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Traversing worlds – Dispersal potential and ecological classification of *Speolepta leptogaster* (Winnertz, 1863) (Diptera, Mycetophilidae)

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Abstract

Speolepta leptogaster (Winnertz, 1863) is frequently occurring in European subterranean environments. As for most cave animals, studies addressing non-anatomical aspects are sparse. Here we present the first molecular study on *S. leptogaster*. We investigated the demographic structure (i.e. COI locus) of 69 specimens from 36 underground populations in Hesse (Central German Uplands) to get first insights into the species' dispersal ability.

In total, 14 haplotypes were revealed. Haplotype diversity was relatively high, whereas nucleotide diversity was low. Furthermore, a significant but low pattern of isolation-by-distance and (a) past population expansion event(s) were detected.

Our genetic results suggest a (good) active dispersal ability for *Speolepta leptogaster*. The occurrence of several surface records of adult specimens corroborates this hypothesis. We discuss the developmental stages of *S. leptogaster* in the context of the ecological classification system and regard the species as a eutroglophile. Evidence has been found to distinguish two larval types. A reconstructed life-cycle of the species is provided.

Keywords

Cave animal, ecotone, phylogeography, mobility, ecological versatility

Introduction

Century and a half have passed since the mycetophilid *Speolepta leptogaster* (Winnertz, 1863) (Diptera, Mycetophilidae) has been described. The species is widely distributed throughout Europe and can complete its entire life-cycle in subterranean environments such as caves, mines or related underground habitats. Originally placed into the genus *Polylepta*, Edwards (1925) established the monotypic genus *Speolepta* in reference to its subterranean ecology and morphology. Due to its enigmatic ecology, obligate subterranean larvae spinning silk-nets and the short adult life spans, *S. leptogaster* received popular attention amongst entomologists (Schmitz 1912, Lengersdorf and Mannheims 1951, Matile 1962, Plachter 1981). As a tribute, the species was chosen as the Cave Animal of the Year 2013 in Germany. Despite all the scientific attention, research primarily focused on morphological investigations of the different developmental stages (egg, larval stages, pupa and imago) (Schmitz 1912, Lengersdorf and Mannheims 1951, Matile 1962, Plachter 1981), rather than on biological or ecological characteristics. As recently as 2012, two other *Speolepta* species have been described, thus increasing the number of known species in this genus up to three (Sevcik et al. 2012). In their taxonomic revision, Sevcik et al. (2012) pointed to neglected investigations concerning the species' dispersal ability. They suggested that "[a]lthough the European *S. leptogaster* normally breeds and completes its life-cycle entirely underground (Matile 1962), adults of both sexes are frequently found far away from caves. […] This suggests good dispersal abilities where some gene flow may be retained between otherwise very isolated cave populations".

Here, we investigate the dispersal ability of *Speolepta leptogaster* by integrating population genetic data of specimens collected from underground sites in the Central German Uplands (Hesse). The study region was chosen since it was covered by permafrost during the Last Glacial Maximum (18,000 – 24,500 ybp) (Clark et al. 2009). A postglacial (re-)colonization of this area (inferring a certain level of mobility) rather than the survival in a central German glacial refugium is likely.

Methods

Material and morphological analyses

In total, 69 specimens of *Speolepta leptogaster* from 37 different caves, bunkers, wells, tunnels and cellars in Hesse (maximum of three per site) and one from a bunker in Poland were analyzed. The samples (Table 1) were collected and identified by members of the Hesse Federation for Cave and Karst Research (Germany) (Reiss et al. 2009) and stored in ethanol. In order to ensure good DNA preservation, all molecularly-processed samples were dated no later than six years old. Prior to DNA isolation, images of all specimens were taken with the camera "Moticam Model Moticam 5". Specimens

Table 1. Dataset of analyzed specimens of *Speolepta leptogaster* and locality information. LS: life stage (L: larva, P: pupa, I: imago); H: haplotype. The geographic coordinates are in Degrees (°), Minutes (**'**) and Decimal seconds (**"**). The locality numbers resemble the numbers given to each natural region by the German Bundesamt für Naturschutz.

#	LS	Coordinates	Biotope	Locality numbers	H	NCBI
1a	L	50°10'13.80"N; 9°24'11.92"E	natural cave	141 Sandsteinspessart	9	KF624625
1b	L	50°10'13.80"N; 9°24'11.92"E	natural cave	141 Sandsteinspessart	1	KF624626
1c	L	50°10'13.80"N; 9°24'11.92"E	natural cave	141 Sandsteinspessart	4	KF624627
2a	L	50°15'49.43"N; 9°31'39.86"E	natural cave	141 Sandsteinspessart	1	KF624628
2b	L	50°15'49.43"N; 9°31'39.86"E	natural cave	141 Sandsteinspessart	1	KF624629
3a	I	50°4'55.99"N; 7°48'56.02"E	mine shaft	304 Westlicher Hintertaunus	1	KF624630
3b	I	50°4'55.99"N; 7°48'56.02"E	mine shaft	304 Westlicher Hintertaunus	1	KF624631
4a	P	50°5'34.37"N; 7°51'6.16"E	mine shaft	304 Westlicher Hintertaunus	8	KF624632
4b	L	50°5'34.37"N; 7°51'6.16"E	mine shaft	304 Westlicher Hintertaunus	8	KF624633
5a	L	50°6'36.32"N; 7°56'49.81"E	mine shaft	304 Westlicher Hintertaunus	2	KF624634
5b	L	50°6'36.32"N; 7°56'49.81"E	mine shaft	304 Westlicher Hintertaunus	2	KF624635
5c	L	50°6'36.32"N; 7°56'49.81"E	mine shaft	304 Westlicher Hintertaunus	2	KF624636
6	L	50°9'19.87"N; 8°4'53.98"E	mine shaft	304 Westlicher Hintertaunus	1	KF624637
7a	L	50°52'40.73"N; 8°28'11.28"E	mine shaft	320 Gladenbacher Bergland	7	KF624638
7b	L	50°52'40.73"N; 8°28'11.28"E	mine shaft	320 Gladenbacher Bergland	7	KF624639
8	L	50°53'21.05"N; 8°25'39.54"E	mine shaft	320 Gladenbacher Bergland	11	KF624640
9	L	50°52'23.23"N; 8°28'37.34"E	rock cellar	320 Gladenbacher Bergland	3	KF624641
10a	L	51°0'4.61"N; 8°35'41.53"E	mine shaft	332 Ostsauerländer Gebirgsrand	2	KF624642
10b	L	51°0'4.61"N; 8°35'41.53"E	mine shaft	332 Ostsauerländer Gebirgsrand	2	KF624643
10c	L	51°0'4.61"N; 8°35'41.53"E	mine shaft	332 Ostsauerländer Gebirgsrand	2	KF624644
11a	L	51°23'34.73"N; 8°41'28.79"E	mine shaft	332 Ostsauerländer Gebirgsrand	1	KF624645
11b	L	51°23'34.73"N; 8°41'28.79"E	mine shaft	332 Ostsauerländer Gebirgsrand	14	KF624646
11c	L	51°23'34.73"N; 8°41'28.79"E	mine shaft	332 Ostsauerländer Gebirgsrand	1	KF624647
12a	L	51°13'56.89"N; 8°54'4.32"E	natural cave	340 Waldecker Tafelland	2	KF624648
12b	L	51°13'56.89"N; 8°54'4.32"E	natural cave	340 Waldecker Tafelland	2	KF624649
13a	L	51°14'25.08"N; 8°54'9.40"E	mine shaft	340 Waldecker Tafelland	1	KF624650
13b	L	51°14'25.08"N; 8°54'9.40"E	mine shaft	340 Waldecker Tafelland	1	KF624651
14	L	51°18'55.58"N; 9°24'26.86"E	undercroft	342 Habichtswälder Bergland	1	KF624652
15a	L	51°7'41.70"N; 8°59'0.17"E	spring	344 Kellerwald	1	KF624653
15b	L	51°7'41.70"N; 8°59'0.17"E	spring	344 Kellerwald	1	KF624654
16	L	51°7'32.66"N; 8°59'33.47"E	spring	344 Kellerwald	1	KF624655
17	L	50°30'3.56"N; 9°7'25.43"E	rock cellar	350 Unterer Vogelsberg	1	KF624656
18a	L	50°31'0.59"N; 9°32'3.73"E	mine shaft	350 Unterer Vogelsberg	1	KF624657
18b	L	50°31'0.59"N; 9°32'3.73"E	mine shaft	350 Unterer Vogelsberg	1	KF624658
19	I	50°27'0.29"N; 9°49'15.92"E	rock cellar	353 Vorder- und Kuppenrhön	2	KF624659
20	L	50°34'21.18"N; 9°57'38.63"E	rock cellar	353 Vorder- und Kuppenrhön	12	KF624660
21	L	50°35'23.96"N; 9°59'54.13"E	rock cellar	353 Vorder- und Kuppenrhön	1	KF624661
22a	P	50°35'57.73"N; 9°59'59.60"E	culvert	353 Vorder- und Kuppenrhön	1	KF624662
22b	P	50°35'57.73"N; 9°59'59.60"E	culvert	353 Vorder- und Kuppenrhön	1	KF624663

