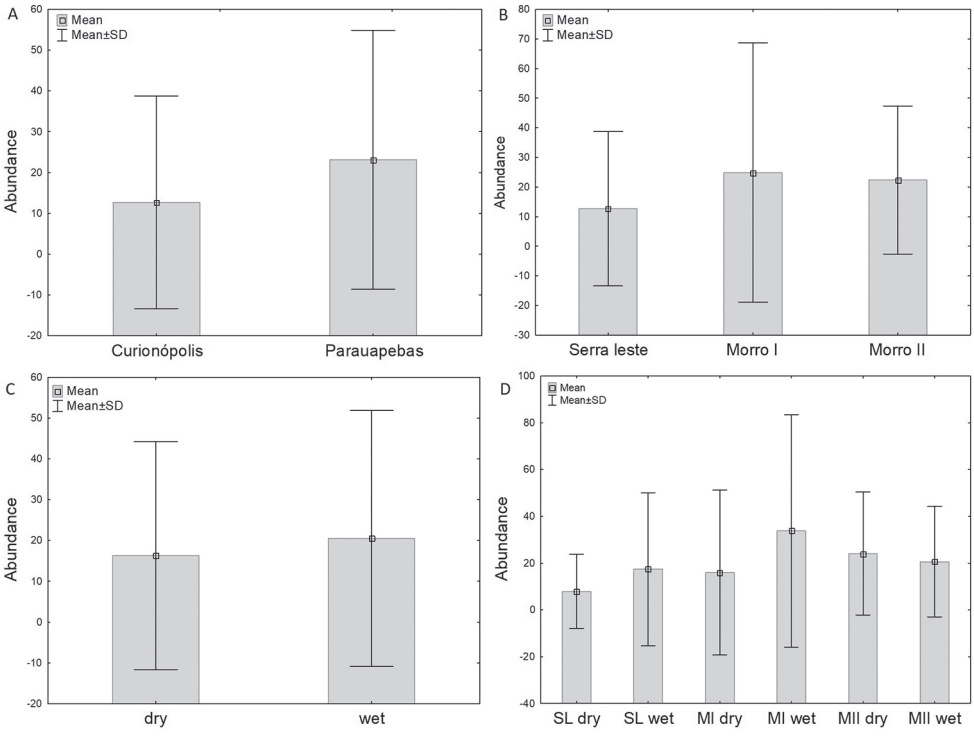

Figure 4. Distribution of abundance of sand flies counted in caves of Parauapebas and Curionópolis (**A**), in the regions of Morro I, Morro II and Serra Leste (**B**) in the dry and rainy periods of the year (**C, D**), Morro (MI), Morro II (MII) and Serra Leste (SL). Negative values do not represent negative abundance but the mean value subtracted from the standard deviation.

in relation to Morro II (mean = 22.3 individuals, sd = 24.98) and Serra Leste (mean = 13.60 individuals, sd = 29.39) (Figure 4B).

Considering all areas, the mean abundance was different between the dry and rainy periods (KW-H (1; 532) = 4.14; p <0.01), with a higher mean abundance in the rainy season (mean = 21 individuals, sd = 33.83) in relation to the dry (mean = 16 individuals, sd = 27.70) (Figure 4C). Considering each area independently, the mean total abundance was different between the dry and rainy periods only in Morro I (KW-H (1; 88) = 6.33; p <0.01), with a higher mean abundance in the rainy period (mean = 33, 79 individuals, sd = 49.7) in relation to the dry (mean = 15.97 individuals, sd = 35.2) (Figure 4D).

Discussion

Despite their restrictive traits, such as the scarcity of food resources, which could preclude the establishment of epigean species, caves can present a diverse and peculiar fauna. In this sense, inventories carried out in caves, particularly of sand flies, are extremely important, as they can contribute to the knowledge of the fauna of these insects, generating data of taxonomic, ecological and epidemiological importance (Pinto et al. 2012).

The use of light traps has been widely used in studies of sand flies in caves (Galati et al. 2010; Alves et al. 2011; Barata and Apolinário 2012; Carvalho et al. 2013). Such methodology is justified by the fact that it allows a longer time of exposure in the collections, characterizing the abundance more efficiently and preserving fragile morphological structures, essential for the identification of these insects at the specific level (head, wings, chest, legs, abdomen). However, the constant lighting can attract individuals who potentially would not be using the caves as a shelter or feeding place. Thus, the use of the manual collection is justified, with the use of brushes moistened with 70% alcohol when rapid assessments are intended and/or to minimize the attractiveness bias of light traps, especially in places closer to the cave entrances. However, care for specimens during collection and transport must be increased to avoid damaging morphological structures (Feitosa and Castellón 2004).

Sciopemyia sordellii was predominant (60.6% of the total number of specimens captured) and had a higher number of occurrences in the caves. Other authors have also recorded this species in caves (Galati et al. 1997; Carvalho et al. 2013), but also in other habitats, as in wild animal dens, tree trunks and tops, tabular roots, domestic animal attachments and in the human home (Aguiar and Medeiros 2003). Despite not being identified as a *Leishmania* vector, Guimarães et al. (2014) found DNA from this parasite in *Sc. sordellii* for the first time in Brazil. However, further studies are needed to clarify the role of this species in the transmission of the parasite, mainly due to its wide distribution and local abundance.

Pintomyia gruta represented 7.86% of the collected sand flies. This species is endemic to the Northeastern part of the country, and until a few years ago, its occurrence was restricted to the caves of Serra dos Carajás (Ryan 1986; Aguiar and Medeiros 2003). In 2015, specimens of *Pi. gruta* were captured in an area close to a hydroelectric

system in the state of Rondônia (Galardo et al. 2015), expanding knowledge about its occurrence and distribution. New records were also made in caves in Rondônia (Ogawa et al. 2016).

Among the most important findings of this work, the first records of *Pi. serrana*, *Tr. brachipyga*, *Tr. dasypodogeton* and *Ev. saulensis* in Brazilian caves must be highlighted. Despite the latter not be considered vector species of *Leishmania*, Araújo Pereira et al. (2017) and Avila et al. (2018) reported their infection in specimens collected in the state of Acre, highlighting the need to investigate the possible role of this species in the transmission of *Leishmania*.

Females of the *Mi. peresi* species, as well as *Ev. saulensis*, feed on ectothermic animals, such as reptiles and amphibians. Possibly, the registration of these species in caves is associated with the presence of reptiles and amphibians, which use this environment as a shelter (Matavelli et al. 2015). It is worth mentioning that the troglophilic habit of *Mi. peresi* has already been reported by Galati et al. (1997).

The species *Micropygomyia* spp. collected in the caves of the municipalities of Curionópolis and Parauapebas did not have their identification confirmed. It is important to highlight that it is probably a new species, which needs to be further studied. For this, new collections should be carried out in these places in search of new specimens for a future description of this species.

Two specimens of *Evandromyia termitophila* were reported in captures in ferruginous caves in Pará. Santos et al. (2011) captured this species close to a cave, but not inside. Therefore, this is not an unprecedented record of this species in Pará, but rather, the first notification of *Ev. termitophila* in caves in the state.

Considering the epidemiological importance of some species, we call attention to the record of *Ny. umbratilis*, which had also been recorded in caves in the State of Amazonas and Rondônia (Alves et al. 2011; Ogawa et al. 2016). *Nyssomyia umbratilis* is a vector for *Leishmania* (*Viannia*) *guyanensis* in Brazil (Lainson et al. 1979; Young and Duncan 1994; Souza et al. 2003; Feitosa and Castellon 2009). Other authors have found specimens of *Ny. umbratilis* infected also by *Leishmania braziliensis* (Arias and Freitas 1978).

Lutzomyia longipalpis species must be highlighted because it is the most important species in the epidemiological cycle of visceral leishmaniasis in Latin America (Brasil 2016). Infectious forms of *Leishmania infantum* have already been identified in this species by many authors (Lainson et al. 1987; Michalsky et al. 2011; Lidani et al. 2017). Due to its great ecological value and adaptive plasticity, it is currently considered an urbanized species, being captured mainly at home and in shelters for domestic animals (Barata et al. 2005; Dias et al. 2007; Michalsky et al. 2009; Chagas et al. 2016). However, the record of *Lu. longipalpis* in caves is still being documented (Galati et al. 2006; Galati et al. 2010; Carvalho et al. 2013; Campos 2017), revealing that some populations can still be found in wild and preserved places, such as some caves of this study.

The differences in composition and the lower richness and abundance found for the Serra Leste caves (Curionópolis), despite being weak may be related to the effect of environmental degradation observed in this region. Studies carried out outside cave

environments have shown a reduction ratio of sand flies in areas with capoeira vegetation, rural and urban areas concerning to forested areas (Araújo et al. 2000; Barros et al. 2000; Carvalho et al. 2000). Such reduction in richness and abundance and differences in species composition are probably because, in general, sand flies prefer shaded environments, with higher humidity and greater availability of organic matter (Aguiar and Medeiros 2003). However, some species may be more tolerant and occur in greater numbers in anthropized areas (Araújo et al. 2000; Carvalho et al. 2000).

In Brazil, a greater number of sand flies has been associated with the rainiest periods of the year (Marinho et al. 2008; Feitosa and Castellon 2009; Barata and Apolinário 2012) and this probably related to the greater availability of blood sources, with a higher rate of oviposition and, thus, providing a greater occurrence of larvae and, later, adults. The higher abundance of these insects in the rainy season, with the presence of proven vectors, suggests a greater risk of leishmaniasis transmission at this time.

The phlebotomine fauna was shown to be rich and abundant in the sampled caves, however, environmental degradation seems to be the main factor in producing changes in composition and richness, especially in the Serra Leste region. In addition, the large number of species in the caves of Morro I and II, in comparison to Serra Leste, reinforces the importance of these places as a shelter for sand flies in degraded areas.

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Supplementary material 1

Table S1

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RESEARCH ARTICLE

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A new troglobitic species of *Allochthonius* (subgenus *Urochthonius*) (Pseudoscorpiones, Pseudotyrannochthoniidae) from Japan

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Abstract

Allochthonius (*Urochthonius*) *yoshizawai* **sp. nov.**, found in Hiura-do Cave, a limestone cave located in the municipality of Kumakogen, Ehime Prefecture, Japan, is described. It can be distinguished from the consubgeneric species mainly by the carapacal chaetotaxy (6–2, 18), by the presence of 6 setae on the cheliceral palm, by the rallum with 11 blades, by the presence of 8 clavate coxal blades on coxae I, and by the decreased number and distinct shape of the chelal teeth. A redescription of the subgenus *Urochthonius*, and keys to the subgenera of *Allochthonius* and to the species and subspecies of *Urochthonius* are also provided, as well as some ecological remarks, a brief discussion on troglomorphisms for the subgenus, and potential threats for this species.

Keywords

Cave, pseudoscorpions, taxonomy, troglomorphism

Introduction

In East Asia, the pseudoscorpion family Pseudotyrannochthoniidae Beier, 1932 is represented by two genera, *Allochthonius* Chamberlin, 1929 and *Pseudotyrannochthonius* Beier, 1930 (Harvey 2013). The genus *Allochthonius* is further divided into two

subgenera: *Allochthonius* Morikawa, 1954, composed of 16 species (Hu and Zhang 2012; Gao and Zhang 2013; Harvey 2013; Zhang and Zhang 2014; Gao et al. 2016), and *Urochthonius* Morikawa, 1954, with three species (Hu and Zhang 2012; Harvey 2013). The subgenus *Allochthonius* is characterized by four-eyed, mostly surface-living species, whereas anophthalmic or two-eyed, mostly cave-dwelling species, are allocated to the subgenus *Urochthonius* (Morikawa, 1960).

During an expedition to caves in Japan (carried out from September 5 to 15, 2017), a single pseudoscorpion was found, belonging to a new species herein described. The single male specimen belonging to the subgenus *Urochthonius* was found in Hiura-do Cave, a limestone cave located in Shikoku Island. The new species is considered troglobitic, and it shows a distinct combination of morphological features. It shares some characteristics with two consubgeneric species, *A.* (*U.*) *ishikawai* Morikawa, 1954 and *A.* (*U.*) *brevitus* Hu & Zhang, 2012.

It is important to point out that the subgeneric division of the genus *Allochthonius*, which is solely based on morphological characters (e.g. the absence or number of eyes) and typical habitat type, appears unstable and thus a taxonomic revision is imperative. However, while we recognize the need of further research on this matter, the description of a new species based on a single specimen, although oftentimes discouraged by researchers, can be of crucial importance, especially when taken into consideration species conservation. Furthermore, it is noteworthy that many troglobitic species, especially predators, can be extremely rare. Due to impacts on cave systems, a species may lose its habitat and become potentially extinct before its formal taxonomic description, as observed by Ferreira et. al. (2020). Accordingly, we hereby propose the description of a newly discovered *Urochthonius* species.

Methods

Study area

Fieldwork was carried out in September 2017 at Hiura-do Cave, a limestone cave located in the municipality of Kumakogen, Ehime Prefecture, Shikoku Island, Japan (Figs 1, 5A–D). A visual search was conducted, and a single male specimen was found walking on a rock wall. It was captured by using a fine brush, and subsequently transferred to a small-labeled plastic vial containing 70% ethanol for preservation.

More details on habitat are covered in a separate section (habitat and threats) later in the paper.

Preparation and analysis

In order to properly observe taxonomic characters, the specimen and its dissected appendages were mounted in temporary cavity slides, using glycerin as medium. Photographs and measurements of body parts were taken with a Zeiss Axio Zoom.V16 stereomicroscope, using the software Zen 2.3. Drawings were prepared with a draw-

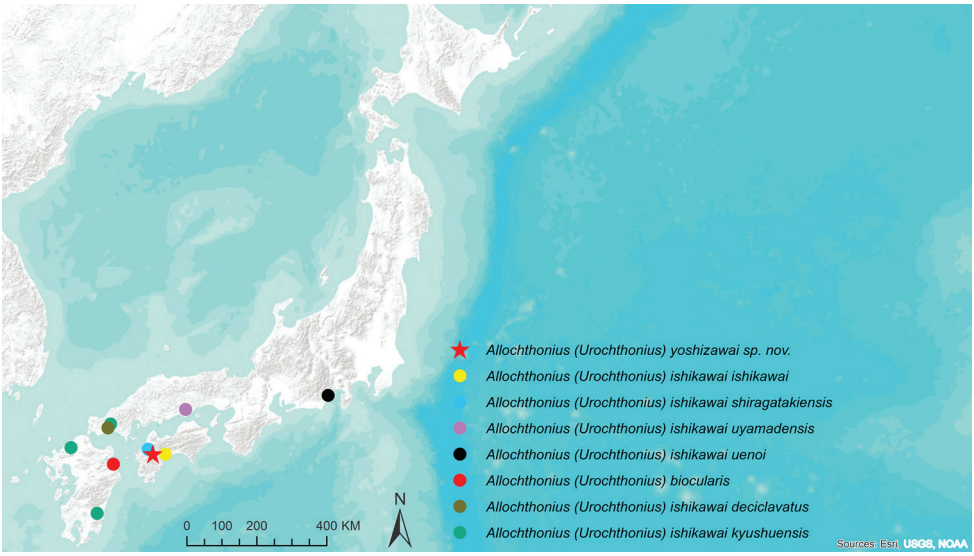

Figure 1. Map of distribution of the representatives of the subgenus *Urochthonius* in Japan.

ing tube on a Leica DM750 microscope equipped with phase contrast. For drawings, Kaiser's glycerol gelatin was used instead of glycerin. This mounting media solidifies at cold temperatures, thus allowing the dissected body parts to be kept in a fixed position.

Description of coloration was based on photographs of the living specimen, which were taken with a Cannon SX50 camera. The terminology used in the description follows Chamberlin (1931), Harvey (1992), Judson (2007), Vachon (1941a, 1941b) and Gabbutt and Vachon (1963). Measurements follow Chamberlin (1931) and represent the average of two measurements taken on different days.

Abbreviations used: For trichobothria: ***ib*** = interior basal; ***isb*** = interior sub-basal; ***ist*** = interior sub-terminal; ***it*** = interior terminal; ***eb*** = exterior basal; ***esb*** = exterior sub-basal; ***est*** = exterior sub-terminal; ***et*** = exterior terminal; ***b*** = basal; ***sb*** = sub-basal; ***st*** = sub-terminal; ***t*** = terminal. **ICHUM** = Invertebrate Collection of the Hokkaido University Museum; **ME** = main entrance, **SE** = secondary entrance.

Results

Family Pseudotyrannochthoniidae Beier, 1932
Genus *Allochthonius* Chamberlin, 1929
Subgenus *Urochthonius* Morikawa, 1954

Type species. *Allochthonius* (*Urochthonius*) *ishikawai* Morikawa, 1954.

Diagnosis (modified from Morikawa 1960). No eyes or, more rarely, two rudimentary eyes present. Epistomal process absent. Fixed chelal finger with 7–20 acute marginal teeth, movable finger with 10–17. Cheliceral palm with 5–6 setae; fixed fin-

ger of chelicera generally with one basal large tooth and a few small teeth before it, with one distal large tooth and several teeth after it, or with several small teeth on the median swelling without any large tooth. Coxal blades of coxa I with a spray of 5–11 clavate processes on a mound. Palps, chelicerae and legs long and slender. Setae of the body also long and slender. Typically cave inhabitants.

***Allochthonius* (*Urochthonius*) *yoshizawai* sp. nov.**
http://zoobank.org/0E7ED7D0-E509-4CC0-BAF3-AB66F22ED6AA
Figures 2–4

Type material. Holotype male (ICHUM-6165), in alcohol: Japan, Ehime Prefecture, Kumakogen, Hiura-do Cave (33°29'20.4"N, 132°55'48.0"E), on the cave wall (dark zone), 5 September 2017, R.L. Ferreira leg.

Etymology. The specific name is given in honor of Dr. Kazunori Yoshizawa, not only due to the assistance provided during fieldwork in Japanese caves, but also to his great contribution to the knowledge of arthropods, especially Psocodea.

Diagnosis. Differing from the other members of subgenus *Urochthonius* by the following combination of characters: carapace with 18 setae (6 on anterior margin, 2 on posterior margin); cheliceral palm with 6 setae, fixed cheliceral finger with large basal tooth, rallum with 11 blades (each with fine barbules, the basal-most blade shorter than the others); coxa I with a spray of 8 clavate coxal blades (subequal in length) on a mound, bisetose intercoxal tubercle present between coxae III and IV; on the fixed chelal finger, 7 (8 on the right chela) acute, narrow, large, widely-spaced teeth; on the movable chelal finger, 10 acute, small, widely-spaced teeth; chelal teeth varying in size.

Description of adult male (female unknown). Troglomorphic habitus (Fig. 2A, B). Body mostly translucent, with a vitreous aspect. Chelae, chelicerae, and tergites light pinkish orange; other parts of body white. Vestitural setae smooth, long and acuminate.

Carapace (Fig. 4B): Nearly square in dorsal outline, 1.1 times longer than broad, slightly constricted posteriorly; anterior margin somewhat straight, but becoming indistinctly concave towards median region; without eyes or eyespots; two weak transverse furrows present, near anterior and posterior margins; chaetotaxy 6: 6: 2: 2: 2 (18).

Figure 2. *Allochthonius* (*U.*) *yoshizawai* sp. nov., male holotype **A** habitus of male **B** live specimen in natural habitat. Scale bar: 2 mm (**A**).

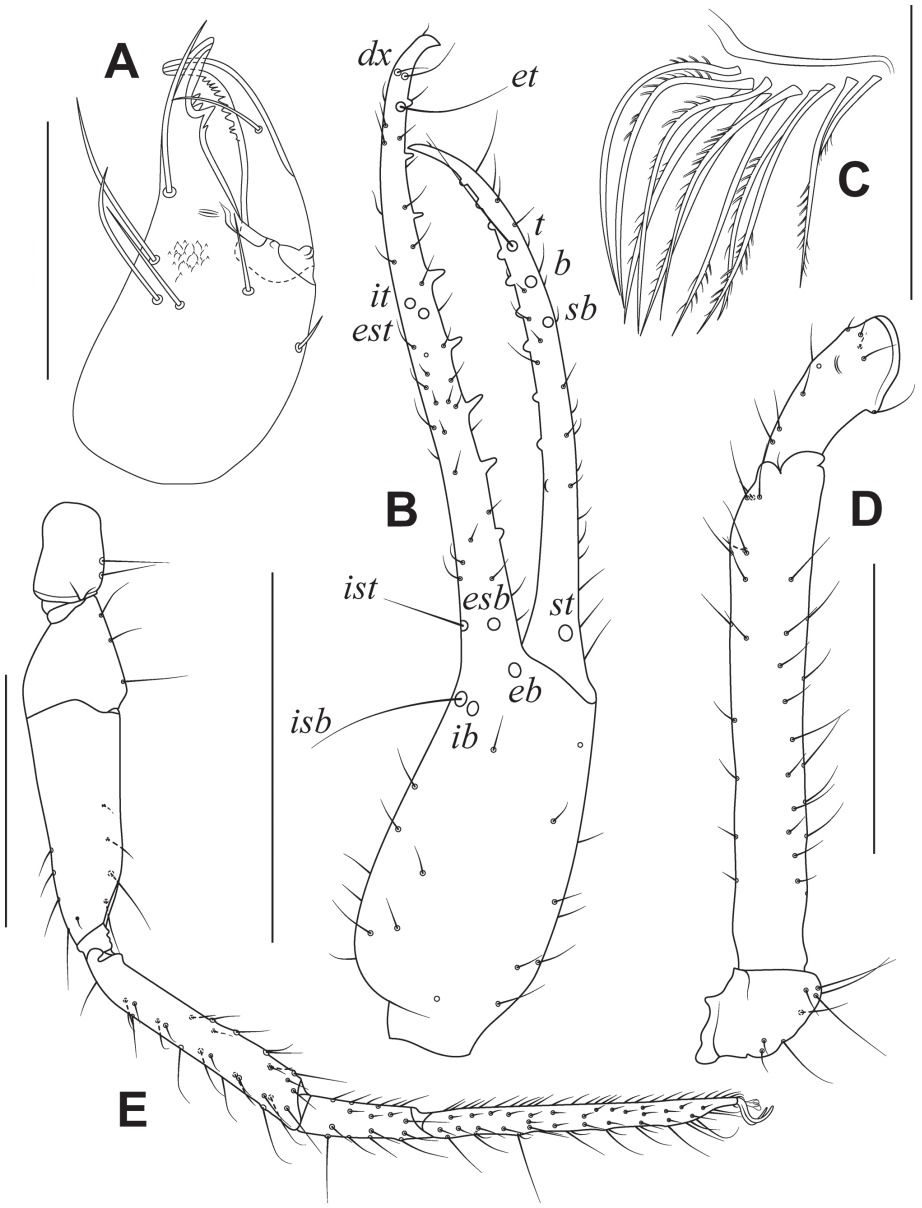

Figure 3. *Allochthonius* (*U.*) *yoshizawai* sp. nov., male holotype **A** right chelicera, showing detail of surface texture, antiaxial (slightly ventral) view **B** right chela, showing trichobothrial pattern and marginal teeth, antiaxial view **C** right cheliceral rallum **D** left palp, dorsal view **E** left leg IV, retrolateral view. Scale bars: 0.25 mm (**A**); 0.5 mm (**B**, **D**, **E**); 0.125 mm (**C**).

Chelicerae (Fig. 3A, C): Surface mostly scaly-reticulate. Hand with 6 setae (including 1 ventral seta); movable finger with 1 subdistal seta; galea absent; fixed finger with 4 apical teeth, including one large basal tooth (third one on right chelicera, fourth

one on left), followed by small denticles (4–12); movable finger with 6–8 teeth of equal length, followed by 7 smaller teeth on left chelicera (a few denticles on right chelicera); rallum (Fig. 3C) composed of 11 blades (7 in one row, 4 in another row) with fine barbules, the basal-most one distinctly shorter than the others (~1/3 length of other blades); serrula exterior with 18 blades, serrula interior of the left chelicera with 15 blades, 16 blades on the right.

Tergites: Undivided; chaetotaxy uniseriate, I–XI 2: 2: 4: 6: 6: 7: 9: 10: 8: 5: 2. Anal operculum without dorsal setae.

Coxae: Palpal: manducatory process with two setae, apical seta reduced; rest of palpal coxae with three setae. Pedal: coxae I each with a spray of 8 clavate blades (Fig. 4D); chaetotaxy I 4, II 4–5, III 5, IV 5–6; intercoxal tubercle present between coxae III and IV, bearing two setae.

Genital operculum of male (Fig. 4A, C): Anterior genital operculum with 6 anterior setae, and one posterior seta; genital opening with 8 setae on the right side, and 10 on the left.

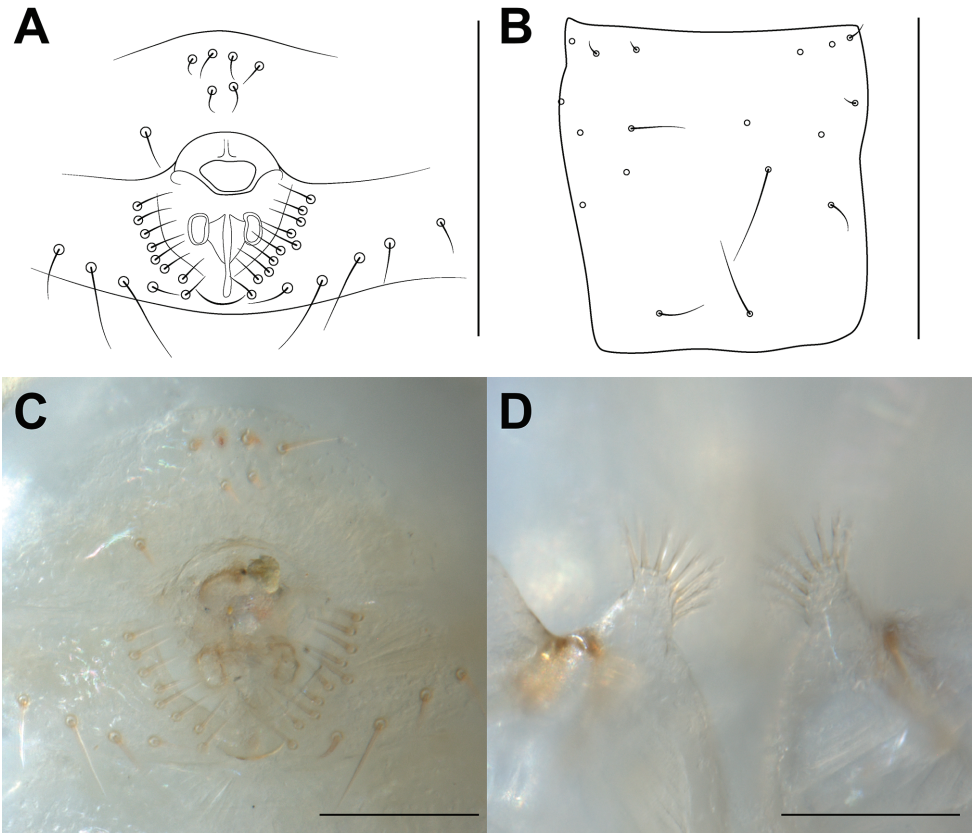

Figure 4. *Allochthonius* (*U.*) *yoshizawai* sp. nov., male holotype. **A** Male genital operculum **B** holotype carapace, showing distribution of setae (most hairs missing) **C** male genital operculum **D** coxal blades on coxae I, ventral view. Scales: 0.25 mm (**A**); 0.5 mm (**B**); 0.1 mm (**C**, **D**).

Sternites: Chaetotaxy II–XI 13: 16: 17: 15: 15: 15: 12: 10:–:2. Anal operculum with one pair of ventral setae.

Palp (Fig. 3B, D): Femur chaetotaxy: 6: 10: 4: 7: 2 (Fig. 3D). Trichobothria *ib* and *isb* located on a small dorsal hump. Trichobothrial pattern (Fig. 3B): trichobothrium *sb* distinctly nearer *b* than *sb*; *it* distad to *est*; trichobothria *ib-isb-eb-esb-ist* clustered at the base of fixed finger. On the left chela trichobothrium *est* is missing. Fingers distinctly curved, movable finger shorter than fixed finger. Fixed finger with 7 (8 on the right chela) acute, large, narrow, widely-spaced, irregular marginal teeth; on the left chela, the fourth distal tooth distinctly larger than the others, two small basal tubercles present; the right fixed finger marginal teeth larger compared with those of the left chela. Movable finger with 10 acute, small, widely-spaced, irregular teeth.

Leg IV (Fig. 3E): Subterminal setae long and acuminate. Arolia shorter than claws, latter slender and smooth. Two tactile setae present, one on the metatarsus and another on the tarsus.

Measurements (length/breadth or, for legs, length/depth in mm, ratios in parentheses): Body length 1.97. Carapace 0.55/0.51 (1.1). Palps: trochanter 0.29/0.16 (1.8), femur 0.91/0.14 (6.5), patella 0.31/0.13 (2.5), hand with pedicel 0.54/0.26 (2.0), movable finger length 0.78, chela with pedicel 1.39 (5.3). Leg I: femur 0.53/0.08 (6.4), patella 0.32/0.07 (4.8), femur/patella (1.6), tibia 0.26/0.06 (4.7), tarsus 0.56/0.06 (10.2). Leg IV: femur+patella 0.77/0.17 (4.4), tibia 0.57/0.09 (6.1), metatarsus 0.28/0.07 (3.8), tarsus 0.64/0.06 (11.5), tarsus/metatarsus (2.3).

Key to subgenera of *Allochthonius*[*]

1 Four eyes well-developed, mostly free-living species....**Subgenus *Allochthonius***
– Eyes completely absent or two rudimentary eyes, mostly cave-dwelling species..... **Subgenus *Urochthonius***

Key to species and subspecies of *Urochthonius*

1 Two rudimentary eyes present.....***A.* (*U.*) *biocularis***
– Eyes absent **2**
2 Palpal femur stout, 3.9 times longer than broad.....***A.* (*U.*) *brevitus***
– Palpal femur slender, 3.9–6.5 times longer than broad.....**3**
3 Cheliceral palm with 6 setae, rallum with 11 blades .....
.....***A.* (*U.*) *yoshizawai* sp. nov.**
– Cheliceral palm with 5 setae, rallum with 10 blades.....**4**
4 Chelal fingers distinctly curved, fixed finger with 9 marginal teeth, movable finger with 11 marginal teeth.....***A.* (*U.*) *ishikawai shiragatakiensis***
– Fixed chelal fingers not so curved, with 13–17 marginal teeth **5**

[*] modified from Morikawa 1960.

5 Anterior margin of carapace with 10 setae.....**6**
– Anterior margin of carapace with 8 setae.....**7**
6 Chelal fingers with 13–14 marginal teeth; cheliceral movable finger with about 13 minute teeth ..... ***A.* (*U.*) *ishikawai deciclavatus***
– Chelal fingers with about 16 marginal teeth; cheliceral movable finger with about 18 minute teeth ..... ***A.* (*U.*) *ishikawai kyushuensis***
7 Body length 1.51–1.97 mm..... ***A.* (*U.*) *ishikawai ishikawai***
– Body length 2.31–2.38 mm.....**8**
8 Carapace chaetotaxy 8–2, 24 ..... ***A.* (*U.*) *ishikawai uenoi***
– Carapace chaetotaxy 8–2, 18 ..... ***A.* (*U.*) *ishikawai uyamadensis***

Habitat and threats

Hiura-do Cave is a limestone cave with approximately 160 meters of horizontal extent and two entrances (Fig. 5B). The secondary entrance (Fig. 5B, SE), although wider than the main entrance (Fig. 5B, ME), is considerably low (<1 m in height), in addition to being located on a rock escarpment, which makes access to the cave interior quite difficult. From the main entrance (Fig. 5A), the conduit presents a descending slope, until reaching a vertical pit, from which is possible to access a lower level. At the deepest part of the cave there is a drainage (Fig. 5D), which springs at the end of the cave and sinks a few meters further. This drainage springs out at the external environment some dozen meters down from the main cave entrance, forming a stream. Most of the cave conduits are formed by exposed limestone, being devoid of sediments (Fig. 5C). The single specimen of *A.* (*U.*) *yoshizawai* sp. nov. was found freely walking on a limestone surface, on the side of the wall, actively crawling in an aphotic area located around 50 meters from the nearest entrance (Fig. 5B). The cave is highly oligotrophic, and only some scarce organic debris deriving from vegetation was observed when it was visited. Neither bat colonies nor guano deposits were observed. Nonetheless, it is important to point out that there may be seasonal variation (i.e. the influx of organic matter may be higher during certain periods of the year).

Potential prey for the pseudoscorpion are mainly springtails (Entomobryomorpha and Onychiuridae), which are relatively abundant in the cave. Other troglobitic species observed in the cave during our visit included, besides the Collembola, the highly troglomorphic carabid beetle *Nipponaphaenops erraticus* Ueno, 1971, the staphylinid beetle *Quedius* sp., the Grylloblattodea *Galloisiana* (an undescribed species), and a Rhagidiidae mite.

The cave presents obvious signs of human visitation (there is an iron ladder installed from the upper to the lower level), but such visitors seem to be mostly speleologists, so no severe impacts were observed in the cave. The external environment is also well preserved, with a forest covering most of the landscape. Considering the well-preserved status of both the cave and the external landscape surrounding the cave, the species seems not to be seriously threatened at the moment.

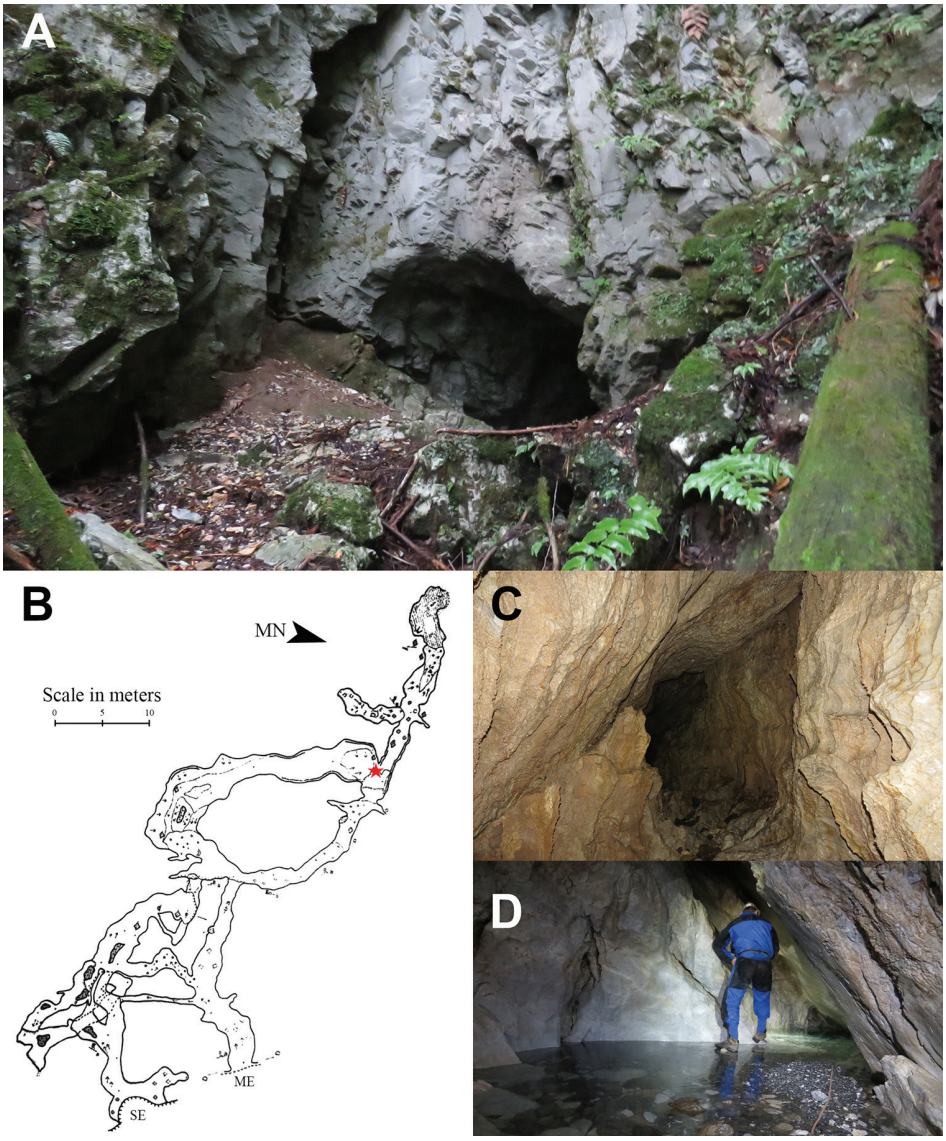

Figure 5. Type locality and habitat of *Allochthonius* (*U.*) *yoshizawai* sp. nov. **A** Main cave entrance **B** map of Hiura-do Cave, showing the site (red star) where the specimen was found, as well as the entrances **C** general aspect of the cave conduit. The specimen was collected crawling on a damp wall **D** drainage system at the deepest portion of the cave.

Discussion

Troglomorphisms and taxonomic traits

Concerning *Urochthonius* spp., it is difficult to state with certainty whether some characteristics represent typical troglomorphisms found in other pseudoscorpions. Two spe-

cies, *A.* (*U.*) *biocularis* Morikawa, 1956 and *A.* (*U.*) *ishikawai*, are cave-dwelling, found in Japan. One of the main differences regarding external morphology between *A.* (*U.*) *biocularis* and *A.* (*U.*) *ishikawai* is that the former bears two anterior rudimentary eyes (Morikawa 1956), whereas the latter is anophthalmic (Morikawa 1954, 1956, 1960). The only epigean species within the subgenus, *A.* (*U.*) *brevitus*, which is recorded from China, is also characterized by the absence of eyes (Hu and Zhang 2012; Zhang and Zhang 2014). Contrastingly, the cave-dwelling representative of the nominal subgenus from Japan, *A.* (*A.*) *opticus troglophilus* Morikawa, 1956, bears four eyes on the carapace – the main diagnostic character for the subgenus (Morikawa 1960). It is clear, therefore, that the genus *Allochthonius* should undergo a major taxonomic revision, including phylogenetic analyses and thorough examination of type material, not only for providing a better understanding of troglomorphisms in the group, but also for assessing the validity of the subgenera (which could eventually be synonymized). In conclusion, the lack of phylogenetic studies precludes any tests whether the absence of eyes represents a morphological specialization to the subterranean environment within the genus.