#	LS	Coordinates	Biotope	Locality numbers	H	NCBI
23a	L	50°30'30.64"N; 9°55'48.65"E	mine shaft	354 Hohe Rhön	1	KF624664
23b	L	50°30'30.64"N; 9°55'48.65"E	mine shaft	354 Hohe Rhön	6	KF624665
23c	L	50°30'30.64"N; 9°55'48.65"E	mine shaft	354 Hohe Rhön	6	KF624666
24a	L	50°28'7.25"N; 9°57'45.40"E	spring	354 Hohe Rhön	1	KF624667
24b	L	50°28'7.25"N; 9°57'45.40"E	spring	354 Hohe Rhön	1	KF624668
25a	L	50°52'35.18"N; 9°42'27.40"E	brick-built cellar	355 Fulda-Haune-Tafelland	1	KF624669
25b	L	50°52'35.18"N; 9°42'27.40"E	brick-built cellar	355 Fulda-Haune-Tafelland	1	KF624670
26a	L	50°53'4.56"N; 9°43'25.10"E	rock cellar	355 Fulda-Haune-Tafelland	1	KF624671
26b	L	50°53'4.56"N; 9°43'25.10"E	rock cellar	355 Fulda-Haune-Tafelland	1	KF624672
27a	L	50°51'35.21"N; 9°45'19.94"E	mine shaft	355 Fulda-Haune-Tafelland	1	KF624673
27b	L	50°51'35.21"N; 9°45'19.94"E	mine shaft	355 Fulda-Haune-Tafelland	13	KF624674
27c	L	50°51'35.21"N; 9°45'19.94"E	mine shaft	355 Fulda-Haune-Tafelland	1	KF624675
28a	L	51°7'34.36"N; 9°47'35.70"E	brick-built tunnel	357 Fulda-Werra-Bergland	1	KF624676
28b	L	51°7'34.36"N; 9°47'35.70"E	brick-built tunnel	357 Fulda-Werra-Bergland	10	KF624677
29	L	51°12'26.24"N; 9°52'16.82"E	mine shaft	357 Fulda-Werra-Bergland	1	KF624678
30	L	51°0'43.13"N; 9°55'45.73"E	mine shaft	357 Fulda-Werra-Bergland	1	KF624679
31a	L	51°0'24.88"N; 9°57'44.68"E	mine shaft	357 Fulda-Werra-Bergland	5	KF624680
31b	L	51°0'24.88"N; 9°57'44.68"E	mine shaft	357 Fulda-Werra-Bergland	5	KF624681
31c	L	51°0'24.88"N; 9°57'44.68"E	mine shaft	357 Fulda-Werra-Bergland	5	KF624682
32a	L	51°13'29.10"N; 9°57'8.68"E	mine shaft	358 Unteres Werratal	1	KF624683
32b	L	51°13'29.10"N; 9°57'8.68"E	mine shaft	358 Unteres Werratal	1	KF624684
33a	L	51°13'27.05"N; 9°57'11.74"E	touristic mine	358 Unteres Werratal	2	KF624685
33b	L	51°13'27.05"N; 9°57'11.74"E	touristic mine	358 Unteres Werratal	2	KF624686
34a	L	51°10'45.44"N; 10°4'15.28"E	mine shaft	358 Unteres Werratal	1	KF624687
34b	L	51°10'45.44"N; 10°4'15.28"E	mine shaft	358 Unteres Werratal	1	KF624688
35	L	51°31'8.11"N; 9°22'39.43"E	bunker complex	361 Oberwälder Land	1	KF624689
36a	L	51°31'8.11"N; 9°22'39.43"E	bunker complex	361 Oberwälder Land	1	KF624690
36b	L	51°31'8.11"N; 9°22'39.43"E	bunker complex	361 Oberwälder Land	1	KF624691
36c	L	51°31'8.11"N; 9°22'39.43"E	bunker complex	361 Oberwälder Land	1	KF624692
37	I	52°24'0"N; 15°31'59.99"E	bunker complex	Nietoperek (Poland)	8	KF624693
Σ 69						

were measured using the measuring function of the image-capturing program "Motic Images Plus 2.0". The bodies of the specimens were examined to determine potential differences in phenotype, which could correspond to different haplotypes.

Molecular analyses

A small piece of the posterior end of the body (approx. one sixth of the total animal) was used for larval samples. In the case of pupae and imagines, a larger portion of the abdomen (approx. ¼) was macerated. DNA isolation was performed according to the instructions of the DNEasy Blood & Tissue Kit (Qiagen Sample and Assay Technologies, Hilden, Deutschland) for the column purification of animal tissue.

A 662 bp fragment of the Cytochrome C Oxidase subunit 1 (COI) gene was amplified using the primers C1-J-2195 5'-TTGATTTTTTGGTCACCCTGAAGT-3' and TL2-N-3014 5'-TCCAATGCACTAATCTGCCATATTA-3' established by Simon et al. (1994). The PCR reactions were executed in a Peqlab Primus Advanced 96 thermo cycler. Each PCR-mix (25 µL) contained 2.5 µL PCR-buffer (10×, without $MgCl_2$), 2.0 µL $MgCl_2$ (100 mM), 0.3 µL dNTPs (20 mM), 1.0 µL of each primer, 0.3 µL Taq-DNA-Polymerase, 1.5 µL BSA (bovine serum albumin, 10 mg/mL), 11.4 µL ddH_2O and 5.0 µL template DNA. PCR conditions were as follows: initial denaturation step 1 min 94 °C, 30 cycles of denaturation (30 sec, 94 °C), annealing (30 sec, 43.7 °C) and elongation (30 sec, 72 °C) and a final elongation step with 7 min at 72 °C. PCR products were bi-directionally sequenced using the Sanger chain termination method (Sanger et al. 1977). Sequencing service was provided by the Laboratory Centre of the Biodiversity and Climate Research Centre (Frankfurt am Main, Germany) with their own sequencing protocols. Editing and assembly of the forward and reverse sequences were done using Geneious v5.4.6. The sequences were manually trimmed to a length of 659 bp to fit the shortest sequence retained. Sequence alignment was performed using the MAFFT v6.814b plug-in for Geneious under default settings and the automatic algorithm option.

Population genetic analyses

The software DnaSP v5 (Librado and Rozas 2009) was used to calculate the number of haplotypes, the total haplotype and nucleotide diversity and to perform neutrality tests. Haplotype diversity (Hd) can result in values between 0 and 1. A value of Hd = 1 implies that two randomly picked samples will always demonstrate two different haplotypes (= 100% diversity). The nucleotide diversity (π) illustrates the average genetic distance between two sequences estimated as an average for all sequences. The value can range from 0 (no changes in all sequences) to a theoretical 1 (every base is replaced). The calculation for Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989) tests can be positive, negative or have a zero value. A significant positive value for Tajima's D may point to over dominant selection or a population bottleneck event. Significant negative values point to a population expansion event or purifying selection. A value of zero or non-significant values cannot reject the neutral model of molecular evolution. Fu's Fs shares the same characteristics but adds the support for genetic hitchhiking with a negative value. Standard settings were used and the sequences were trimmed to the same length. The program TCS 1.21 (Clement et

al. 2000) was used to reconstruct a haplotype network by Statistical Parsimony (Templeton et al. 1992). Networks were created by a 95% connection probability.

A Mantel-test (Mantel 1967) was used to test for correlation between geographic distance vs. genetic distance. The geographic distance matrix was calculated with the Geographic Distance Matrix Generator 1.2.3 (Ersts 2014) using a WGS84 spheroid and geodesic distances in km. Genetic distances were calculated as p-distances using the software MEGA 5.2 (Tamura et al. 2011) and the pairwise deletion option. The Mantel-test was performed in XLSTAT 2013 (Addinsoft) using the linear Pearson product-moment correlation coefficient (r) and 10,000 permutations. A value of r = 0 implies no linear correlation, whereas a minimal / maximal value of -1 / +1 indicates total negative / positive correlation.

Results

Demographic structure of *S. leptogaster* in Hesse

No gaps were present in the COI-alignment. In total, 14 haplotypes (H1-H14) were revealed. Haplotype diversity was high with a value of Hd = 0.7017, whereas nucleotide diversity was low with π = 0.00165 (Table 2). The haplotype network (Figure 1) indicated a highly frequent haplotype, H1, which consisted of 39 samples (57%) and a second relatively frequent haplotype, H2, which comprised 11 samples (16%). Eight haplotypes were singletons (H3, H4, H9-H14), two consisted of two (H6 and H7) and two of three samples (H5 and H8). There were only four haplotypes (H4, H8, H9 and H11) which were not directly connected to the most frequent haplotype H1.

When the different haplotypes are placed in a geographical context (Figure 2), H1 can be revealed throughout the study region of Hesse. Haplotype 2 demonstrates a similar distribution except in the central underground sites and the most northern parts of Hesse. Haplotype 8 occurs once in southern Hesse and in the sampling site in Poland. All other haplotypes have been found within only a single locality. At a few localities several haplotypes co-occurred: In north-western Hesse H14+H1 and H2+H1; in north-eastern Hesse H9+H1, H2+H1 and H13+H1 and in south-eastern Hesse, H10+H6+H1.

Table 2. Overview of statistical values. Estimates are given for neutrality tests (Fu's Fs, Tajima's D), haplotype (Hd), nucletide diversity (π) and Pearson's r in a Mantel-test (geographic distance vs. genetic distance).

estimates	value	p-value
Fu's Fs	-10.28	$p < 0.001$
Tajima's D	-1.89	$p = 0.004$
Hd	0.7017	
π	0.00165	
r	0.17	$p < 0.0001$

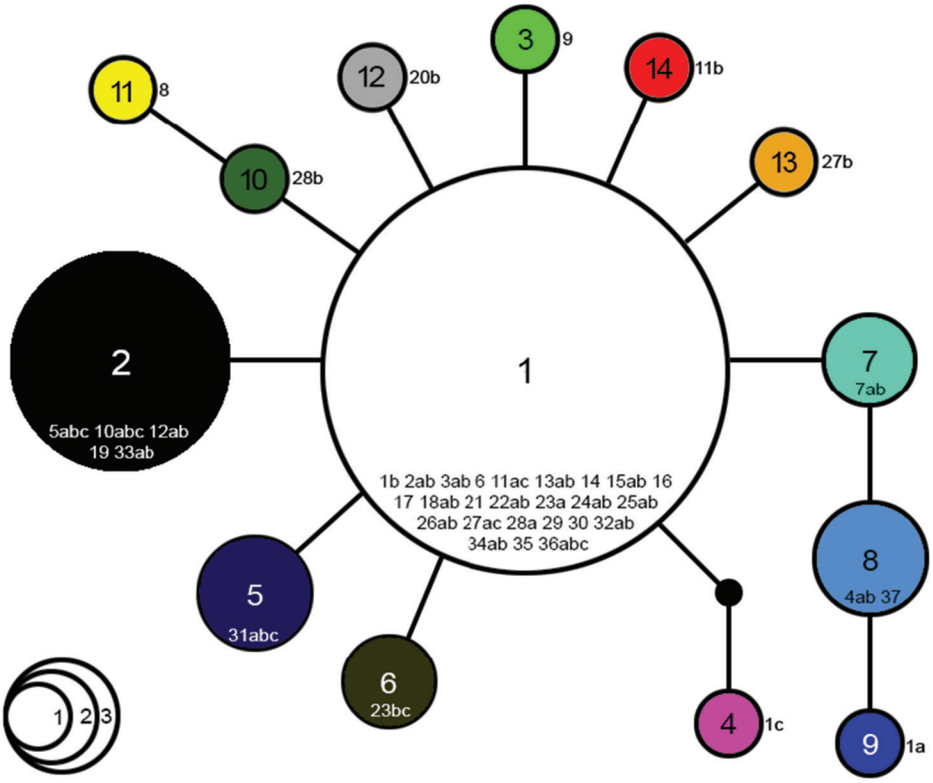

Figure 1. CO1 haplotype network for *Speolepta leptogaster*. Haplotypes are numbered in sequence with their volume proportional to their frequency in the total dataset. Lines interconnecting the haplotypes illustrate the mutational course and the number of mutational steps between them. Numbers with letters within or alongside circles refer to Table 1.

The results of both neutrality tests were significantly negative ($p < 0.01$) and point to (a) past population expansion event(s) (Table 2). In our dataset, 28.5% of all mutations (4/14) were non-synonymous leading to a change of the respective amino acid.

The Mantel-test ($r = 0.17$, $p < 0.0001$) reveals a significant positive low correlation between genetic distance and geographic distance (Table 2).

Discussion

Dispersal ability

The region of the Central German Uplands (including our target area of Hesse) was covered with permafrost during the Last Glacial Maximum (18,000 – 24,500 years ago) (Clark et al. 2009). It is widely accepted, that ecologically diverse species were able to (re-)colonize this area when the climatic conditions became more suitable after

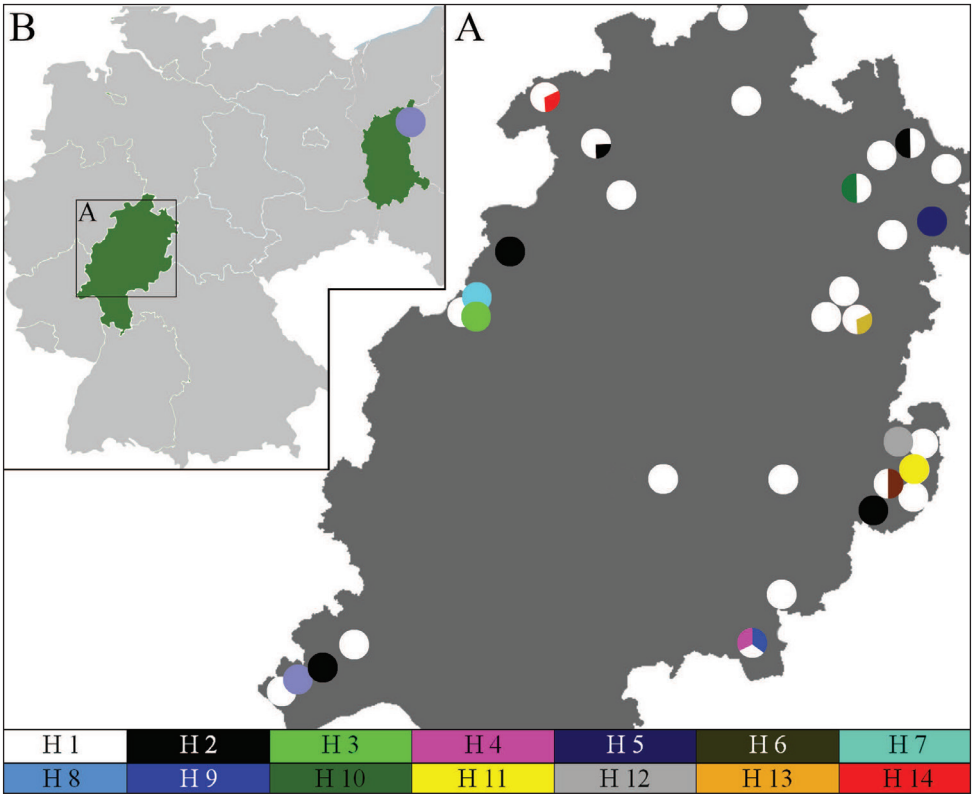

Figure 2. Spatial pattern of haplotypes of *Speolepta leptogaster* in Hesse**.** The haplotype (H) distribution of *S. leptogaster* within Hesse (**A**) with a comparison group in Poland (**B**) is depicted as a circle for every underground locality with colored sections for the different haplotypes. To be depicted in a reasonable manner, multiple localities were reduced to one circle if they were situated nearby (up to 4 km) and had the same color.

the ice shields had retracted northward again (Taberlet et al. 1998, Hewitt 1999, Petit et al. 2002). Our genetic analyses are in agreement with a similar postglacial scenario for *Speolepta leptogaster.* The recent demographic structure of this species in Hesse is best explained by (a) past population expansion event(s). Most probably and after population bottlenecks, the low-frequency and most often cave endemic haplotypes have evolved from the high-frequency central haplotypes already present in the ancestral gene pool of the species (i.e. the founder effect). The significant but weak pattern of isolation-by-distance can be interpreted in terms of a certain level of connectivity between populations, which may be explained by dispersal of adult specimens. An interesting finding is the identity of haplotypes found in Poland and southern Hesse. Yet, a pan-European sampling is needed to address any further hypotheses (e.g. passive anthropogenic transportation vs. active surface-dispersal).

In general, our results imply good dispersal ability for *Speolepta leptogaster*. This is further supported by surface records of adult specimens compiled from the literature

Box 1. Original description of *Speolepta leptogaster* (Winnertz, 1863). Translated from German. Originally this species has been described under the name of *Polylepta leptogaster*.

The habitus is very similar to the *Bolitophila*. Body color brown. Mouth-rim slightly pulled forward and garlanded with hairs. The filamentous antennae are about 1.33 times as long as head and thorax together. The flagellum links are 3 to 4 times as long as broad. The haltere is whitish with a black-brown tip. The abdomen is very slim and cylinder-shaped, about 5 to 6 times as long as the very short thorax and constricted at the base. The coxa and femur yellow, tibia more brownish, tarsus light brown. The feet of the front legs are 2.33 times as long as the tibia, the tibia slightly shorter than the metatarsus (9 : 9 ¾) with lanceolate basis. Wings slightly greyish nearly colorless; the subcosta proceeding over the cubitus up to the tip of the wing, the supporting vein broken off in front of the lateral vein, the marginal lateral vein pulled far back, the discal cell trapezoid-shaped, 1.5–2 times as long as broad, the style of the upper fork about half the length of the upper prong, the basis of the rear cell under the middle of the wing on the far side of the discal cell, the axilla vein not sturdy, broken off on the opposite side of the rear cell. I only captured a female of this very rare species once in august in a swampy, forested area. A second female is located at the Royal Museum in Leyden, which differs from mine in the way that the discal cell, which is 1.5 times as long as broad in my specimen, is 2 times as long as broad. Apart from that, they completely matched.