Nonetheless, *A.* (*U.*) *brevitus* exhibits an array of characteristics that sets it apart from the hypogean species. *Allochthonius* (*U.*) *brevitus* is characterized by having generally stouter appendages. Particularly in reference to its palpal femur ratio (3.9 times longer than broad) (Hu and Zhang 2012; Zhang and Zhang 2014), it is significantly smaller when taken into account the range shown by the consubgeneric species (5.1–6.5 times longer than broad) (Morikawa 1954, 1956, 1960). Additionally, in terms of coloration, the body is mostly light yellowish (except for the carapace and tergites, which are strong yellowish brown), and the chelicerae and palps are reddish (Hu and Zhang 2012; Zhang and Zhang 2014). On the other hand, cave-dwelling *Urochthonius* species show a pronounced reduction in body color as can be inferred from the descriptions of *A.* (*U.*) *ishikawai uenoi* Morikawa, 1956, *A.* (*U.*) *biocularis*, and *A.* (*U.*) *ishikawai ishikawai* Morikawa, 1954 (Morikawa 1954, 1956). Paleness and more elongate, slender appendages represent common troglomorphic traits found in pseudoscorpions (Heurtault 1994). Therefore, we argue that the aforementioned characters could be pointed out as the main morphological specializations presented by cavernicolous *Urochthonius* species.

Furthermore, although it may simply represent a dispersal-aiding trait, the considerably high level of chelal finger curvature could potentially represent a troglomorphism for the cave-dwelling species in the subgenus. When comparing the chelal fingers of hypogean species and the single epigean representative, *A.* (*U.*) *brevitus*, the former present generally curved fingers (Morikawa 1954, 1956, 1960), whereas the latter shows straight fingers, only slightly curved distally (Hu and Zhang 2012; Zhang and Zhang 2014). This higher curvature (see in Figure 2B the wide gap between the curved fingers of the chelae, especially in the right chela) may enable the troglobitic species to capture both bigger and smaller prey. When considering the usually low population densities of cave invertebrates in general, such a trait could be adaptive, allowing them to feed on a wider range of potential prey, which may be important in an oligotrophic environment.

We note some inconsistencies in the measurements and ratios in the descriptions of *A.* (*U.*) *biocularis* and *A.* (*U.*) *ishikawai kyushuensis* Morikawa, 1960, regarding the palpal femur (Morikawa 1956, 1960). In reference to *A.* (*U.*) *biocularis*, a range of 5.3–5.7 times longer than broad is mentioned, but measurements are only specified for the holotype (Morikawa 1956). Concerning *A.* (*U.*) *ishikawai kyushuensis*, the values for length and breadth provided for the palpal femur of the holotype are, respectively, 1.13 mm and 0.07 mm. Comparing to the dimensions given for the remaining types (female allotype, 1.29/0.20; male paratype, 1.02/0.18; female paratype, 1.35/0.22), a breadth of 0.07 mm appears to be considerably narrow. Additionally, the ratios that can be obtained by using the previous values, which make up a range of 5.7–16.1 times longer than broad, are different from those found in the description: "*palpal femur 5.7–6.7 times* (*in male*) *and 6.1–7.5 times* (*in female*)" (Morikawa 1960). Furthermore, concerning leg I, except for *A.* (*U.*) *brevitus*, no values for length and breadth or ratios can be found in the descriptions of *Urochthonius* species. Also, only the descriptions of *A.* (*U.*)*ishikawai ishikawai*, *A.* (*U.*) *ishikawai uyamadensis* Morikawa, 1954 and *A.* (*U.*) *brevitus* include measurement data for leg IV. Hence, we opted for not comparing dimensions extensively between *Urochthonius* species and subspecies.

Allochthonius (*U.*) *yoshizawai* sp. nov. is markedly pale, as evidenced by the mostly translucent cuticle of the holotype, and also presents slender appendages (e.g. palpal femur 6.5 times longer than broad). The new species presents a combination of characters based on which distinction from the consubgeneric species can be easily made. Differences related to the carapacal chaetotaxy, number of setae on the cheliceral palm, number of rallum blades, and number of palp chela marginal teeth can be indicated.

Carapacal chaetotaxy: Urochthonius species show a range of 18–28 setae on the carapace (Morikawa 1954, 1956, 1960; Hu and Zhang 2012). In the new species, a total of 18 carapacal setae (6 on anterior margin, 2 on posterior margin) can be found. *Allochthonius* (*U.*) *ishikawai uyamadensis* exhibits the same number of setae on the carapace, however, differently from *A.* (*U.*) *yoshizawai* sp. nov., it has 8 setae on the anterior margin (Morikawa 1954).

Cheliceral traits: Allochthonius (*U.*) *brevitus* has 6 setae on the cheliceral palm (Hu and Zhang 2012), as with the new species. Contrastingly, all *A.* (*U.*) *ishikawai* subspecies have 5 setae on the cheliceral palm (Morikawa 1954, 1956, 1960). In *A.* (*U.*) *ishikawai* subspecies, the rallum includes 10 pinnate blades (Morikawa 1954, 1956, 1960); in *A.* (*U.*) *yoshizawai* sp. nov., there are 11 clavate blades on the rallum of the singular known specimen. *Allochthonius* (*U.*) *brevitus* shows the same number of rallum blades as the new species (Hu and Zhang 2012).

Number of chelal teeth: Allochthonius (*U.*) *ishikawai* subspecies have a range of 9–17 teeth on the fixed chelal finger, and 11–17 teeth on the movable finger (Morikawa 1954, 1956, 1960). Regarding *A.* (*U.*) *brevitus*, 20 teeth can be found on the fixed finger, 17 on the movable (Hu and Zhang 2012). Finally, the new species has 7 (8 on the right chela) teeth on the fixed finger, 10 on the movable. A similar number (fixed finger: 9, movable finger: 11) was identified in *A.* (*U.*) *ishikawai shiragatakiensis* Morikawa, 1954. Accordingly, both taxa bear less than half the number of teeth generally shown by the congeners.

On the troglobitic status of *Urochthonius* species

With regard to the subgenus *Urochthonius*, the troglobitic status of its representatives has not been considered in previous works. As outlined earlier, important morphological specializations to the subterranean environment (e.g. paleness) can be recognized in *Urochthonius* cavernicolous species. Hence, we argue that *A.* (*U.*) *yoshizawai* sp. nov. and the consubgeneric cave-dwellers are troglobitic.

Even when taking into account that *A.* (*U.*) *ishikawai kyushuensis* was recorded from six caves located in Kyushu and Honshu islands, inference of the troglobitic status for the subgenus as a whole is still plausible. Sendra et al. (2018) described a troglobitic species of campodeid dipluran collected from caves located in Shikoku and Kyushu islands. *Pacificampa nipponica* Sendra, 2018, was found in two caves, each one located in a different island. It is known that these islands, currently separate, used to be connected during the last glacial age (Sendra et al. 2018). In this regard, we can conclude that although a certain species inhabits more than one cave, even when distant from each other, it can still be assigned as troglobitic.

Acknowledgments

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Attheyella (*Canthosella*) *thailandica* sp. nov. (Copepoda, Harpacticoida, Canthocamptidae) from caves in Thailand

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Abstract

During this sampling campaign, the canthocamptid *Attheyella* (*Canthosella*) *thailandica* **sp. nov.** was collected from various caves in Thailand. The new species is widely distributed in the country and favours habitats, such as phytotelmata and wet soil. *Attheyella* (*Canthosella*) *thailandica* **sp. nov.** is the second member of the genus to be found in Thailand, after *Attheyella* (*Canthosella*) *vietnamica* Borutzky (1967), which is most similar to it. Amongst Asian species, both *A.* (*C.*) *thailandica* **sp. nov.** and *A.* (*C.*) *vietnamica* have identical setal formulae, with a greater number of armatures on the distal endopods of legs 2–4. However, *A.* (*C.*) *thailandica* **sp. nov.** markedly differs from *A.* (*C.*) *vietnamica* in the insertion point of the dorsal seta and the presence of inner spinules on the caudal ramus. Additionally, the leg 4 endopod is two-segmented in *A.* (*C.*) *thailandica* **sp. nov.**, but one-segmented in *A.* (*C.*) *vietnamica*.

Keywords

Morphology, phytotelmata, southeast Asia, taxonomy, wet soil

Introduction

The genus *Attheyella* Brady, 1880 is found in a wide range of habitats in various water bodies and often in groundwater (Chang and Kim 1992). The subgenus *Attheyella* (*Canthosella*) Chappuis, 1931 is one of the five subgenera of *Attheyella* and contained

15 valid species: *A.* (*C.*) *acanthophora* (Delachaux, 1924); *A.* (*C.*) *aliena* Noodt, 1956; *A.* (*C.*) *antillica* (Petkovski, 1973); *A.* (*C.*) *chocoensis* Gaviria & Defaye, 2012; *A.* (*C.*) *fluviatilis* Chappuis, 1931; *A.* (*C.*) *kalima* (Delachaux, 1924); *A.* (*C.*) *lacustris* Chappuis, 1931; *A.* (*C.*) *mervini* Janetzky, Martinez Arbizu & Reid, 1996; *A.* (*C.*) *muscicola* (Chappuis, 1928); *A.* (*C.*) *pilagaensis* Janetzky, Martinez Arbizu & Reid, 1996; *A.* (*C.*) *silvicola* Löffler, 1973; *A.* (*C.*) *siolii* (Kiefer, 1967); *A.* (*C.*) *striblingi* (Reid, 1990); *A.* (*C.*) *vera* Por & Hadel, 1986 and *A.* (*C.*) *vietnamica* Borutzky, 1967 (Walter and Boxshall 2020). To date, six species of the subgenus *Canthosella* have been originally described from southeast Asia (SEA): three species from Indonesia [*A.* (*C.*) *fluviatilis*, *A.* (*C.*) *lacustris* and *A.* (*C.*) *muscicola*] and one species each from Malaysia [*A.* (*C.*) *silvicola*], Vietnam [*A.* (*C.*) *vietnamica*] and Thailand [*A.* (*C.*) *thailandica* sp. nov.], respectively. In Thailand, the first *Attheyella* species found in a cave, *A.* (*C.*) *vietnamica*, has being collected in northern Thailand (Watiroyram et al. 2015) and the second species of the genus found in a cave of the country is described herein as *A.* (*C.*) *thailandica* sp. nov.

Material and methods

This research mainly focused on cave-dwelling copepods in freshwater from the epikarst zone and related habitats – especially water dripping from rocks and plants at cave entrances (Fig. 1). Samples were collected using a filtering bottle with a mesh size of 60 µm and were preserved immediately in 70% ethanol. In the laboratory, samples were rinsed with tap water through a sieve with 60 µm mesh size. Adult specimens were sorted under an Olympus SZ51 stereomicroscope at 40× magnification and were placed in a mixture of glycerol and 70% ethanol (ratio ~ 1:10 v/v) to pure glycerol. Animals were dissected and prepared on a glycerine-mounted slide under a stereomicroscope at 40–100× magnification. The specimens were mounted in pure glycerine on a glass slide and were sealed under a cover glass with transparent nail varnish. Whole specimens were stored in 70% ethanol.

All appendages and body ornamentation were examined with 1000× magnification under an Olympus CX31 compound microscope. Drawings were made using an Olympus U-DA drawing tube mounted on the microscope. Final versions of the drawings were done using the CorelDRAW 12.0 graphic programme.

Specimens for scanning electron microscopy (SEM) were dehydrated in an ethanol series (50%, 70%, 80%, 90%, 95%, 100% and 100%) for 15 min at each concentration. Specimens were dried in a critical point dryer and mounted on stubs. Mounted specimens were coated with gold in a sputter-coater. SEM photographs were carried out using a LEO 1450VP scanning electron microscope.

Abbreviations used are: Enp, endopod; Exp, exopod; Exp/Enp-n, exopodal segment n/endopodal segment n; P1–P6, legs 1–6; s, spine; a, aesthetasc; NHMUK, the Natural History Museum (United Kingdom); NPU, Nakhon Phanom University, Faculty of Science (Thailand).

The descriptive terminology follows Huys and Boxshall (1991).

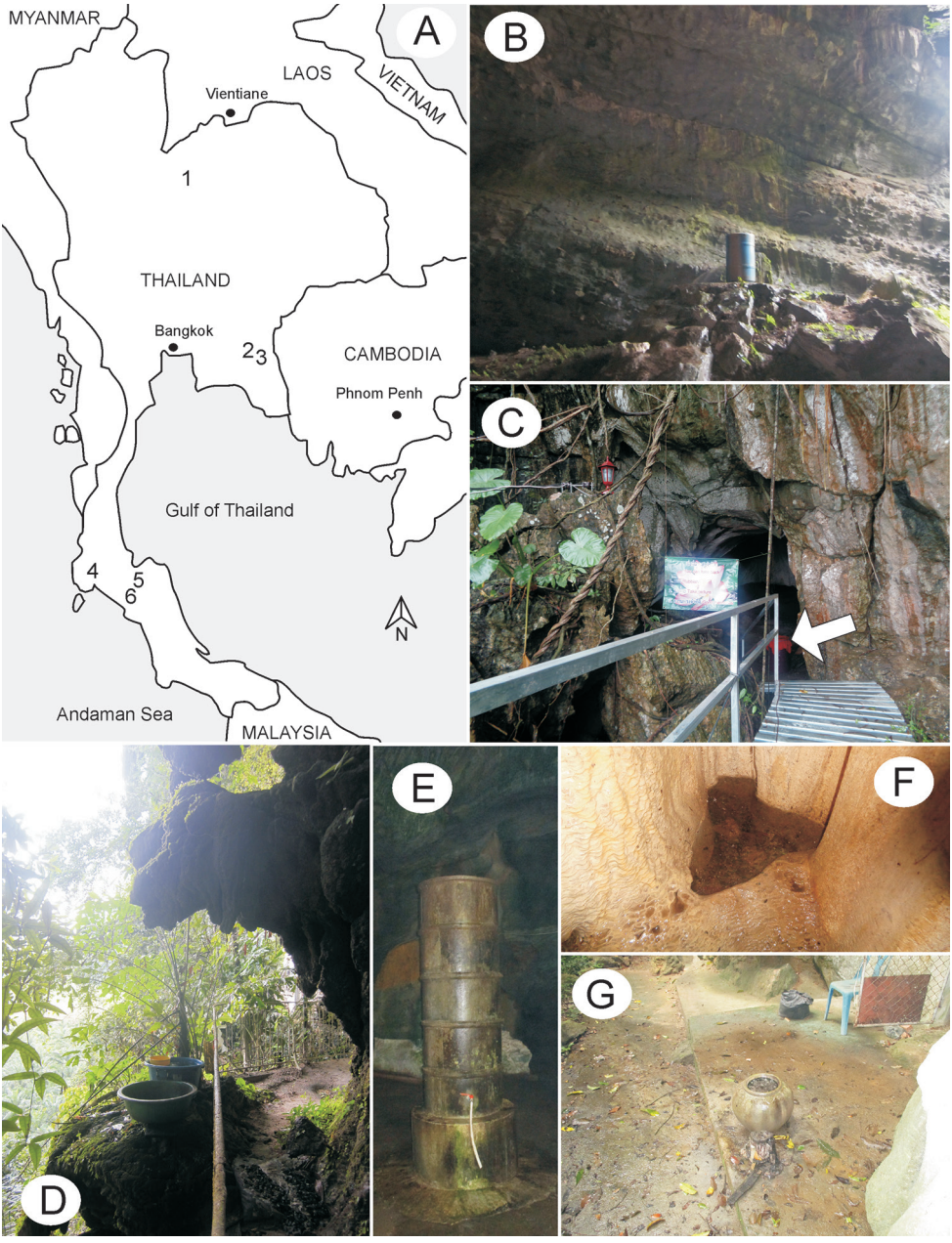

Figure 1. Distribution of *Attheyella* (*Canthosella*) *thailandica* sp. nov. **A** black circle = capital city, Arabic numbers refer to sampling caves: 1, Huang Po cave; 2, Khao Chakan cave; 3, Plub Pleung Thong cave; 4, Payanakarat cave; 5, Pra Hor cave; 6, Mae-nang Songsri cave **B** Huang Po cave **C** Plub Pleung Thong cave (white arrow indicates water container with the new species) **D** Payanakarat cave **E** Mae-nang Songsri cave **F** Khao Chakan cave **G** Pra Hor cave.

Taxonomic section

Family Canthocamptidae Brady, 1880
Genus *Attheyella* Brady, 1880
Subgenus *Canthosella* Chappuis, 1931

***Attheyella* (*Canthosella*) *thailandica* sp. nov.**
http://zoobank.org/CE4F2A69-0F23-4EB7-B84B-33A29FC75F8E
Figures 2–7

Other localities. Mae-nang Songsri cave, Hin Tok Subdistrict, Ron Phibun District, Nakhon Si Thammarat Province, southern Thailand: 08°14'45"N, 99°52'01"E, 45 m altitude, 29 October 2015; Payanakarat cave, Tham Thong Lang Subdistrict, Thap Put District, Phang Nga Province, southern Thailand: 08°31'11"N, 98°33'57"E, 140 m altitude, 5 November 2014; Khao Chakan cave, Khao Chakan Subdistrict, Khao Chakan District, Sa Kaeo Province, eastern Thailand: 13°39'36"N, 102°05'04"E, 120 m altitude, 1 September 2017; Plub Pleung Thong cave, Wang Mai Subdistrict, Wang Sombun District, Sa Kaeo Province, eastern Thailand: 13°26'50"N, 102°13'03"E, 223 m altitude, 31 August 2017. All samples were collected by the author.

Type localities. Huang Po cave, Thung Na Lao Subdistrict, Khon San District, Chaiyaphum Province, north-eastern Thailand: 16°35'25"N, 101°49'28"E, 384 m altitude, 16 October 2017; Pra Hor cave, Tham Yai Subdistrict, Thung Song District, Nakhon Si Thammarat Province, southern Thailand: 08°06'49"N, 99°43'59"E, 101 m altitude, 29 October 2015. Samples were collected by the author.

Etymology. The specific name of the new species, '*thailandica*', refers to Thailand, where the species was collected.

Type specimens. ***Holotype***: one adult female dissected and mounted on one slide, NHMUK 2020.56; ***Allotype***: one adult male dissected and mounted on one slide, NHMUK 2020.57; ***Paratypes***: three adult females and three adult males not dissected and stored in a 1.5 ml microtube with 70% ethanol, NHMUK 2020.58-63; one adult female dissected and mounted on one slide, NPU 2020-003; one adult male dissected and mounted on one slide, NPU 2020-004; three adult females and three adult males not dissected and stored in a 1.5 ml microtube with 70% ethanol, NPU 2020-005.

Description of adult female (holotype). ***Body*** (Fig. 2A) cylindrical, with mean length 510 μm, measured from rostrum to distal rim of caudal rami (n = 5, range 500–530 μm). Cephalothorax with narrow, saddle-shaped, well discernible integumentary window; several sensilla scattered on dorso-lateral surface. Prosome and urosome (segments 1–4) with free posterior margins smooth dorsally; rows of minute spinules dorsally on prosomites 2–4, urosomites 1–2. Genital double-somite completely fused (Figs 2B, 4A), about 0.5× as long as wide, with row of strong dorso-lateral and ventral spinules along distal margin, mid-ventral bell-shaped copulatory pore, duct and receptacles. Urosomite 3 with row of spinules ventrally inserted near posterior edge, row interrupted mid-ventrally. Urosomite

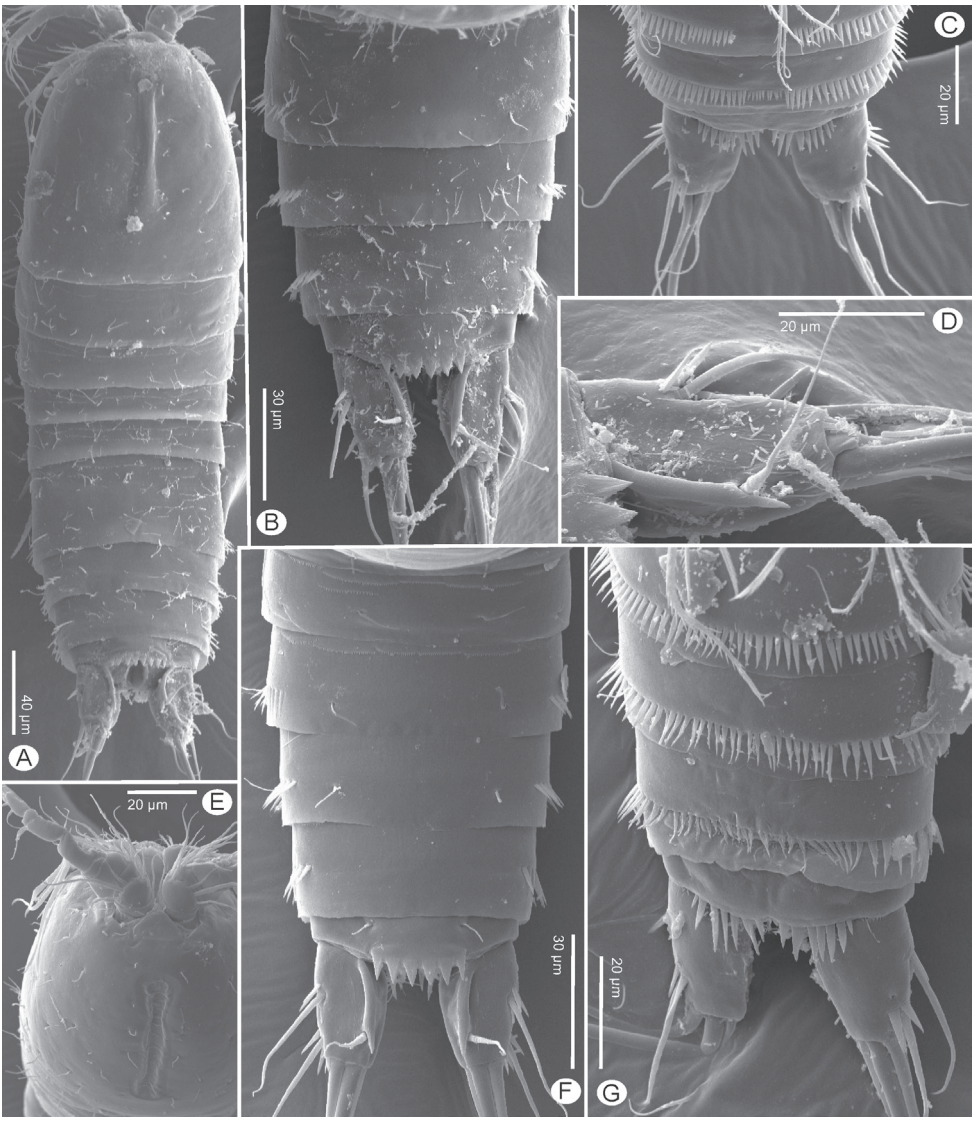

Figure 2. *Attheyella* (*Canthosella*) *thailandica* sp. nov., female (**A–D**) and male (**E–G**): **A** habitus, dorsal view **B** urosome without urosomite 1, dorsal view **C** urosomite 3–5, ventral view **D** caudal ramus, dorsal view **E** cephalothorax, dorsal view **F** urosome without urosomite 1, dorsal view **G** urosome without urosomite 1, ventral view.

4 with continuous row of spinules ventrally near posterior margin. Anal somite (Figs 2B, C, 4A, B) with one pair of sensilla dorsally above base of anal operculum; seven to ten spinules (nine in holotype) ventrally near insertion of each caudal ramus; anal operculum concave and well-developed, with six strong spinules on free posterior margin.

Caudal ramus (Figs 2A–D, 4A, B) conical, about 2.0× longer than wide, inner margin unornamented; longitudinal keel located along dorso-inner margin, ending in

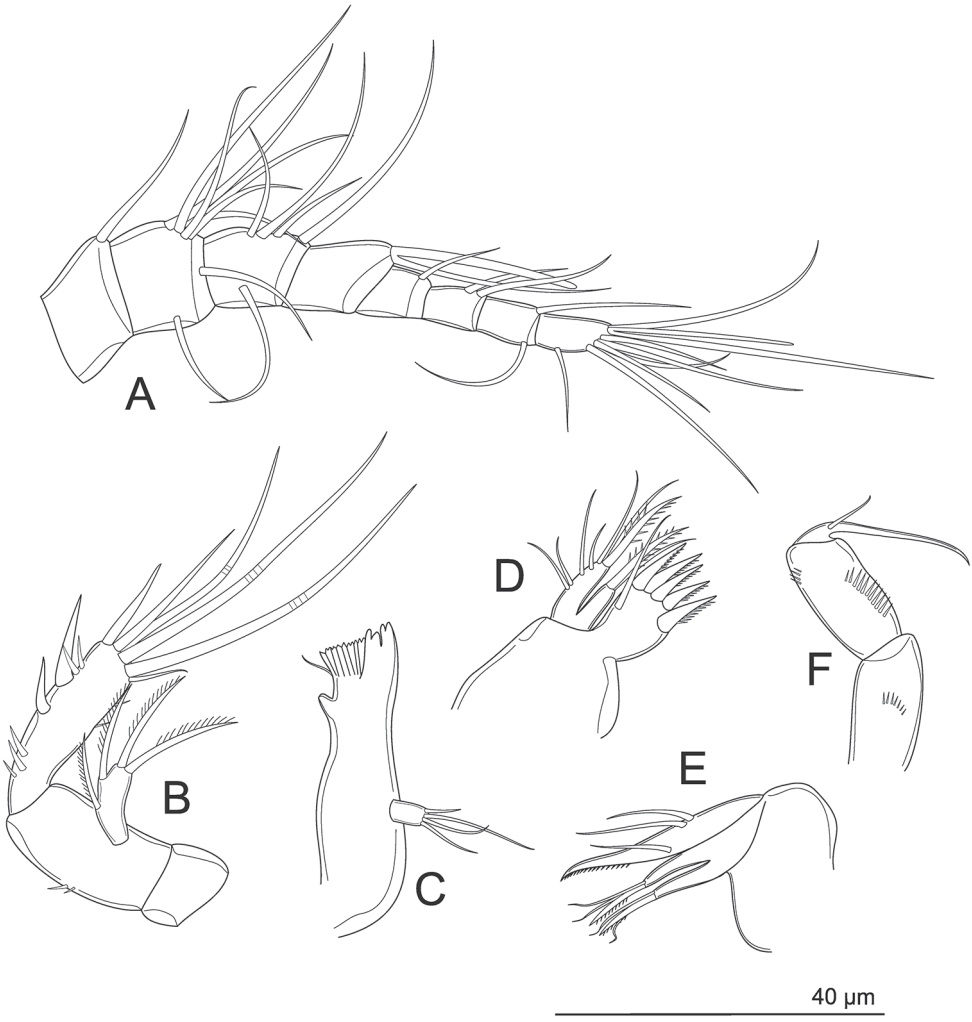

Figure 3. *Attheyella* (*Canthosella*) *thailandica* sp. nov., female: **A** antennule **B** antenna **C** mandible **D** maxillule **E** maxilla **F** maxilliped.

acute tip. Ramus with seven setae (setae I–VII), all smooth, except setae IV and V. Anterolateral accessory seta (I) small, inserted near seta II. Anterolateral seta (II) inserted at two-quarters of ramus, accompanied by two spinules, about 1.5× longer than ramus length. Posterolateral seta (III) inserted at three-quarters of ramus, accompanied by two to three (two in holotype) spinules, about 1.3× longer than ramus length. Outer apical seta (IV) unipinnate, without a breaking plane, about 1.7× longer than ramus length. Inner apical seta (V) longest, bipinnate, without a breaking plane, more than 5.0× longer than ramus length. Inner accessory seta (VI) shortest, about as long as ramus length. Dorsal seta (VII) articulated, inserted on distal end of dorsal keel at three-quarters length of ramus, about 1.6× longer than ramus length.

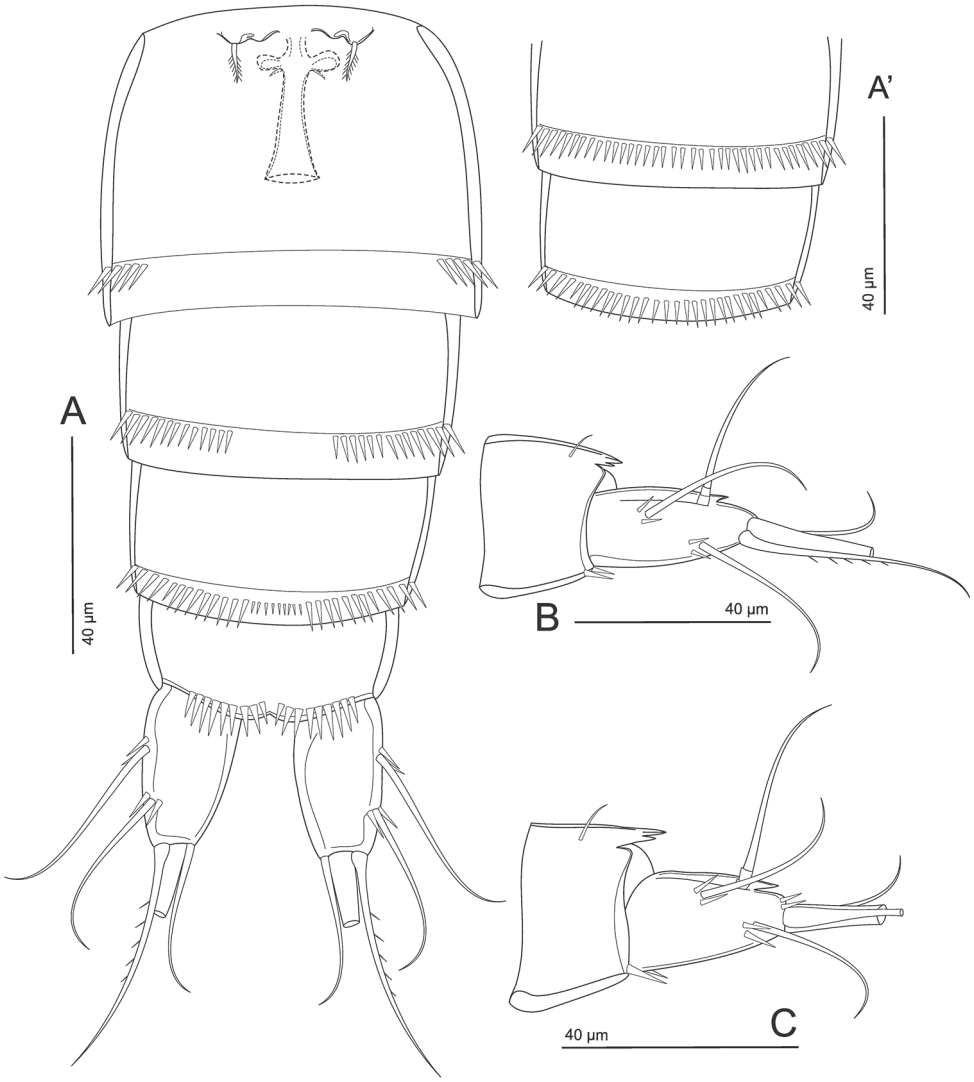

Figure 4. *Attheyella* (*Canthosella*) *thailandica* sp. nov., female (**A, B**) and male (**C**): **A** urosome without urosomite 1, ventral view (**A'** variation on urosomite 3–4, from type locality) **B, C** anal somite and caudal ramus, lateral view.

Antennule (Fig. 3A) eight-segmented, with setal formulae as follow: 1(I), 8(II), 5(III), 1+a(IV) with conjoined bases, 1(V), 2(VI), 2(VII), 7 and 1+a(VIII) with conjoined bases (Arabic and Roman numerals refer to number of setae and segment number, respectively). Aesthetasc cylindrical; both aesthetascs fused at their bases with a seta forming an acrotheck. All setae slender, smooth.

Antenna (Fig. 3B) with small, unarmed coxa. Allobasis elongated, with two spinules on abexopodal margin. Exp one-segmented, with two inner and two apical unipinnate setae. Enp one-segmented, with two outer spines, one apical spine and five

apical setae (two normal and three geniculated setae); inner and outer margins ornamented with two rows of spinules.

Mandible (Fig. 3C) with two large teeth, seven small teeth distally and one lateral seta on gnathobase, with a small knob on the disto-lateral margin. Palp one-segmented, with four apical setae.

Maxillule (Fig. 3D) with five apical spines and one anterior seta on praecoxal arthrite. Coxal endite with one spine and one seta apically. Basis with one spine, two setae apically. Exp and Enp reduced, represented by four lateral setae on basis.

Maxilla (Fig. 3E) with two endites on syncoxa, each endite with three apical setae. Basis elongated, drawn out into a claw, with one proximal accessory seta. Enp reduced and represented by two setae.

Maxilliped (Fig. 3F) with unarmed coxa, ornamented with row of spinules on median surface. Basis with two groups of spinules at inner and outer margins. Enp one-segmented, transformed into claw-like segment, accompanied by one small seta inserted proximally.

P1–P4 with three-segmented Exp and two-segmented Enp. The armature formula is as follows (Arabic and Roman numerals indicate number of setae and spines, respectively; not including spinules):

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

*the formula for the male P3Enp: Enp-1–3 is 0-0, 0-1, 0-2-0, respectively.

P1 (Fig. 5A) basis with short, strong outer spiniform spine and long, slender, smooth inner seta. Enp shorter than Exp; Enp-1 reaching to middle of Exp-2, with one inner unipinnate-tipped seta, ornamented with strong outer spinules and inner setules. Enp-2 with one smooth distal inner seta, one distal inner geniculated seta and one distal outer unipinnate seta; ornamented with strong outer spinules. Exp-1–2 with one distal outer spiniform spine; both segments ornamented with strong outer spinules. Exp-3 with one distal outer spiniform spine, two apical setae (inner seta geniculated, outer one unipinnate) and one distal inner geniculated seta; ornamented with few distal outer spinules.

P2 (Fig. 5B) basis with short, strong outer spine. Enp as long as Exp-1; Enp-1 shorter than wide, unarmed. Enp-2 with two elements apically; outer spine slightly longer than segment; inner seta bipinnate, long, extending beyond Exp. Exp-1 with one enlarged distal outer spine; ornamented with outer spinules. Exp-2 with one distal outer spine and one smooth distal inner seta. Exp-3 with two distal outer spines, two apical setae (inner seta bipinnate, outer one unipinnate) and one smooth inner seta; ornamented with disto-outer spinules.

P3 (Fig. 5C) basis with long, smooth, slender outer seta. Enp as long as Exp-1; Enp-1 shorter than wide, unarmed. Enp-2 with three elements apically; outer spine and innermost seta shorter than segment, subequal in length; middle seta bipinnate,

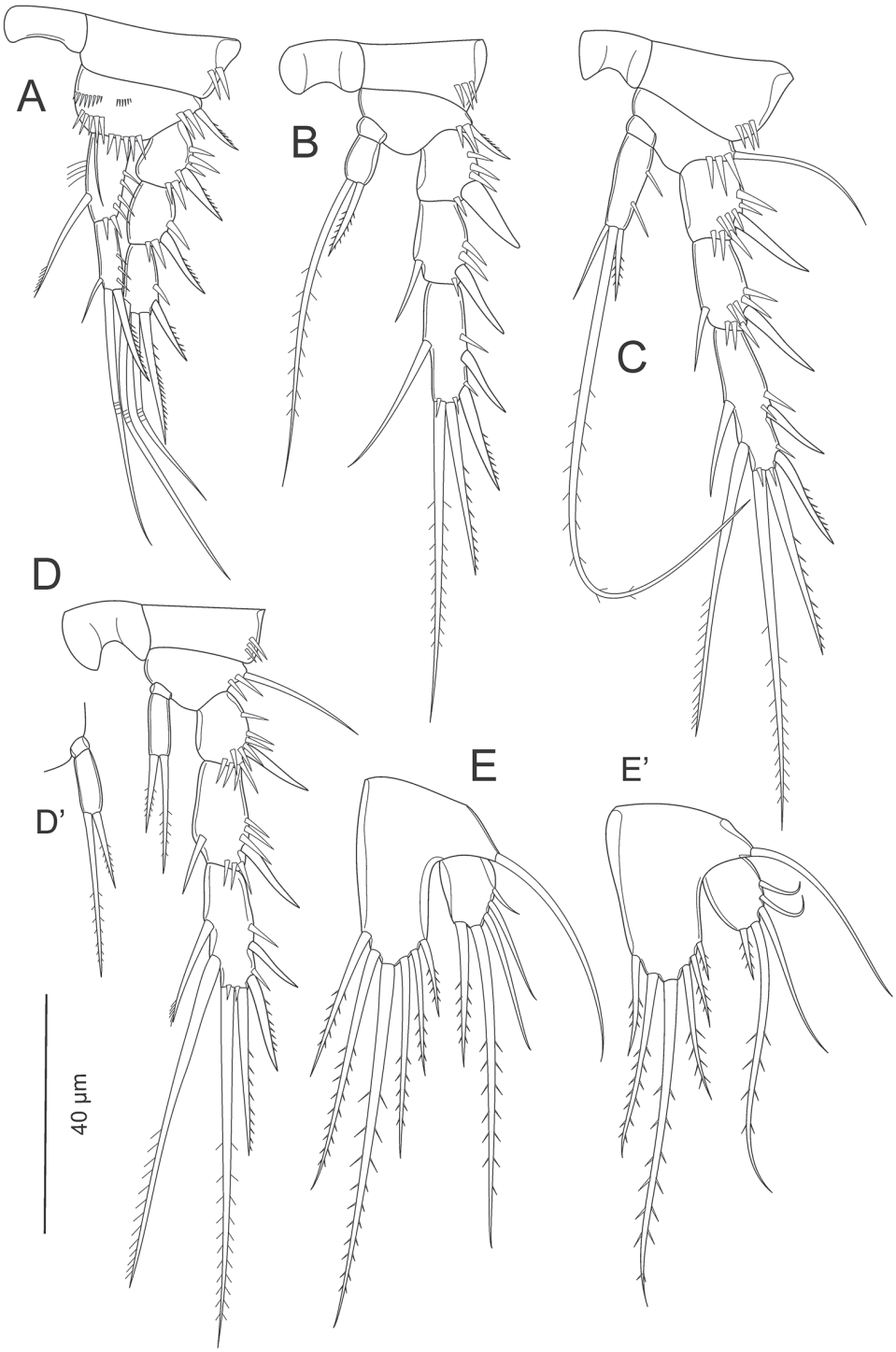

Figure 5. *Attheyella* (*Canthosella*) *thailandica* sp. nov., female: **A** P1 **B** P2 **C** P3 **D** P4 (**D'** variation) **E** P5 (**E'** variation, specimen from Pra Hor cave).

reaching beyond Exp. Exp-1–2 with one distal outer spine, ornamented with spinules along disto-outer margin; Exp-2 with one additional smooth distal inner seta. Exp-3 with two distal outer spines, two apical setae (inner seta bipinnate, outer one unipinnate) and two inner setae (distal seta unipinnate, about 3.0× longer than proximal seta); ornamented with spinules along disto-outer margin.