(Table 3). Although all developmental stages can be found in caves throughout the year, there are more findings of surface imagines in summer than in winter. Whether this is due to the biology of *S. leptogaster* or to the smaller number of traps being laid out in winter cannot be determined.

Ecological classification

Animals associated with underground habitats can be classified into different categories. Those classifications (i.e. ecological, behavioral, morphological) have been under constant change and revision. Multiple authors created new categories as well as split up old ones (Shiner 1854, Racovitza 1907, Vandel 1965, Hamilton-Smith 1971, Sket 2008). However, the main ecological categories to which we refer here have remained more or less unchanged: eutrogloxenes (normally not living in caves), subtroglophiles (partially living in caves, but without permanent populations), eutroglophiles (able to complete several underground generations) and eutroglobionts (solely living in caves). As a result of this ecological continuum of cave-association, i.e. from "only found by chance" to "being obligate subterranean", probable cave-associated (e.g. morphological and behavioral) adaptations are multifarious.

Since the biological diversity of subterranean species and ecology of subterranean habitats is high (Moseley 2008), every categorical and thus limited ecological classification system will elicit problems in classifying all cave organisms. This is particularly challenged by a "species from both worlds" living in a transitional environment or ecotone, as which caves must be considered (Moseley 2008). The situation is even more complicated

Table 3. Surface records of *Speolepta leptogaster*. N: number of specimens found, f: female, m: male.

Region	Date of collection	Habitat/collection	N	Reference
		swampy forest area	1 f	Winnertz (1863)
Germany, Birgsau, südlich von Oberstdorf, im Stillachtal	18–27 Sept. 1975	light trap	1 m	Plassmann (1977), first description *S. dissona*
Germany, Saxon Switzerland 1.5 km SE of Ober-vogelgesang	10–21 May 1997	deciduous forest, Malaise trap	1 f, 1 m	U. Kallweit (unpublished)
S-Germany, Grenzach	11 May 2008		1 f	B. Rulik (unpublished)
Germany, Harz mountains	26 Oct. 2004	natural mature spruce forest, yellow pan trap	2 m	U. Kallweit (unpublished)
Germany, Harz mountains	25 June 2004	natural mature spruce forest, Malaise trap	1 m	U. Kallweit (unpublished)
Germany, Hesse, Fulda, Mittelbergquelle	10 Oct. 2004	hand collection	1 f	Zaenker (2008)
Germany, Hesse, Auersbergquelle 21	18 Sept. 2009	sweep net	1 f	Zaenker unpublished
Germany, Hesse, Lützeler Sang-Quelle 2	25 Oct. 2007	sweep net	1 m	Zaenker (2008)
Norway, Kvinnherad, Rosendal, riverside at Avlsgården, Baroniet	11–15 May 1990	Malaise trap	1 f, 2 m	Ševčík et al. (2012)
Norway, Bergen, Haukeland	9 May–28 June 1991	Malaise trap	3 m	Ševčík et al. (2012)
Norway, Bømlo, Vorland, Langevåg	11 Feb 2002–12 Feb 2003	Malaise trap	7 f, 5 m	Ševčík et al. (2012)
Norway, Etne, Skånevik skyttarbane	3 Sept. 2009	sweep net	1 f, 1 m	Ševčík et al. (2012)
Norway, Fjell, Vindenes	5 Sept. 1978	light trap	1 f	Ševčík et al. (2012)
Norway, Os, Raudli	23–30 May 1991		1 f, 1 m	Ševčík et al. (2012)
Norway	June–Sept. 1991	Malaise trap	17 f, 11 m	Ševčík et al. (2012)
Norway, Os, Sæleli	20–27 June 1991	Malaise trap	1 m	Ševčík et al. (2012)
Norway, Øygarden, Dalsvann, Alvøy	5 June 1987	light trap	1 f	Ševčík et al. (2012)
Norway, Sveio, Førde, Solheimshaugen	3–10 June 1991	Malaise trap	2 f	Ševčík et al. (2012)
Norway, Sveio, Førde, Solheimshaugen	15 June 1991	sweep net	1 f, 1 m	Ševčík et al. 2012

Region	Date of collection	Habitat/collection	N	Reference
Norway, Sunndal, Jordalsgrenda, Jordalsøra, Hamrene	31 Mai–13 Jul. & 26 Aug.–6 Sept. 2004	Malaise trap	9 f, 14 m	Ševčík et al. (2012)
Norway, Sunndal, Jordalsgrenda, Jordalsøra, Hamrene	14 June–3 Jul. & 12–25 Aug. 2005	window trap	1 f, 1 m	Ševčík et al. (2012)
Norway, Aurland, Vassbygdvatnet (lower end of lake)	4 Aug. 1969	sweep net	1 f	Ševčík et al. (2012)
Norway, Porsgrunn, Hitterødbekken	13 June–11 Jul. 1988	Malaise trap	3 f	Ševčík et al. (2012)
Sweden, Jokkmokk, Kaltisbäcken 1km NNE Messaure	19–30 June 1968	air suction trap	1 f, 3 m	Ševčík et al. (2012)
Sweden, Lund, Høje Å (stream) at Värpinge	23–28 May 2004	yellow pan trap	1 m	Ševčík et al. (2012)
Sweden, Klippan, Skäralid NR (ravine with stream)	26 Sept. 1983	sweep net	1 m	Ševčík et al. (2012)
Sweden, Högsby kommun, Getebro	29 Jul–31 Aug. 2004	Malaise trap	1 f	Ševčík et al. (2012)
Faroe Islands, Streymoy, Kvivik	13–17 Jul. 1990	Malaise trap	2 m	Ševčík et al. (2012)
Czech Republic, Bohemia, Jizerské Hory Mts, Jedlový důl	6–28 Jul. & 1–22 Sept. 2005	Malaise trap	3 m	Ševčík et al. (2012)
Czech Republic, Bohemia, Mt. Poledník	29 Aug. -5 Okt. 2004	Malaise trap	1 m	Ševčík et al. (2012)
Czech Republic, Bohemia, Krkonoše Mts., Bílé Labe	16–30 Aug. 2007	Malaise trap	1 m	Ševčík et al. (2012)
Czech Republic, Bohemia, Labský důl	24–27 Jul. 2006	Malaise trap	1 m	Ševčík et al. (2012)
Czech Republic, Moravia & Silesia, Hrubý Jeseník Mts, Velká kotlina	9–26 June 2006	Malaise trap	1 m	Ševčík et al. (2012)
Czech Republic, Moravia & Silesia, Rejvíz	20 May–1 Jul. 2005	peat-bog, Malaise trap	1 f	Ševčík et al. (2012)
Slovakia, Poľana Mts., Hrončecký grún	7 May–4 Jul. 2006	yellow pan trap	1 m	Ševčík et al. (2012)
Slovakia, Kyslinky–Pod Dudášom	15 June 2009		1 m	Ševčík et al. (2012)
Slovakia, Predná Poľana Mt.–Bystré waterfall	17 June 2009		1 m	Ševčík et al. (2012)
Slovakia, Spády waterfall	18 June & 4 Jul. 2009		2 m	Ševčík et al. (2012)
			[52 f, 64 m]	

by the fact that *Speolepta leptogaster* belongs to the holometabolic insects with different developmental stages demonstrating different ecological requirements. This species is thus a prime example that an ecological classification system for cave-dwelling *species,* based on four categories may be too ambiguous particularly in cases where different *life stages* may behave ecologically distinct. Here we discuss the species three effective life stages, including the larva, pupa and imago in respect to the ecological classification system. We characterize the single developmental stages and potentially the whole species.

The larvae hatch and live solely within subterranean habitats reaching from microcavities to macrocaverns (or caves). They are incapable of surviving on the surface, which is related to their preference for a highly water-saturated atmosphere and adaptation to oxygen respiration. They lack a trachea system but are able to respire oxygen through a very thin cuticula spanning the entire body surface (Schmitz 1912). Author's observations (SZ and AW) point to a behavior of avoiding strong air currents. Since the pupal stage is immobile and directly succeeds the larval developmental stage, it can only occur in the same habitat. The imago however, possesses elongated legs (sometimes regarded as a troglomorphy) (Schönborn 2003) and demonstrates a sluggish flight. At the same time, imagines do not feed and probably only survive a few days to weeks, thus complicating the inference of their ability to survive outside the subterranean habitat. However, several surface records of imagines (Table 3) and sightings of larvae in the transition and entrance zones of underground sites (Zaenker 2008, Weber 2012) are known. Furthermore, it seems that adult specimens travel between caves during the night.

Conclusively, not all developmental stages are capable of surviving outside the subterranean zone. Thus, the completion of a life-cycle aboveground will be incomplete – eliminating the eutrogloxenes as a potential ecological category for *Speolepta leptogaster*. Due to the short life-span of adults rarely leaving the subterranean zone, the complete life-cycle (or a very large proportion) is within the subterranean environment – leaving the eutroglophiles and eutroglobionts. From an evolutionary point of view and for long-term survival, *S. leptogaster* should be classified as a eutroglophile, i.e. being able to complete several underground generations but having the ability of surface dispersal. But pinpointing *S. leptogaster* to a single ecological category clearly underestimates the ecological versatility of the species.

Potential life-cycle

Even after 150 years, some facts about the biology of *Speolepta leptogaster* are still unknown. Specifically, these include the number of larval stages and the durations of the different life stages. Although Schmitz (1912) claimed that he had successfully bred *S. leptogaster* in captivity, he provided no information about the duration of any of the life stages. Still, a comparison with related Mycetophilidae (including the ecologically-similar keroplatid *Arachnocampa luminosa* (Skuse, 1890)) (Baker 2010, Li et al. 2011) may enable a vague picture of the potential life-cycle and the life-spans of the different

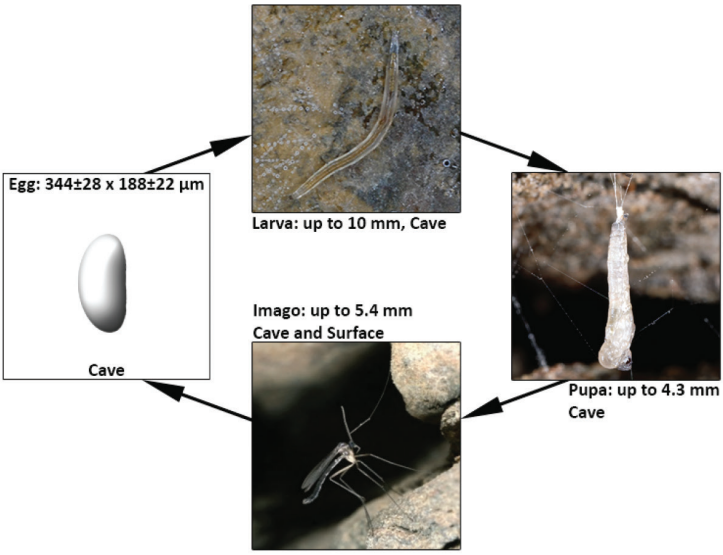

Figure 3. Potential life-cycle of *Speolepta leptogaster*; egg-drawing modified after the descriptions of Plachter (1981).

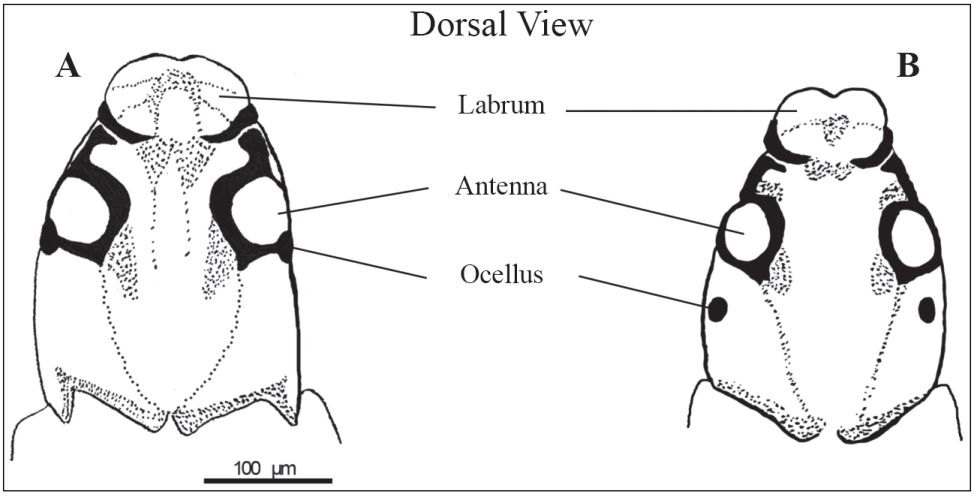

Figure 4. Comparison between two larval stages of *Speolepta leptogaster*. **A** depicts the larger and probably older larva type A which on average is 10 mm long **B** depicts the smaller larva B type which is 5–10 mm long. Dotted regions depict areas of increased pigmentation, black regions illustrate maximum pigmentation.

developmental stages: a) egg: approx. 2 weeks; b) larval stages: 6 – 12 months; c) pupa: approx. 2 weeks and d) imago: 4 – 20 days (Figure 3).

Two types of larvae were present in our material. They can be distinguished by a small deviance of the ocelli position and by body measurements (Figure 4). In type

A, the ocellus is situated adjacent to the antenna whereas in type B, the ocellus is approximately 15 to 20 µm behind the antenna. The head capsule of larvae type B was thinner (A: about 210 µm, B: about 190 µm), slightly shorter (A: about 300 µm, B: about 280 µm) and generally more pointed than in type A. The labrum of larvae type B displays a deeper indentation in the middle of the frontal end. At the proximal end of the head area where the vermiform body begins, the larvae type A displays an edged transition, whereas with larvae type B**,** it is more semicircular. In the sample set, three larvae were found that appeared as depicted in type B. Two of which had only half the total length of the average length of a normal larva (4.7 mm and 5.0 mm compared to 10 mm). A third specimen was of normal size. Since different larval stages between hatching and pupating are reported in other Mycetophilidae (Madwar 1937), this might explain the morphological discrepancy of larval type A and B in *Speolepta leptogaster*.

Conclusions

Since this work presents the first step towards understanding the dispersal potential of the ecologically versatile species *Speolepta leptogaster* within a small area, subsequent studies should incorporate a larger, pan-European sampling to address diversification patterns for this abundant cave species. Its ecological diversity in congruence with frequent subterranean and sporadic surface animals further challenges the eco-categorical classification system applied for subterranean species.

Acknowledgements

Images of pupa and the male imago were taken by Klaus Bogon. We thank all members of the Hesse Federation for Cave and Karst Research for sample collection, Adrienne Jochum for proofreading the manuscript and two anonymous reviewers for their constructive comments.

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RESEARCH ARTICLE

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Troglofauna in the vadose zone: comparison of scraping and trapping results and sampling adequacy

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Abstract

Most sampling of troglofauna occurs in caves but troglofauna species are widespread across the vadose zone in Western Australia in iron ore deposits and calcretes. Other than in karstic calcrete, the subterranean spaces in the Western Australian vadose zone are small and often of similar size to the troglofauna inhabiting them. Here we describe how troglofauna can be sampled in the vadose zone using a technique called scraping, in which a haul net is dropped down a hole drilled for geological exploration. We analysed of the results of 10,895 sampling events in which both the scraping and trapping techniques were used. In the Pilbara region of Western Australia, where most of the fieldwork occurred, scraping collected approximately three-quarters more troglofaunal animals than trapping and more than twice as many troglofauna species per sample. Most orders of troglofauna were collected in greater numbers by scraping than trapping. However, the yields from both troglofauna sampling techniques are low and, even when the results of both techniques are combined to constitute a single unit of sample effort, the currently prescribed effort for environmental impact assessment will document only about half the species present at a site. It is suggested that a larger number of samples should be collected.

Keywords

Troblobite, subterranean fauna, Australia, sampling method, environmental impact assessment

Introduction

The study of troglofauna still occurs predominantly in caves across most of the world (e.g. Schneider and Culver 2004, Culver et al. 2006, Skubała et al. 2013). However, in Western Australia there has been focus on the occurrence of animals in the smaller spaces distributed, often at considerable depths, in vadose zones across much of the landscape in arid areas (Guzik et al. 2010). The habitat in the upper parts of these non-karstic vadose zones, where subsurface colluvium and weathered conglomerates are present, may be considered to comprise Juberthie et al.'s (1981) milieu souterrain superficiel (MSS). At depth, the vadose zone comprises various types of bedrock in which spaces are mostly the result of fracturing and weathering (Fig. 1).

Sampling of troglofauna in the vadose zone is challenging, especially in deeper rocky areas. Most of the sampling undertaken in Western Australia is for the purpose of assessing the potential impacts of mining on the conservation of troglofauna (EPA 2007, 2013) and occurs in drill holes installed for geological exploration (Fig. 2B). The geologies most frequently sampled for troglofauna are iron ore formations, granitoids and mafic rocks hosting gold deposits, and calcretes associated with potential water supplies or containing minerals such as uranium. Drill holes in iron formations may extend more than 100 m below the ground surface (e.g. Biota 2006, Bennelongia 2010).

Until recently, troglofauna were collected from drill holes using traps baited with leaf litter (EPA 2007). Capture rates from these traps in the Pilbara region (Fig. 3) tended to be very low, with yields of 0.25 troglofaunal animals per trap regarded as satisfactory sampling (Subterranean Ecology 2007). Given that the Pilbara is rich in troglofauna and may support more than 45 species in a few square kilometres (Bennelongia 2010), there has been concern that modest trapping efforts are unlikely to document most of the troglofauna species present in an area.