P4 (Fig. 5D) basis and Exp as in P3, but Exp-3 shorter. Enp as long as Exp-1; Enp-1 small, shorter than wide, unarmed. Enp-2 with two bipinnate setae apically, both longer than segment; inner seta shorter than outer seta.

P5 (Fig. 5E) without ornamentation on surface. Basal seta smooth, slender. Baseoendopod separated from Exp, well-developed, exceeding Exp, with six spiniform setae; third inner seta longest apically; remaining setae decreasing in length to outer and inner margins of Enp. Exp sub-oval, with five setae, second inner seta longest; two innermost setae spiniform; three outer remaining setae smooth and decreasing in length to margin of Exp.

P6 (Fig. 4A) reduced to a single bipinnate seta inserted on the small plate, anterior to the seminal receptacle on the first half of genital double-somite.

Adult females with single egg sac containing 12–15 eggs (holotype: 12 eggs).

Description of adult male (allotype). ***Body*** length 510 μm (Fig. 2E–G) from rostrum to distal rim of caudal ramus, 470–590 μm (n = 5), smaller than female. Prosome (including antenna and mouthparts), anal somite and caudal ramus similar to those of female. Genital somite (Figs 2F, 6B) without row of posterior spinules; urosomites 3–5 with continuous posterior spinules along ventral to dorso-lateral sides.

Antennule (Figs 6–7A) ten-segmented, geniculated between segments 7 and 8. Segment 4 small, beneath segment 3. Setal formulae: 1(I), 7(II), 5(III), 2(IV), 3 and 1+a(V), 2(VI), 1(VII), 0(VIII), 0(IX), 7 and 1+a(X); aesthetasc on segments 5 and 10 fused to the base of seta, forming an acrotheck. All setae smooth.

P1, P4 (Figs 6B, 7D) and P2–3 Exp similar to those in female. P2 (Fig. 7B) Enp slightly longer than Exp-1; Enp-1 shorter than wide, unarmed. Enp-2 with long bipinnate seta apically, with two to three spinules on outer margin. P3 (Fig. 7C) Enp three-segmented; Enp-1 shorter than wide, unarmed. Enp-2 with thin inner apophysis with harpoon-like tip, long, exceeding beyond Exp. Enp-3 with two apical setae; outer seta bipinnate, longer than Exp; inner seta smooth, thin, shorter than segment. P4 (Fig. 7D) Enp two-segmented; Enp-1 small, shorter than wide, unarmed; Enp-2 shorter than Exp-1, about 2.5× longer than wide, with two bipinnate setae apically (outer seta longer than inner one; inner seta slightly longer than segment).

P5 (Figs 6C, 7E) separated from somite, baseoendopod of left and right sides fused medially. Basal seta long, slender and smooth. Baseoendopod separated from Exp, reaching one-half of Exp, with two spiniform setae; inner seta over 4.0× longer than outer seta. Exp with four setae; second inner seta longest, followed by second outer seta, innermost seta and outermost seta, respectively; two inner setae bipinnate, two outer setae smooth.

Variability. (a) The free distal margin of the anal operculum varies from six to ten spinules in females and six to eight spinules in males, a characteristic which is,

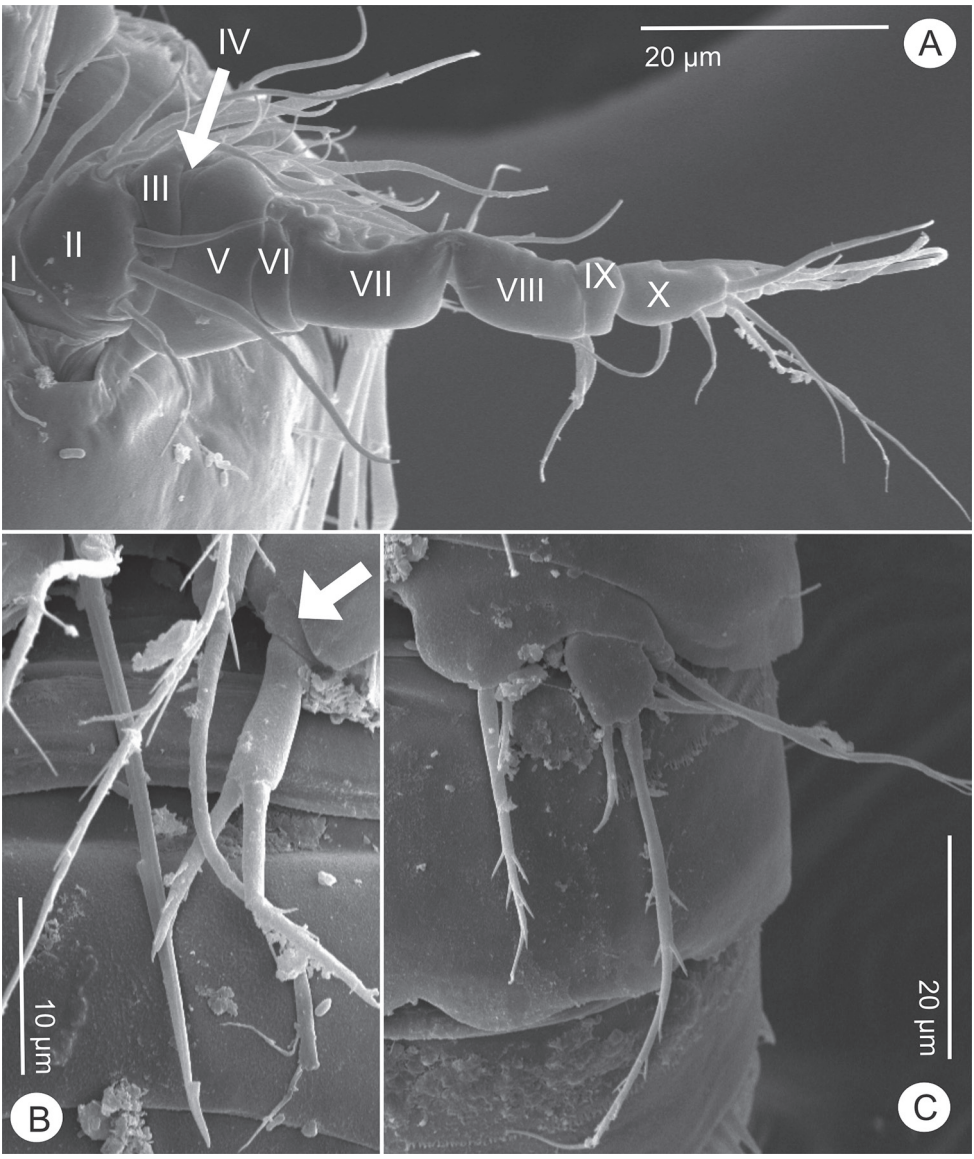

Figure 6. *Attheyella* (*Canthosella*) *thailandica* sp. nov., male: **A** antennule (white arrow indicates segment IV) **B** P4 Enp (white arrow indicates Enp-1) **C** P5 and P6.

perhaps, useless for differentiating amongst species, as mentioned by Gaviria and Defaye (2012). (b) The posterior margin of urosomite 3 in female has a continuous row of ventral spinules (Fig. 4A'; one of the other five examined specimens collected from the type locality). (c) The P2–P3 Enp-2 has a different number of spinules along the outer margin, with two spinules on P2 and three spinules on P3 in females (not shown in Figure; one of the other five female from the type locality) and with two spinules on P2 in males (Fig. 7B'; one of the other five examined specimens collected from the

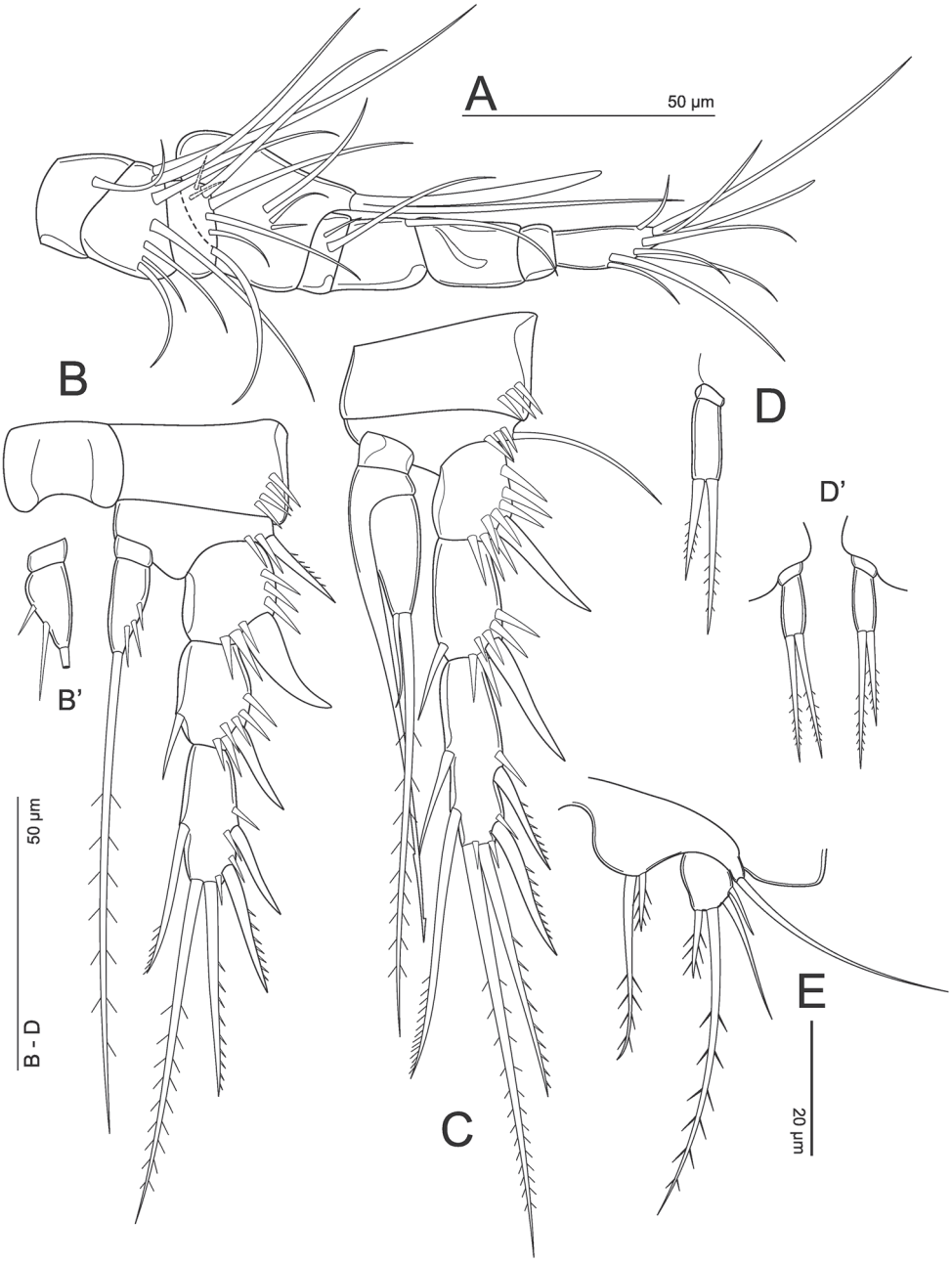

Figure 7. *Attheyella* (*Canthosella*) *thailandica* sp. nov., male: **A** antennule **B** P2 (**B'** variation, specimen from type locality) **C** P3 **D** P4 Enp (**D'** variation, specimen from Pra Hor cave) **E** P5.

type locality). (d) The seta size of the P4 Enp-2 in both sexes, the outer seta about 2.0× longer than the inner seta in female and the inner seta longer than or equal in length to outer seta in male (Figs 5D', 7D'; two of the other five examined specimens collected

from Pra Hor cave). (e) The female P5 in the southern population shows mostly shorter Exp and End setae (Fig. 5E'; two other females from Pra Hor cave).

Ecology. *Attheyella* (*C.*) *thailandica* sp. nov. is usually found in pools of water at cave entrances, where water seeps through soil and plants before flowing down into the cave (see Fig. 1B, D, G). In this research, some samples were found in a pool at the twilight zone of a cave (Fig. 1C) and in a pool in the dark zone of a cave, which was directly fed by the dripping water from stalactites (Fig. 1F). The favourite habitats of *A.* (*C.*) *thailandica* sp. nov. in the present study are likely to be phytotelmata or wet soils, as is already known from most species of *Attheyella* (subgenus *Canthosella*) (Reid 2001). At times, *A.* (*C.*) *thailandica* sp. nov. has been found together with *Bryocyclop muscicola* (Menzel, 1926); both are recognised as stygophile species and are widely distributed in Thailand (Watiroyram 2018; present study).

Discussion

Attheyella (*C.*) *thailandica* sp. nov. has been identified as belonging to the subgenus *Canthosella* Chappuis, 1931 because it shares the following characteristics with other members of the subgenus: posterior margin of somites smooth; caudal ramus longer than wide; antenna with one-segmented Enp; P1–P4 with two-segmented Enp (P1 Enp shorter than Exp, P4 Enp-1 much smaller than other legs of Enp-1); P2–P4 Enp-2 with at least two elements; male P3 with three-segmented Enp bearing an inner apophysis on Enp-2; male P4 Exp-3 without transformed spine; P2–P4 Exp-2–3 with at least one inner seta and one outer spine; female P5 baseoendopod well-developed (reaching beyond Exp) bearing six setae, while Exp bears five setae; male P5 with two setae on baseoendopod and four setae on Exp.

However, *A.* (*C.*) *thailandica* sp. nov. shows morphological differences from other species of the subgenus, related to the ornamentation of the caudal rami. Most species of *Attheyella* (*Canthosella*), except *A.* (*C.*) *antillica* and *A.* (*C.*) *mervini*, carry spinules on the inner margin of the caudal ramus (at least in the female). This margin is bare in both sexes of *A.* (*C.*) *thailandica* sp. nov.

At present, the *Canthosella* subgenus shows two lineages, which include ten American and six SEA species. They can easily be differentiated by the number of armatures on their P2–P4 legs. In contrast to SEA species, all American species, except *A.* (*C.*) *acanthophora*, show a higher number of setae on the P2–P4 Enp-2 in both sexes. Females of these American species have three to four, three to five or two elements on P2–P4, respectively – except for the P4 of *A.* (*C.*) *antillica*, which has one seta. Females of SEA species show one to two setae, one to three setae or one seta on P2–P4, respectively – except for the P4 of *A.* (*C.*) *vietnamica* and *A.* (*C.*) *thailandica* sp. nov., which carry two setae. Additionally, the male P3 Enp-3 of the American species shows two apical setae – except in *A.* (*C.*) *aliena*, which has only one seta. Males of the SEA species have only one seta on the male P3 Enp-3, except for *A.* (*C.*) *thailandica* sp. nov., which has two setae. However, a minute seta located close to a long distal seta on

Table 1. Morphological differences of the new species and the SEA species of the subgenus *Canthosella* Chappuis, 1931.

Species Characteristics	*A.* (*C.*) *fluviatilis*	*A.* (*C.*) *lacustris*	*A.* (*C.*) *muscicola*	*A.* (*C.*) *silvicola*	*A.* (*C.*) *vietnamica*	*A.* (*C.*) *thailandica* sp. nov.
Female						
Anal operculum	With 8 spinules	With 7 spinules	With 7–10 spinules	With 5–8 spinules	With 6 spinules	With 6–10 spinules
Caudal ramus (CU)						
– shape	Conical	Sub-rectangular	Oval	Conical	Conical	Conical
– Length/wide ratio	< 1.3	1.5	> 1.7	1.5	1.5	> 1.7
– Inner median margin	With spinules	With spinules	With spinules	With spinules	With spinules	Without spinules
– Insertion point of seta VII	??	??	??	3/4 of CU	Distal end of CU	3/4 of CU
P2–P4						
– segmentation	2.2.2	2.2.2	2.2.2	2.2.2	2.2.1	2.2.2
– Armature on distal Enp	1.1.1	2.2[1].1	2.2[2].1	2.2.1	2.3.2	2.3.2
P5						
– Baseoendopod	With spinules With 1 apical seta	With spinules With 1 apical seta	Without spinules With 1 apical seta	Without spinules With 1 apical seta	Without spinules With 2 apical seta	Without spinules With 1 apical seta
Male						
– Armature on P3 Enp-3	1	unknown	1	1	1	2
– Armature on P2 and P4 distal Enp	1[3].1	unknown	1.1	1.2[4]	1.2	1.2

[1] = Well (2007) holds that *A.* (*C.*) *lacustris* with three spines and setae, counting two apical setae and one inner seta (though the latter appears to be a spinule rather than a seta). However, Well (2007) does not count those similar elements on the inner margin of the P3 Enp-2 in *A.* (*C.*) *fluviatilis*. Therefore, the present study notes that *A.* (*C.*) *lacustris* has two apical elements. [2] = *A.* (*C.*) *muscicola* has one apical seta and one outer seta, in contrast to *A.* (*C.*) *lacustris* with two apical setae. [3] = Well (2007) holds that *A.* (*C.*) *fluviatilis* has three spines and setae, probably by counting one apical seta and two small inner setae as described in Chappuis (1931). However, Well (2007) does not generally count armatures (like spinules) on the inner margin of *Attheyella* species described in Chappuis (1931), and the male P2 Enp-2 usually has the same number of armatures on its segment (or fewer) than females. Therefore, the present study notes that *A.* (*C.*) *fluviatilis* has one apical seta. [4] = An original description notes, in the leg formula, that the male P4 of *A.* (*C.*) *silvicola* has a two-segmented Enp, but it is presented as one-segmented Enp in the related Figure. Well (2007) notes that the male has a one-segmented Enp.

the male P3 Enp-3 could easily have been overlooked in previous descriptions (P.H.C. Corgosinho, personal communication).

Amongst the six SEA species, *A.* (*C.*) *thailandica* sp. nov. is most similar to *A.* (*C.*) *vietnamica* for the following reasons (Table 1). (a) The P3 Enp-2 of a female in these species bears three elements, while it only bears two elements in *A.* (*C.*) *lacustris*, *A.* (*C.*) *muscicola* and *A.* (*C.*) *silvicola* and has only one seta in *A.* (*C.*) *fluviatilis*. (b). The P4 Enp in both sexes bears two apical setae, while it bears only one seta in all other individuals, except the male of *A.* (*C.*) *silvicola*, which has two setae. (c) The P2 Exp-1 in both sexes of these species has an enlarged outer spine, but this spine is normal in the other species; this characteristic is presumed to be a synapomorphy, which can be used to define closely-related species.

Nevertheless, *A.* (*C.*) *thailandica* sp. nov. also shows strong morphological differences from *A.* (*C.*) *vietnamica* in the following aspects. (a) The P4 Enp is two-segmented in both sexes of *A.* (*C.*) *thailandica* sp. nov. (Fig. 6B), but one-segmented in *A.* (*C.*) *vietnamica*. (b) The female caudal ramus of the new species has no spinules on the inner margin, but these spinules are present in *A.* (*C.*) *vietnamica*. (c) The new species has a

dorsal seta located at three-quarters of the length of the caudal ramus, while this seta is located almost at the distal end in *A.* (*C.*) *vietnamica*. d) The male of the new species has two setae on the P3 Enp-3, while the male of *A.* (*C.*) *vietnamica* has one seta. e) *A.* (*C.*) *thailandica* sp. nov. exhibits a reduced Enp (especially P3) in both sexes, which are shorter in *A.* (*C.*) *vietnamica*. f).The morphology of P5 differs in several ways.

In the female of *A.* (*C.*) *thailandica* sp. nov., the innermost seta of the Exp is located at the (sub)distal margin, while it is obviously located at the inner margin in *A.* (*C.*) *vietnamica*. The new species has a third inner seta located at the apex of the baseoendopod, while this third inner seta and the third outer seta are located apically in *A.* (*C.*) *vietnamica*. The male of *A.* (*C.*) *thailandica* sp. nov. has a more developed P5 baseoendopod, in contrast to *A.* (*C.*) *vietnamica*, whose P5 baseoendopod reaches to one-third of the Exp. Thus, *A.* (*C.*) *thailandica* sp. nov. can be established as its own taxonomical unit new to science.

A key to worldwide species of the subgenus *Canthosella* Chappuis, 1931

Females

(female unknown for *A.* (*C.*) *siolii* and *A.* (*C.*) *striblingi*)

1 P4 Enp one-segmented **2**
– P4 Enp two-segmented **4**
2 Caudal ramus with inner margin produced into curved process ..... ***A.* (*C.*) *acanthophora***
– Caudal ramus with normal inner margin **3**
3 P2 Enp-2 with two seta and spine ..... ***A.* (*C.*) *vietnamica***
– P2 Enp-2 with four setae and spine ..... ***A.* (*C.*) *kalima***
4 P4 Enp-2 with one seta **5**
– P4 Enp-2 with 2–3 setae **9**
5 Caudal ramus with spinules along dorso-inner margin **6**
– Caudal ramus without spinules along dorso-inner margin ... ***A.* (*C.*) *antillica***
6 P2 Enp-2 with one seta ..... ***A.* (*C.*) *fluviatilis***
– P2 Enp-2 with two seta and spine **7**
7 P3 Enp-2 with one apical seta and one outer seta ..... ***A.* (*C.*) *muscicola***
– P3 Enp-2 with two apical setae **8**
8 P5 baseoendopod with spinules along inner margin ..... ***A.* (*C.*) *lacustris***
– P5 baseoendopod without spinules along inner margin ..... ***A.* (*C.*) *silvicola***
9 P4 Enp-2 with two seta and spine **10**
– P4 Enp-2 with three setae and spine ..... ***A.* (*C.*) *pilagaensis***
10 P2 Enp-2 with two seta and spine ..... ***A.* (*C.*) *thailandica* sp. nov.**
– P2 Enp-2 with four setae and spine **11**
11 P3 Enp-2 with four setae and spine **12**
– P3 Enp-2 with five setae and spine **13**

12 Caudal ramus with spinules along dorso-inner margin.....***A.* (*C.*) *vera***
– Caudal ramus without spinules along dorso-inner margin.....
.....***A.* (*C.*) *mervini***
13 Caudal ramus with dorsal seta at 1/2 of length.....***A.* (*C.*) *chocoensis***
– Caudal ramus with dorsal seta at 3/4 of length.....***A.* (*C.*) *aliena***

Males

(male unknown for *A.* (*C.*) *kalima*, *A.* (*C.*) *lacustris* and *A.* (*C.*) *pilagaensis*)

1 P5 baseoendopod with two setae.....**2**
– P5 baseoendopod unarmed.....**7**
2 Caudal ramus with inner margin produced into curved process.....
.....***A.* (*C.*) *acanthophora***
– Caudal ramus with normal inner margin.....**3**
3 P4 Enp with one apical seta.....**4**
– P4 Enp with two apical seta and spine.....**5**
4 P2 Enp-2 with one seta.....***A.* (*C.*) *muscicola***
– P2 Enp-2 with three setae and spine.....***A.* (*C.*) *fluviatilis***
5 P3 Enp-3 with one seta.....**6**
– P3 Enp-3 with two setae.....***A.*(*C.*) *thailandica* sp. nov.**
6 P4 Enp with two subequal apical setae.....***A.* (*C.*) *silvicola***
– P4 Enp with outer apical seta longer than 2.0× that of inner apical seta.....
.....***A.* (*C.*) *vietnamica***
7 P4 Enp with one apical seta.....***A.* (*C.*) *antillica***
– P4 Enp with two apical seta and spine.....**8**
8 P2 Enp-2 with three setae and spine.....**9**
– P2 Enp-2 with four setae and spine.....**10**
9 P3 with two-segmented Enp.....***A.* (*C.*) *mervini***
– P3 with three-segmented Enp.....***A.* (*C.*) *siolii***
10 Caudal ramus with spinules along dorso-inner margin.....**11**
– Caudal ramus without spinules along dorso-inner margin.....**12**
11 P3 Enp-3 with one apical seta.....***A.* (*C.*) *aliena***
– P3 Enp-3 with two apical setae.....***A.* (*C.*) *vera***
12 Caudal ramus slightly longer than wide.....***A.* (*C.*) *chocoensis***
– Caudal ramus about 2.0× longer than wide.....***A.* (*C.*) *striblingi***

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Living in isolation for almost 40 years: molecular divergence of the 28S rDNA and COI sequences between French and Polish populations of the cave beetle *Speonomus normandi hydrophilus* (Jeannel, 1907)

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Abstract

The paper gives the results of the first studies on the molecular divergence between native and non-native populations of *Speonomus normandi hydrophilus* (Jeannel, 1907). This species is endemic to Massif Arize in the Central Pyrenees (France), and represents highly specialised organisms that live underground. In 1982, one hundred specimens of *S. normandi hydrophilus* had been experimentally introduced into the Dzwonnica Cave (Poland). Since then, a numerous population has developed in the Towarna-Dzwonnica cave system, and the neighbouring Cabanowa Cave. After almost 40 years of isolation between native and non-native populations, the genetic variations were examined using the COI and 28S rDNA genes. Analyses of the haplotypes of 28S showed one common haplogroup, which confirms the origin of the Polish group. The differentiation of haplotypes for the COI marker was high for both the French and Polish populations. Altogether 18 haplotypes of this marker have been detected, 12 in the French population and 9 in the Polish. However, only a portion of the haplotypes is shared between the native and introduced population.

Keywords

Coleoptera, intentionally introduced species, mitochondrial DNA, nuclear DNA, population genetics, troglobites

Introduction

Recently, due to human activities, such as high-speed transport systems and increased shipping, insect species are moving across ecosystems, countries, and continents (e.g., Gogala 2003; Harmat et al. 2006; Lis et al. 2008; Lis and Whitehead 2019). Most species are introduced accidentally to new areas, including the Asian tiger mosquito *Aedes albopictus* (Skuse, 1895), spotted lanternfly *Lycorma delicatula* (White, 1845), and brown marmorated stink bug *Halyomorpha halys* (Stål, 1855). However, some species have been intentionally introduced for various reasons. Several bee species have been introduced as honey producers, i.e., the eastern honey bee *Apis cerana* Fabricius, 1793 from Asia to New Guinea; the honey bee *Apis mellifera* Linnaeus, 1761 from Europe to North America; and the East African lowland honey bee *A. mellifera scutellata* Lepeletier, 1836 from Africa to, for an example, Brazil. Biological pest control is another reason for introducing species into a new environment. For example, the ladybird *Rodolia cardinalis* (Mulsant, 1850) was deliberately transferred from Australia to California to fight the cottony cushion scale *Icerya purchasi* Maskell, 1878, the citrus tree pest (Tomalak and Sosnowska 2008). Another example is the introduction of a butterfly *Cactoblastis cactorum* (Berg, 1885) from Mexico to Australia to combat the prickly pear. The caterpillar of this butterfly species successfully destroyed the unwanted plant (Tomalak and Sosnowska 2008). Species that were accidentally or intentionally introduced to a new environment, created sometimes new populations. They are an important topic in studies of genetic variation, genetic drift, and rapidness of genome changes (Estoup and Clegg 2003; Zepeda-Paulo et al. 2010).

Although the role of isolation in natural populations of cave-dwelling invertebrates has been estimated several times (e.g. Allegrucci et al. 1997; Strecker et al. 2003; Ribera et al. 2010; Kruckenhauser et al. 2011; Bradic et al. 2012; Pérez-Moreno et al. 2017), there exists only a few study on populations intentionally moved from their natural habitat to be implemented in a quite new, non-native cave habitat (Coiffait 1968; Tercafs and Brouwir 1991; Juberthie and Gers 1992; Dethier et al. 2002). Nevertheless, no data on the genetic divergence in intentionally introduced populations isolated for decades have yet been provided.

In the present paper, we provide results on the genetic divergence between native and introduced populations of a cave beetle *Speonomus normandi hydrophilus* (Jeannel 1907), which was intentionally transferred from the French Central Pyrenees to a cave in Poland (Skalski 1994).

S. normandi hydrophilus is a troglobitic species endemic to the Central Pyrenees in France and native populations have been studied by Crouau-Roy (1986, 1990) and Crouau-Roy et al.(1992) in both ecological and genetic aspects. Specimens of the analyzed beetle were brought from France to Poland in 1982 by Skalski and experimentally introduced into the Dzwonnica Cave: the Kraków-Częstochowa Upland, Poland (Skalski 1994). That experiment aimed to study an adaptation of highly specialized species into a new ecosystem, i.e. different biocenosis, climate conditions, and sediments (Skalski 1994).

Materials and methods

Study area

The Bastardech cave "Gouffre de Bastardech" (Ariège) is a small cave with a steep entrance that lies in the Central Pyrenees (France) at an altitude of 630 m a.s.l. (Fig. 1). Its narrow passages are sloping and form two floors, and the walls and floors are covered by sediment (Coiffait 1968).

The Dzwonnica cave, where specimens of *S. normandi hydrophilus* were intentionally introduced, lies in the northern part of the Kraków-Częstochowa Upland, Poland (Fig. 1). As in the native cave, it is also a small cave with a horizontal, narrow passage. It is connected to another cave, the Towarna Cave by a small passage; the latter is situated at 330 m a.s.l (Zygmunt 2013).

Organism

Speonomus normandi hydrophilus (Jeannel 1907) (Coleoptera: Leiodidae), belongs to the tribe Leptodirini, which contains about 900 species (Jeannel 1924; Perreau 2004; Cieslak et al. 2014). This subspecies is endemic to the western part of the Arize Massif in the Central Pyrenees and is a highly specialized organism that lives underground in caves and the mesovoid shallow substratum (Crouau-Roy 1992).

In 1982, Skalski collected 50 males and 50 females of this species from the Bastardech cave in the French Pyrenees. These specimens of both sexes were separately

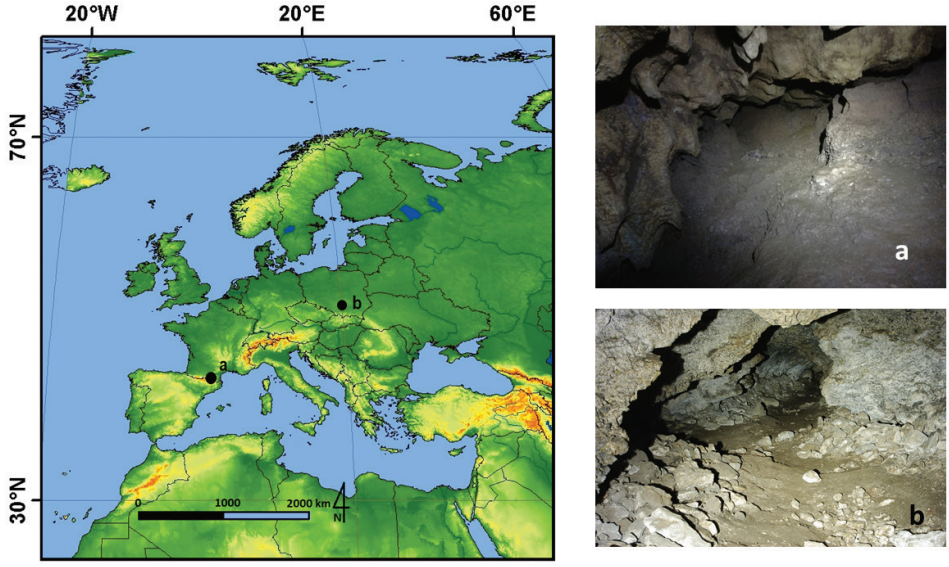


Figure 1. Map of Europe showing the location of sampling sites **a** the Bastardech Cave (France) and its interior **b** the Towarna & Dzwonnica Caves (Poland) and its interior.

inserted into a thermos with ice transported to Poland in two boxes containing cheese. Subsequently, they were introduced to the deep zone of the Dzwonnica cave in Poland. After 12 years, Skalski published that specimens of *S. normandi hydrophilus* had adapted to a new environment and descendants were frequently observed near the place of introduction (Skalski 1994). Nowadays, abundant populations of this species live in three closely situated caves in the Towarne Mts., i.e., the Dzwonnica and the Towarna caves (Klasiński 2006; Dumnicka and Płotek 2013; Kocot-Zalewska 2016) and the Cabanowa cave (unpublished data). The Dzwonnica and Towarna Caves are connected by a passage. The connection between Dzwonnica and Cabanowa is not available for humans but undoubtedly there are rocks crevices penetrable for small organisms.

Specimen sampling

A total of 100 individuals of *S. normandi hydrophilus*, 50 specimens in the Towarna-Dzwonnica caves system (Poland, 49°14'11"N, 19°51'52"E) and 50 specimens in Bastardech Cave (France, 42°57'38"N, 1°14'30"E) were collected by direct searching. In the Bastardech Cave, one week before the sampling, cheese was put onto the cave floor as bait. In the Towarna-Dzwonnica cave system specimens were collected directly without any bait. All samples were stored in 96% ethanol at -20 °C.

Molecular markers

The 549 bp COI fragment was amplified using primer pair Pat and Jerry (Simon et al. 1994). The studied nuclear marker was a 549 bp internal fragment of a large ribosomal unit (LSU), which was amplified using the Ka and Kb primer pair (Ribera et al. 2010). Primers pairs are listed in Table 1.

DNA extraction and amplification

The total genomic DNA was extracted from thorax muscle tissues using a Sherlock AX kit (A&A Biotechnology) following the manufacturer protocol PCR amplifications were performed in a volume of 50 µl using ready-to-use mix PCR Mix Plus (A&A Biotechnology) and primer pairs.

The PCR reaction for the 28S rDNA consisted of initial denaturation for 3 min at 95 °C, followed by 34 cycles of 15 sec at 94 °C, 30 sec at 50 °C, and 40 sec at 72 °C. The final elongation step was 7 min at 72 °C. PCR amplification of the COI consisted

Table 1. Primers used for amplification and sequencing of the 28S and COI genes.

Marker	Primer name	Sequence (5' → 3')	Source
28S	Ka	ACACGGACCAAGGAGTCTAGCATG	Ribera et al. (2010)
	Kb	CGTCCTGCTGTCTTAAGTTAC	Ribera et al. (2010)
COI	Jerry	CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
	Pat	TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)

of initial denaturation for 3 min at 94 °C, followed by 34 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 45 sec at 72 °C, and final elongation for 10 min at 72 °C.

The purification of amplicons and sequencing were performed at A&A Biotechnology, Gdynia, Poland. All sequences were obtained in the forward and reverse directions. The obtained sequences were checked using the BLAST tool (https://blast.ncbi.nlm.nih.gov/) to detect possible contamination. All received sequences showed high similarities to sequences of *S. normandi hydrophilus* already deposited into GenBank. The sequences were deposited in GenBank under accession numbers: MW187125–MW187145 and MW187545–MW187565 for the 28S gene and MW187329–MW187352 and MW187448–MW187476 for the COI gene.

Molecular analyses

Each obtained sequence was manually edited for accuracy using FinchTV v. 1.4.0 (Geospiza Inc.) and aligned using ClustalW (with default parameters) in the MEGA X software (Kumar et al. 2018). The ends of sequence reads were trimmed to avoid the influence of missing data resulting from incomplete sequences. The COI sequences were translated to protein sequences to check stop codon in the middle of the sequences and frameshifts. The number of haplotypes (H), haplotype diversity (*hd*), number of segregating sites (S), number of variable sites (V), nucleotide diversity (ND), total number of nucleotide differences (TM), and average number of nucleotide differences (*k*) were computed with DnaSP v.6 (Rozas et al. 2017).The genetic distance between individuals was calculated using a p-distance model in MEGA X software (Kumar et al. 2018).Tajima's D (Tajima 1989) test was performed using DnaSP v.6 (Rozas et al. 2017). The haplotype networks for each gene were constructed using a Median-Joining Network method (Bandelt et. al. 1999) in PopART v.1.7 software (Leigh and Bryant 2015). The visualization of the relationships among haplotypes contains two additional sequences for each gene obtained from GenBank: HG915551.1, AM229403.1 for 28S and HG915401.1, LN849271.1 for COI, respectively.

Results

We successfully obtained 42 sequences (549 bp) of the 28S rDNA and 53 sequences (549 bp) of the COI. In total, 18 haplotypes of the COI were identified among the analyzed specimens. The native Pyrenean population was characterized by 12 different haplotypes, whereas the Polish had nine. Overall, the haplotype diversity of the COI gene (hd) for all specimens was 0.8287 and was 0.8005 for the native French population and 0.8442 for the Polish one.

The average number of nucleotide differences (k) was 5.492, 5.428, and 5.558 for all specimens, the French, and the Polish population, respectively. The total number of the mutations (TM) of all analyzed sequences was 25 and was 21 and 17 for the native Pyrenean population and the Polish one, respectively. All obtained genetic diversity indices of the COI gene are presented in Table 2.