The primary objective of this paper is to describe, and to document the capture efficiency of a new troglofauna sampling technique called scraping. The implications of low troglofauna capture rates for the completeness of environmental impact assessments are also examined. As a second set of objectives, we briefly describe the troglofauna we have collected in the Pilbara, give some ecological information to improve knowledge of a region with high subterranean fauna conservation values, and highlight the potential richness of non-karstic vadose zones as troglofauna habitat.

Methods

Scraping

The scraping method of collecting troglofauna was developed by modifying a haul net used to sample stygofauna in wells. The principle is simple: a cone-shaped net is dropped down a drill hole and dragged back up against the wall of the hole. Troglofauna crawling on the wall are 'scraped' into the net and collected.

Figure 1. Diamond drilled geological core showing structure of the subterranean habitat from surface to 40 m depth.

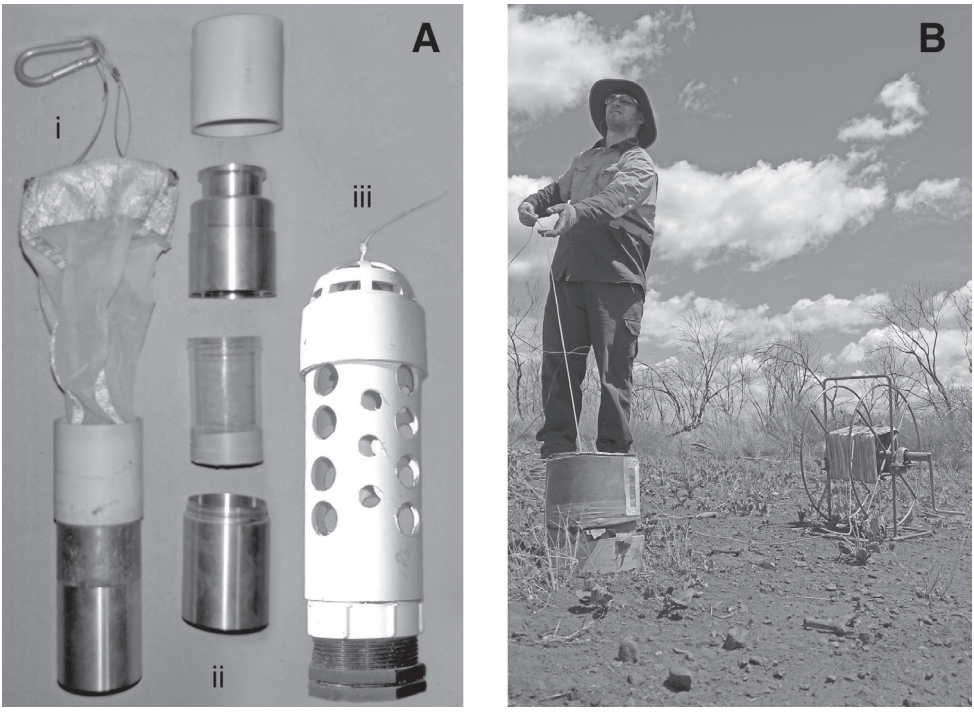

Figure 2. Troglofauna sampling equipment. **A** net for scraping and trap: i, net assembled; ii, collar, catch tube and protective brass case disassembled; iii, trap **B** scraping a drill hole in the Pilbara.

Different diameter nets are used for scraping according to the size of holes being sampled, with the ideal net diameter being about 60% of the diameter of the drill hole. The net itself consists of a metal ring, a cone-shaped net of 150 micron mesh and a

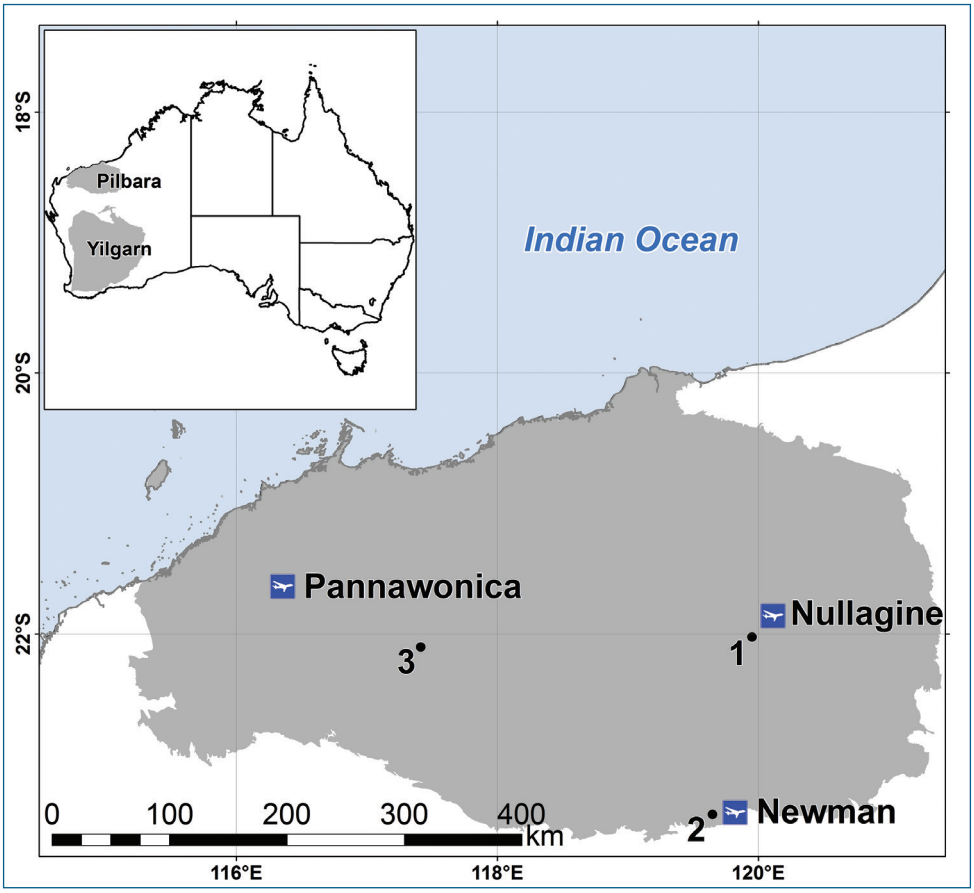

Figure 3. Pilbara and Yilgarn regions of Western Australia, showing some towns in the Pilbara and Areas 1, 2 and 3 where species accumulation curves were calculated.

polycarbonate catching vial (Fig. 2A). The leading edge of the net, which is wrapped over the metal ring, can be reinforced with Kevlar to reduce wear as the net is retrieved. A cylindrical brass weight is attached to the narrow base of the net using cable ties and the 120 mm polycarbonate sample collecting vial is screwed into the brass weight, which has an internal thread. A protective metal base can be fitted around the base of the vial as shown in Fig. 2A.

When collecting data on the troglofauna yield of scraping, the net was lowered to the base of the drill hole and retrieved four times, with the net being dragged against a different sector of the hole during each retrieval. A short metal cylinder was fitted into the collar at the top of the holes while sampling to reduce friction and wear on the nylon cord (see Fig. 2B). In the sampling analysed in this paper, scraping occurred immediately before setting a troglofauna trap.

After each retrieval of the net, the contents of the polycarbonate vial, including sand and stones from the sides of the drill hole, were emptied into a sample jar. The contents of

the jar were preserved in 100% ethanol at 4°C after completion of four hauls. In the laboratory, samples were elutriated to separate animals from heavier sediment and screened into size fractions using Endecotts sieves (250, 90 and 53 μm) to remove debris and improve searching efficiency. Samples were then sorted under a dissecting microscope.

Trapping

The troglofauna traps used were developed from the design of Biota (2006) and consisted of a short length of PVC tube of 50 mm internal diameter. Holes were drilled in the upper part of the tube to allow access of fauna (Fig. 2A). Prior to setting the trap, it was half-filled with wetted leaf litter that had previously been sterilised by microwaving. The trap was left in place eight weeks before being retrieved, with trap contents being emptied into a plastic bag and freighted to the laboratory. In every fourth drill hole, two traps were set (about one third of the distance between surface and bottom of the hole and a few metres above the bottom); in the remaining holes a single deep trap was set a few metres above the bottom of the hole.

In the laboratory, the contents of each plastic bag were placed in a Tullgren funnel under 25 watt incandescent lights for 72 h. Most troglofauna moved down through the funnel and dropped into the vial of ethanol below. However, leaf litter was quickly checked under a microscope for any remaining animals and more thorough searching conducted if animals were present.

Drill holes

The geological exploration holes sampled for troglofauna were drilled with a reverse circulation process, whereby rock is broken up by a pneumatic hammer and the rock chips are sent to the surface by air pressure. After drilling, holes were fitted with a short, capped PVC collar extending approximately 1.5 m below ground surface. The purpose of the collar was to prevent collapse of the hole near the surface where substrates are most unstable. The remainder of the hole was open to the surrounding rock matrix. Most holes were 150 mm in diameter and drilled between six months and several years prior to sampling. Approximately 30% of the holes sampled were <30 m deep and 70% were <50 m deep but 2% of holes were >300 m deep. All drill holes sampled in the Pilbara were vertical but about a quarter of the holes sampled in the Yilgarn were inclined 30° from vertical.

Identifications

With the exceptions of nematodes, oligochaetes, mites and collembolans, all invertebrate animals exhibiting some troglomorphies were considered to be potential troglofauna and

were identified to species or morphospecies level. While a few species could be identified using available keys, most of the time the characters employed in keys for surface taxa were used to construct morphospecies taxonomy. In addition to the use of keys, expert taxonomists were consulted and many specimens were examined genetically to help determine species boundaries (see Acknowledgments).

Nematodes, oligochaetes, mites and collembolans were excluded from study partly because existing information provided insufficient basis to separate Pilbara and Yilgarn species of troglofauna from surface relatives but also because it was not a regulatory requirement to identify these groups (EPA 2007).

Data analysis

The data analysed in this paper came from a large number of sampling events when both a scrape and a trap sample were collected from the drill hole at the same time. Given that the trap was set immediately after scraping, it was regarded as a contemporaneous sample, even though it was retrieved eight weeks later. Sampling occurred in 65 different areas within the Pilbara (90% of effort) and the eastern Yilgarn regions of Western Australia (Fig. 3), with most drill holes being sampled twice in different seasons. The areas varied in size from about 2–400 km^2 but were mostly <10 km^2. When two traps were set in one drill hole, trapping results were combined prior to making a comparison with the equivalent scrape sample.

Two types of analysis occurred using the entire dataset. First, the total numbers of species and animals collected in trap and scrape samples were compared. Second, the numbers of animals in traps and scrapes were compared for various orders represented by ≥6 specimens. The differences between numbers of animals in traps and scrapes were expressed as bias factors by dividing the number of specimens caught in the higher yielding sampling technique by the number caught in the lower yielding one and converting this ratio to its $base_{10}$ logarithm. When the higher yielding technique was trapping, the logarithm was assigned a negative value. Chi-squared tests were used to examine the significance of differences in numbers of animals collected by the two techniques.

In a third analysis, based on data from three areas in the Pilbara, species accumulation curves for scraping, trapping and combination sampling (i.e. collection of both trap and scrape samples) were calculated for each area using EstimateS software (Colwell et al. 2012). The three areas contained iron ore deposits and were near the towns of Nullagine (Area 1, c. 40 km^2), Newman (Area 2, 2.5 km^2) and Pannawonica (Area 3, 4.5 km^2) (Fig. 3). The proportions of troglofauna species collected by each sampling technique were calculated by comparing the yield of the technique against the ICE metric estimate of the total number of species present based on combination sampling.

In addition to the above analyses of sampling efficiency, we used convex hulls to calculate the ranges of all species represented in the entire dataset by records from ≥3 drill holes. We also examined the depth below ground surface at which species were

trapped, using records from single traps or the deeper trap if two traps were set in a drill hole. Chi-squared goodness of fit tests were used to test for variations in occurrence of invertebrate orders with depth.

Results

A total of 9882 individual specimens considered to be troglofauna, representing 658 species, were collected in the 10,895 troglofauna sampling events. Of these, 9252 individuals of 566 species were collected from the Pilbara and 630 individuals of 92 species were collected from the Yilgarn (six species were considered to occur in both areas). Diplurans, isopods, beetles, pseudoscorpions and schizomids are the more speciose troglofauna groups in the Pilbara and Yilgarn (Fig. 4).

Scraping collected 76% more troglofaunal specimens than trapping (5115 vs. 2907) and more than twice as many species per sample (0.25 vs. 0.11, Table 1) in the 9815 sampling events in the Pilbara. Differences between the two techniques were less pronounced in the Yilgarn, with the scraping component of 1080 sampling events collecting 29%

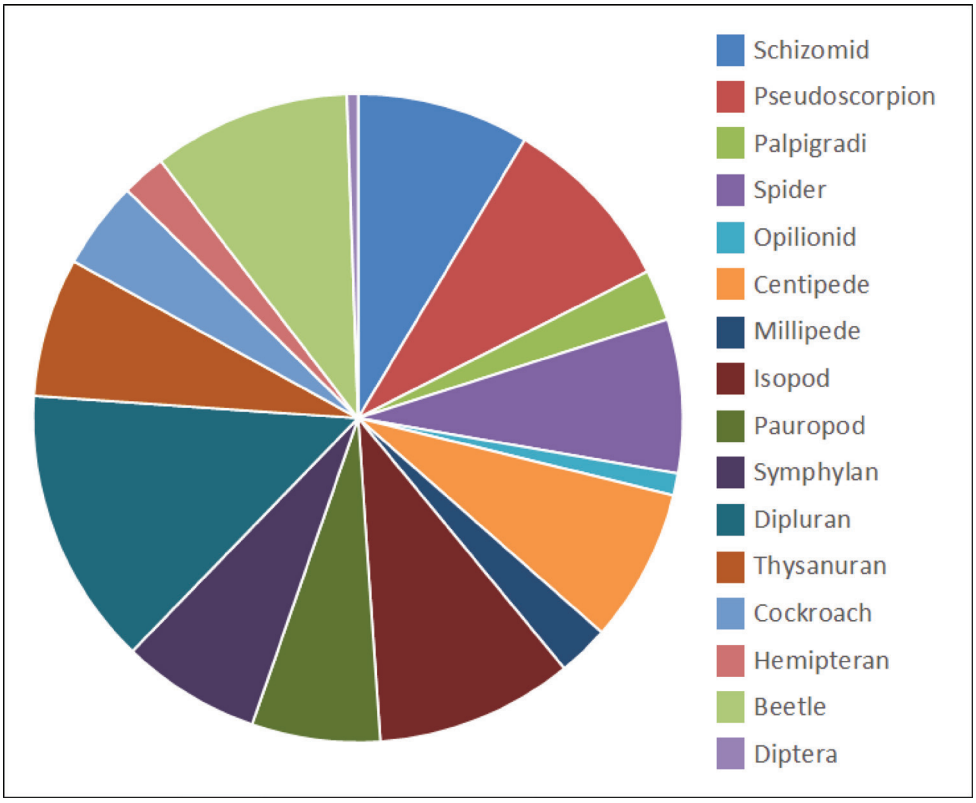

Figure 4. Taxonomic composition of troglofauna in the Pilbara and Yilgarn. Orders in legend are shown clockwise from the top of the pie chart.

Table 1. Numbers of troglofauna collected by scraping and trapping in the Pilbara and Yilgarn.

	Pilbara		Yilgarn		Total	
	Scrape	**Trap**	**Scrape**	**Trap**	**Scrape**	**Trap**
Total animals	5115	2907	208	292	5323	3199
Animals per sample	0.52	0.30	0.19	0.27	0.49	0.30
Species per sample	0.25	0.11	0.10	0.09	0.23	0.10

fewer specimens than trapping but 10% more species. The lower success of scraping in the Yilgarn was probably partly the result of many Yilgarn drill holes being inclined and logistically difficult to sample by scraping (although retrieving traps was also difficult).

Bias among orders

Altogether, 20 orders of troglofauna represented by ≥6 individuals were collected from the Pilbara (excluding nematodes, oligochaetes, mites and collembolans). Thirteen orders yielded substantially more specimens in scrapes (1.2–100 times more) than traps (Fig. 5, Table 2). These orders included symphylans, pauropods and palpigrads, which were almost exclusively collected in scrape samples. Coleoptera were collected in equal numbers in scrapes and traps, while millipedes, isopods and dipterans were more abundant in traps. However, among the four millipede orders, the higher abundances in traps were significant only for Polydesmida and Spirobolida.

Dipterans collected in the Pilbara nearly all belonged to the family Sciaridae, which were collected as larvae or recently hatched adults. Nearly all were caught in traps. Eggs were also found in traps, which appeared to constitute favourable breeding habitat for sciarids, with eggs hatching and producing larvae and even adults while the traps were in place.

The results of sampling in the Yilgarn were similar to those in the Pilbara, with 10 orders represented by ≥6 animals and six of these collected mostly in scrapes (Fig. 5). Coleoptera, the centipede order Scolopendromorpha and millipede order Polyxenida were collected in approximately equal numbers in scrapes and traps, while isopods were more abundant in traps.

Many of the animals collected in scrapes, especially hemipterans, were found in root mats broken off by the net and scraping may be a particularly efficient way of sampling this microhabitat.