In the case of the nuclear 28S rDNA subunit, three haplotypes were identified, among them, three were detected in the Pyrenean population and a single haplotype within the Polish one. Overall, the haplotype diversity of the 28S rDNA (*hd*) for all analyzed specimens was 0.180. The native French population was characterized by a diversity value of 0.338, while the introduced population had no haplotype diversity. The average number of nucleotide differences (k) was 0.184 for both populations and was 0.352 for the French population. There were no nucleotide differences detected within the Polish population of the 28S rDNA marker. The total number of mutations (TM) was two in the Pyrenean population. No mutations were detected among the population introduced to the Dzwonnica cave in Poland. All obtained genetic diversity indices of the nuclear 28S rDNA marker are presented in Table 3.

The genetic distance between populations for nuclear 28S rDNA and mitochondrial COI markers are presented in Table 4.

The haplotype network generated for 28S gene demonstrate presence of one haplogroup. The most common haplotype (Hap 1) is present in all specimen from Poland, three from France and specimens sequences obtained from GenBank HG915551.1 and AM229403.1 (specimens from Riverenert, not far from Bastardech cave).

Table 2. Genetic diversity indices of the COI gene calculated for the studied specimens.

Population	ns	H	*hd*	S	V	ND	TM	*k*	Tajima's D:
All	53	18	0.8287	25	25	0.01000	25	5.492	-0.00998
France	29	12	0.8005	21	21	0.00989	21	5.428	0.00989
Poland	24	9	0.8442	17	17	0.01012	17	5.558	0.79194

ns – number of analyzed sequences; H- number of haplotypes; *hd* - haplotype diversity, S- number of polymorphic (segregating) sites; V – number of variable sites; ND - nucleotide diversity; TM - total number of mutations; *k* - the average number of nucleotide differences.

Table 3. Genetic diversity indices of the 28S marker calculated for the studied specimens.

Population	ns	H	*hd*	S	V	ND	TM	*k*	Tajima's D:
All	42	3	0.180	2	2	0.00033	2	0.184	-1.12813
France	21	3	0.338	2	2	0.00064	2	0.352	-0.84329
Poland	21	1	0.000	0	0	–	0	0.000	*

ns – number of analyzed sequences; H- number of haplotypes; *hd* - haplotype diversity, S- number of polymorphic (segregating) sites; V – number of variable sites; ND - nucleotide diversity; TM - total number of mutations; *k* - average number of nucleotide differences. Tajima's D statistical significance: Not significant, P > 0.10.*- The DnaSP was unable to conduct Tajima's test.

Table 4. Average pairwise genetic distance, based on the p-distance method, of 28S and COI sequences.

Marker	Population	Overall average pairwise genetic distance
28S	France	0.0009715
	Poland	0.0000000
	All	0.0003343
COI	France	0.0098881
	Poland	0.0101238
	All	0.0100037

The haplotype network analysis for COI gene reveals two distinct haplogroups separated by seven mutational changes. The largest haplogroup (Haplogroup 1) contains 14 different haplotypes and is divided into two subgroups. The subgroup 1 contains the most common haplotype (Hap 2) which is shared among ten specimens from France, seven from Poland and one specimen obtained from GenBank (HG915401.1), from Riverenert. The second subgroup of Haplogroup 1 contains haplotype (Hap 8) which is shared among six specimens from Poland and one from France. The second haplogroup contains the second most common haplotype (Hap 3) which is shared among nine specimens from France and four from Poland. The haplotype Hap19 is referred to the sequence obtained from GenBank LN849271.1, from a cave (Ruisseau souterrain d´Aulot, Saint Girons) located ca 7 km from Bastardech cave in a straight line.

Discussion

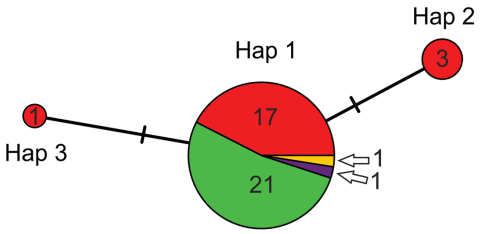
The introduction of *S. normandi hydrophilus* to a new environment is not the first experiment of its kind. In the 20th century, many cavernicolous species were experimentally introduced to new caves (Coiffait 1968; Bouillon and Hubart 1982; Tercafs and Brouwir 1991), including three species of the genus *Speonomus* (i.e., *S. diecki*, *S. stygius*, *S. longicornis*). However, this paper gives the first results of genetic variation between the native and introduced populations among *Speonomus* species.

The molecular analyses were carried out using one mitochondrial and one nuclear marker, similar to other studies on genetic variation between closely related species (for example, Niemiller et al. 2008; Faille et al. 2013; Rizzo et al. 2013; Cieslak et al. 2014; Pérez-Moreno et al. 2017) and among one species (for example, Faille et al. 2015; Boyd et al. 2020).

The results obtained in this study showed that nuclear 28S is less diverse than mitochondrial COI, which is unsurprising. Three haplotypes of the 28S marker were detected in the French population but only one in the Polish. Analyses of the haplotype network (Fig. 2) showed one common haplogroup, which confirms the origin of the Polish group.

The variations of haplotypes for the COI marker was high for both the French and Polish populations (0.80 and 0.84, respectively). The most common haplotypes were Hap 2 and Hap 3, which were shared between specimens from both populations (Fig. 3) and formed two major haplogroups.

The high variability in the French population might be explained by the fact that *Speonomus normandi hydrophilus* inhabits caves and the mesovoid shallow substratum in an area of c.a. 30 × 40 km (Crouau-Roy et al. 1992). Many local subpopulations exist, which are not entirely isolated. Thus, some gene flow, although limited, could be observed (Crouau-Roy 1986). Crouau-Roy (1986) findings are reflected in our result of haplotype network for the COI marker (Fig. 3), in which two major groups of haplotypes are visible.



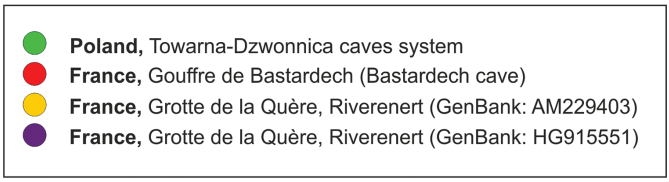


Figure 2. Median-joining haplotype network for 28S sequences. Different colours correspond to geographic origin and circle size is proportional to the number of individuals with the same haplotype. The number of individuals with a specific haplotype is in a circle. Hatch marks along edges represent the number of mutations between nodes.

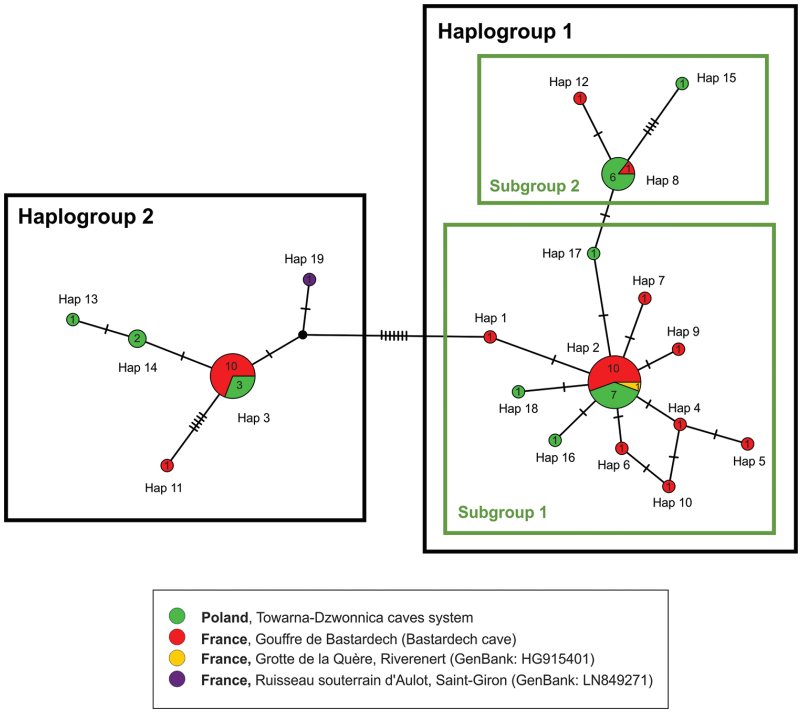


Figure 3. Median-joining haplotype network for COI sequences. Different colours correspond to geographic origin and circle size is proportional to the number of individuals with the same haplotype. The number of individuals with a specific haplotype is in a circle. Hatch marks along edges represent the number of mutations between nodes.

The high diversity of the COI marker among the native population indicates that founder specimens transplanted to Polish cave were also diversified. Currently, the Polish population shares only a portion of the haplotypes with the native French population. Moreover, the presence of specific Polish population haplotypes may be the result of adaptation. Microevolutionary changes in the mtDNA could be stress-induced (Kranthi et al. 2006), and conditions in a new environment can be considered stress factors, which might have been expressed in the mtDNA sequences. For example, air temperature could have had an important influence on current haplotype diversity. The research provided by Crouau-Roy et al. (1992) about populations occurrences in caves and mesovoid shallow substratum (MSS) indicated that caves populations live in a more stable temperature between 7.5 to 12 °C, while MSS populations inhabit areas with a broader temperature spectrum. However, in both population groups, seasonal fluctuations of abundance were observed. Cave specimens were observed throughout the year, whereas the MSS environment were not observed in some months (Crouau-Roy et al. 1992). Stable temperature is present in the Towarna Cave (Kocot-Zalewska 2016). During the research conducted in this cave, 88.3% of the collected specimens inhabited a space with a temperature amplitude 1.8 °C, 11% lived in a place with a temperature amplitude 8.2 °C, and only 0.7% of the collection was caught in place with amplitude 14.6 °C (Kocot-Zalewska 2016). On the other hand, temperature changes may not be the significant survival factor for subterranean organisms. However, the range of tolerated temperatures is limited (Rizzo et al. 2015). Thus, environmental change could lead to the survival of just part of the introduced specimens to the Dzwonnica Cave or it could be a lack of reproduction success among part of the population. Moreover, the observation of the high variability of the COI marker also follows the inheritance of the maternal mitochondrial genome and its fast mutation, which affects the rapidly spreading changes in the native and non-native populations (Kondo et al. 1990; Skibinski et al. 1994; Zhang and Hewitt 2003).

The total number of mutations in the COI was 25, and it was almost equal in both the French and Polish groups (21 ver. 17, respectively). Tajima's D statistic is positive when there is an excess of high-frequency mutations, for example, after a population contraction or under balancing selection. Tajima's D statistic is negative when there is an excess of low-frequency mutations, for instance, after a population expansion, a recent selective sweep, weak negative selection, or when samples come from an admixed population (Stajich and Hahn 2005). In this study, the Tajima's D test was positive for the COI marker results. It had a high value among the Polish group, which might be an effect of high-frequency mutations after a reduction in population (Stajich and Hahn 2005; Jackson et al. 2015). This hypothesis is strongly supported by a small number of specimens who founded a new population (just one hundred individuals) in a new environment. However, this assumption was based only on the COI marker. No polymorphism was found in the 28S rDNA sequences of the Polish population, and therefore, the Tajima test could not be used.

With the observed lower number of haplotypes in the introduced population, the obtained results were not surprising. In the research of Li et al. (2011), five populations

of silver carp (*Hypophthalmichthys molitrix*) were compared between native (China) and introduced (USA, Hungary) habitats. All native populations had a higher variety of haplotypes in comparison to non-natives. Compared to another study on introduced species, a low genetic variation was observed. The amphibian species *Eleutherodactylus johnstonei* (Barbour 1914) was introduced from the Caribbean to Colombia 25 years ago. The recent study, based on two mitochondrial markers, has shown very low genetic variations within the implanted population. There was no variation in 12S rRNA and three haplotypes in the D-loop marker were detected. Interestingly, the two recorded haplotypes diverged by one and two mutations from the most common haplotype (Leonhardt et al. 2019).

Gene flow is not always strongly limited between subterranean populations. In a cave cricket from the Rhaphidophoridae family, *Dolichopoda schiavazzii* Capra, 1934, twelve populations from caves and man-made subterranean environments in the Apennine Peninsula, as well as on the islands Elba and Pianosa, were studied (Allegrucci et al. 1997). Gene flow was not observed between the island and continental populations; however, between the two populations that occur in the caves on the Monte Argentario promontory, substantial gene flow was noticed. It is believed that genetic variation was influenced more by the degree of isolation and dependence on climatic factors than geographical distance (Allegrucci et al. 1997). A similar conclusion was observed in fish populations from the Cyprinidae family *Garrabarreimiae* Fowler & Steinitz, 1956 inhabiting cave water and populations inhabiting surface water in Oman (Kruckenhauser et al. 2011). A substantial genetic difference between the populations was explained by a founder effect in the cave. However, some gene flow was observed, which was explained by the occasional contact between the subterranean and surface populations. On the other hand, the results obtained by Boyd et al. on cave beetles (2020) suggest that isolation by distance influences genetic structure.

The obtained results support a widely accepted theory that underground living species, as well as introduced ones, have a lower genetic variation in comparison to ancestral populations. For a better understanding of changes that have recently occurred in the introduced *Speonomus normandi hydrophilus*, it seems necessary to use microsatellite markers or NGS technology (Fumagalli et al. 2013, 2014) in further studies. These techniques and approaches allow tracking recent changes in the genome resulting, for example, from changes in the population size (Garza and Williamson 2008), colonisation of a new area (Estoup and Clegg 2003; Zepeda-Paulo 2010), or gene flow (Bradic et al. 2012).

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RESEARCH ARTICLE

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Two new stygobiotic species of *Horatia* Bourguignat, 1887 (Hydrobiidae) from Croatia

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Abstract

In this paper we describe two new species of the freshwater snails of genus *Horatia*. A new stygobiotic species of *Horatia* Bourguignat, 1887 is described from Izvor Beguša in Croatia. It occurs in sympatry with the crenobiotic *H. klecakiana* Bourguignat, 1887, but is morphologically and molecularly distinct. It is characterized by the terminal part of the body whorl separated from the columella, and neither eyes nor any pigment on the soft parts. It is a stygobiont gastropod, known so far only from one living specimen and several empty shells, thus its soft part morphology and anatomy remain unknown. Another new species of stygobiotic *Horatia* was found inside the cave Mali Rumin, its description is based solely on numerous empty shells from the cave sediments.

Keywords

Cytochrome oxidase, open coil, phylogeny, stygobiont gastropod, sympatry

Introduction

Bourguignat (1887) described the genus *Horatia*, with its type species *H. klecakiana* Bourguignat, 1887, from "sorgente près de Ribaric, dans la vallée de Cetina" in Croatia (Fig. 1A). Radoman (1983) identified this type locality with the Vrijovac spring in the source area of the Cetina River. The Bourguignat's syntype is figured at the Fig. 1A, together with seven nominal species from upper Cetina Basin described by him in 1887. All these species (Fig. 1) were later synonymized by Brusina (1907) with *H. klecakiana* (Radoman 1965, 1966, 1973, 1983), as Bourguignat obviously underestimated the high variability of the species (Glöer and Reuselaars 2020). *Horatia* was the first nominal genus described for the European Hydrobiidae with valvatiform shell (Bodon et al. 2001). Taylor (1966) established Horatiini as a tribe in Hydrobiidae, within the subfamily Cochliopinae, and Bole (1993) established a distinct family Horatiidae. Radoman (1973) included *Horatia* in Orientalinidae. Kabat and Hershler (1993) presented a review of understanding of this genus in the literature. Szarowska and Falniowski (2014) revised the phylogenetic position of *Horatia* applying molecular data, as a sister clade of *Sadleriana* Clessin, 1890. Glöer and Reuselaars (2020) questioned the identification of *Horatia* in Szarowska and Falniowski (2014), as having "closed umbilicus". However, the umbilicus presented in the photographs in Szarowska and Falniowski (2014) is exactly identical with the one presented by Glöer and Reuselaars (2020) for their new species *H. podvisensis* Glöer et Reuselaars, 2020, and with the one of a syntype of *H. klecakiana* (Fig. 1A). Radoman (1983) listed three species of *Horatia* from the former Yugoslavia: *H. klecakiana*, *H. novoselensis* Radoman, 1966, and *H. macedonica* (Kuščer, 1937) (Fig. 2). Species of *Horatia* were reviewed by Schütt (1961), Boeters (1974, 1998) and Bole (1993). The stygobiotic *Horatia knorri* Schütt, 1961 from Spring Ombla near Dubrovnik was later synonymised by the same author (Schütt 2000) with *Pseudamnicola troglobia* Bole, 1961. However, the morphology of the shell of *H. knorri* suggests its species distinctness from *P. troglobia*, as well as its uncertain generic assignment, only provisionally within the genus *Horatia* (Hirschfelder 2017). All the gastropods from Caucasus previously assigned to the genus *Horatia* were transferred to the new genera *Pontohoratia* Vinarski, Palatov & Glöer, 2014 and *Motsametia* Vinarski, Palatov & Glöer, 2014. The hitherto known species of *Horatia* occur in springs (thus being crenobiotic) at western Balkan Peninsula (Fig. 2), having eyes and pigmented mantle, but no species has been hitherto confirmed from stygobiont habitats.

In 2020 we collected one live specimen (Figs 3, 4A) and several empty shells of *Horatia* in Izvor Ruda Beguša, cave just above the spring zone and in the spring lake, sieved from sandy sediment at the spring and cave bottom, 13 km ESE of Sinj, Split District, Croatia. It occurred in sympatry with a few living specimens of *H. klecakiana*. The specimen was evidently a stygobiont, with neither eyes nor pigment, and with the characteristic shell, evidently different from the ones found in the other species of *Horatia*. Another stygobiotic species, represented by numerous empty shells and shell fragments was found inside the cave Mali Rumin, Split District, Croatia (in summer 2020), and earlier in the active spring sediments in the same locality (in spring 2017).

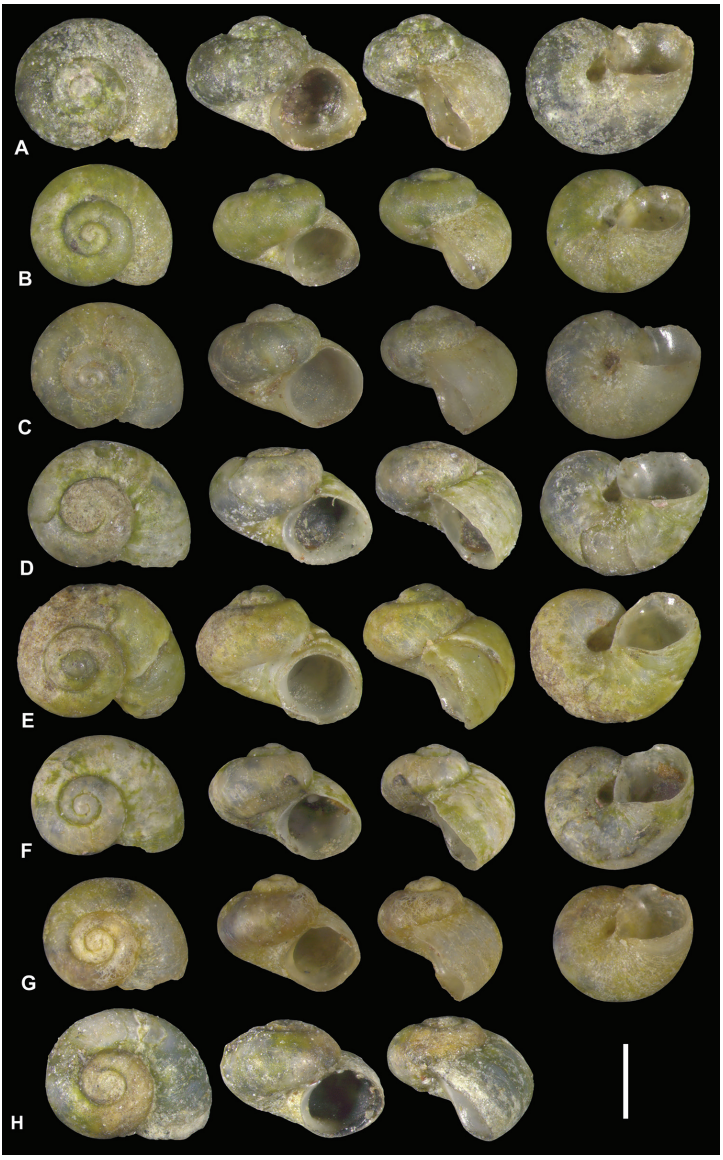

Figure 1. Type material of taxa described by Bourguignat, 1887 **A** syntype of *H. klecakiana* MHNG -110592. Croatie, ex Yougoslavie, Ribarić, Vallée de La Cettina (=Cetina) (=Vrijovac spring in the source area of the Cetina River), Vriovac spring in Paško polje, near to village Cetina. The following species are junior synonyms of *H. klecakiana*: **B** syntype of *H. verlikana* MHNG 110600 Marais entre Verlika (=Vrlika=Verlicca) et Ribaric **C** syntype of *H. palustris* MHNG 110597 Fontaine à Ervac (=Hrvace) **D** syntype of *H. obtusa* MHNG 110596 Sorgente de La Cetina **E** syntype of *H. letourneuxi* MHNG 110593 Fontaine du moulin à Ervac (=Hrvace) **F** syntype of *H. fontinalis* MHNG 110589, Sorgente de La Cettina **G** syntype of *H. albanica* MHNG 110587, Source du moulin Cetina, à Durazzo (=Durrës), not Durrës in Albania, likely the misspelling of the watermill name in village Cetina **H** syntype of *H. obliquq* MHNG 110594 Fontaine du moulin à Ervac (=Hrvace). (Photo MHNG by Estée Bochud and Eike Neubert, kindly provided by Emmanuel Tardy).

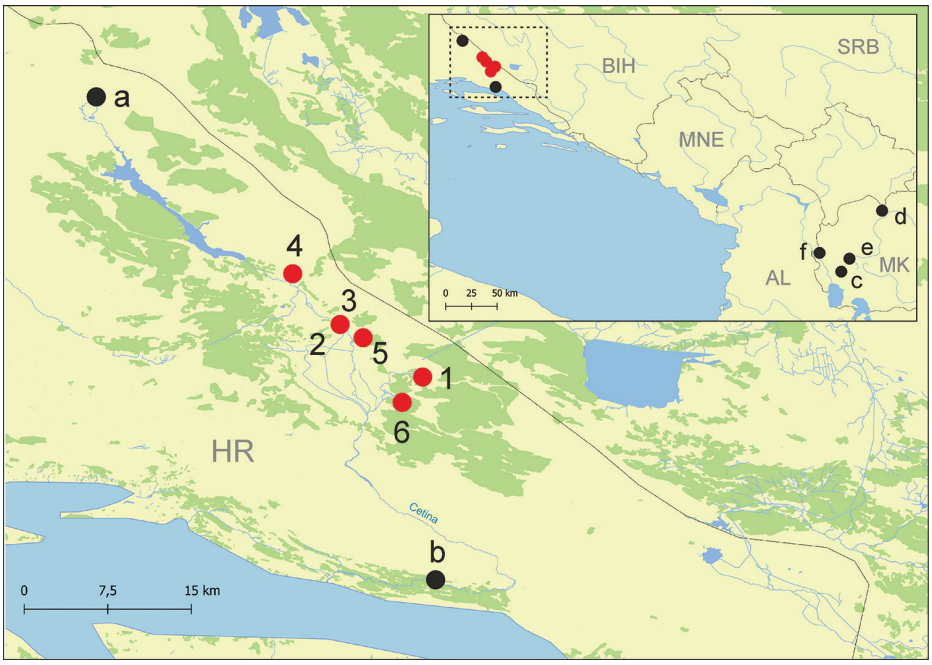

Figure 2. Localities of *Horatia*. Red circles and numbers indicate new *Horatia* localities given in the material description. Black circles and letters: **a** *H. klecakiana* – locus typicus **b** *H. klecakiana* – Kučiće (Szarowska and Falniowski 2014) **c** *H. novoselensis* – locus typicus **d** *H. macedonica* – locus typicus **e** *H. podvisensis* – locus typicus **f** *H. podvisensis* – the other locality (Glöer and Reuselaars 2020). For description of localities 1–6 - see Materials and methods

Material and methods

The live *Horatia* snails and empty shells were collected by A. Falniowski, J. Grego M. Olšavský and J. Olšavská at August 5-th 2020 and by J. Grego, G. Jakab and B. Šmída during March 17-th 2017 at following localities (Figs 2, 3):

1. Izvor Ruda – Beguša, Ruda, sand at the stream bottom below the spring lake, 13 km ESE of Sinj, Split District, Croatia, 43°40'06.6"N, 16°47'45.6"E – *H. ozimeci* sp. nov. and *H. klecakiana* (Fig. 3E).
2. Vrelo Kosinac 1, Sinjski Obrovac, Sinj District, Croatia, 43°43'40.89"N, 16°42'2.29"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3A).
3. Vrelo Kosinac 2, Sinjski Obrovac, Sinj District, Croatia, 43°43'43.11"N, 16°42'0.83"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3B).
4. Mali Rumin cave, Rumin, Sinj District, Croatia, 43°46'50.88"N, 16°38'56.75"E – *H. stygorumina* sp. nov. in cave sandy sediment and *H. klecakiana* at sediment of the cave entrance (Fig. 3C).
5. Gala spring, opposite to Crkva Svih Svetih, Gala, Sinj District, Croatia, 43°42'43.00"N, 16°43'39.88"E – *H. klecakiana* (Fig. 3D).

Figure 3. Sampled localities in Sinj District, Croatia **A** Sinjski Obrovac, Vrelo Kosinac 1 **B** Sinjski Obrovac, Vrelo Kosinac 2 **C** Rumin, cave Mali Rumin **D** Gala, Vrelo Gala **E** Ruda, Izvor Ruda Beguša **F** Grab, Grabske Mlinice, Izvor Grab.

6. Grabske Mlinice, Grab, Sinj District, Croatia, 43°38'27.26"N, 16°46'13.57"E – *H. klecakiana* and *H.* cf. *ozimeci* (Fig. 3F).

The snails were collected from the sediment with a 500 μm sieve and fixed in 80% analytically pure ethanol, replaced two times, and kept in -20 °C temperature in a refrigerator. The shells were photographed with a Canon EOS 50D digital camera, under

Table 1. Taxa used for phylogenetic analyses with their GenBank accession numbers and references.

Species	COI GB numbers	References
Agrafia wiktori Szarowska & Falniowski, 2011	JF906762	Szarowska and Falniowski 2011
Alzoniella finalina Giusti & Bodon, 1984	AF367650	Wilke et al. 2001
Anagastina zetavalis (Radoman, 1973)	EF070616	Szarowska 2006
Avenionia brevis berenguieri (Draparnaud, 1805)	AF367638	Wilke et al. 2001
Belgrandiella kuesteri (Boeters, 1970)	MG551325	Osikowski et al. 2018
Belgrandia thermalis (Linnaeus, 1767)	AF367648	Wilke et al. 2001
Dalmatinella fluviatilis Radoman, 1973	KC344541	Falniowski and Szarowska 2013
Daphniola louisi Falniowski & Szarowska, 2000	KM887915	Szarowska et al. 2014a
Ecrobia ventrosa (Montagu, 1803)	KX355839	Osikowski et al. 2016
Fissuria boui Boeters, 1981	AF367654	Wilke et al. 2001
Graziana alpestris (Frauenfeld, 1863)	AF367641	Wilke et al. 2001
Graecoarganiella parnassiana Falniowski & Szarowska, 2011	JN202352	Falniowski and Szarowska 2011
Grossuana angeltsekovi Glöer & Georgiev, 2009	KU201090	Falniowski et al. 2016
Hauffenia tellinii (Pollonera, 1898)	KY087861	Rysiewska et al. 2017
Horatia klecakiana Bourguignat 1887	KJ159128	Szarowska and Falniowski 2014
Iglica gracilis (Clessin, 1882)	MH720985	Hofman et al. 2018
Islamia zermanica (Radoman, 1973)	KU662362	Beran et al. 2016
Montenegrospeum bogici (Pešić & Glöer, 2012)	KM875510	Falniowski et al. 2014
Paladilhiopsis grobbeni Kuscer, 1928	MH720991	Hofman et al. 2018
Pseudorientalia sp.	KJ920490	Szarowska et al. 2014b
Radomaniola curta (Küster, 1853)	KC011814	Falniowski et al. 2012
Sarajana apfelbecki (Brancsik, 1888)	MN031432	Hofman et al. 2019
Tanousia zrmanjae (Brusina, 1866)	KU041812	Beran et al. 2015

a Nikon SMZ18 microscope with dark field; measurements of the shell were taken using IMAGEJ image analysis software (Rueden et al. 2017).

DNA was extracted from whole specimens; tissues were hydrated in tris-EDTA (TE) buffer (3 × 10 min); then total genomic DNA was extracted with the Sherlock extraction kit (A&A Biotechnology), and the final product was dissolved in 20 µl of TE buffer. The extracted DNA was stored at -80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland).

Mitochondrial cytochrome oxidase subunit I (COI) locus was sequenced. Details of PCR conditions, primers used and sequencing were given in Szarowska et al. (2016). Sequences were initially aligned in the MUSCLE (Edgar 2004) Programme implemented in MEGA 7 (Kumar et al. 2016) and then checked in BIOEDIT 7.1.3.0 (Hall 1999). Uncorrected p-distances were calculated in MEGA 7. The estimation of the proportion of invariant sites and the saturation test for entire data sets (Xia 2000; Xia et al. 2003) were performed using DAMBE (Xia 2013). In the phylogenetic analysis, additional sequences from GenBank were used as reference (Table 1). The data were analysed using approaches based on Bayesian Inference (BI) and Maximum Likelihood (ML). In the BI analysis, the GTR + I + Γ model of nucleotide substitution was applied. Model was selected using MRMODELTEST 2.3 (Nylander 2004). The analyses were run using MRBAYES v. 3.2.3 (Ronquist et al. 2012) with default of most priors. Two simultaneous analyses were performed, each with 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of the trees were discarded as burn-in. The

analyses were summarised as a 50% majority-rule tree. Convergence was checked in TRACER v. 1.5 (Rambaut and Drummond 2009). The Maximum Likelihood analysis was conducted in RAxML v. 8.2.12 (Stamatakis 2014) using the 'RAxML-HPC v.8 on XSEDE (8.2.12)' tool via the CIPRES Science Gateway (Miller et al. 2010). We applied the GTR model which is the only nucleotide substitution model implemented in RAxML, whose parameters were estimated by RAxML (Stamatakis 2014).

For comparison purposes the pictures of type material of taxa described by Bourguignat, 1887, were used. The pictures were kindly provided by Emmanuel Tardy (MHNG).

Abbreviations

JG Jozef Grego collection;
MHNG Muséum d'histoire naturellem ville Genéve;
NHMW Naturhistorisches Museum Wien, Austria;
OZRM Croatian Natural History Museum – Opća zbirka recentnih mekušaca, Zagreb, Croatia;
SBMNH Santa Barbara Museum of Natural History, California, USA.

Systematic part

Family Hydrobiidae Stimpson, 1865
Subfamily Horatiinae D. W. Taylor, 1966
Genus *Horatia* Bourguignat, 1887

***Horatia ozimeci* Grego & Falniowski, sp. nov.**
http://zoobank.org/77EA36ED-B25F-4E95-9DC5-20C8B3513B40
Figs 4A–E, 5A

Type locality. Spring Izvor Ruda – Beguša, Ruda, sand at the stream bottom below the spring lake, 13 km ESE of Sinj, Split district, Croatia; 43°40'06.6"N, 16°47'45.6"E.

Holotype. Dry shell with operculum (Fig. 4B), J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11600.

Paratypes. The single live collected paratype (Fig. 4A) has been destroyed for DNA extraction; GenBank number: MW448545.

From type locality: J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg August 5-th 2020, OZRM 11601/1 specimen; NHMW 113607/1 specimen, JG 1542/28 specimens (Fig. 4C–E); Type locality, J. Grego, G. Jakab and B. Šmída leg. March 17-th 2017, JG F0724/24 specimens.

Diagnosis. Shell minute, valvatoid, distinguished from the other species of *Horatia* by its body whorl separated at its terminal sector from the penultimate one (scalarity at this part), the circular and complete peristome and extremely wide umbilicus showing earlier whorls inside.

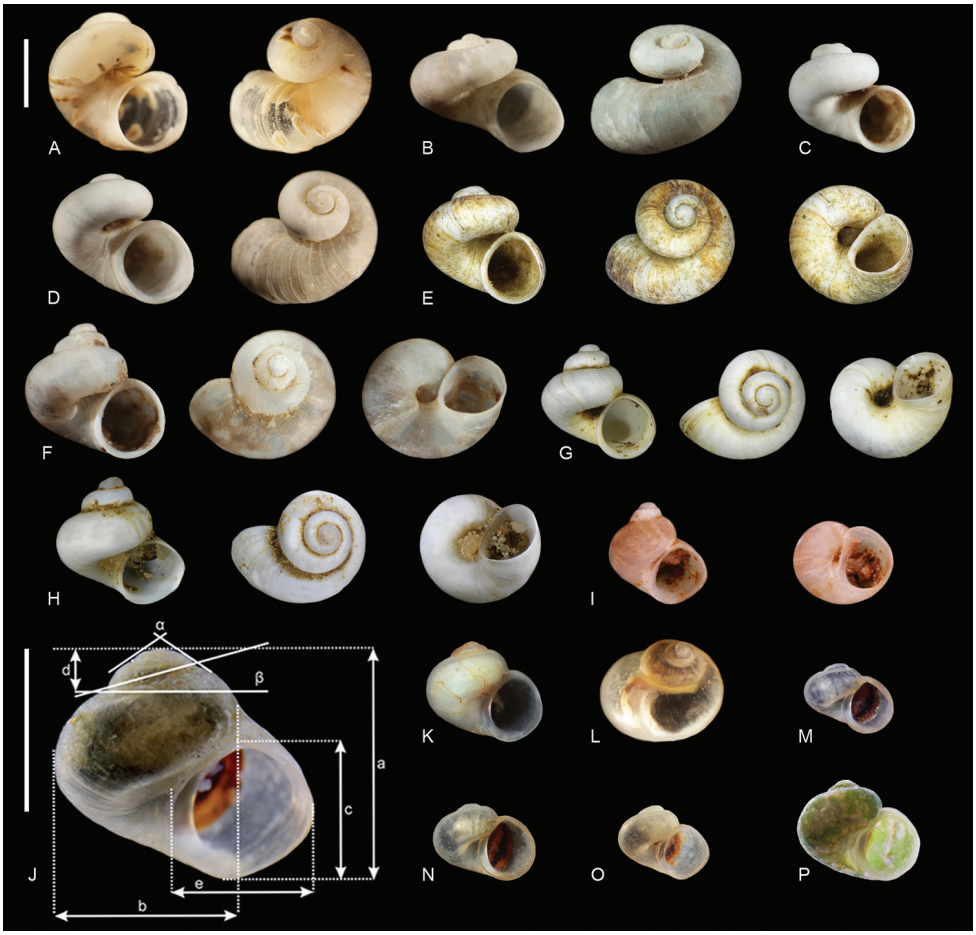

Figure 4. Shell variability of two *Horatia* species **A–E** *H. ozimeci* **A** sequenced specimen (2H76) **B** holotype OZRM 11603 **C–E** paratypes **E** NHMW 113607, (Photo NHMW by Ivo Gallmetzer) **F–H** *H. stygorumina* **F** holotype OZRM 11600 **G–H** paratypes NHMW 113608, (Photo NHMW by Gallmetzer) **I** Cf. *Horatia knorri* Schütt, 1961, spring of Ombla in Komolac, holotype SMF 164247 (Photo SMF by Sigrid Hof) **J–P** sequenced specimens of *H. klecakiana* **J** Vrelo Kosinac 2 **K** *H. klecakiana*, Gala spring **L** *H. klecakiana*, Ruda Beguša **M, N, P** *H. klecakiana*, Vrelo Kosinac 1 **O** *H. klecakiana*, Grabske Mlinice. The shell measurements are also shown: a shell height, b body whorl breadth, c aperture height, d spire height, e aperture breadth, α apex angle, β angle between body whorl suture and horizontal surface. Scale bars: 1 mm.

Description. ***Shell*** (Fig. 4B) 2.14 mm high and 1.62 mm broad, valvatoid, whitish, translucent, rather thin-walled, consisted of about 3 ½ whorls, growing rapidly and separated by moderately deep suture, more prominent closer to the aperture: the terminal part of the body whorl completely separated from the penultimate one. Spire low and narrow, body whorl large. Aperture prosocline, circular in shape, peristome complete and separated from the columella, swollen, umbilicus extremely wide, with

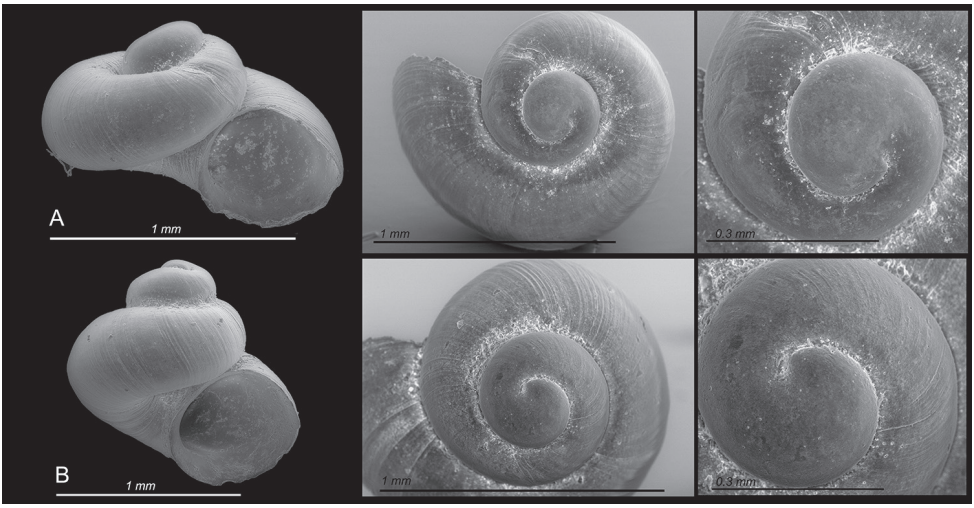

Figure 5. SEM images of *H. ozimeci* (**A**) juvenile specimen from the type locality (SBMNH 635080) and *H. stygorumina* (**B**) juvenile shell from cave Mali Rumin (SBMNH 635079). SEM SBMNH by Vanessa Delnavaz.

the earlier whorls visible inside. Shell surface smooth, with growth lines delicate but visible. Operculum reddish-brown, translucent, paucispiral.

Shell variability restricted (Fig. 4A, C–E), and shell less variable than in *H. klecakiana* (Figs 1, 4J–P).

Protoconch (Fig. 5A) smooth, similar as in *H. klecakiana*.

Measurements of holotype, illustrated paratypes, and shells of *H. klecakiana*: Table 2.

Soft parts morphology and anatomy. The body white, pigmentless, with no eyes. The anatomy unknown.

Derivatio nominis. The specific epithet *ozimeci* refers to our friend Mr.sc. Roman Ozimec, a biospeleologist from Bast, Croatia, deeply devoted to the study and protection of the subterranean habitats in the Dinarides and Balkans.

Distribution and habitat. Known from the type locality. The type locality is a karst spring lake surrounded by three outflow caves intermittently draining the karst conduit at high water saturation. The spring draining water from sinkholes at Buško Jezero (Bosna and Hercegovina) and supports the river Ruda, a left tributary of Cetina River. The following Hydrobiidae species were detected in the habitat: *Horatia klecakiana*, *Orientalina curta germari* (Frauenfeld, 1863), *Montenegrospeum sketi* Grego & Glöer, 2018, *Kerkia jadertina sinjana* (Kuščer, 1933).

Molecular distinctness and relationships of *Horatia ozimeci*. We obtained eight new sequences of COI (409 bp, GenBank Accession Numbers MW448545–MW448552). The tests by Xia et al. (2003) revealed no saturation. The topologies of the resulting phylograms were identical in both the ML and BI. All the seven sequences of *H. klecakiana*, collected at five localities, were identical. *H. ozimeci* formed a sister clade with *H. klecakiana* (bootstrap support 100%, Bayesian probability 1.0), confirming the

Table 2. Shell measurements of the *Horatia*: I–IV *H. ozimeci* (I sequenced specimen, II holotype, in bold; III–IV paratypes); V–VII sequenced specimens of *H. klecakiana*; VIII *H. stygorumina*, holotype, in bold. Measurements are shown in Fig. 4J.

	I	**II**	III	IV	*H. ozimeci*	V	VI	VII	**VIII**
a	2.14	**1.70**	2.02	1.71	1.89 ± 0.22	1.61	1.51	1.61	**2.08**
b	1.62	**1.58**	1.56	1.38	1.54 ± 0.11	1.33	1.19	1.26	**1.57**
c	1.22	**1.12**	1.29	1.05	1.17 ± 0.11	1.14	0.88	1.08	**1.24**
d	0.23	**0.32**	0.18	0.26	0.25 ± 0.06	0.17	0.26	0.25	**0.49**
e	1.05	**1.05**	1.18	0.95	1.06 ± 0.09	0.97	0.91	0.92	**1.08**
a	131	**131**	123	124	127.25 ± 4.35	112	114	113	**104**
β	4	**9**	8	9	7.50 ± 2.38	6	11	10	**8**

congenerity of the two taxa (Fig. 6). Within genus *Horatia* the p-distance between the taxa was 0.074. This well supported clade belongs to the Horatiinae, subfamily of Hydrobiidae. Deeper relationships within the Horatiinae remain unresolved, because of the lack of acceptable support for deeper nodes, which is typical of the phylograms based on COI.

Horatia stygorumina Grego & Rysiewska, sp. nov.

http://zoobank.org/36A97AFE-52B6-4F52-A17E-348C5CF604F3
Figure 4F–H, 5B

Type locality. Mali Rumin cave, Rumin, Sinj District, Croatia, 43°46'50.88"N, 16°38'56.75"E.

Holotype. Dry specimen, J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11602 (Fig. 4F).

Paratypes. From Type locality: J. Grego, A. Falniowski, M. Olšavský and J. Olšavská leg., August 5-th 2020, OZRM 11603/1 specimen; NHMW 113608/1 specimen, JG F1526/160 specimen; Type locality, J. Grego, G. Jakab and B. Šmída leg. March 17-th 2017, JG F0736/26 specimens.

Other material. Morphologically similar stygobiotic *Horatia* shells were found in following spring localities (springs at left tributaries of Upper Cetina River: Vrelo Kosinac 1, Sinjski Obrovac, Sinj district, Croatia, 43°43'40.89"N, 16°42'2.29"E JG/8; Vrelo Kosinac 2, Sinjski Obrovac, Sinj District, Croatia, 43°43'43.11"N, 16°42'0.83"E JG/4; Grabske Mlinice, Grab, Sinj District, Croatia, 43°38'27.26"N, 16°46'13.57"E JG/3). For the time being we treat those as *H.* cf. *stygorumina*.

Diagnosis. Shell minute, trochiform, distinguished from the other species of *Horatia* by its very wide umbilicus showing earlier whorls inside. From the *H. ozimeci* s. nov. distinguished by higher conical spire and suture reaching the aperture, as well by more swollen protoconch and by slight sinuation at labral columellar margin. From stygobiotic cf. *Horatia knorri* different by much wider umbilicus and by aperture shape less declined from the columella in its labral profile.

Description. ***Shell*** (Fig. 4F) 2.08 mm high and 1.57 mm broad, conical, fresh shells milky white and translucent, with 3 rounded inflated whorls and deep suture,

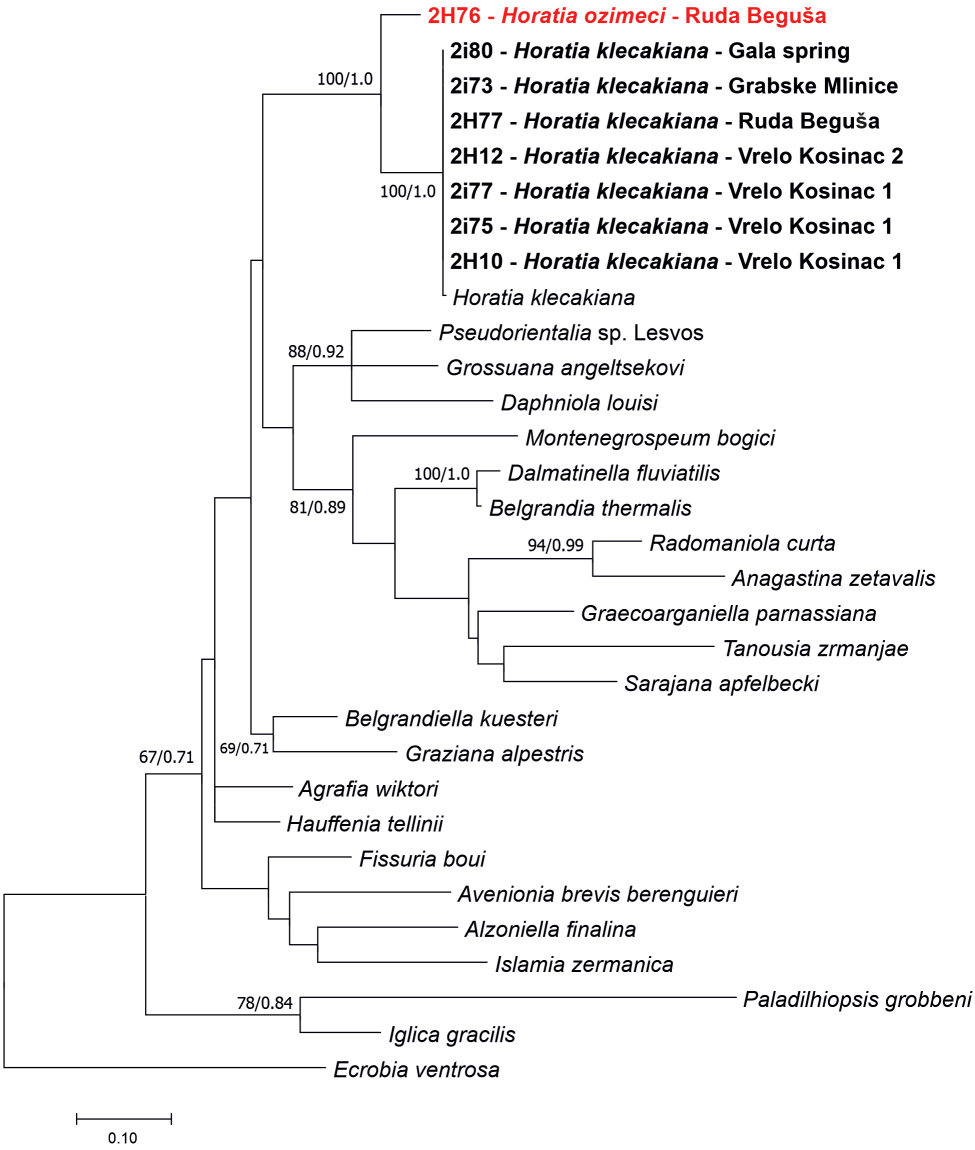

Figure 6. Maximum Likelihood tree inferred from mitochondrial COI. Bootstrap support above 60% and corresponding Bayesian probabilities are given. Bold indicates newly obtained sequences.

the whorls regularly tapering towards the aperture. Spire conically elevated, body whorl prominent. Smooth shell surface covered with blunt regular axial growth lines more prominent at the body whorl. Aperture almost rounded, tear-shaped, shortly attached to the body whorl by weak grove. Labral lateral profile backward protruded almost straight, columellar margin slightly sinuated at its middle. Umbilicus open.

Measurements of holotype of *H. stygorumina*: Table 2.

Soft parts morphology and anatomy. Not known.

Derivatio nominis. The specific epithet *stygorumina* is derived from the stygobiont habitat and from the type locality: cave Mali Rumin also referring to the name of the nearby settlement Rumin.

Distribution and habitat. Known only from the type locality, where empty shells can be found in the cave sediments, as well as in the sandy sediments of the intermittent spring. The shells are washed out from their stygobiont habitat. The cave is 50 m long, with two branches, acting as an intermittent overflow of the larger permanent spring Vrilo Rumin, situated 730 m east-southeast. Both springs are draining karstwater from middle part of Livansko Polje Basin (Bosnia and Herzegovina) Under Dinara Mts. towards upper Cetina River Valley. The following Hydrobiidae were detected in the locality: *Horatia klecakiana*, *Orientalina curta germari*, *Kerkia jadertina sinjana* (Kuščer, 1933) and a Montesieriid species of *Paladilhiopsis* Pavlović, 1913 and/or *Lanzaia* Brusina, 1906.

Discussion

All four valid species of *Horatia* described so far inhabit springs, but they have eyes and more or less pigmented soft parts, and can be classified as crenobiotic, at most as stygophiles (as defined by Culver and Pipan 2009, 2014). *H. ozimeci* sp. nov., with neither eyes nor any pigment, seems the first typically stygobiont *Horatia*. This single live specimen was most probably washed out from the cave together with few empty shells. Already Bourguignat (1887) noted the high variability of the shell of *H. klecakiana* (Fig. 1); later Radoman (1966, 1983) described and illustrated also the high variability of the penis in this species. The most characteristic feature of the shell of *H. ozimeci* is its partial scalarity – the open coil at the terminal part of the body whorl. There have been found also empty shells with the higher, conically elevated spire and more whorls visible within the umbilicus. Few empty shells found in the type locality were entirely scalariform, but they need not belong to *H. ozimeci*. Scalarity is characteristic for a few truncatelloidean species, e.g. *Gocea ochridiana* Hadžišče, 1956 from the Ochrid Lake (Radoman 1983), and several species from the Baikal Lake (Sitnikova et al. 2001; Clewing et al. 2015) but sometimes may be phenotypically determined (e.g. parasites, untypical chemistry: e.g. Fretter and Graham 1962), but is also typical feature of some species. In our case this morphological character is accompanied by molecular distinctness.

So far *H. klecakiana* was found only in the Cetina Valley and Livansko Polje Basin (Fig. 2). One of our sites was placed in the Ruda Valley and the second at Grab valley (both are left tributary of Cetina River); thus they are the first *H. klecakiana* localities outside the Cetina Valley and Livansko Polje.

High bootstrap support and p-distance between *H. ozimeci* and *H. klecakiana* confirm that they are two distinct species, belonging to the same genus *Horatia*. The evidence of species distinctness is especially strong since the two taxa occur in sympatry (inside the spring zone). The complete lack of polymorphism in the studied fragment

of COI in the specimens of *H. klecakiana* from its five sequenced populations additionally strengthens the molecular difference between *H. klecakiana* and *H. ozimeci* as delimiting distinct species.

The second stygobiotic species *H. stygorumina* sp. nov. is known only as empty shells from cave sediments of its type locality, 17 km from the locality of *H. ozimeci* sp. nov. Despite the similarities in the shell morphology of both species, suggesting their congeneric position, the second species differs from *H. ozimeci* sp. nov. by more elevated conical spire and more close-set last whorl. The stygobiotic *Horatia* forms can be found in most of the large springs at left tributary of upper Cetina River (springs: Rumin, Kosinac, Gala, Beguša, Grab). It may suggest possibly a similar evolutionary adaptation as we can see in the geographically close *H. ozimeci* sp. nov.

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Forty-year natural history study of *Bahalana geracei* Carpenter, 1981, an anchialine cave-dwelling isopod (Crustacea, Isopoda, Cirolanidae) from San Salvador Island, Bahamas: reproduction, growth, longevity, and population structure

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Abstract

Almost nothing has been reported on the natural history of any of the world's 92 species of cave cirolanids, including those from saltwater caves (anchialine). Over 1400 specimens of *Bahalana geracei* Carpenter, 1981 were collected in two caves from 1978–2018; size-frequency data provided insight into population structure. Some specimens were maintained alive over multiple years to study rarely reported activities for cave cirolanids: feeding, molting, growth, longevity, and reproduction. Photographs document these phenomena. Mating occurred after gravid females shed both halves of reproductive molts. Females can have multiple broods (iteroparous) with ~2.0–3.5 years per reproductive cycle: egg production (~9–24 months), mating, brooding (5–6 months), release of 6–55 mancas (2.3–3.3 mm long), and oostegite molt (~2–13 months after manca release). Estimated lifetime fecundity is 58 mancas per female; probable range is 20–120. In Lighthouse Cave, females outnumbered males (~4:1), grew larger (16.8 vs. 9.5 mm), and lived longer. Growth rates were slow: ~1–2 years for three instars of post-marsupial manca development (from ~2.3–4.0 mm); estimated adult growth rate was 0.8 mm/year (1.6 molts/year) for males, and 0.5 mm/year (1.5 molts/year) for females. Longevity estimates for females are 25–28 years with 23–30 instars, vs. 6–8 years for males with 13–15 instars. Males from Major's Cave were nearly as numerous and as large (14.8 mm) as females; estimated longevity for males is >20 years. Longevity estimates of >20 years appear to be the longest for any isopod species. Female longevity probably increased by being starvation resistant, surviving multiple broods, cannibalizing smaller *B. geracei*, and living in a low-stress environment. Populations appear to be stable, relatively large, and not currently threatened.

Keywords
Age compression, cannibalism, fecundity, gestation, iteroparous, mancas, molting, starvation resistance, stygobitic

Table of contents

Introduction

According to Bruce et al. (2017) the isopod family Cirolanidae Dana, 1852 is one of the largest families of free-living isopods, with more than 500 species in 62 genera, including about 91 described species that are stygobitic (aquatic and strictly subterranean). Messana (2020) recently described *Catailana whitteni*, a new genus and species of stygobiotic cirolanid from caves in China, making a new total of about 92 stygobitic species in 63 genera. Eight stygobitic species are from The Bahamas including six species of *Bahalana*. The type species *Bahalana geracei* Carpenter, 1981 is known with certainty only from caves on San Salvador Island. Almost nothing has been reported on feeding, growth, and reproduction of any of these 92 species. In discussing our growing need to protect cave invertebrates, Hutchins et al. (2010) noted that, "the basic ecology and life history of most subterranean species are unknown because access to their habitat is technically challenging, and especially because their lengthy life span and low population density render ecological studies difficult." Culver and Pipan (2019) pointed out the difficulties and rareness of breeding cave stygobites and, "Among crustaceans the only case of captive breeding known to us is that of Fong (1989) with the amphipod *G. minus*", in reference to *Gammarus minus* Say, 1818. In addition, Magniez (1975) reported success with the cave Stenasellid isopod *Stenasellus virei* Dollfus, 1897 that he, "bred in the laboratory for many years (1960–1974)."

Studies of almost all cave species naturally begin with collecting and preserving a few specimens for taxonomic descriptions and/or DNA studies. In most cases, additional specimens are never collected and kept alive for observation and attempted culturing. For example, Hutchins et al. (2010) collected 70 specimens of *Antrolana lira* Bowman, 1964 from nine sites for genetic data to analyze population structure, and "all specimens were preserved immediately in 95%-100% ethanol." Such studies are extremely valuable to support conservation initiatives, but immediate preservation obviously limits study of their natural history. As a result of these challenges, most published discussions on ecology of anchialine cave species are limited to salinity and a list of other animals found in the same caves.

The current long-term study of *B. geracei* was made possible by an extraordinary set of circumstances. First, because of my previous experience in culturing and describing new species of freshwater cave invertebrates (e.g., Carpenter 1970a, b), I was excited when the director of the Bahamian Field Station, Donald T. Gerace, agreed to lead my marine biology class to Lighthouse Cave in 1978. As soon as we started finding isopods and other unusual cave animals, the strong potential for new discoveries became apparent. I was fortunate to be able to teach marine biology courses on San Salvador Island almost every year for over 20 years (1977–2000), which gave us the opportunity to explore Lighthouse Cave as an example of an unusual marine habitat and for students to do research on several cave species. After my retirement in 2001, several former students and research colleagues kindly volunteered to help continue this cave research (2001–2020).

Fortunately, Lighthouse Cave is easily accessible from the Gerace Research Centre (formerly the Bahamian Field Station), the cave water is shallow enough to explore without scuba, and the population of *B. geracei* is usually moderately high and not endangered. Since specimens are relatively easy to maintain in the laboratory for long periods and are translucent enough to reveal sexual condition and digestive processes, they were ideal for students to use for research projects that have contributed to this study.

Although this study encompasses more than 40 years, collection data from several years are not included for a variety of reasons. Sometimes no specimens were found, or our research concentrated on other cave animals (e.g., other isopod species, remipedes, and brittle stars); some years I did not visit San Salvador Island because I taught courses in other locations (e.g., Australia or Ecuador), or my research associates or I had health issues or family obligations. Even when field studies were not carried out, laboratory culturing and research continued.

Three approaches were used in this study: (1) collecting over 1400 specimens (most were returned to the caves) during a 40-year period to provide insight into population structure based largely on size-frequency distributions, (2) maintaining some specimens over multiple years to learn about behavior, feeding, molting, growth, and longevity, and (3) observing life cycle stages and reproductive events: egg development, mating, gestation, release of mancas (offspring) from the marsupium, and development of post-marsupial mancas – all phenomena that have been rarely or never reported for cave cirolanids. These three approaches are covered in reverse order: first reproduction and life cycle development, then growth and longevity, and last population structure.

With 40 years of data and observations recorded in hundreds of pages of notes, it has been challenging to decide what to include in this paper. Some of my observations are of phenomena so rare that they may seem trivial, but they may also be the most interesting and valuable if they are the first times ever reported for this elusive group. Even with over 1400 specimens, several of the population phenomena examined (e.g., number of months between molts for specific sizes and reproductive conditions) do not have sufficient numbers to warrant traditional statistical tests, but they still provide evidence to support growth and longevity patterns. The section on "Growth rates and longevity" is one of the longest because it has so many components and because it is important to explain how my longevity estimates were calculated, since any claims of extreme longevity will likely be scrutinized and questioned. Results of this unusually extensive long-term study are presented with the hopes that it will also provide insight into the lives of other cave cirolanids and other crustaceans.

Materials and methods

Study areas

San Salvador Island is a small island (about 16 km by 8 km) in the eastern part of The Bahamas archipelago, 24°06'N, 74°29'W (Fig. 1A). It sits atop a shallow-water carbon-

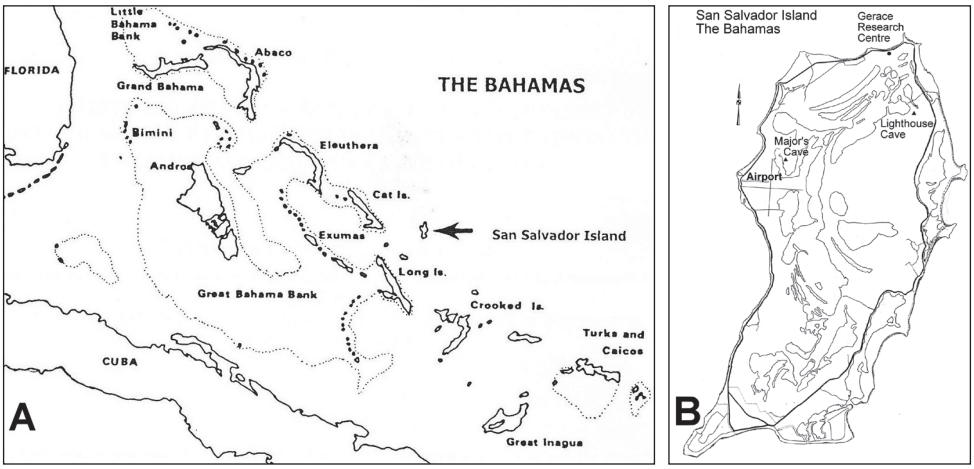

Figure 1. Maps showing **A** location of San Salvador Island in The Bahamas and **B** San Salvador Island with locations of Lighthouse Cave and Major's Cave.

ate bank isolated from other banks, like the Grand Bahama Bank, by deep water (Yager and Carpenter 1999). Many caves in The Bahamas are close to the ocean, so they contain salt water. They fit the definition of anchialine, which was originally described as a habitat consisting of "pools with no surface connection with the sea, containing salt or brackish water, which fluctuates with the tides" (Holthuis 1973). Due to the use of the restrictive word "pools", this definition was modified by Stock et al. (1986) as, "Anchialine habitats consist of bodies of haline waters, usually with a restricted exposure to open air, always with more or less extensive subterranean connections to the sea, and showing noticeable marine as well as terrestrial influences." Bishop et al. (2015), proposed a broader definition of anchialine as, "a tidally-influenced subterranean estuary located within crevicular and cavernous karst and volcanic terrains that extends inland to the limit of seawater penetration."

Many anchialine caves in The Bahamas and other locations around the world have a saltwater layer below a substantial freshwater layer, so cave divers need to dive through the freshwater layer and halocline to study the marine waters and its inhabitants below. In contrast, the anchialine caves on San Salvador Island do not have this stratification; instead, they have salt water or brackish water all the way to the surface. As such, these caves do not conform well with the new definition by Bishop et al. (2015) because they do not have the influence of a freshwater stream or river that is typical of estuaries. However, they may still be influenced by the mixing of oceanic salt water with meteoric fresh water that penetrates through the soil and/or with underground freshwater aquifers, even if the freshwater habitats are not readily accessible for humans to explore. The anchialine caves on San Salvador Island might best be described by combining the first two definitions: Anchialine habitats consist of salt or brackish bodies of water which fluctuate with the tides, and have subterranean connections to the sea, but no surface connection.

Two caves were used in this long-term study (Fig. 1B): Lighthouse Cave and Major's Cave. The more important one is Lighthouse Cave where we collected *B. geracei* in at least 23 of the years from 1978 to 2018. This cave is located about ½ km from the Dixon Hill Lighthouse (northeastern side of the island) and about 1 km from the ocean (Carpenter 1981); it is about 3 km from the Gerace Research Centre and is a popular field trip for many courses taught at GRC. The geology of Lighthouse Cave was described by Mylroie (1980) and the hydrology by Davis and Johnson (1988). According to Carpenter (1981), "The cave consists mainly of one room about 40 m in diameter with a large pile of breakdown rocks in the middle mostly surrounded by quiet water up to 1 m deep. Close to the entrance, which is a narrow hole near the roof, is a small room about 10 m in diameter where the isopods were found." Since then, specimens of *B. geracei* have been found throughout the cave's rooms and channels. Tidal fluctuations of about 0.5 to 1 m occur through complex conduit systems; tides are delayed (compared to ocean tides) by ~45 minutes. Surprisingly, Davis and Johnson (1988) reported that "the tidal range is greater (sometimes by as much as 0.5 m) than that in the ocean." We found it much easier to collect isopods and other marine animals near low tides. "The water in Lighthouse Cave is usually near full ocean salinity (~35 ppt), but sometimes it drops to ~20 ppt or lower after heavy rains (my samples on 18 June 2013 varied from 20–32 ppt depending on location in the cave)", Carpenter (2016). In laboratory experiments, *B. geracei* easily tolerated salinities down to 15 ppt and survived for several days when near 11 ppt.

Besides *B. geracei*, other aquatic life in Lighthouse Cave includes: the asellote gnathostenetroid isopod *Neostenetroides stocki* Carpenter & Magniez, 1982, the red shrimp *Barbouria cubensis* (Von Martens, 1872), several copepod and ostracod species, several sponge species (see van Soest and Sass 1981), tube worms (*Spirorbis* sp.), the mangrove rivulus fish *Kryptolebias marmoratus* (Poey, 1880) and occasional microbial colonies (probably a species of *Beggiatoa* or *Thiothrix*). Terrestrial life includes numerous bats and cockroaches (*Periplaneta americana* Linnaeus, 1758), evaniid wasps that parasitize cockroach egg cases, four isopod species, the pseudoscorpion *Paraliochthonius carpenteri* Muchmore, 1984, two snail species, land crabs and rats.

Major's Cave is on the northwest side of the island near the San Salvador Island International Airport, about 6 km southwest of Lighthouse Cave, and is considerably more challenging to access. It was discovered in 1997 by men working on the runway extension. A faunal survey was conducted by professors and students from Siena College (Loudonville, NY) and Le Moyne College (Syracuse, NY) on 15 June 1997, during which Dr. Nancy Elliott (Siena College) collected and preserved two remipedes from the surface of a pool. Every year from 1997 to 2004 my marine biology classes and research associates visited Major's Cave, primarily to search for and study the remipedes. Jill Yager and I described the remipede as a new species, *Speleonectes epilimnius* Yager & Carpenter, 1999. In the same issue of Crustaceana, I published a companion paper on the behavior and ecology of this species; this is the only species ever found at the surface of anchialine waters (rather than below a halocline), which allowed me to keep a few specimens alive long enough to study feeding, grooming, and resting

behaviors; a description of Major's Cave is included in the section on "Habitat and fauna" (Carpenter 1999). Salinity is usually about 24 ppt (~70% of ocean salinity). Other marine animals found in this cave include copepods, marsh crabs *Armases miersii* (Rathburn, 1897), and *B. geracei*. Between 1997–2004 we collected enough *B. geracei* specimens to determine that the populations in Major's Cave and Lighthouse Cave are remarkably different from each other (especially regarding sizes and numbers of males), which are described in the section on Life cycle and population structure.

Sampling

Collections were almost always made in June or July, but also in January 1980, 1999, and 2013.Collecting and export permits were required (requested and granted) starting around 2007. My marine biology classes and research groups usually collected in at least one cave once or twice during each trip to San Salvador Island; we spent about one hour collecting, usually at low tides when most collecting areas were less than 1 m deep. Most collecting in Lighthouse Cave was done in a small side room near the entrance; this left the population in the remainder of the cave relatively unaffected. There were usually 6–10 collectors, but this varied from 2–20, which strongly affected the number of specimens collected; previous experience and natural collecting skills also contributed to success.

Several collecting techniques were tried over the years. Baited traps tended to catch other animals such as red shrimps (*B. cubensis*) and ostracods. Black aquarium nets with long handles were most effective in collecting the white *B. geracei* that were easily seen either swimming or resting on the dark silt-covered substrate or rocks. A variety of flashlights were used; strong underwater flashlights increased chances of finding small specimens. Specimens were transferred to individual containers (usually 35 mm film cannisters) to avoid cannibalism that often occurred when two specimens were put together. Containers were nearly filled with cave water to reduce turbulence during transport to the field station. They were then kept in my laboratory/bedroom, where air conditioning was maintained near cave temperature (~25–26 °C). Each specimen was examined alive under a dissecting microscope (7–40×) to determine size, gender, manca stage, and sexual condition for females (bearing eggs or oostegites or neither). Oostegites, visible as shiny plates (Figs 3D, 4A–E), are flexible flaps that extend from the coxae (first segments) of pereopods (legs) 1–5 to form the marsupium or brood pouch; if oostegites were present, but no eggs or developing embryos or mancas, these females had released their mancas within the previous few months. Males were easily identified in dorsal view by white sperm-packed sperm ducts (vasa deferentia) extending from pereonites 5–7 (Figs 3A, B, 7C, D) to ventrally located penes (Figs 3B, 5F); sometimes males needed to be confirmed by finding the clear penes and/or an appendix masculina (Fig. 3B) on a pleopod 2. Each specimen was numbered in the order in which it was examined, often starting with the largest ones. Measurements of body length (front of head to tip of telson) were made to the nearest 0.1 mm using an ocular micrometer and/or ruler or grid beneath specimens (Fig. 5B).

Collection data are summarized in Table 1 and Fig. 2. Most specimens were returned to the caves after examination to reduce our effect on the populations. Some were kept for long-term observation in Kentucky, especially if they were likely to provide additional information on growth or reproduction.

Culture methods

Specimens kept for long-term observation and experimentation were maintained in clear plastic jars or translucent food storage containers with tight fitting lids and 20–100 ml of salt water (near 35 ppt) at a depth of only 1–2 cm. This shallow depth provided a high surface area to volume ratio for better oxygen exchange, since no extra aeration or filtration was used. Small rocks or pieces of dry wall sanding screens were used as substrate to facilitate molting. When females were releasing their mancas, they were housed in jars with a horizontal sanding screen held 1–2 cm above the substrate so mancas could crawl through and avoid being trampled (Fig. 4F). Mud from the caves was sometimes used as substrate, but specimens did well in containers without supplemental substrates. Although *B. geracei* do not seem to be light sensitive, they were stored in a dark room or incubator. Sometimes they were kept at ambient room temperatures ~20–27 °C, but usually close to cave temperatures of 25–26 °C with supplemental heating. From 1979 to 2001 specimens were kept in a research laboratory at Northern Kentucky University where students could learn maintenance techniques and perform experiments on *B. geracei* and other cave species. Since retiring from NKU in 2001, it has been convenient to keep specimens at my home.

Each animal was kept in a separate container to avoid cannibalism and to provide data on individual feeding, molting, growth, and egg production; of course, breeding experiments required short-term exceptions to this practice of separation. Jars were labeled with each adult specimen's collection number and year (e.g., female #5, 2018) to facilitate multi-year tracking. Mancas were kept in jars labeled with the date of birth (release from marsupium), plus a letter if more than one was released on that date (e.g., 7-27-20A). Adults were routinely offered food every 3–6 weeks, which was the typical time for digestion. Mancas were offered food every 1–3 weeks. After each feeding, containers were cleaned with a paper towel, and newly aerated water was added. Many different food items were eaten including brine shrimp, ghost shrimp, crab, crayfish, California black worms, earthworms, cockroaches, dragonfly nymphs, mayfly nymphs, mosquitoes (larvae, pupae, and adults), centipedes, spiders, terrestrial isopods, asellote isopods from Lighthouse Cave, frog tadpoles, and cooked meat (shrimp, lobster, fish, chicken, and turkey).

All photographs of *B. geracei* in this paper are of live specimens (Figs 3A–7E) except for the shed exuvium (Fig. 7F) using various Nikon cameras with built-in flashes and a 60 mm micro-Nikkor lens, either shot through a dissecting microscope or directly. An accessory flash helped illuminate microscope photographs. Each figure is labeled with the isopod's orientation (dorsal, ventral, or lateral), size (body length in mm), identification number, year of collection, and date photograph was taken.

Table 1. Numbers and sizes of *Bahalana geracei* from Lighthouse Cave (1978–2018).

Year	Mancas / Sizes [mm]	Males / Sizes [mm]	Females / Sizes [mm]	Total
1978	0 / 0–0	1 / 8.0	4 / 13.6–15.0	5
1979	1 / 4.0	2 / 8.0	4 / 12.0–14.0	7
1992	0 / 0–0	1 / 6.0	27 / 5.0–16.0	28
1993	3 / 3.0–3.3	4 / 5.8–7.9	31 / 4.5–16.0	38
1994	10 / 2.5–3.8	9 / 4.5–8.3	60 / 4.8–16.0	79
1995	3 / 3.3–3.9	7 / 4.4–7.5	48 / 4.7–16.5	58
1996	7 / 2.5–3.3	19 / 4.6–7.1	62 / 4.2–16.8	88
1997	2 / 3.7–3.8	27 / 4.0–8.3	61 / 3.8–13.3	90
1999	10 / 2.6–4.2	32 / 3.6–8.0	80 / 4.0–15.5	122
2000	7 / 2.6–3.9	22 / 4.2–9.5	68 / 5.0–16.2	97
2001	5 / 2.3–3.8	21 / 4.5–7.0	112 / 4.5–16.0	138
2002	6 / 2.5–3.5	15 / 4.8–8.0	63 / 4.5–14.8	84
2003	11 / 2.5–3.8	24 / 4.0–8.5	72 / 4.0–14.7	107
2004	1 / 3.2	4 / 3.5–7.0	58 / 4.5–16.5	63
2005	3 / 2.4–3.9	3 / 6.0–8.2	55 / 4.0–16.0	61
2006	4 / 2.6–4.0	8 / 5.0–8.0	60 / 4.0–13.0	72
2007	8 / 2.8–4.0	17 / 3.5–7.0	70 / 4.0–16.5	95
2008	8 / 2.8–4.0	3 / 5.5–7.3	19 / 4.3–14.5	30
2011	0 / 0–0	6 / 4.0–7.0	12 / 6.0–16.0	18
2013	0 / 0–0	0 / 0–0	6 / 6.8–9.0	6
2013	0 / 0–0	0 / 0–0	7 / 5.0–13.0	7
2014	0 / 0–0	0 / 0–0	10 / 8.8–13.8	10
2016	0 / 0–0	7 / 5.0–7.5	12 / 4.0–14.0	19
2018	3 / 3.2–4.0	12 / 3.5–7.0	46 / 4.5–16.0	61
Totals	92 / 2.3–4.0	244 / 3.5–9.5	1047 / 3.8–16.8	1383

Results

Reproduction and development

Until this study, little has been reported on any aspect of reproduction in cave cirolanids. Fortunately, I was able to observe all stages of the life cycle of *B. geracei*. These include: mancas (with three stages: M1, M2, and M3) that had recently been released from brooding females; males that were young pre-reproductive juveniles and older mature breeders; and females that were pre-reproductive juveniles, egg-bearers, brooders, oostegite-bearers, inter-cycle females, and post-reproductive females. Numbers and sizes of specimens in these stages that were collected in Lighthouse Cave from 1978–2018 are summarized in Table 1 and Fig. 2. Details of these data are discussed later in the sections on "Growth rates and longevity" and "Population structure." However, first I will describe details of the reproduction and development observed in the laboratory, accompanied by photos (Figs 3A–6F), to give visual images of the life cycle stages.

Although the description of the life cycle could start at any stage, I decided to start with: (1) egg-bearers that had not yet undergone reproductive molts to produce marsupia with oostegites, followed by (2) breeding procedures that led to mating and fertilization of eggs, (3) brooding of embryos and mancas inside marsupia, (4) release of mancas, (5) post-marsupial manca development, (6) oostegite-bearing females, and (7) females that were collected with eggs or mancas still in their marsupia.

Size ranges in mm	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9	7.0-7.9	8.0-8.9	9.0-9.9	10.0-10.9	11.0-11.9	12.0-12.9	13.0-13.9	14.0-14.9	15.0-15.9	16.0-16.9	Totals and %
Males	4	45	87	66	30	11	1								244 = 19%
Egg-bearers	0	2	44	137	99	41	13	7	4	2	2	2	0	1	354 = 27%
Oost.-bearers	0	0	6	49	47	18	3	5	1	4	13	11	8	2	167 = 13%
Non-breeders	2	55	73	80	61	54	28	27	19	21	29	37	21	19	526 = 41%
All females	2	57	123	266	207	113	44	39	24	27	44	50	29	22	1047 = 81%
															M+F = 1291

Figure 2. Post-manca specimens of *Bahalana geracei* from Lighthouse Cave (1978-2028), color coded for quantities within reproductive conditions, **Blue** = males in top row: 2 smallest size classes (3 & 4 mm) were pre-reproductive (light blue), males peaked in 5 mm class then declined rapidly in next 4 size classes, **Green** = smallest egg-bearers (4 mm class) and oostegite-bearers (5 mm class), **Pink** = smallest non-breeders (3, 4, & 5 mm classes) were presumed to be pre-reproductive, **Yellow** = peak numbers for females were in 6 mm class, **Red** = lowest numbers for females were in 11 mm class, **Gray** = females persisted in largest classes (13, 14, 15, & 16 mm) in all stages.

Egg-bearers

As seen in Fig. 2, 354 egg-bearing females (without oostegites) were collected in this study. Egg size for each female was estimated as small, medium, or large. Some of these females were kept for long-term study to determine length of time for egg production and for possible breeding (Fig. 3A, C–F). It took about 9–24 months for females to grow eggs to maturity.

The two smallest egg-bearing females (Fig. 2, green) were 4.5 and 4.8 mm (probably 2nd juvenile instars) with many more (n = 44) in the 5 mm class and the most egg-bearers (137 of 266 females = 52%) in the 6 mm class, followed by gradual declines over the next 10 classes. Egg-bearing females (n = 354) represented ~27% of all the 1047 females collected. This is an extraordinarily high number, since collections of most cave cirolanid species have never included any specimens reported as "ovigerous", and this certainly contributed to the relatively high population in *B. geracei*.

Incidentally, for this study I avoid using the vague term "ovigerous", which has been applied to females with either eggs or embryos inside ovaries, or pereon, or marsupium.

Breeding procedures and mating

Isopods typically molt in two stages, including the reproductive molt (parturial molt). According to Wilson (1991), the gravid female "first molts the exoskeleton posterior to the fifth thoracic segment", mates when her exoskeleton is soft, and "the female will then molt the anterior half of the body and deploy the oostegites that form the brood pouch." But the process is different in *B. geracei*. Several times captive females were observed to have had molted only their posterior halves, males were put with them for

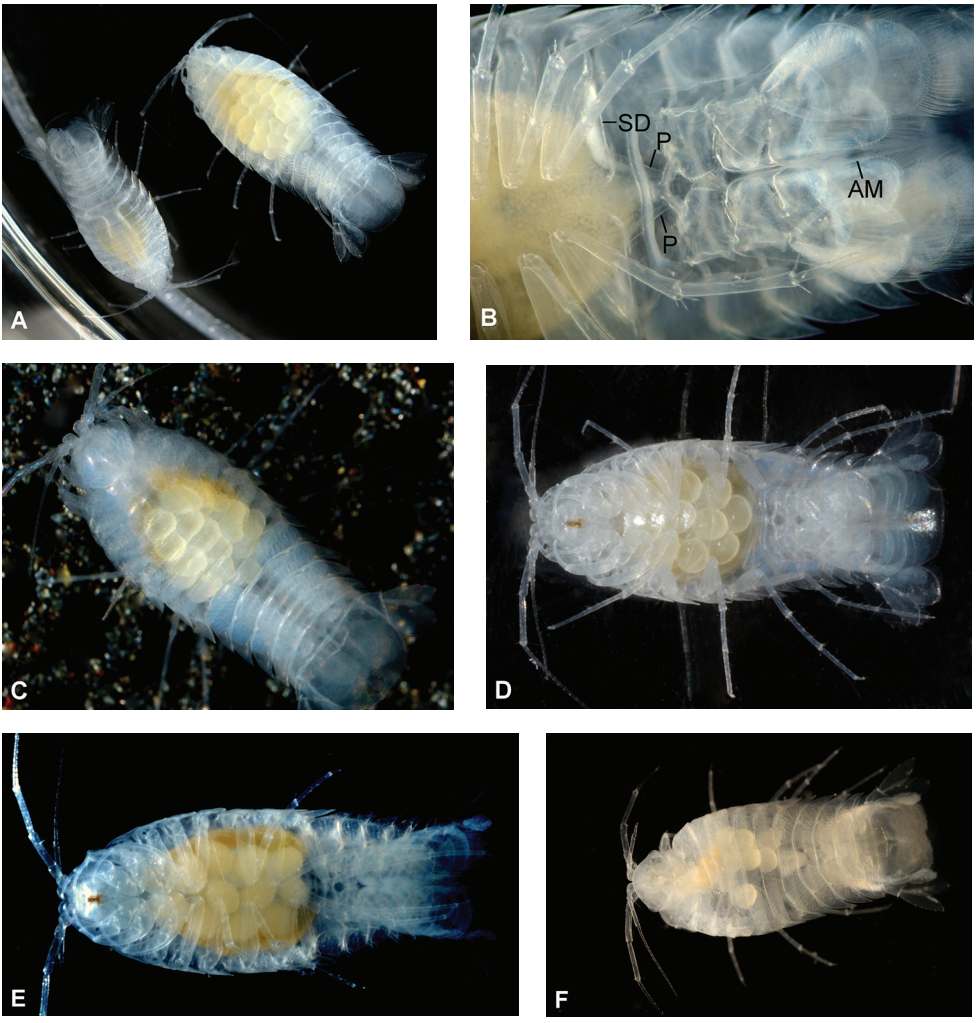

Figure 3. Male and female *Bahalana geracei* **A** dorsal view; 5.8 mm ♂ #5 (2016) on left with white sperm ducts; 8.0 mm ♀ #6 (2016) on right with ~20 eggs ~0.5 mm diameter; she was later cannibalized by 6.0 mm ♂ #11 (2016); 1 Oct. 2016 **B** ventral view; 8.5 mm ♂ #5 (2016) with white sperm-filled ducts (SD), penes (P), and appendix masculina (AM); 24 August 2020 **C** dorsal view; 7.5 mm ♀ #33 (2018) with ~12 eggs ~0.6 mm diameter, after reproductive molt and before mating; 20 May 2019 **D** ventral view; 7.5 mm ♀ #33 (2018) with ~12 round eggs ~0.65 mm diameter, in marsupium 3 weeks after mating; 13 June 2019 **E** ventral view; 7.5 mm ♀ #33 (2016) with ~12 elongated embryos in marsupium, 6 weeks after mating; 6 July 2019 **F** dorsal view; 8.0 mm ♀ #6 (2016) with a few eggs after being cannibalized by 6.0 mm ♂ #11 (2016); 1 Oct. 2016.

possible mating, but neither the males nor females showed interest in mating; the male was then removed. This was my first indication that female *B. geracei* mate only after molting the anterior half (Fig. 7F), which is usually ~4 days after the posterior half. Sometimes this led to successful mating, and sometimes not. If mating did not occur

and eggs were not fertilized, eggs inside her pereon gradually deteriorated into two white masses and were reabsorbed. Such white masses were never observed in freshly collected females, which indicates females in the caves probably had little trouble attracting mates at the appropriate time.

It was challenging to breed *B. geracei* since all adult males and females were normally kept in individual containers to avoid cannibalism. To reduce the chances of cannibalism during a mating encounter, males were fed before being placed with a female. On one occasion, 6.0 mm male #11 (2016) was placed with 8.0 mm female #6 (2016) when timing seemed to be right for mating (after molting posterior and anterior halves), but he unexpectedly attacked her and ate some of her eggs before he could be removed (Fig. 3F). Even though this male was smaller, and he had eaten 13 days before their encounter, he was apparently more interested in feeding than mating; in subsequent attempts, even smaller males were paired with females whenever possible, and food was offered to them within 5 days of pairing.

Sometimes actual mating was not observed, but the pair was left unattended for several hours or days after her anterior molt, and females subsequently produced successful broods. These successes allowed for observation and photography of females incubating eggs and embryos during their incubation periods of 5.5–6.0 months, release of mancas, and their subsequent development.

According to Wilson's (1991) report on isopod genitalia, "The details of copulation are generally unclear because it occurs so quickly (Ridley 1983)." Apparently, there are no published records of copulation in any cave cirolanids. As mentioned above, many isopod species copulate during the female's biphasic reproductive molt, but several times male *B. geracei* were unsuccessfully paired with females before her anterior half was molted; all successful matings occurred after the anterior half was molted (up to 8 days afterwards) and oostegites were deployed. This provided a narrow window of opportunity for me to find females when they might be receptive. Fortunately, this also provided an opportunity for me to control the circumstances for mating, to observe the actual mating activity at least three times, and to record it on film once.

The first successful captive breeding event for *B. geracei* was with 6.2 mm female #49 (1995) collected with large eggs in Lighthouse Cave, 4 July 1995. By 15 November 1995 she had molted both halves, so 6.6 mm male #27 (1995) was put into her container. Six months later, on 12 May 1996 (Mother's Day in the U.S.), #49 released 3 mancas, 2 more the next day, and 4 more on 15 May 1996; development of these 9 mancas are described at the end of the section on Post-marsupial manca development. During #49's 6-month gestation (described in next section on Gestation) her activity level decreased; she remained stationary on a vertical screen for 18 days straight. But she was active enough to eat four small meals and grew to 7.0 mm. She molted 51 days after manca release, then again 4 months after that.

The second successful breeder was #88 (1996), a 13.2 mm female with no discernible eggs when collected in Lighthouse Cave, 15 July 1996. By 30 July 1997 (1 year after collection) she had molted both halves, now 13.8 mm with oostegites and

~14–16 eggs on each side. Two days later I added 6.3 mm male #72 (1997) and filmed his mating behavior. He swam past her twice then climbed onto her right side, rapidly tapped her antennae while his head was near the top of her head, moved to her left side and tucked his abdomen near her 5th pereopod for about 3 seconds, moved back to her right side and pushed her 6th and 7th pereopods posteriorly, mated for about 10 seconds with his abdomen tucked under her while thrusting his pleon and rapidly beating his pleopods. After mating, he rested on her side nearly 10 minutes; at one time he put his head near the ventral part of pereopod 5 for about 30 seconds, possibly to check sperm. After dismounting, he rested near her side for a few minutes until she slowly moved away. Under a dissecting microscope, sperm were visible inside his sperm ducts and on her 5th pereonite. Microscopic examination the next day revealed sperm inside her spermathecae and eggs inside her marsupium (in contrast to #49 described above). For the next three weeks she periodically pushed against her oostegites with her "elbows" of pereopods 1 to move ~18 eggs forward and backward inside her marsupium, while rapidly ventilating with her maxillipeds (~50 times/15 seconds); then she flexed her body to remove some excess water from the marsupium. Small sperm packets remained visible in her spermathecae for the next three weeks. Surprisingly, her movement of eggs back and forth gradually pushed all of them out the posterior end of her marsupium, and no embryos or mancas were produced. Some of these mating behaviors are compared to other crustaceans in the discussion section.

Gestation

The successful mating of female #49 (1995) described above resulted in an unusual gestation. During incubation, I examined her marsupium using mirrors, fiber optic lights and a microscope. Side views showed the marsupium expanding and contracting with fluid (aiding the circulation created by beating maxillae), but eggs, embryos or mancas were never visible inside her marsupium (Fig. 4A). Instead, they appeared to be retained inside her pereon (Fig. 4C). This was totally unexpected and did not match the normal incubation inside the marsupium of most other isopods (Johnson et al. 2001; Wilson 1991), nor of my later successful *B. geracei* brooders described below (more on this in the discussion section).

On 6 July 2018, 61 specimens of *B. geracei* were collected in Lighthouse Cave; 17 females were egg-bearers; eight were retained for further study. During the next 17 months, three completed their reproductive cycles. Males were added to each of the females after their reproductive molts were completed; no mating was observed, but a male was left with each one for several days. Photos (Figs 4B, D–F, 5A) document these reproductive events over 12–17 months. Additional photos document growth and development of mancas (Fig. 5A–F). Here are timelines for these reproductive events (egg development through manca release) for these three females:

1. #5 (2018) 11.0 mm with medium eggs, ate 5 times, reproductive molt on 10 February 2019 (7 months after collection), ate 4 times during gestation (5.5 months), 55 mancas released in 11 days (27 July to 6 August 2019), 2.3–2.8 mm (Figs 4B, D, E, 5A, B).

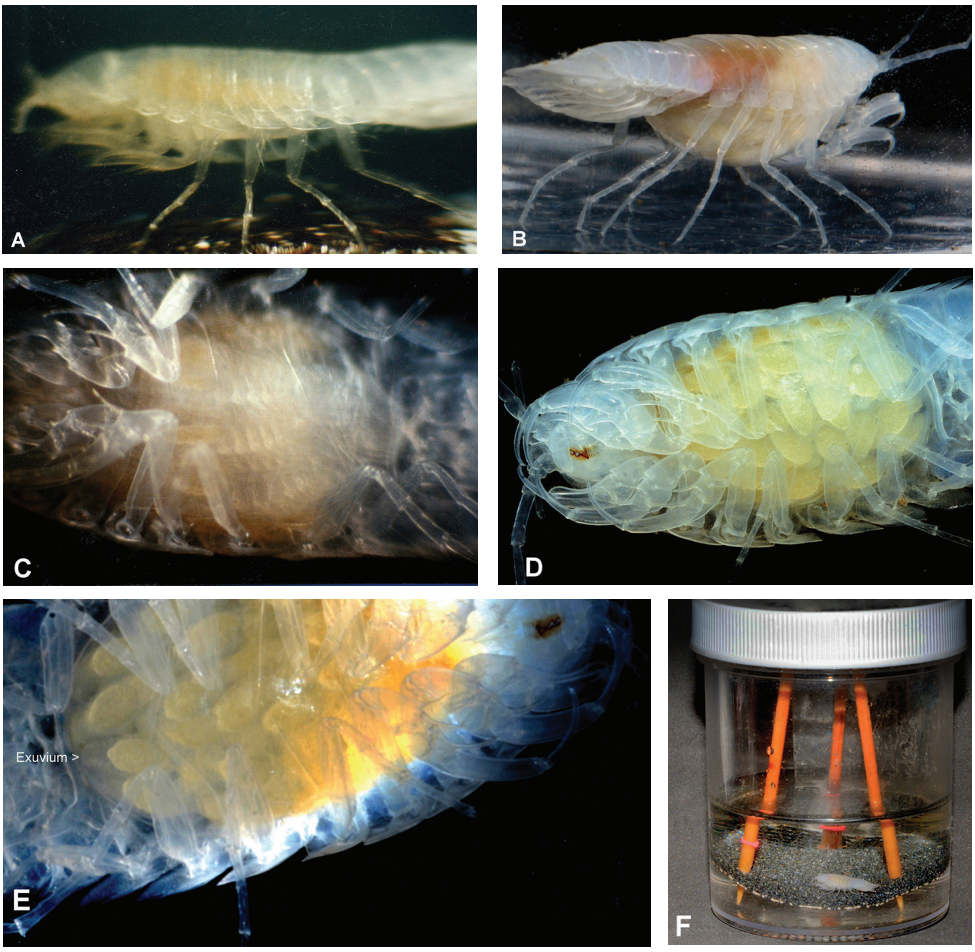

Figure 4. *Bahalana geracei* gestation **A** lateral view; 6.2 mm ♀ #49 (1995), marsupium with water partly expelled and no mancas; May 1996 **B** lateral view; 13.0 mm ♀ #5 (2018) with mancas in marsupium, brown gut 14 days after eating worm; 31 July 2019 **C** ventral view; 6.2 mm ♀ #49 (1995) with mancas inside perion; May 1996 **D** ventral view; 13.0 mm ♀ #5 (2018) with developing embryos inside marsupium, 3 months after mating; 11 May 2019 **E** ventral view; 13.0 mm ♀ #5 (2018) with developing embryos, some at posterior end with exuvia, 4 months after mating; 8 June 2019 **F** maternity jar with screen holding 13.0 mm ♀ #5 (2018) 1–2 cm above bottom of jar; 3 August 2019.

2. #33 (2018) 7.5 mm with large eggs, ate 5 times, reproductive molt 20 May 2019 (10 months after collection), ate 4 times during gestation (5.5 months), 13 mancas released in 18 days (2 November to 19 November 2019), 2.3–3.0 mm (Fig. 3C–E).

3. #35 (2018) 7.0 mm with large eggs, ate 10 times, reproductive molt 29 June 2019 (11.5 months after collection), ate 2 times during gestation (5.7 months), 6 mancas released in 8 days (19 December to 26 December 2019), 3.0–3.3 mm.

The number of mancas released each day ranged from 0 to as many as 17 for #5, 4 for #33, and 2 for #35. Since #5 had so many growing eggs and embryos, her length

increased ~18% from 11.0 mm when caught to 13.0 mm before releasing mancas; #33 increased ~20% from 7.5 to 9.0 mm, and #35 increased only ~3% from 7.0 to 7.2 mm.

As is typical of isopods (Johnson et al. 2001), the overall trend for the six females with reliable records was for smaller females to have much smaller broods than larger females. The four smallest females in the 6–9 mm range had 6–13 mancas: 6 for 7.2 mm #35 (2018), 9 for 7.0 mm #49 (1965), 12 for 7.2 mm #92 (2000), and 13 for 9.0 mm #33 (2018). The two largest females in the 13–15 mm range had 32–55 mancas: 32 for 14.8 mm #1 (2002) and 55 for 13.0 mm #5 (2018). For details on 7.2 mm #92 (2002) and 14.8 mm #1 (2002) see section below on Brooders with eggs or mancas.

Here are additional details regarding eggs and brood sizes for various females, including #5, #33, and #35. Eggs were usually round while developing inside the pereon (Fig. 3C) and when first placed in the marsupium (Fig. 3D); within three weeks they became elliptical embryos (Fig. 3E). Egg diameters were ~0.5 mm for large females such as 8.0 mm #6 (2016) (Fig. 3A) and 13 mm #5 (2018). For two smaller females, 7.5 mm #33 (Fig. 3C, D) and 7.0 mm #35, eggs were slightly larger (~0.60 to 0.65 mm).

Egg sizes and brood sizes of *B. geracei* were compared to marine cirolanids with comparable body lengths (5–16 mm) found in table 3 in Johnson et al. (2001). Only two such species were listed with egg diameters: *Cirolana carinata* (0.43 mm) and *Excirolana chiltoni* (0.6–0.9 mm); so, *B. geracei*'s egg diameters of ~0.5–0.65 mm were not unusual for cirolanids of this size.

Johnson et al. (2001) listed the following brood sizes for ten comparable species: *Cirolana harfordi* (18–68), *Cirolana imposita* (15–33), *Cirolana parva* (11–28), *Cirolana carinata* (14–45), *Eurydice longicornis* (34–59), *Eurydice natalensis* (13–25), *Excirolana chiltoni* (10–55), *Excirolana japonica* (17–68), *Pseudolana cocinna* (7–45), and *Pseudolana towrae* (18–24). The lower number $\bar{X}$ for these 10 is 15.7; the higher number $\bar{X}$ is 45.0. So, *B. geracei*'s brood sizes of 6–55 ($\bar{X}$ = 21.2, n = 6) appear to be in the lower end of the range for cirolanids of this size, although my sample size is small.

According to Johnson et al. (2001), "Three molts occur while the embryos are still in the brood pouch in isopods. The three molts include hatching from the egg membranes, a postnaupliar molt, and a larval ecdysis just prior to release from the brood pouch."

Apparently, mancas from female #5 (2018) molted late in development since shed exuvia could be seen inside her marsupium (Fig. 4E), and she released remains of exuvia into the water along with mancas; some of these exuvia were shed in one piece (i.e., monophasic). The marsupial molts seen in Fig. 4E were photographed ~6 weeks before release, which suggests they were probably postnaupliar molts, although they could also have been larval ecdyses.

Post-marsupial manca development

Most isopod species, including *B. geracei*, go through three instars or manca stages (M1, M2, and M3) before the 7th pair of pereopods becomes formed and functional (Wilson 1981); then I call them "juveniles." Manca 1 (M1) is the first instar upon release from the marsupium; there was no sign of 7th pereopods or external male genitalia. After their first molt they were in the manca 2 stage (M2); they still had no clear

sign of 7th pereopods, but males had a pair of tiny penes. Since M1's varied in size from 2.3–3.3 mm, and molts resulted in a size increase of 0.3–0.5 mm, M1 and M2 individuals overlapped in size and there was no way to distinguish between M1 and M2 females in the overlapping size range; M2's were identified as male M2's if penes were visible (but no 7th pereopod). In manca 3 stage (M3), 7th pereopods were partially developed, non-functional, and held across the body beneath pereopods 6 (Fig. 5E, F). Some M3 males collected in Lighthouse Cave had sperm in their sperm ducts.

Because manca stages are difficult to tell apart, published reports on field collections (including type series for descriptions of new species) often recognize all post-marsupial instars simply as "mancas" or "immatures;" (e.g., Botosaneanu and Illife 1997, 2003a; Bruce 2008). Fortunately, two *B. geracei* mancas from female #5 (2018) survived long enough to go through stages M1, M2, and M3 to develop into juveniles. Here are more details of the three manca stages in this species, based on mancas from #49 (1995), #5 (2018), #33 (2018), and #35 (2018).

In the above section on Gestation, the unusual 6-month incubation for #49 (1995) was described. When she released her 9 mancas, body lengths were 2.5–2.7mm. They began eating at 12 days and ate regularly every 1–3 weeks until fasting for 1–2 months before molting. Five mancas survived long enough to molt from M1 to M2 in 111–296 days old ($\bar{X}$ =169 days); these molts increased body lengths by 0.3–0.5 mm; they lived another 105–300 days without molting to M3, eventually dying at 10–20 months old.

Large 13 mm female #5 (2018) released her first 5 mancas on 27 July 2019 while being prepared for photographs under a dissecting microscope, so she and 3 mancas were photographed together (Fig. 5A). She released 50 more mancas over the next 10 days. Some were kept in individual containers; sometimes all from one day were kept together in one jar to observe interactions and to reduce maintenance activities. Newly released mancas held their antennae and pereopods straight against their bodies (Fig. 5B); several were dead when released, or so weak that they moved little and died in a few days. Each had a white hepatopancreas (gastric caeca or midgut gland) that contained enough nourishment for several days (Fig. 5B–D). First meals began at 6–15 days old (Fig. 5C, D). Food was offered at intervals of 1–3 weeks and included California black worms (Figs 6A, B), pieces of shrimp (Fig. 5C), brine shrimp (Figs 5D, 6C, D), centipedes, spiders, and live 1–2 mm long *N. stocki* isopods from Lighthouse Cave; there was one case of cannibalism. The clear exoskeleton allowed for observation of food intake, including the eyes of brine shrimp clearly visible in the stomach of one (Fig. 6D). Shrimp pieces and brine shrimp often turned red inside their stomachs (Fig. 6E); later the hepatopancreas turned red and remained red for several days (Figs 5E, F, 6F). Feces appeared in hind gut within 1 day after eating; a fecal string was sometimes passed a few days later.

Nine mancas from #5 (2018) survived long enough to molt to M2; most of them died shortly after molting. One healthy survivor molted from M2 to M3 in 59 days, then from M3 to juvenile (J1) in another 58 days. Another one molted from M2 to M3 in 84 days, then from M3 to J1 in 65 days. Thus, the time spent in each stage for

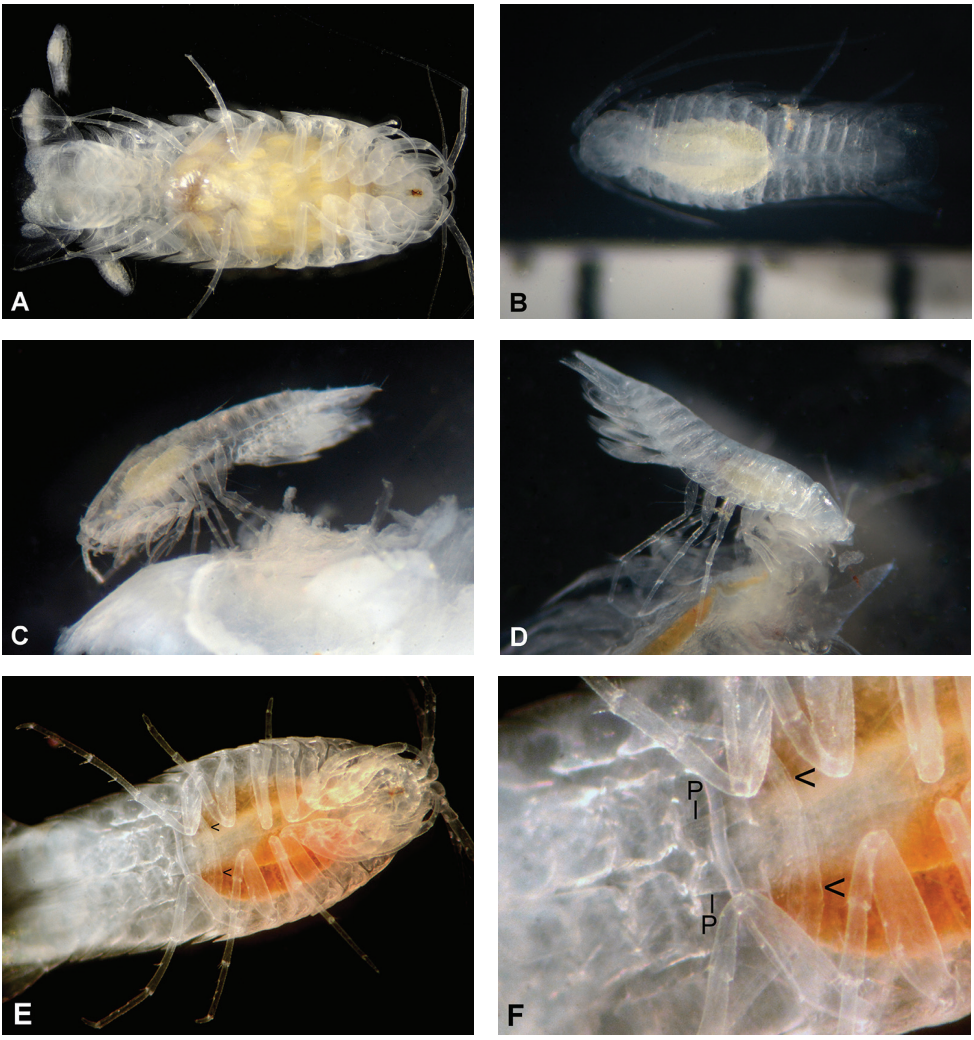

Figure 5. *Bahalana geracei* manca development **A** ventral view; 13.0 mm ♀ #5 (2018) releasing first mancas 2.3 mm long; 27 July 2019 **B** dorsal view; manca (M1) 2.3 mm long, just released from female #5 (2018); note white hepatopancreas and most appendages held along sides; 27 July 2019 **C** lateral view; 2.5 mm manca (M1) #7-31A (2019) eating first meal (shrimp); 5 August 2019 **D** lateral view; manca (M1) eating brine shrimp; 11 August 2019 **E** ventral view; 3.5 mm ♂ manca (M3) #8-6 (2019) with red hepatopancreas from eating shrimp; arrows at developing 7th pereopods crossed under 6th pereopods; 18 April 2020 **F** ventral view; 3.5 mm ♂ manca (M3) #8-6 (2019) with red hepatopancreas; arrows at developing 7th pereopods, P's point to penes; 18 April 2020.

these 9 mancas were: M1 65–123 days ($\bar{X}$ = 103.2 days, n = 9), M2 59–84 days ($\bar{X}$ = 71.5 days, n = 2), M3 58–65 days ($\bar{X}$ = 61.5 days, n = 2), total 254–268 days ($\bar{X}$ = 261 days, n = 2). This is a long time for isopod manca development and is compared to other species in the section on Life cycle stages.

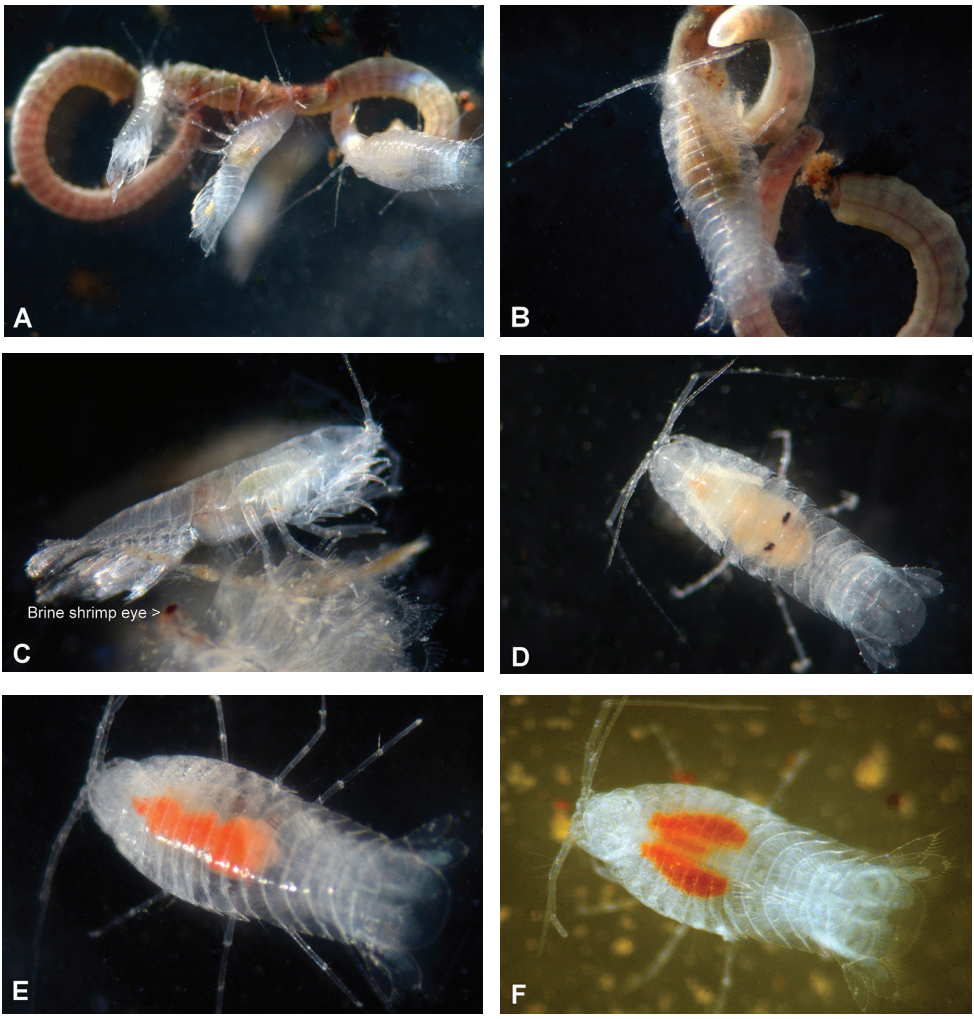

Figure 6. *Bahalana geracei* mancas feeding **A** lateral and dorsal views; ~2.5 mm mancas (M1) eating California black worm; 27 August 2019 **B** dorsal view; ~2.5 mm manca (M1) with full gut from eating worm; 27 August 2019 **C** lateral view; ~2.5 mm manca (M1) #7-30 (2019) eating brine shrimp; eye at arrow was eaten and shows in next photo; 11 August 2019 **D** dorsal view; ~2.5 mm manca (M1) #7-30 (2019) with dark brine shrimp eyes in stomach; 11 August 2019 **E** dorsal view; ~2.5 mm manca (M1) #7-27A (2019) with red gut after eating cooked shrimp; 21 October 2019 **F** dorsal view; ~2.5 mm manca (M1) #7-27A (2019) with red hepatopancreas 10 days after eating cooked shrimp; 31 October 2019.

Unfortunately, many mancas refused to eat anything, and others stopped eating after a few meals. Fasting was often related to preparation for molting; mancas usually fasted for 1–5 weeks before molting and 1–2 weeks afterwards. It took 1–4 days ($\bar{X}$ ~ 2.0, n = 9) between molting posterior and anterior halves; once molting was monophasic to leave a complete exuvium. First molts occurred after eating 3–8 meals. Molting seems to be a challenging process required for isopod growth, especially for mancas. Of the 13 mancas from #33 and 6 mancas from #35, none lived long enough to complete their first molts.

Even mancas released on the same day varied in size from 2.3 to 3.3 mm, which provided opportunities for cannibalism. Molts resulted in size increases of 0.3–0.5 mm.

Irregular molting and fasting created additional cannibalism opportunities for mancas that completed their molts to become larger than their smaller (and sometimes fasting) siblings housed with them. Cannibalism was observed only three times for mancas from #5, #33, and #35. After three months all surviving mancas were housed separately.

Oostegite-bearers

After mancas are released from a female's marsupium, she retains her oostegites for several months until her next molt, which I call an "oostegite molt." Oostegite-bearers have rarely been observed in other cave cirolanids. Botosaneanu et al. (1986) pointed out that, "Concerning the reproduction, it is interesting to note that ovigerous females or females with brood plates or pouches, were apparently never found in the subterranean species (this was expressly noted, for instance, for *Antrolana*, *Bahalana*, some *Typhlocirolana*…); this phenomenon still awaits explanation (one published explanation being that ovigerous females are very rare and secretive, rarely foraging in areas accessible to sampling)." In their description of a single oostegite-bearing specimen of their new species *Zulialana coalescens* Botosaneanu & Viloria, 1993, the authors re-emphasized the rareness of this phenomenon by noting that, "this is one of the very few known cases of subterranean cirolanids where specimens with oostegites were found (to the best of our knowledge the only already known case being that of *Skotobaena*)."

In this study of *B. geracei* it was surprisingly common to find females with oostegites. In fact, out of 1047 adult females collected in Lighthouse Cave, 167 (= 16.0%) were oostegite-bearers (Fig. 2). These females had released mancas from marsupia within the previous few months, and some molted their oostegites 3–13 months later in culture (Table 5). The six smallest oostegite-bearers (green) were in the 5 mm class with many more (n = 49) in the 6 mm class, followed by declines to a low of one in the 11 mm class, then a surprising increase in the last 5 size classes. Since oostegite-bearers were found in all size classes larger than 5.9 mm, this is a strong indication that females were capable of having multiple broods over their long lifetimes. More individuals probably stayed in their larger instars longer (including oostegite-bearing) because of slower molt cycles.

Non-breeders

About 50% of all females collected (526 of 1047) did not have detectable eggs or oostegites, so they were considered non-breeders. This group included: (1) pre-reproductive females that were too young and small to produce detectable eggs, (2) inter-cycle females that had completed a reproductive cycle (including release of mancas and shedding of oostegites) and had not yet produced a new set of detectable eggs, and (3) post-reproductive females that were larger/older and seemed to have stopped reproducing. The smallest females in the 3, 4, & 5 mm classes (pink) were presumed to be pre-reproductive,

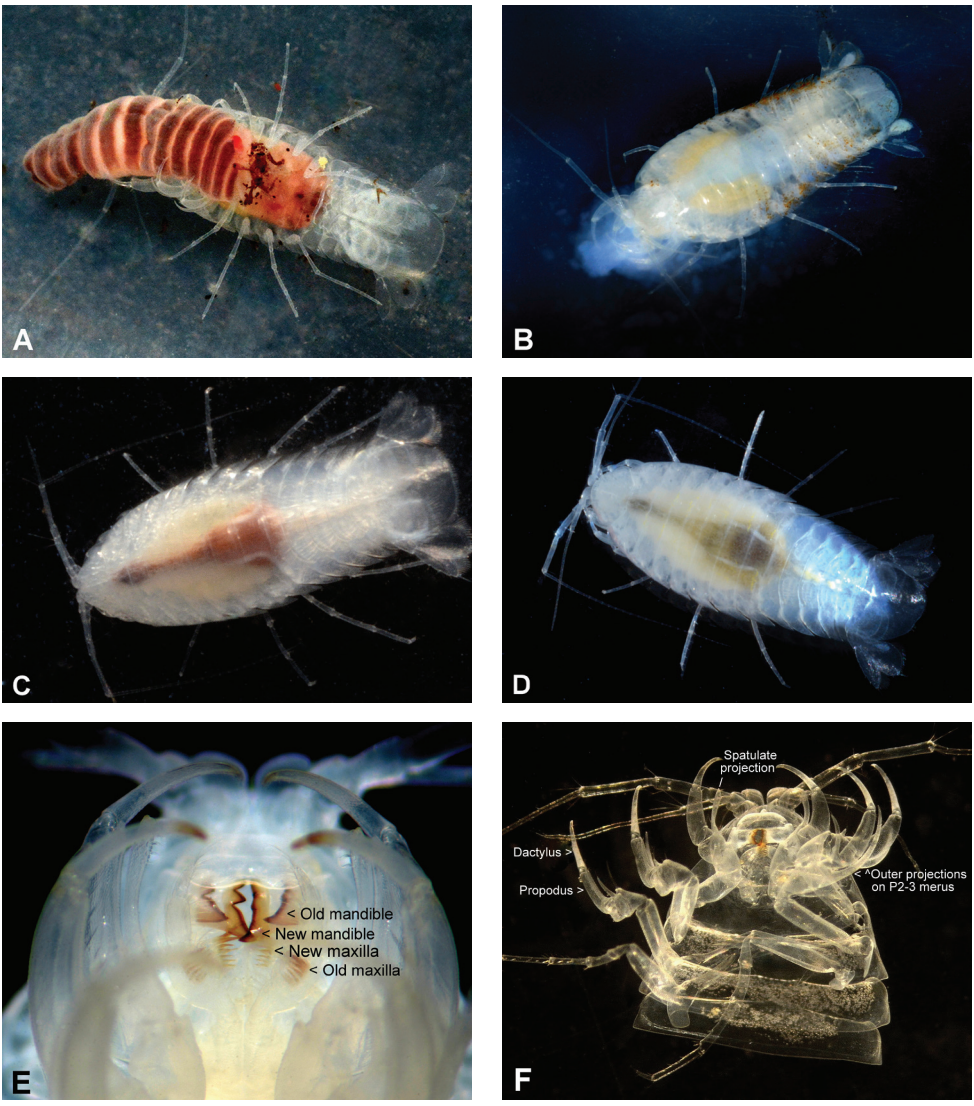

Figure 7. *Bahalana geracei* adults, feeding and molting **A** ventral view; 6.0 mm ♀ #7 (2016), pereopods 1–3 holding earthworm, worm in gut; 7 January 2017 **B** dorsal view; 9.0 mm ♀ #1 (2013), pereopods 1–3 holding shrimp piece forward to eat; shrimp in gut; 21 January 2013 **C** dorsal view; 5.5 mm ♂ #18 (2016), 4 weeks after eating earthworm, visible in gut; 26 November 2017 **D** dorsal view; 8.7 mm ♂ #37 (2018), 4 weeks after eating centipede; white fecal pellets forming in hind gut; 30 May 2020 **E** ventral view; 9.5 mm ♀ #10 (2018), double mandibles and maxillae before molting; 10 July 2020 **F** ventral view; anterior exuvium from 6.3 mm ♀ #23 (2018); 24 September 2019.

although some might have been producing eggs that were too small to be detected. The 6 mm class (yellow) had the greatest number of non-breeders (80) and likely consisted of a mix of pre-reproductive females and inter-cycle females. The largest non-breeders likely

consisted of inter-cycle and post-reproductive stages; the number of large inter-cycle females may have increased with size partly because this recovery stage should require more energy and time after larger broods. Non-breeders and other females gradually declined to lows in the 11 mm range (red), presumably because of mortality.

Brooders from caves

There have been few reports of cave cirolanid females brooding eggs or mancas within their marsupia. In their description of *Yucatalana robustispina* (Botosaneanu & Iliffe, 1999) the authors mentioned that a "female allotype has a well-developed marsupium in which 3 very large eggs were found." Botosaneanu and Iliffe (2000) later remarked that one additional female specimen of *Y. robustispina* was caught and "deserves a special mention, because it has 10 pulli in its marsupium–a remarkably high number for a stygobitic cirolanid." Messana (2020) reported that one female in his type series of *Catailana whitteni* was "ovigerous, 15.4 × 4.6 mm, bearing 9 eggs in brood pouch."

Brooding female *B. geracei* were also rare in our cave collections, so they are not shown in Fig. 2. Out of 1047 females collected in Lighthouse Cave, only four were incubating eggs or mancas. Here are notes regarding each of them, recorded soon after they were collected:

1. 17 July 2000, #92. 7.2 mm ♀ with ~12 large eggs in marsupium; 6 Nov. 2000, #92 in mud-bottomed container had 11 mancas, most are healthy and active; mother appears to have 2 mancas inside (consistent with original estimate of ~12 large eggs on 17 July); mother was never observed to dig into mud and remained moderately active, so the hypothesis of pregnant ♀♀ being rare due to hiding in substrate still needs confirmation.
2. 22 July 2002, #1. 14.8 mm ♀ with 11 mancas in film can; had 21 more in next 4 days.
3. 16 July 2003, #67. 7.0 mm ♀ with oostegites & 2 mancas (2.5 mm) in film can.
4. 11 July 2006, #41. 8.4 mm ♀ with 1 manca in marsupium & 2 (2.6 mm) in film can.

Probable explanations for why so few brooders were collected are covered in the discussion section on Gestation. Fortunately, considerable information about brooders was obtained from successful breeding in captivity, described above in Gestation. They are also included later in Table 7 Life cycle stages.

Feeding behaviors

Feeding in culture and in caves

Individual records were kept for dozens of *B. geracei* specimens that were collected in the caves, then raised under laboratory conditions for up to seven years. They were measured

approximately every six months and/or after a molt. Measurements were made before feeding because a full meal could increase length by ~20%. Food was offered every ~3–6 weeks even when food was still visible inside them, and they often still accepted the food.

As mentioned in culture methods and in descriptions of manca feeding, *B. geracei* ate a large variety of foods. Live food such as California black worms, earthworms, ghost shrimp, and brine shrimp were attacked and eaten while still alive. Species in the genus *Bahalana* can be distinguished from all others in the family Cirolanidae because pereopods 1–3 (P1–3) are prehensile with the two distal segments (dactylus and propodus) elongated and with long projections on several segments (especially the merus) (Fig. 7F). Photographs showed that P1–3 were used to grasp and manipulate prey (Fig. 7A, B), while mandibles pulled food into the mouth. Pointed tips of P1–3 often penetrated prey tissue, but projections on the outer side of P2–3 were often held away from prey (see lower side of worm in Fig. 7A). Other possible functions for lateral projections are described in the Discussion section.

When dead food, such as a piece of shrimp, was placed close to a *B. geracei*'s head it was often attacked right away. If food was placed further away, isopods usually increased searching activity until the food was found, sometimes while "dancing" rapidly with head down near substrate and tail up, apparently following a scent trail. However, it was not unusual for them to wait 30 minutes or more before eating. Individuals took about 1–30 minutes to complete their meals, which roughly corresponded to the amount of food ingested. Food intake and the digestive processes were easily monitored since *B. geracei* exoskeletons are relatively clear. Dark food such as earthworms, spiders, and centipedes could be seen in enlarged digestive tracts, sometimes for several weeks (Fig. 7C, D). When shrimp of any kind (commercial, brine, ghost, or *B. cubensis*) was eaten, the hepatopancreas often turned red or orange as digestion proceeded (Fig. 6F).

Digestion time varied widely depending on size and type of meal. If a small liquid meal was eaten (e.g., body fluids from prey) it was processed as quickly as 2–4 days, and fecal pellets were not formed. More often the food consumed consisted of muscle (e.g., cooked shrimp) or other internal and external body parts (Fig. 7C, D), which often took 3–8 weeks to digest and for the gut to clear; fecal pellets started forming in a few days, and fecal pellets or strings were passed about 2–8 weeks after eating.

Experiments were performed to observe interactions between *B. geracei* and other crustaceans that live in Lighthouse Cave. When 14.3 mm female #39 (1995) was placed in a bucket with a 6 cm red shrimp *B. cubensis*, within 3 minutes the shrimp grabbed the isopod, but a few seconds later the isopod pulled off the shrimp's leg and ate on it for 15 minutes, which turned its gut red. However, when 7.2 mm female #53 (1996) was left overnight with a 5 cm *B. cubensis*, the shrimp ate the inside of the isopod, leaving an empty exoskeleton. In other trials, live small (1–2 mm) *N. stocki* isopods from Lighthouse Cave were readily eaten by *B. geracei* adults and mancas, which turned the gut white or gray.

In some years (n = 13) I made notes when freshly collected specimens clearly had food in their guts. In 7 years, only 2–7% of specimens had food; in 6 years, 17–50% had food. Three days after hurricane Bertha hit in 1996, salinity in Lighthouse Cave dropped to ~25 ppt, and 37 of 88 specimens (= 42%) had food in gut, possibly from

food that washed in. The color of the gut hinted at probable food consumed: white for another *B. geracei* or a *N. stocki* isopod; red, pink, or orange for *B. cubensis*; brown or black from terrestrial arthropods (e.g., insects or spiders). A few specimens were dissected to examine gut contents, but this usually revealed nothing identifiable. However, specimens raised in the laboratory that ate pieces of arthropods (e.g., brine shrimp and centipedes) sometimes passed feces with remains of exoskeletons.

Cannibalism

Cannibalism has often been reported in isopods. Wong and Moore (1995) described the cirolanid *Natatolana borealis* (Lilljeborg, 1851) as a voracious omnivorous scavenger, and "Cannibalism of damaged or moulting individuals was observed frequently in the laboratory." Jormalainen and Shuster (1997) studied cannibalism in the freshwater sphaeromatid Socorro isopod *Thermosphaeroma thermophilum* (Richardson, 1897) and found that, "In laboratory containers without refuges, males cannibalized females, males and females cannibalized mancas, and mancas cannibalized each other, even in the presence of alternative food." Studies performed by one of my students, Ron Bitner, showed similar cannibalistic behavior for *B. geracei*; when 2–4 individuals were housed together, all sizes and genders were susceptible to cannibalism by individuals of the same size or larger (n = 6). However, in 10 other trials, 2–4 specimens of various sizes and genders were together >1 month without cannibalism (unpublished observations presented at 1997 Kentucky Academy of Science meeting).

Only once was cannibalism observed directly in Lighthouse Cave. On 4 July 1995, I saw a large *B. geracei* on a rock, but it did not start swimming when touched with an aquarium net. When it was maneuvered into the net, I saw it was holding and eating another *B. geracei*. Later examination showed the cannibal was an 11.0 mm female, while the victim was a 6.5 mm female (still barely alive). On another occasion (27 July 1999), while 14.0 mm female #102 (1999) was being measured soon after capture, I noticed her gut was full and white; dissection revealed the remains of a small *B. geracei* inside her stomach. Large female isopods could be more important predators than mangrove rivulus fish. A broader perspective is covered in the discussion section on Cannibalism.

Molting, fasting and starvation resistance

Most laboratory-raised *B. geracei* adults fed regularly, usually every month, but some refused food for several consecutive months, then started eating again. Others died after several months of fasting, probably because they had trouble with some aspect of molting; this was especially true for mancas, juveniles, and large adults. This emphasizes a common problem in keeping isopods and other crustaceans alive for long periods–they often have problems molting. This has been noted by other researchers such as Vogt (2018), who stated that in his "laboratory population of marbled crayfish, more than 85% of the adults died during ecdysis." Molting problems may be compounded for *B. geracei* by the long extensions on pereopods 1–3.

It was routine for adult *B. geracei* to fast for 1–3 months before a molt, when feeding structures could not function; for instance, several days before a molt, double mandibles and maxillae appeared, as seen in Fig. 7E. The time between molting posterior and anterior halves was ~2–7 days. Fasting persisted for 1–3 weeks after molting while feeding structures hardened. The fasting routine associated with molting gives crustaceans some natural resistance to starvation.

A dramatic example of starvation resistance in *B. geracei* came in June 2015 as I was cleaning film cannisters for my 2015 cave trip; I found one cannister that still had a 7.3 mm female isopod in it from a field trip at least two years before. This cannister had the usual 35 ml of saltwater and had had no water changes or aeration. This female later ate and seemed to have no negative effects from this extended fasting experience. Another extreme example is the deep-sea isopod that fasted for >5 years in a Japanese aquarium (for details, see Growth rates and longevity, below). In general, older/larger individuals have more reserves so they can probably survive pre-molt fasts much longer. Starvation resistance is particularly important for brooding females so they can apparently remain safely hidden as they fast for six months. The broad impact of this phenomenon on crustaceans in general, and especially on cave crustaceans, is described in the discussion section on Starvation resistance.

Growth rates and longevity

Growth rates in general

For many years people have asked me, "How long do your isopods live?" My reply has been, "I estimate they could live as long as 20–35 years, since the growth rates in all stages of their life cycle are extremely slow." But several variables make it difficult to accurately determine growth rates and longevity for long-lived crustaceans like *B. geracei*. These variables include: (1) higher temperatures usually create faster growth, which is probably not a major variable in this study, since lab temperatures were usually close to cave temperatures at ~25 °C, (2) food is in low supply in caves, but abundant in culture, (3) length of female molt cycles vary with their reproductive condition and age, (4) multiple broods allow for longer life spans, (5) young isopods molt much more often than older ones that may go more than a year without molting, and (6) starvation resistance permits some older slow-growing individuals to appear to be young because they remain small.

This last variable can create misleading estimates of age because a large range of ages can be in the same size class due to variations in molt and growth rates. I call this phenomenon "age compression." It can have the strongest effects in larger size ranges because growth and molt cycles become progressively slower due to reproductive costs and age, and at variable rates. Smaller size ranges were probably affected by age compression, too. For instance, "all females" in Fig. 2 increased from 123 in the 5 mm class to 266 in the 6 mm class, probably because the age range for 5.0–5.9 mm females was ~2–3 years, while the age range for 6.0–6.9 females was probably ~3–6 years.

According to Vogt (2018), "Precise data on longevity can be obtained only by rearing in captivity from hatching to death and by long-term marking with internal tags. In practice, most life span data are calculated from growth models based on length-frequency distribution, mark and recapture, and the analysis of molt increment, intermolt duration, and reproduction parameters." Vogt (2018) also stated that, "These indirect aging techniques have a small probability of error at younger ages but a large one at older ages. Therefore, in long-lived species, they give only a rough estimate of life span (Hartnoll 2001; Vogt 2012a)." This is largely due to age compression.

All the above methods were used in this study except for long-term marking with internal tags, which were not used because of the small size of *B. geracei*. I was not able to keep any specimens alive for an entire life span of >20 years, but many individuals of different sizes and reproductive conditions survived for several years to give a reasonably accurate picture of their lives as presented in Tables 1–7. Vogt (2018) pointed out that longevity can be expressed in several ways: age of oldest specimen, age of oldest cohort, mean age of oldest 10% of population, or maximum age estimated by growth models. The last method was used for this study.

Determining precise longevity in *B. geracei* was complicated because body length measurements varied considerably depending on when measurements were taken relative to feeding, molting, and stage of reproduction. A large meal could increase body size by 20%, followed by gradual return to normal over 1–2 months of digestion (so, specimens measured upon capture sometimes shrank over the next few weeks). Size sometimes increased by ~20% immediately after molting as water was absorbed to expand the new exoskeleton, then part of that gain was lost over the next few days. Females also increased length by ~10–20% while growing eggs and embryos, then lost some of that increase when mancas were released. All these variables were considered in developing and analyzing the following estimates of growth and longevity.

In the early years of this study (1993–1996) estimates of longevity were based on morphometrics: observed changes with each molt (n = 44) and length of intermolt periods ($\bar{X}$~12 months). For instance, the number of telson setae on the posterior end ranged from 11 in mancas (M1) to 60 in large females, increasing by 1–5/molt ($\bar{X}$~2); with an average of 1 molt/year, the increase of 49 telson setae (60–11 = 49) from smallest to largest individuals, divided by 2 setae/molt, gave an estimate of 49/2 = 24.5 years to develop 49 additional setae. A similar estimate of 28 years longevity was based on increases in the number of flagellar articles in antenna 1: 0–2 articles/molt ($\bar{X}$~0.5), range of 9–23 (increase of 14 articles/life), so 14 articles/0.5 articles/molt = 28 molts = 28 years. A third estimate of 35 years longevity was based on increases in the number of flagellar articles in antenna 2: 0–5/molt ($\bar{X}$~1), range of 15–50 (increase of 35/life), so 35 articles/1 article/molt per year = 35 years to produce 35 additional articles. It now appears that these estimates of ~24.5 to 35 years are more reasonable for females, rather than males and females combined, because my sample population had a slightly disproportionate number of females which had longer intermolt periods.

Table 2. Molt and growth records for male *Bahalana geracei* from Lighthouse Cave arranged by size.

Size [mm]	Specimen, year	No. of molts	Months between each molt	Total months	Months/ molt	Molts/ year	Total size increase [mm]	Increase/ molt [mm]	Increase/ year [mm]
5.0	#32, 2018	3	6.0, 5.0, 8.0	19 mo. = 1.6 yr.	6.3	1.9	5.0–6.4=1.4	0.47	0.9
5.5	#4, 2016	5	8.0, 6.0, 4.5, 5.0, 5.0	28.5 mo = 2.4 yr.	5.7	2.1	5.5–8.3=2.8	0.56	1.2
5.5	#30, 2018	4	7.0, 5.0, 5.0, 6.0	23 mo. = 1.9 yr.	5.8	2.1	5.5–7.0=1.5	0.38	0.8
5.5	#58, 2018	3	6.0, 5.0, 8.0	19 mo. = 1.6 yr.	6.3	1.9	5.5–6.5=1.0	0.33	0.6
5.8	#5, 2016	5	5.0, 14.0, 9.0, 9.0, 12.0	49 mo. = 4.1 yr.	9.8	1.2	5.8–8.5=2.7	0.54	0.7
6.0	#21, 2018	2	6.0, 13.0	19 mo. = 1.6 yr.	9.5	1.3	6.0–8.0=2.0	1.00	1.3
6.0	#61, 2018	2	5.0, 15.0	20 mo. = 1.7 yr.	10	1.2	6.0–7.2=1.2	0.60	0.7
6.3	#52, 1995	3	3.0, 6.0, 4.5	13.5 mo = 1.1 yr.	4.5	2.7	6.3–8.0=1.7	0.57	1.5
6.5	#54, 1994	2	14.0, 8.0	22 mo. = 1.8 yr.	11	1.1	6.5–7.7=1.2	0.60	0.7
7.0	#3, 2016	7	8.0, 8.0, 6.0, 4.0, 5.0, 6.0, 7.0	44 mo. = 3.7 yr.	6.3	1.9	7.0–10.0=3.0	0.43	0.8
7.0	#28, 2018	2	12.0, 8.0	20 mo. = 1.7 yr.	10	1.2	7.0–7.5=0.5	0.25	0.3
7.0	#37, 2018	2	9.0, 12.0	21 mo. = 1.8 yr.	10.5	1.1	7.0–8.7=1.7	0.85	0.9
7.5	#12, 2016	4	12.0, 11.0, 13.0, 8.0	44 mo. = 3.7 yr.	11	1.1	7.5–9.0=1.5	0.38	0.4
Totals	n = 13	n= 44	Avg = 7.8				Avg = 1.6	Avg=0.54	Avg=0.8

Growth rates and longevity for males

Many more molt and growth data are now available to provide better analyses, including separate growth rates and longevity estimates for males and females. Table 2 shows molt and growth records for 13 adult males (5.0–7.5 mm long when collected), and each had 2–7 molts (total n = 44); these males were arranged by size to examine the effect of size on molt rates. Months between molts (column 4) were 3–15; the average months/molt (column 6) increased with size: 6.8 months for 5.0–5.8 mm males (n = 5), 8.8 months for 6.0–6.5 mm males (n = 4), and 9.5 months for 7.0–7.5 mm (n = 4). Months/molt were converted to molts/year ($\overline{X}$ = 1.6) in column 7. Total size increases (column 8) were from times of collection to last molts. Average size increases/molt (column 9) ranged from 0.25 to 1.0 mm/molt ($\overline{X}$ = 0.54 mm). Size increases/year (column 10) ranged from 0.3 to 1.5 mm ($\overline{X}$ = 0.83 mm) and were less ($\overline{X}$ = 0.60 mm) for the four largest males (7.0–7.5 mm).

"Increased size/molt" multiplied by "molts/year" yields "increased size/year", which is a logical way to express growth rates. So, how does this relate to longevity? Although 7.0 mm #3 (2016) in Table 2 grew to 10.0 mm in captivity, the size range of adult males collected from Lighthouse Cave was 5.0–9.5 mm; at an average size increase of 0.8 mm/year (column 10), this 4.5 mm growth could occur in ~5–6 years, with ~8–9 molts (4.5 mm growth/0.54 mm/molt = 8.3 molts). Note that fast growers like 6.3 mm #52 (1995) might grow 4.5 mm in only 3 years at his rate of 1.5 mm increase/year, while slow growers like 7.0 mm #28 (2018) might take 15 years to grow 4.5 mm at the rate of 0.3 mm/year. The average length of mancas released from marsupia (instar M1) was ~2.5 mm; length increased by ~0.3–0.5 mm/molt in the next four molts (to instars M2, M3, J1, J2) to approach the 5.0 mm size in Table 2; this early growth occurred as fast as 1–2 years in culture. So, it appears that male *B. geracei* from Lighthouse Cave (at least in culture) would likely live a total of ~6–8 years (probable range is ~4–17 years) with ~13–15 instars (5–6 pre-adult, plus 8–9 adult).

Table 3. Molt records for egg-bearing female *Bahalana geracei* from Lighthouse Cave arranged by size.

Size [mm]	Specimen, year	Reproductive condition	Months to reproductive molt
6.0	#23, 2018	Egg-Bearer	4
6.0	#76, 1994	Egg-Bearer	14
6.0	#47, 1995	Egg-Bearer	3
6.2	#49, 1995	Egg-Bearer	4
7.0	#35, 2018	Egg-Bearer	11.5
7.1	#15, 1993	Egg-Bearer	12
7.1	#60, 1996	Egg-Bearer	8
7.2	#59, 1996	Egg-Bearer	15
7.5	#33, 2018	Egg-Bearer	10
7.7	#18, 1993	Egg-Bearer	24
8.1	#20, 1996	Egg-Bearer	11
9.0	#28, 1993	Egg-Bearer	15
11.0	#5, 2018	Egg-Bearer	7
13.2	#88, 1996	Egg-Bearer	12
15.7	#69, 1996	Egg-Bearer	15
Totals			n = 15, Avg = 11.0

To support this probable range of ~4–17 years, please note three males in Table 2: 5.8 mm #5, 7.0 mm #3, and 7.5 mm #12. These three were retained (along with four other males) from our 30 June 2016 collection in Lighthouse Cave. All three were still alive in October 2020 (= 4.3 years in captivity) after 5–7 molts. Based on above growth rates, they were probably 3–10 years old (with 5–10 instars) when collected, which would now make them 7–14 years old, with ~10–15 instars. Some males probably live even longer in the caves, with irregular food availability resulting in longer molt cycles.

In the next section on Life cycle and population structure, I point out that several male *B. geracei* from Major's Cave grew much larger than those in Lighthouse Cave; the largest was 14.8 mm. If growth rates for males are the same for both caves, and if males in Major's Cave grow an additional 5.3 mm (to 14.8 mm in Major's Cave vs. 9.5 mm in Lighthouse Cave), this might take another 6.6 years at an average increase of 0.8 mm/year. That would give a truly extraordinary longevity for males in Major's Cave of ~12–15 years (probable range is ~10–24 years) with ~23–25 instars. However, molt intervals increased with size and age (typical of crustaceans, as noted by Gilligan et al. 2007), resulting in gradual decreases in annual growth rates/year from an $\bar{X}$ of 0.8 for all Lighthouse Cave males to an $\bar{X}$ of 0.6 for the four largest Lighthouse Cave males (Table 2); so, growth rates for large males in Major's Cave was probably slower and the resulting longevity longer than the above estimates. However, whatever allowed these males to grow larger (e.g., a better food supply) might also have allowed them to grow faster, so the longevity estimate for Major's Cave males is still open.

Growth rates and longevity for females

Determining molt and growth rates for female *B. geracei* was more complicated than for males because of longer life spans and long reproductive cycles with various stages and types of molts. Females had three types of molts. They began life the same as

Table 4. Molt records for oostegite-bearing *Bahalana geracei* from Lighthouse Cave arranged by size.

Size	Specimen, year	Reproductive condition	Months to oostegite molt
6.2	#28, 1995	Oostegite-Bearer	3.5
6.5	#15, 2016	Oostegite-Bearer	8
7.0	#15, 1999	Oostegite-Bearer	7
7.5	#86, 1996	Oostegite-Bearer	3
12.0	#31, 1993	Oostegite-Bearer	13
15.6	#35, 1995	Oostegite-Bearer	9
15.7	#37, 1996	Oostegite-Bearer	13
			n = 7, Avg = 8.07
6.0	#76, 1994	Egg-Bearer->Oost-Bearer	10
6.0	#47, 1995	Egg-Bearer->Oost-Bearer	6
6.0	#7, 2016	Egg-Bearer->Oost-Bearer	6
6.9	#52, 1996	Egg-Bearer->Oost-Bearer	5
7.1	#60, 1996	Egg-Bearer->Oost-Bearer	11
7.7	#18, 1993	Egg-Bearer->Oost-Bearer	11
8.5	#8, 1992	Egg-Bearer->Oost-Bearer	6
9.0	#28, 1993	Egg-Bearer->Oost-Bearer	8
16.3	#37, 1995	Egg-Bearer->Oost-Bearer	12
			n = 9, Avg = 8.33

males, starting with manca 1 (M1) (~2.5 mm) and increasing by ~0.3–0.5 mm/molt with regular growth molts to the next four instars (M2, M3, J1, J2) to approach the 5.0 mm size. They started producing eggs at ~4.0–4.9 mm (see Fig. 2), which took ~9–24 months before their reproductive (parturial) molts that produced oostegites forming the marsupia. This was sometimes followed by mating, then brooding for 5.5–6.0 months. After mancas were released, oostegite molts produced new oostegite-free exoskeletons; this happened ~2–16 months after brooding (longer for larger females). A few oostegite-bearers also had eggs, indicating they had started producing eggs for the next reproductive cycle before their oostegite molts.

Data to show these complex molt and reproductive cycles are presented in Tables 3–5. The summary at the end of this section gives an estimated life span for females of ~25–28 years, with a total of ~23–30 instars. Although the following details are somewhat tedious, it is important for me to present my data, methods, and rationale for these extraordinary estimates for future discussions and comparisons.

Table 3 shows the number of months after 15 egg-bearers completed egg production and had reproductive molts ($\overline{X}$ = 11 months). These females had been bearing eggs for several months (exact number undetermined) before being collected, which partly explains the wide range of 3–24 months (plus, the trend of more months for larger females), so the actual time spent in this stage is likely to be ~9–24 months, with 16 months as a possible average.

Table 4 shows two sets of oostegite-bearers; the first seven specimens had oostegites when collected (having released mancas an undetermined number of months before), then underwent oostegite molts 3–13 months later in captivity ($\overline{X}$ = 8.07 months). The next nine specimens were egg-bearers when collected, they had reproductive molts, did not mate, and then had their oostegite molts 5–12 months later ($\overline{X}$ = 8.33). In both sets the number of months increased with size. The oostegite molts for those

Table 5. Molt and growth records for non-breeding *Bahalana geracei* from Lighthouse Cave arranged by size; G = growth molts, R = reproductive molts, O = oostegite molts.

Size [mm]	Specimen, year	Molt no.	Months between molts	Total months	Mo./ Molt	Molts/ Year	Total size increase [mm]	Increase/Molt [mm]	Increase/year [mm]
			Pre-reproductive						
3.9	#57, 1995	4	3(G), 6(G), 8(G), 5G)	22 mo = 1.8 yr	5.5	2.2	3.9–5.6 = 1.7	0.4	0.80
4.2	#3, 1996	1	11(G)	11 mo = 0.9 yr	11	1.1	4.2–4.4 = 0.2	0.2	0.20
4.5	#35, 1993	1	13(G)	13 mo = 1.1 yr	13	0.9	4.5–4.8 = 0.3	0.3	0.25
5.8	#36, 1993	1	13(G)	13 mo = 1.1 yr	13	0.9	5.8–6.5 = 0.7	0.7	0.70
	n = 4	n = 7	**Avg. for 7 growth molts = 8.4 mo.**					Avg = 0.4 mm	Avg = 0.49
			Inter-cycle						
6.5	#15, 2016	4	8(O), 15(R), 7(O), 7(G)	37 mo = 3.1 yr	9.2	1.3	6.5–9.0 = 2.5	0.6	0.80
8.8	#50, 1995	3	4(G), 7(G), 9(R)	20 mo = 1.7 yr	6.7	0.6	8.8–9.4 = 0.6	0.2	0.35
	n = 2	n = 7	**Avg for 3 growth molts = 6.0 mo.**					Avg = 0.4 mm	Avg = 0.58
Totals			**Avg for all 10 growth molts = 7.7 mo.**						
			Post-reproductive						
11.0	#21, 1995	0	10 meals, no molts	23 mo = 1.9 yr					0.0
14.3	#39, 1995	0	12 meals, no molts	14 mo = 1.2 yr					0.0
15.3	#38, 1995	0	12 meals, no molts	14 mo = 1.2 yr					0.0
16.5	#22, 1995	0	15 meals, no molts	24 mo = 2.0 yr					0.0
16.8	#71, 1996	0	10 meals, no molts	17 mo = 1.4 yr					0.0
	n = 5	n = 0							

that did not mate (second set) probably occurred sooner after reproductive molts than might be expected because they did not spend 5–6 months brooding, and because they could reabsorb nutrients from their unfertilized eggs, rather than spending more energy brooding.

Table 5 shows molt and growth records for a few females designated as non-breeders, since they were not egg-bearers, oostegite-bearers, or brooders. This table includes: (1) pre-reproductive females that were too young and small to produce detectable eggs, (2) inter-cycle females that had completed a reproductive cycle (including release of mancas and shedding of oostegites) and had not yet produced a new set of detectable eggs, and (3) post-reproductive females that were larger/older and probably had stopped reproducing.

The first set in Table 5 shows four small (3.9–5.8 mm) pre-reproductive females that had a combined total of seven growth molts (no reproductive or oostegite molts). The smallest one (3.9 mm #57, 1995) was collected as a manca (M3), went through four more instars (J1, J2, J3, J4) in 22 months and grew 1.7 mm to yield a growth rate of 0.8 mm/year (surprisingly similar to growth rates cited above for males and oostegite-bearers). The other three pre-reproductive females each molted only once in 11–13 months, with size increases of only 0.2–0.7 mm/year.

The second set in Table 5 shows molt and growth records for two mid-sized inter-cycle females that had a mix of molts (G = growth molts, R = reproductive molts, and O = oostegite molts). The first one is 6.5 mm #15 (2016), which is very important

because it accurately shows the time for several stages of the life cycle. She was collected with oostegites, which she shed in 8 months (so she is also listed in Table 4 Oostegite-bearers). Within 6 months, she produced eggs that were clearly visible; she had her reproductive molt 9 months later (15 months after her oostegite molt), so she was recognizable as an egg-bearer for only 9 months. Note that there were no growth molts before more eggs were produced, so she did not enter an inter-cycle stage. She had a second oostegite molt 7 months after her reproductive molt, followed by a growth molt after another 7 months; she then produced more eggs that were visible in 5 months and later deteriorated. So, this last time she did have an inter-cycle stage of 7 months. She grew 2.5 mm during this 3.5-year process (0.7 mm/year). So, except for brooding, this female went through two complete reproductive cycles in 3.5 years, which is strong evidence that females are capable of multiple broods (iteroparous), one right after the other without any growth molts in between.

The other inter-cycle female was 8.8 mm #50 (1995), collected without eggs or oostegites; she had two consecutive growth molts (at 4 and 7 months), followed by egg production and a reproductive molt after 9 months; she grew only 0.6 mm in 1.7 years (0.35 mm/year). This 8.8 mm inter-cycle female is probably the best representative of non-breeders in the 8 mm size range, and the 11 months (4 + 7) preparing for her two growth molts may be a good estimate of the time inter-cycle females often spend recovering from brooding, at least near the 8 mm range.

The third set in Table 5 lists five relatively large females (11.0–16.8 mm) that never had eggs, oostegites, or molts of any kind during their 14–24 months in captivity. These were active females that fed regularly (10–15 meals apiece). I use them as examples of post-reproductive females because it seems unlikely that they would have produced more eggs (based on the low percentage of egg-bearers in these size ranges), and it shows that some large/old females may have extremely long intermolt periods that strongly extend their life spans.

Figure 2 (see section on Reproduction and development) provides a way to support the above estimates for how much time females of a specific size (e.g., the prime breeding ranges) were likely to spend in each reproductive condition, since the times should be roughly proportional to the numbers collected in each condition. For instance, if a female spends 16 months as an egg-bearer and 8 months as an oostegite-bearer, she is twice as likely to be captured as an egg-bearer vs. an oostegite-bearer. In the three size ranges with the most egg-bearers and oostegite-bearers (6.0, 7.0, 8.0 mm), 586 females were collected: 277 egg-bearers (137 + 99 + 41 in the 6, 7, and 8 mm size ranges, respectively) (277/586 = 47%), 114 oostegite-bearers (= 19%), and 195 non-breeders (= 33%). These numbers are roughly proportional to the average times estimated for each reproductive condition in Tables 3–5 (with a total of 16 + 8 + 11 months = 35 months): 16 months for egg-bearing (16/35 = 46%), 8 months for oostegite-bearing (8/35 = 23%), and 11 months for recovering inter-cycle females (11/35 = 31%).

If we add 6 months for brooding (after 16 egg-bearing months), that should give a reasonable estimate for an entire reproductive cycle: 16 + 6 + 8 + 11 = 41 months, or nearly 3.5 years! However, it would likely be considerably shorter in younger/smaller

reproductive females that tend to have shorter intermolt periods. For instance, 6.2 mm female #49 (1995) (described above in the section on Mating) molted 51 days after releasing mancas (oostegite molt) and again 4 months after that (growth molt), so her cycle could have been: 16 (egg-bearing) + 6 (brooding) + 2 (oostegite-bearing) + 4 (inter-cycle recovery) = 28 months. Thus, a range of ~2.0–3.5 years seems to be a reasonable estimate for female *B. geracei* reproductive cycles.

If we can determine the growth rate during a reproductive cycle, that should tell us how many broods are likely in a long-lived female and ultimately provide insight into longevity. If the average increase/molt during an entire reproductive cycle was near the average for males, females would average ~0.5 mm/molt X 3 molts/cycle = 1.5 mm in 2.0–3.5 years. However, it is likely that growth during a female's reproductive cycle would be slower than growth for males since brooders fast for ~6 months, and a major portion of food consumed during the cycle would go to egg and embryo development. Gilligan et al. (2007) noted that in most crayfish, "mature females divert energy to egg production as opposed to growth and therefore grow more slowly than mature males." Overall, it seems reasonable to estimate ~1.0 mm growth for a reproductive cycle lasting ~2.0 years (= 0.5 mm/year; 1.5 molts/year) for smaller *B. geracei* females.

In the above description of Table 5 and growth rates I described the important sequence for 6.5 mm #15 (2016) that was oostegite-bearing when collected, so she had already had one brood; she then produced another set of eggs (without an inter-cycle growth molt), had a reproductive molt, followed by an oostegite molt and a growth molt, before producing more eggs. This tells us that oostegite-bearers in the 5–6 mm size ranges can have at least two consecutive broods, with the second brood being released by oostegite-bearers in the 7 mm range. It is likely that these reproductive cycles each take ~2.0–3.5 years.

In Fig. 2 we can see that the next size range (8.0–8.9 mm) had only 18 oostegite-bearers, compared to 49 in the 6 mm range and 47 in the 7 mm range; this was a major decline of 62%. There was also a 45% decline in the entire female population (only 113 in the 8 mm class compared to 207 in the 7 mm class, presumably from increased mortality). This may indicate that only about half the females had a third consecutive brood soon after their first two, possibly due to the physical toll of producing two broods, including two six-month long fasts.

The eight size ranges >8.9 mm continued to show decreases in the percentage of egg-bearers, as females either died or spent more time as oostegite-bearers, or in the inter-cycle recovery stage, or eventually as post-reproductive. So, most females probably had one reproductive cycle in the 6 mm range, one in the 7 mm range, about half probably had a 3rd brood in the 8 mm range, and some had additional broods in the 9–17 mm ranges as indicated by the 47 large oostegite-bearers.

So, if most females produced 2–3 broods while ~6.0–8.9 mm long, what was the probable growth rate and longevity for the remainder of their lives at 9.0–16.9 mm? This is an important part of the life cycle, since it represents a substantial part of the population (out of 1047 females collected in Lighthouse Cave, 279 were in the 9.0–16.9 mm ranges = 27%); it is also where many females spent the longest parts of their lives, since growing and molting processes are slowed. But it was also difficult to

determine growth rates in these size ranges because large females had lower survival rates in captivity and molts were less common.

Tables 3–5 show the few molt records available for large females, along with those of smaller females. The four large egg-bearers (9.0, 11.0, 13.2, & 15.7 mm) in Table 3 had reproductive molts at 15, 7, 12, and 15 months ($\bar{X}$ = 12.2 months). In Table 4 the three large females collected as oostegite-bearers (12.0, 15.6, & 15.7 mm) had oostegite molts at 13, 9, and 13 months ($\bar{X}$ = 11.7 months); the two egg-bearers that had oostegite molts (9.0 & 16.3 mm) molted 8 and 12 months after their reproductive molts ($\bar{X}$ = 10.0 months). And the five large females (11.0 to 16.8 mm) in Table 5 never molted while in captivity for 23, 14, 14, 24, and 17 months ($\bar{X}$ = 18.4 months). This mix of 14 intermolt periods probably gives a good overall picture of instar lengths for large females, with an average of 14.0 months: (15 +7 + 12 + 15) + (13 + 9 + 13) + (8 + 12) + (23 + 14 + 14 + 24 + 17) = 196; 196/14 = 14.0 months).

This average instar length of 14.0 months is nearly twice (actually 1.8 times) the average instar length of 7.8 months for males (Table 2, column 4 = months between molts); males had an average increase/molt of 0.54 mm (Table 2, column 9) and an average increase of 0.8 mm/year (Table 2, column 10). So, the average increase/year for large females would likely be ~1/2 of 0.8 mm/year = ~0.4 mm/year. That would mean that if large females grew 7.9 mm (from 9.0 to 16.9 mm) that would take 7.9/0.4 = 19.75 years, and 7.9 mm of growth at the rate of 0.54 mm/molt = 14.6 molts.

To summarize, longevity estimates for female *B. geracei* are exceptional. Longevity is estimated to be 25–28 years: 2–3 years pre-reproductive (2.5 mm-6.0 mm) + 4–6 years producing 2–3 broods (6.0–8.5 mm) + up to 19 years mostly post-reproductive. Females could probably have a total of 23–30 instars: 6–8 pre-adult + 5–7 for 2–3 reproductive cycles + 12–15 while mostly post-reproductive. These are extraordinary estimates for any isopod species, but especially for one living in warm water (25–26 °C). However, growth rates for *B. geracei* maintained in captivity and fed regularly were probably faster than for those animals living in the caves with low food supply, so the life span could be even longer than the above estimates. Possible explanations for such long life spans are analyzed in the discussion section on Growth rates and longevity.

Life cycle and population structure

Many aspects of the *B. geracei* life cycle have been covered in preceding sections. Now I want to further compare the numbers for each stage and give an overview of the population. These are best covered by elaborating on Table 1 (introduced after Methods and materials), on new Tables 6, 7, and on Fig. 8.

Table 1 Numbers and sizes from Lighthouse Cave

This table summarizes 23 years of collections of *B. geracei* in Lighthouse Cave from 1978–2018, with numbers and sizes of mancas, males, and females. (There are two

entries for 2013 because collections were made in January and June.) Totals for each year are shown on the right side. In most years we were able to collect >50 specimens, which is unusually high for stygobitic cirolanids; possible explanations for this are in the discussion on Population size. The population appeared to be reasonably stable in most year, with similar proportions from year to year for mancas, males, and females.

Reproduction appeared to be continuous and probably not seasonal, based on the nearly constant presence of females in all stages of the reproductive cycle, except for brooders that stay hidden. Even though the isopods tended to not swim very often or very far, the population was not confined to the room where we typically collected them. When we collected in that same room a second or third time within a few days, sizeable samples were still collected, indicating considerable movement of isopods from other parts of the cave. Also, we usually saw many when we explored other parts of the cave. Although Lighthouse Cave is relatively confining for us as collectors, isopods can probably move freely through the water table and porous limestone to other parts of the island, including other caves.

Fluctuations in numbers of specimens collected each year seemed to be due mostly to the number of collectors and our proficiency, rather than to large changes in the population size. There are good reasons why fewer than 11 specimens were collected in four years. In our first visit to Lighthouse Cave in 1978 we did not have proper collecting equipment, and the five specimens (used for the type series) were caught with our hands (without nets) as they swam toward the surface. In 1979 we had collecting equipment, but our flashlights were relatively weak. In 2013 and 2014 specimens were unusually difficult to find; in June 2013 there were so many white microbial clumps and strands growing on almost everything (rocks, dirt, and sponges) and floating free in the water, that it was hard to identify the white *B. geracei* unless they were swimming. The deteriorated water quality was a concern for many of us at the Gerace Research Centre. It was thought that it may have been associated with too many visitors with sunscreens or insect repellants, so cave explorers were advised to refrain from using these chemicals in the future. Fortunately, water quality and *B. geracei* populations returned to normal by 2016.

Table 1 includes all manca stages combined (M1, M2, and M3) in the second column. They ranged in size from 2.3–4.0 mm, which were similar to sizes of mancas raised from laboratory broods; size ranges for the three manca stages are shown in Table 7. The total number of mancas collected was 92 = 6.6% of all 1383 Lighthouse Cave specimens. Since mancas are the smallest stages, they are much harder to find, and the numbers collected are not a good measure of birth rates.

One of the most striking patterns shown in Table 1 is that every year females were larger and more numerous than males. The percentage of males in the total adult population ranged from 0% (2013 and 2014) to 36.8% (7 males + 12 females in 2016). The total for all years was 1291 adults with 244 males (= 19%). Only one of these 244 males was >8.5 mm; that was 9.5 mm #19 (2000). The consistency of these

female-biased ratios suggested that some basic biological phenomena were at work. However, it eventually became clear that the size of males was not strictly limited by an inherent biological phenomenon (e.g., genetics), since a few males kept in captivity for several years grew to >9.5 mm.

Furthermore, samples of *B. geracei* from Major's Cave showed that males can be nearly as numerous and grow to be nearly as large as females. On 28–29 July 1999 we collected 35 *B. geracei* in Major's Cave: 10 mancas (3 M1-M2, 7 M3), 10 adult males, and 15 adult females (10 males out of 25 adults = 40%); this was a higher percentage than in any collection in Lighthouse Cave. Even more dramatic were the sizes of these 10 males (4.2, 7.0, 7.0, 8.0, 9.6, 10.6, 11.0, 12.2, 12.5, and 14.8 mm; $\bar{X}$ = 9.7 mm); that is, 6 of these 10 males were larger than any ever found in Lighthouse Cave! The 15 females ranged in size from 4.8–16.0 mm, $\bar{X}$ = 10.2 mm.

Table 6 Major's Cave males and females

This table combines data of *B. geracei* collected in Major's Cave from 1999, with collections from 2000–2004, making a total of 21 males of 52 adults = 40%. Figure 8, Graph A (Lighthouse Cave) and Graph B (Major's Cave) compare the dramatic differences in the two populations, indicating the strong influence the environment can have. One key difference in the two caves is that mangrove rivulus fish are known predators of isopods in Lighthouse Cave, while these fish have not been found in Major's Cave. Other possible explanations for these differences are found in the discussion section on Life cycle and population structure.

Figure 8 A Lighthouse Cave males and females

Another interesting pattern for the Lighthouse Cave population is shown in this graph. The size distribution follows a normal distribution by size (bell-shaped curve) until the dip at 11.0–11.9 mm, which is then followed by increases in the largest sizes. This puzzling pattern has been shown in some shrimp species (see Conides et al. 1994, Relini and Relini 1998), but researchers did not give adequate explanations. I hypothesize that four factors may be responsible for this pattern in *B. geracei*: (1) larger females should have a survival advantage by cannibalizing smaller *B. geracei*, (2) larger females may have less stress from brooding if they are post-reproductive, (3) mangrove rivulus

Table 6. Number of post-manca specimens of *Bahalana geracei* from Major's Cave (1999–2004) by 1 mm size ranges and sex.

Sex	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9	11.0–11.9	12.0–12.9	13.0–13.9	14.0–14.9	15.0–15.9	16.0–16.9	Total & %
Males	1	2	2		4	2	1	2	4	2		1			21=40%
Females		1		2	4	9	4	4	2	2	2			1	31=60%
Total M+F	1	3	2	2	8	11	5	6	6	4	2	1		1	52=100%

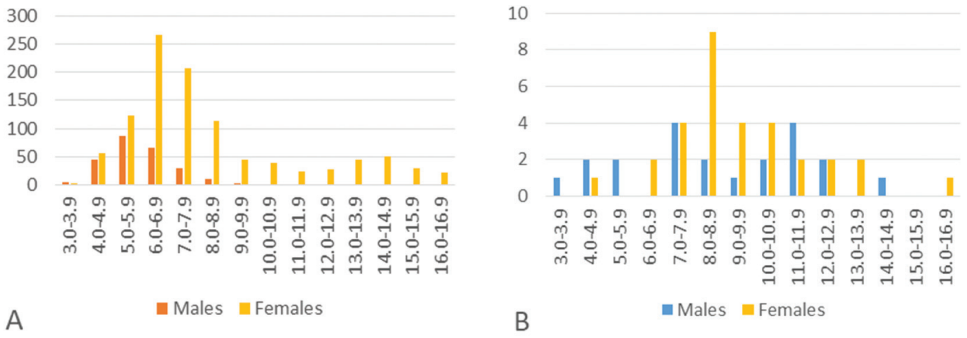

Figure 8. Size distribution of male and female *Bahalana geracei* in **A** Lighthouse Cave (1978–2018) and **B** Major's Cave (1999–2004) with more and larger males (blue).

Table 7. Life cycle stages of *Bahalana geracei* from Lighthouse Cave, 1978–2018.

Stage	Manca 1	Manca 2	Manca 3	Juv. 1	Juv. 2	Male breeders	Egg-bearers	Brooders	Oost.-bearers	Inter-cycles	Post-repro.
Size range [mm]	2.3–3.3	2.6–3.8	3.0–4.3	3.5–4.8	4.0–5.3	4.5–9.5	4.5–16.5	5.8–16.5	5.8–16.5	5.8–16.5	9.0–16.8
Instar no.	1	2	3	4	5	6–15	6–30	7–30	7–30	8–30	14–30
Time in stage	2–10 mo.	2–10 mo.	2–10 mo.	3–10 mo.	3–12 mo.	4–14 mo/ instar	6–24 mo.	5.5–6 mo.	2–13 mo.	7–18 mo/ instar	7–24 mo/ instar
Approx. age	0–10 mo.	2–20 mo.	4–24 mo.	6–30 mo.	9–36 mo.	1–17 yrs.	2–26 yrs.	3–26 yrs.	3–26 yrs.	3–26 yrs.	16–26 yrs.

fish, which appear to be a major predator of the isopods in Lighthouse Cave, may be gape-limited and have difficulty eating larger isopods, and (4) age compression has the strongest effect in the largest size ranges (see Growth rates in general).

Table 7 Life cycle stages

This table summarizes data for all life cycle stages for *B. geracei* from Lighthouse Cave (1978–2018); for each stage it includes estimates for size range, instar number(s), time in stage, and approximate age based on hundreds of observations of live laboratory specimens. It should be stressed that the time spent in each stage varied widely from a few weeks in the first few instars to years in the oldest/largest instars. So, estimates of minimum ages (bottom lines) were mostly determined by minimum times it took to go through all instars to that point.

One important point is that all life cycle stages for *B. geracei* took longer than in other isopods. For instance, in most terrestrial isopods all three manca stages are completed in a few days (compared to >6 months for *B. geracei*); Zecchini and Montesanto (2019) reported mean duration for mancas of *Armadillidium granulatum* Brandt, 1833 as M1 = 6 hours, M2 = 15 days, and M3 = 32 days. Koop (1979) reported that *Ligia dilatata* Brandt, 1833 produce their first brood at ~11 months (vs.2–3 yrs. in *B. geracei*). Johnson (1976) reported that in the intertidal isopod

Cirolana harfordi (Lockington, 1877), "females produce 1 or 2 broods of 18–68 young during their 2-year life-span" and "marsupial incubation lasts 3 or 4 months." Having longer durations for every stage for *B. geracei* (plus having multiple broods) results in a much longer life span than for all other isopods reported, as seen in Table 8 in the discussion section on Growth rates and longevity. Females may also live longer than males because they apparently spend six months of each reproductive cycle brooding in isolation, so they are not susceptible to predation (including cannibalism). On the other hand, the physical stresses of brooding probably cause post-brooding impairment in some brooders, leading to the strong declines in "all females" from 113 in the 8 mm class to only 44 in the 9 mm class (Fig. 2).

Estimates for the total number of instars in Table 7 (up to 30 for females) are quite large for any isopod species and are directly related to *B. geracei*'s extreme longevity. It appears that most cirolanid species have fewer than 12 post-marsupial instars; for example, *Natatolana borealis* (Lilljeborg, 1851) has up to 11 instars, 2–3 broods, and a life span of 2.5 years (Johansen 1996, Wong and Moore 1996). However, some large crustaceans have even more instars. According to Vogt (2018), "Many decapods molt more than 20 times in their lifetime, and the Murray crayfish *Euastacus armatus* even molts up to 80 times in its 28 years of life (Gilligan et al. 2007)."

Fecundity

The numbers of egg-bearers and oostegite-bearers (and presumed brooders) for *B. geracei* appear to be very high for a stygobite, so fecundity should also be high. Rockwood (2015) described fecundity as "the mean number of offspring produced per individual (usually female) in the population, per unit time." According to Vogt (2018), "Most crustacea reproduce throughout their entire adult life span, and there is a positive correlation between body size (which itself is positively correlated with age) and clutch size." These traits seem to be held by *B. geracei*.

Most researchers estimate fecundity by counting eggs or mancas per brood, as indicated in my earlier section on Gestation. These are usually based on many females bearing eggs or mancas; instead, I will use oostegite-bearers. For *B. geracei*, I think it is best to base fecundity on the mean number of mancas produced in a female's lifetime, rather than per brood or per year (since cycles take ~2–3.5 years), and rather than egg number because eggs are difficult to count accurately in live animals. According to Johnson et al. (2001), "Within a species, the general trend for brood size to increase with the size of females is almost universal." Thus, it should be expected that both brood size and fecundity should be highly variable for *B. geracei*, since females can have two or more broods that vary greatly in size.

Using the distribution of oostegite-bearing females in Fig. 2 and data for my six brooders (see Gestation) the number of mancas/brood can be estimated for females of various sizes and the probabilities of producing 2, 3, or 4 broods. During this study we collected 167 oostegite-bearers (5.8–16.9 mm) that had released mancas in the

previous few months. Most of them (120 of 167 = 72%) were in the four small size ranges (5, 6, 7, & 8 mm) (Fig. 2). From the methods explained above, it seems likely that most females produced a total of ~20 mancas in their two broods combined during their prime breeding ages (3–8 years) and sizes (5.0–7.9 mm). About half of them probably produced a 3rd brood in the 8.0–9.9 ranges. The mean manca number was 10 for my four brooders in the 6–9 mm ranges (#'s 49, 35, 92, & 33; details in Gestation).

This species seems to be unusual because 44 of 167 oostegite-bearers (= 26%) survived into the upper half of the size ranges (10.0–16.9 mm), with moderately high numbers even in the last four size ranges of Fig. 2. So, there is a 26% probability that adult females produced a 4th additional brood (and possibly a 5th or 6th) sometime later in life when they are 10.0–16.9 mm. I did not have any brooders in the mid-size ranges (10, 11, & 12 mm); my two largest brooders (13.0 mm #5 of 2018, and 14.8 mm #1 of 2002) are in the larger sizes. Brood size for #5 was 55. Brood size for #1 was uncertain because she was collected in the cave, she had 11 mancas in her film can when examined and 21 more in the next 4 days, but she had probably released some before being collected; since she was larger than #5, her brood size was probably as large or larger, so a conservative average for these 2 broods is ~50, which I will use as an estimate for all broods from oostegite-bearers 10.0–16.9 mm. If these are somewhat reasonable assumptions, we can estimate the number of mancas/brood and the number of broods for all 167 as follows:

1. 167 females would probably produce 10 mancas × 2 broods = 167 × 20 = 3340.
2. 83 females (= 167 × 1/2) would probably produce a 3rd brood of 50 = 4150.
3. 43 (= 167 × 0.26) would probably produce a 4th brood of 50 = 2150.

The total for all these is 3340 + 4150 + 2150 = 9640 mancas. 9640 mancas/167 = 58 mancas/oostegite-bearer. It is probably reasonable to think similar fecundity would come to fruition for the other non-oostegite-bearers, including egg-bearers and non-breeders. So, a probable range of fecundity for *B. geracei* is 20–120 mancas per female per lifetime, with a mean of 58. The significance of this surprisingly high fecundity is found in the discussion section on Fecundity.

Discussion

Reproduction and development

There are few reports on marine isopods that have studied the complete reproductive sequence of egg production (time and numbers), breeding, incubation time, and manca development. However, combining data from a variety of reports such as Johnson et al. (2001) can provide information on individual aspects of reproduction to compare to my observations on *B. geracei*.

Mating

It is particularly interesting that *B. geracei* had successful matings only after both the posterior and anterior halves were molted, instead of after the posterior half and before the anterior half as described by Wilson (1991), Johnson et al. (2001), and Wilson and Humphrey (2020). However, Wilson (1991) also pointed out several variations in mating patterns (e.g., precopula or mate pairing, and long-term retention of sperm in spermathecae); the copulatory behavior is best known for the Oniscidea (terrestrial isopods) and Asellota. With so much diversity in isopods, and so few observations of actual mating, I wonder if the pattern of mating after the anterior molt that I observed in *B. geracei* might be common in some groups (e.g., Cirolanids in caves and in other habitats).

The specific mating behaviors observed in *B. geracei* (described earlier in Breeding procedures and mating) appear to be similar to those described by Johnson et al. (2001) for several other pericaridan crustaceans: "Increased activity or directional orientation in males when in close proximity of females nearing their ovigerous molt has been reported in gammarids . . . , mysids . . ., and tanaids." The reason for such directional orientation in males, which I have observed several times in *B. geracei* pairing events, is probably related to exchange of pheromones. Johnson et al. (2001) noted that, "Lyes (1979) showed that female *Gammarus duebeni* release a pheromone in their urine that is received by the male second antennae." Johnson et al. (2001) also noted that, "Other structures on the antennae including male-specific sensory aesthetascs on some isopods . . . may be used to detect female pheromones, but experimental confirmation is needed." It is worth noting that in large *B. geracei* males (~7.5–8.0 mm), antenna 1 is ~40% longer, with ~40% more articles, than in females of comparable size, and they have more and larger aesthetascs than females.

One other note on mating behavior is that palpation with antennae that I observed has also been observed in other crustaceans, such as the amphipod *Eogammarus confervicolus* (Stimpson, 1856). Johnson et al. (2001) described the behavior as, "Once the female has been located, she is usually grasped by the male and then examined by repeated contact or palpation with antennae and other appendages (Heinz 1932; Dunham et al. 1986). . . .The stimulus involved may be a 'contact pheromone' (Michel 1986; Borowsky and Borowsky 1987) where the chemical is on the surface of the female rather than in solution."

Gestation

While most isopods use marsupial brooding, several groups developed internal brooding inside the female's pereon (Johnson et al. 2001). Thompson (2014) reported that "*Cirolana harfordi* individuals from New South Wales, Australia were found to incubate embryos and mancas inside the pereon (thoracic) cavity." Thompson (2014) also pointed out that Johnson (1976) described the reproduction of *C. harfordi* in American specimens as "marsupial incubation of eggs and later stages but did not provide any evidence to support that description." In addition, Klapow (1970) found that 7 species of *Excirolana* also carry embryos and mancas inside the pereon. Thompson (2014) and Klapow (1970) imply that incubating inside the pereon is characteristic of the species they examined (or

the entire genus). Brooding inside the pereon of *Annina lacustris* Budde-Lund, 1908 was also observed by Messana (1990). So, my observation of #49 (1995) incubating inside her pereon now seems plausible, and the definite marsupial incubation of my other specimens implies that the location of incubation may be flexible in some species such as *B. geracei*.

It is rare to find or collect brooding females of any cirolanid isopod species, and the favored explanation is that they hide in the sediment to protect themselves and their brood. One bit of supporting evidence is that, of the thousands of giant *Bathynomus giganteus* Milne-Edwards, 1879 isopods collected by researchers, Barradas-Ortiz et al. (2003) noted that only three brooders have ever been collected, and all were collected from sediment with trawl dredges or nets, rather than by attraction to baited traps. According to Johnson et al. (2001), "Brooding female isopods and tanaids may feed little if at all. The volume of the growing embryos compresses the female's internal organs, including the gut, which would hinder food intake. In addition, mouthparts are so reduced or modified in some brooding females that they cannot feed." Johnson et al. (2001) described the highly modified maxillipeds of brooding females in *B. giganteus* as "oostegites" that help keep embryos inside the marsupium, which may make feeding difficult or impossible. In *B. geracei*, the maxillipeds are slightly modified to circulate water in the marsupium, but all the brooding females I observed in culture (n = 4) fed more than once during incubation (see Fig. 3B). Since we seldom collected brooders, it seems likely that they do not actively hunt for food in the caves. My laboratory observations of brooders indicate that they don't bury into the substrate to hide, although they might still do that in the caves. They might also hide under rock ledges or inside crevices or go to inaccessible deeper areas of the cave, but where they stay when brooding is still a mystery. Fasting for 6 months or more during incubation probably takes a toll on the long-term health for brooders, even though some survive to produce more broods during their long lives.

Feeding behaviors

Feeding behaviors and structures

As mentioned earlier, species in the genus *Bahalana* can be distinguished from all others in the family Cirolanidae because pereopods 1–3 (P1–3) are prehensile with the two distal segments (dactylus and propodus) elongated and with long projections on several segments (especially the merus) (Fig. 7F). Many cirolanid isopod species have substantial spines and projections, especially on the palmar side of feeding pereopods to help hold and manipulate food. In *B. geracei* the spatulate projection on the palmar side of P1 (Fig. 7E, F) bear 4–7 teeth and is used to position food near the mouth. However, the long projections on the outer margins of P2–3 on *Bahalana* species are the most extreme of any cirolanid, which elicits the question, "Why are they so well developed in this particular group?" As mentioned in the earlier section on Feeding behaviors (in Results section), photographs showed that the pointed tips of P1–3 often penetrated prey tissue, but the projections on the outer side of P2–3 were often held away from prey (visible on lower side of worm in Fig. 7A). So, if these lateral projections are used only secondarily in handling prey, might they also have other functions?

In the section describing the increase in the proportion of larger females (>11.9 mm) shown in Fig. 8, I suggested that one possible explanation was that mangrove rivulus fish may be gape-limited predators that have difficulty eating larger isopods. It follows then that having long lateral extensions on P2–3 might help protect *B. geracei* of all sizes from potential predators, including fish, larger cannibalistic *B. geracei*, *B. cubensis* shrimp, and possibly even remipedes, which are known to occur in some of the same caves as species of *Bahalana*. Messana (1990) also hypothesized a correlation between fish predation and the stygobitic stenasellid *Acanthastenasellus forficuloides* Chelazzi and Messana,1985, which is extremely spiny "due to lateral expansion of the tergites." Messana (1990) suggested these isopods evolved their fearful armor "after the arrival of the ancestors of modern stygobitic fish in Somalian underground waters" as a means of protection from them. So, I speculate that the prominent lateral projections on P2–3 of *Bahalana* species may have evolved as a defense against predators (including cannibals), as well as being useful for feeding and possibly grooming.

Cannibalism

Cannibalism is common in carnivores, and especially in the young of precocial species in which parents provide no food or protection for them. As noted by Elgar and Crespi (1992), "The parent that produces a clutch which is partly consumed by her offspring is providing nutrition." This is a common and effective life strategy that provides survival assistance to the most vulnerable stages of a life cycle. The most commonly available food sources for offspring are usually siblings and other members of their cohort because they are the right size and are abundant in their surroundings, relative to other animals; this appears to be the case for *B. geracei* mancas. Elgar and Crespi (1992) suggested that cannibalism is responsible for a significant proportion of mortality in many species. Fox (1975), in describing two-year old pike and four-year old pike, said LeCren (1965) "calculated that cannibalism could account for all mortality among the younger class." Two *B. geracei* mancas that I raised from birth ate 21 and 26 meals in their first year. If they had similar eating frequencies in the caves, and if most of their meals were other mancas, that would certainly have a huge impact on the mortality of an average brood of ~20 (calculated in Gestation section of Results). Perhaps this strong potential impact of cannibalism may have promoted a predator-prey arms race to produce the prominent lateral projections on P2–3 that could be used for both attacks by cannibals and defense against them.

Starvation resistance

Since fasting before and after each molt is routine for crustaceans, they could be considered naturally resistant to starvation. This may help explain why crustaceans are the most abundant group of anchialine animals, although Pérez-Moreno et al. 2016 suggested that, "The reason for the high diversity of crustaceans, the endemism of higher taxa to anchialine systems, and their preponderance over other higher taxa is unknown (Stock, 1995; Sket, 1999)." Enhanced starvation resistance is a general characteristic of

Table 8. Longevity in cave and surface animals.

Longevity	Species	Taxon	Habitat	References
>20 years	*Bahalana geracei* Carpenter, 1981	Isopoda, Cirolanidae	SW cave	This study
>10 years	*Aega antarctica* Hodgson, 1910	Isopoda, Aegidae	SW fish parasite	Wägele 1990
>6 years	*Bathynomus* sp. Milne-Edwards, 1879	Isopoda, Cirolanidae	SW deep sea	Krulwich 2014
3 years	*Mesidotea entomon* Richardson, 1905	Isopoda, Chaetiliidae	SW brackish	Leonardsson 1986
2.5 years	*Natatolana borealis* (Lilljeborg, 1851)	Isopoda, Cirolanidae	SW sea loch	Wong and Moore 1996
2 years	*Cirolana harfordi* (Lockington, 1877)	Isopoda, Cirolanidae	SW beach	Johnson 1976
<2 years	*Cyathura carinata* (Kroyer, 1847)	Isopoda, Anthuridae	SW estuary	Marques et al. 1994
15 years	*Stenasellus virei* Dolfus, 1897	Isopoda, Stenasellidae	FW cave	Magniez 1975
2 years	Asellus aquaticus (Linnaeus, 1758)	Isopoda, Asellidae	FW surface	Magniez 1975
8 years	*Venezillo tenerifensis* Dalens, 1984	Isopoda, Oniscidea	Terrestrial cave	Zimmer and Topp 1999
5–10 years	*Armadillo officinalis* Dumeril, 1816	Isopoda, Oniscidea	Terrest. Surface	Warburg and Cohen 1992
3–4 years	*Porcellio dilatatus* Brandt, 1833	Isopoda, Oniscidea	Terrest. Surface	Heeley 1941
1–2 years	*Porcellio laevis* Latreille, 1804	Isopoda, Oniscidea	Terrest. Surface	Nair 1978
38 years	*Orconectes australis australis* (Rhoades, 1941)	Decapoda, Cambaridae	FW cave	Vogt 2018
22+ years	*Orconectes australis* (Rhoades, 1941)	Decapoda, Cambaridae	FW cave	Venarsky et al. 2012
2–3 years	*Orconectes placidus* (Hagen, 1970)	Decapoda, Cambaridae	FW surface	Taylor 2003
16 years	*Procambarus erythrops* Relyea & Sutton, 1975	Decapoda, Cambaridae	FW cave	Streever 1996
<2 years	*Procambarus clarkii* (Girard, 1852)	Decapoda, Cambaridae	FW surface	Huner 2002
1.6 years	*Bryocamptus pyronaicus* (Chappuis, 1923)	Copepoda, Harpacticodida	FW cave	Rouch 1968
0.7 years	*Bryocamptus zschokkei* (Schmeil, 1893)	Copepoda, Harpacticodida	FW surface	Rouch 1968
7 years	*Amblyopsis spelaea* DeKay, 1842	Osteichthyes, Amblyopsidae	FW cave	Poulson 1963
1.3 years	Chologaster cornuta Agassiz, 1853	Osteichthyes, Amblyopsidae	FW surface	Poulson 1963

cave animals, apparently as an adaptation to food supplies that are low or periodically absent (Culver and Pipan 2019). Hervant and Renault (2002) compared long-term fasting effects on a hypogean isopod species, *Stenasellus virei* Dolfus, 1897, to an epigean species, *Asellus aquaticus* (Linnaeus, 1758), and found that the hypogean species "showed lower magnitudes of response to long-term fasting than the surface-dwelling *A. aquaticus*, with a 7.3-fold slower rate of relative mass loss."

Growth rates and longevity

Apparently, my estimates of >20 years longevity for *B. geracei* are the longest for any isopod species in any habitat, so it is important to compare them to estimates for other species of isopods and for non-isopod taxa. Table 8 lists longevity estimates for eight cave species compared to surface species in the same or similar taxa. In every case, the cave species have much greater longevity than their surface counterparts. This table is divided into six sections based on taxa and habitats.

The first section compares seven saltwater (SW) isopod species from a variety of habitats. *Bahalana geracei* is the only SW cave isopod known to have longevity estimates, and these estimates are at least twice as long as for other isopods living in SW surface habitats. Curiously, the next longest longevity record I could find for a SW isopod was for the Antarctic fish parasite *Aega antarctica* Hodgson, 1910; Wägele (1990)

reported keeping it "in aquaria for more than 2 years", and that "females spawn at an age of more than 10 years." Since the host fish provides protection from predators, perhaps that is a key to this isopod's longevity. Table 8 includes five other SW isopod species from three different families and five different habitats for comparison.

Giant deep-sea isopods like *Bathynomus giganteus* (or other *Bathynomus* species) should be prime candidates for longevity records because they live in cold water, and it should take a long time to grow to 17–50 cm. Unfortunately, there are few records on growth, molting, or longevity for this group. According to an NPR blog report by Krulwich (2014), a giant deep-sea isopod like *Bathynomus giganteus* lived at Japan's Toba Aquarium where it apparently fed regularly for over a year, then fasted for 1868 days (>5.1 years) before dying. This is my basis for including it in Table 8 with a life expectancy of >6 years; however, since Krulwich (2014) described it as "big, almost a foot long, weighing over 2 pounds", it may have been several years old when it arrived at the aquarium. It is also another extreme example of starvation resistance that relates to longevity.

The second section compares two freshwater (FW) isopod species. As noted in my introduction, Magniez (1975) reported his successful breeding of the Stenasellid isopod, *Stenasellus virei*. He estimated a life span of 15 years for *S. virei*, which he said is 10–20 times longer than for an epigean Asellid of the same size, *Asellus aquaticus* (Linnaeus, 1758). The estimated lifespan of 15 years for *S. virei* is the next longest for any isopod after *B. geracei*.

The third section compares terrestrial isopods. Apparently, longevity has been studied much more in terrestrial isopods than in aquatic species because they are easier to maintain over long periods. Vogt (2018) reported on life spans of various groups of crustacea and said, "Isopods have life spans between one and ten years", and cited Warburg (2011) who "compiled longevity data for 14 terrestrial species and concluded that most live less than three years; only three species exceeded an age of five years." The longest-lived species (*Armadillo officinalis* Duméril, 1816) and two other species with more typical life spans are included in Table 8 for comparison to the terrestrial cave isopod *Venezillo tenerifensis* Dalens, 1984. Zimmer and Topp (1999) used growth curves of two adults (kept for four years) and their 12 offspring (from four consecutive broods) to calculate longevity of this cave isopod at ~8 years.

The fourth section compares longevity for five species of freshwater (FW) crayfish: two long-lived FW cave species of *Orconectes* (longevities of 38 and 22+ years) to a surface species of *Orconectes* (2–3 years), and a long-lived *Procambarus* cave species (16 years) to a surface *Procambarus* (<2 years). Venarsky et al. (2012) pointed out in their analysis of longevity of *Orconectes australis* that this cave species lives "4 to 20× longer than any other crayfish within the same genus." The cave *Procambarus* species appears to live several times longer than the surface *Procambarus* as well.

The fifth section shows that the FW cave copepod *Bryocamptus* appears to live 2–3 times longer than the surface species. And the sixth section compares longevity for two amblyopsid fish. According to Culver and Pipan (2019), Poulson (1963) found in his comparison of three stygobiont vs. two non-stygobiont amblyopsid fish that the three cave species had a doubling of life span and "at least a 50% increase in the maximum number of broods as a result of increased longevity, among other traits."

This pattern of greater longevity for cave species appears to be consistent across various taxa and habitats: FW isopods, terrestrial isopods, FW crayfish, FW copepods, and FW fish. So, it is not surprising that *B. geracei* would have greater longevity than saltwater isopods in various surface habitats. The longevity of this stygobitic isopod species is probably not unique among anchialine isopods. It just happens to be the only one seriously studied so far.

So, why do cave species tend to have greater longevity? Vogt (2018) pointed out that, "Cave animals usually experience low and erratic food supplies, as well as constantly low temperature and low oxygen. These factors were shown to result in reduction of metabolism, motility and growth rate, later onset of maturity, and irregular reproduction when compared to epigean relatives (Streever 1996, Venarsky et al. 2012)." However, this does not fully explain greater longevity.

The cave environment is often considered to be very harsh. Benvenuto et al. (2015) in their paper on crustaceans of extreme environments said, "Crustaceans have colonized and filled almost every type of niche available, including the most inhospitable places on our planet, such as Antarctic lakes, subterranean waters, hydrothermal vents, xeric deserts, hypersaline lakes, and highly acidic habitats." We are so dependent on our own sight that subterranean habitats may seem extreme and inhospitable due to the lack of light, but many species have adapted to the absence of light and primary production with slow metabolic rates, starvation resistance, and enhanced chemoreception to find food and mates. For those species that have adapted, the cave environment is not at all inhospitable, but is instead relatively stress free compared to most surface environments.

Cave animals are fortunate that they don't have to respond to the stresses of extreme weather conditions (heat, cold, storms, wind, drought), annual migrations, daily searches for food, nearly constant noise, and the social stresses of courtship, caring for offspring, competing within social hierarchies, defending territories to protect food and mating opportunities, and being constantly alert for predators. It appears that this concept of a low-stress environment as a major factor in increasing longevity for cave animals has been largely overlooked. Stress may also help explain the difficulties in keeping long-lived cave animals alive in captivity for long periods; our laboratory environments and maintenance practices probably add considerable stresses to our captive animals, even though we provide adequate food and protection from predators.

Life cycle and population structure

Males vs. females

The preponderance of female to male *B. geracei* in Lighthouse Cave collections has long been a mystery, with several possible explanations. One that I have long favored is that males are more active since they have to search for receptive females, so they are more likely to be eaten by other *B. geracei* or other predators that live in Lighthouse Cave such as the mangrove rivulus, *K. marmoratus*; rivulus prey on *B. geracei* in laboratory experiments, and the feces of rivulus caught in Lighthouse Cave frequently have remains of *B. geracei*. In contrast, we never found mangrove rivulus in Major's Cave, which has more

and larger males. Also, the number of mancas in Major's Cave was considerably higher (14 of 66 = 21%) than in Lighthouse Cave (92 of 1383 = 6.7%), which may indicate less predation pressure. It is worth noting that collections of almost all populations of cave cirolanids have more females, sometimes many more; e.g., Bruce et al. (2017) reported collecting 37 females and only one male *Lucayalana troglexuma* (Botosaneanu & Iliffe), 1997 in Hatchet Bay Cave on Eleuthera Island, Bahamas. Whatever causes the imbalance in *B. geracei* is probably causing similar imbalances in other species, and selective predation and cannibalism on males seems most likely.

As noted earlier in Table 6 and Fig. 8, females in Major's Cave outnumbered males, but only 3:2. Major's Cave has a different set of potential predators including: remipedes (*Speleonectes epilimnius*) and marsh crabs (*Armases miersii*). Our laboratory experiments showed that marsh crabs are effective predators on *B. geracei*, including large females. Another possible explanation (besides differences in predation) is differences in types or quantities of prey for the isopods; more food could be available for isopods in Major's Cave, which might reduce cannibalism and allow males to survive longer. Another explanation could be simply that male *B. geracei* survive better with the lower salinity of Major's Cave. Vogt (2018) gives examples of other species where, "differences in longevity were also found between populations of the same species in the same geographical area or even in the same water body."

Population size

The population of *B. geracei* in Lighthouse Cave appears to be remarkably high compared to those of most other stygobitic cirolanids, many of which have been collected by cave divers; several of these species have been so sparse, they resulted in type series having <5 specimens (e.g.,1 ♂ *Bahalana exumina* Botosaneanu & Iliffe, 2002; 1 ♀ *Exumalana reptans* Botosaneanu & Iliffe, 2003b; 1 ♂ *Bahalana abacoana* Botosaneanu & Iliffe, 2006). There are several possible reasons for such differences in population sizes, including the great variation in habitats within anchialine environments. Cave divers who explore anchialine habitats usually swim mid-water to avoid hitting stalactites on the ceiling or stirring up the bottom substrate, both areas that may be preferred by some anchialine animals. Areas of scuba exploration are often long distances from access to the surface where food might be brought in by bats and rainwater.

In contrast, the populations of *B. geracei* in Lighthouse Cave and Major's Cave may be relatively large because the caves have entrances large enough to allow substantial populations of bats. Evidence is lacking that *B. geracei* eat bat guano directly, but guano certainly supports large populations of terrestrial animals that probably fall into the water as food for isopods. In addition, *B. geracei* eagerly consumed small asellote isopods, *N. stocki*, which eat Lighthouse Cave detritus almost continuously (personal observation). Thus, they appear to provide an important link between nutrients in the detritus-based food chain and *B. geracei* and other carnivores.

A few other large populations of cave cirolanids have been reported. As mentioned above in Males vs. females, Bruce et al. (2017) reported that 38 specimens were collect-

ed from Hatchet Bay Cave, Eleuthera Island, Bahamas, for their taxonomic study of *L. troglexuma*; 1 male (6.9 mm) and 37 females (sizes and reproductive conditions not mentioned) were collected from baited traps set at 1–3 m for two hours, then preserved in 95% ethanol for DNA studies. Hatchet Bay Cave is similar to Lighthouse Cave, in that it is a walk-in cave (rather than mostly submerged) with an entrance large enough for colonies of bats, and it has pools of water open to the air (Bruce et al. 2017).

Romero (2009) pointed out that, "A popular misconception about cave biodiversity and biomass is that such environments are always poor in both. Although it is true that many hypogean environments are small, lack primary producers, and have a depauperate fauna when compared with the epigean environment, it is not uncommon to find tropical caves with ceilings literally covered by bats, the soil covered by myriads of invertebrates, and water teeming with aquatic life, including hundreds if not thousands of fish in a single pool. The origin of this misconception stems from the fact that most cave research has been conducted in temperate caves (USA, Europe) where biodiversity and biomass are rather poor."

It is probably significant that most anchialine cirolanids in the Western Hemisphere are from tropical and semi-tropical environments, so the food supply provided by the surrounding terrestrial environment should be more substantial and more reliable than in temperate locations. Iliffe and Botosaneanu (2006) provided valuable insight into the distribution patterns of subterranean Cirolanidae, including the high biodiversity of stygobitic cirolanids in the peri-Caribbean and Mexican Realm.

Low oxygen levels may be an even more important factor for population size (and for the evolution of low metabolic rates for cave animals) than low food supply. As pointed out by Culver and Pipan (2019), "Aquifers isolated from the surface tend to have low-oxygen concentration because there is no way to replenish oxygen used up by aerobic respiration of the organisms living in the aquifer and relatively little food unless chemoautotrophy occurs." Bishop et al. (2004) measured the extremely low oxygen levels in the lower levels of two stratified anchialine caves and presented strong evidence that "a unique community of macrofauna has adapted to cope with constant low oxygen conditions and a depauperate food supply." In contrast, the caves on San Salvador Island do not have this stratification, and the substantial entrances to the two caves in this study provide access to oxygen exchange with the surface as water moves back and forth twice daily with the tides. Oxygen levels in Lighthouse Cave and Major's Cave are moderately high at ~2–6 mg/l. This, along with a better food supply, may help explain the relatively high populations, compared to those of anchialine cirolanids that live in stratified anchialine caves and are accessed only by cave divers swimming considerable distances from entrances.

Differences in water chemistry with depth may partially explain which animals live at various depths within anchialine habitats. Furthermore, water samples change markedly when they are brought to the surface from depth. While diving in Mexican caves in 1992, I observed that when a collecting bottle was filled with water at 20 m, then opened later at the surface, compressed gasses (including carbon dioxide) escaped, which markedly changed the pH and allowed calcium carbonate to precipitate. These changes in water chemistry created problems in keeping delicate animals such as remipedes alive for long-term observation.

Fecundity

It appears that *B. geracei*'s fecundity is surprisingly high, especially for a cave isopod. Culver et al. (1995) listed "lowered fecundity" as one of several traits (troglomorphisms) commonly found in cave organisms when compared to surface-dwelling organisms. On the other hand, when Poulson (1963) compared stygobitic and non-stygobitic amblyopsid fish, he found that stygobionts have "a doubling of life span, at least a 40% increase in egg size, and at least a 50% increase in the maximum number of broods as a result of increased longevity." Female *B. geracei* follow this pattern of high number of broods, which greatly increases fecundity.

Unfortunately, it is difficult to make good comparisons of fecundity in other species since there are few reports on cirolanids that include data on mancas per brood in females of different sizes, along with probabilities of having 2, 3, or 4 broods. Hopefully, these estimates and the ways I arrived at them will be useful for others.

Population studies (including fecundity) are often done to provide guidance for conservation work. Fortunately, *B. geracei* populations seem to be relatively large and stable. The Gerace Research Centre has done a good job restricting collecting in sensitive areas on San Salvador Island, including Lighthouse Cave. Optimistically, their conservation initiatives will continue to protect all the island's anchialine habitats. It is hoped that this information about *B. geracei* can provide a basis for conservation work on other cave crustaceans, but considerable caution should be used because population dynamics can vary considerably from species to species and cave to cave as illustrated by the remarkable differences between Lighthouse Cave and Major's Cave populations.

Conclusions

The study of the natural history of cave cirolanids and many other animals has largely been eclipsed by the large number of taxonomic descriptions of fascinating species, including *Bahalana geracei*. It is certainly important to study the taxonomic diversity of various groups and the relationships within them, partly to develop strategies to help them survive threats to their environments. One other way to help protect them is to learn about their behaviors, reproduction, and population dynamics. I feel very fortunate that I have had the opportunity to make dozens of trips to The Bahamas and to study the lives of several species of anchialine animals. Hopefully, this first extensive natural history study of a cave cirolanid will encourage other researchers to study the lives of other cave isopods for extended periods to compare to *B. geracei*. To study reproduction in any long-lived cave species, it may be easier to obtain females with eggs by keeping them alive and feeding them over long periods, compared to trying to find egg-bearing females in the caves where food is less available. It may be wise to carry out such long-term studies as side projects, along with shorter-term ones that students can participate in during their relatively short college careers. Understandably, the first goal of many invertebrate zoologists and entomologists is to identify and classify their animals. However, it is easy to underestimate the excitement and importance of studying live animals.

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