Species accumulation curves

The greater efficiency of scraping, compared to trapping, as a means of documenting the troglofauna of an area was confirmed when species accumulation curves were plotted for three areas in the Pilbara. Scraping yielded 33–57% more species than trapping (Fig. 6). However, both scraping and trapping collected low numbers of animals, so

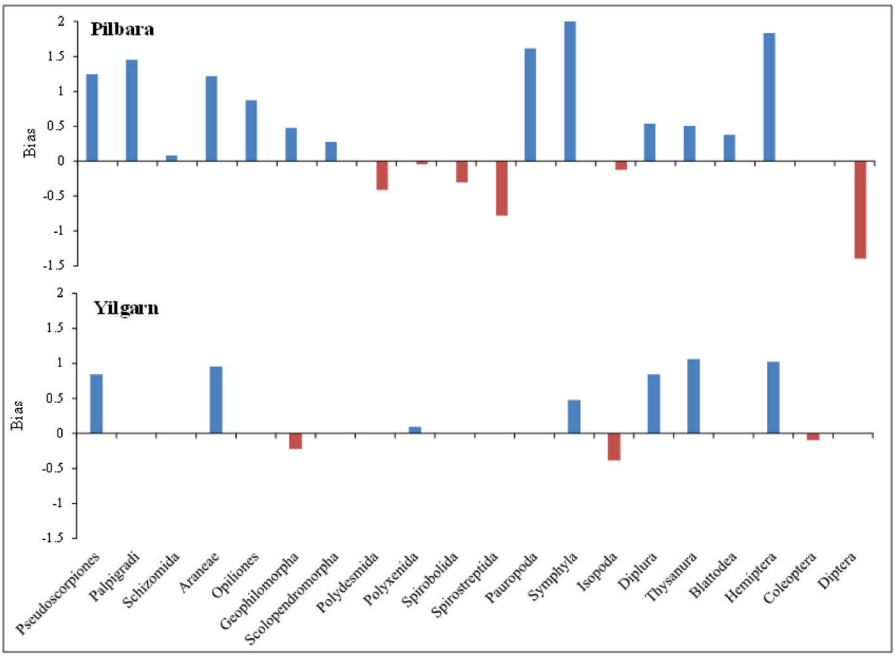

Figure 5. Bias in capture of different orders of troglofauna in the Pilbara using scraping and trapping.

Table 2. Differences between orders in numbers of animals collected by scraping and trapping. *P* values for χ^2 goodness of fit testing assuming equal numbers of animals in traps and scrapes. NS, non-significant.

	Pilbara			Yilgarn		
	Trap	**Scrape**	***P***	**Trap**	**Scrape**	***P***
Pseudoscorpiones	9	158	0.001	0	6	NS
Palpigradi	4	114	0.001			
Schizomida	258	309	0.05			
Araneae	11	183	0.001	1	9	0.05
Opiliones	2	15	0.01			
Geophilomorpha	6	18	0.05	5	3	NS
Scolopendromorpha	19	36	0.05			
Polydesmida	49	19	0.001			
Polyxenida	360	325	NS	4	5	NS
Spirobolida	4	2	NS			
Spirostreptida	36	6	0.001			
Pauropoda	3	124	0.001			
Symphyla	2	204	0.001	6	18	0.05
Isopoda	312	234	0.01	263	109	0.001
Diplura	47	162	0.001	0	6	NS
Thysanura	122	392	0.001	2	23	0.001
Blattodea	465	1117	0.001			
Hemiptera	20	1370	0.001	2	21	0.001
Coleoptera	288	288	NS	5	4	NS
Diptera	889	36	0.001			

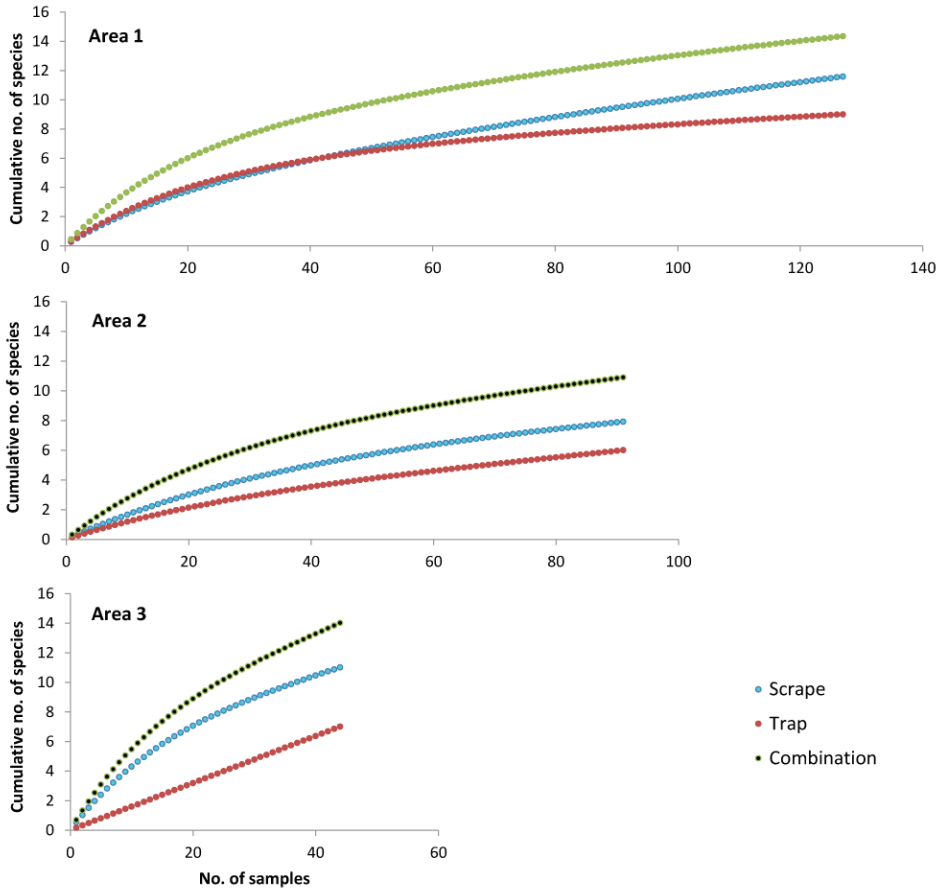

Figure 6. Cumulative numbers of species collected by different trapping protocols in three different areas in the Pilbara (see Fig. 3 for locations). A sample consists of one scraping event, one trapping event (with one or two traps), or the combined results of one scraping and one trapping event in the same hole.

that combining the results of trapping and scraping for each drill hole yielded 25–38% more species than were recorded by scraping alone.

While combination sampling (i.e. collecting both scrape and trap samples) is more efficient than either scraping or trapping alone, a very large sampling effort may still be required with combination sampling to collect most (e.g. 80%) of the troglofauna species present in an area. The 127 combination samples from Area 1 and 91 combination samples from Area 2 collected only 69% and 66%, respectively, of the estimated troglofauna species in these areas. Area 1 yielded 66 animals and 5 singletons (species represented by a single individual), whereas Area 2 yielded 145 animals and 3 singletons. Sampling appeared to collect additional species faster at Area 3 but the fauna there was also richer, so that only 58% of species were collected by 44 combination samples (Fig. 6). The 59 animals collected included 5 singletons. No species was collected from more than one area, despite a small number of species being wide-ranging in the Pilbara (see below).

Table 3. Percentage of various troglofauna groups in trap samples from different depths. Polydesmida, Polyxenida, Spirobolida and Spirostreptida combined as Diplopoda. No traps set at >80 m depth.

	<10 m	**10–19m**	**20–39 m**	**40–80 m**
Schizomida	21	22	19	10
Diplopoda	16	13	14	17
Isopoda	22	15	7	8
Diplura	2	4	6	5
Blattodea	21	25	24	31
Coleoptera	4	5	6	8
Diptera	2	5	10	9
Other	12	11	14	12
No of species records	110	317	288	119

Terrestrial fauna

Both trapping and scraping mostly collected animals inhabiting the surface soil and gravel layers rather than troglofauna. Approximately 97.7 and 93.6% of specimens collected by trapping and scraping, respectively, were classified as surface species rather than troglofauna. While some species classified as surface may be living at depth (see Discussion), the high proportion of surface fauna in the traps and scrapes was the probably mostly the result of two inter-related processes. First, the drill hole was likely to have acted as a conduit for surface fauna to explore the vadose zone by travelling down the outside of the collar onto the walls of the hole. Second, the drill hole was also likely to have acted as a pit trap for much of surface fauna exploring it.

Species ecology

Examination of the depths at which species of different groups were collected showed that all groups recorded frequently in traps were found at depths >40 m (Table 3). No groups showed significant variation in occurrence with depth, although isopods showed a possible tendency to occur more frequently in shallow depths, dipterans to be more common at >20 m depth and schizomids to prefer depths of <40 m.

The ranges of species represented by few records are likely to be underestimated by our convex hull calculations. Nevertheless, it appeared that troglofauna species in the Pilbara and Yilgarn predominantly had very small ranges. Of the 230 species recorded in ≥3 drill holes, 77% had calculated ranges of <10 km^2 and only 4% had ranges >10,000 km^2. Groups with particularly small calculated ranges included isopods, spiders, schizomids and harvestmen (although the latter was represented by only two species) (Table 4). The ranges estimated here for schizomid species fit well with estimates for schizomids elsewhere in the Pilbara (Harvey et al. 2008).

All groups other than schizomids and harvestmen contained some moderately or very widespread species and, in many cases, these may represent troglophiles. The

Table 4. Median ranges of species collected in ≥3 drill holes. The spread of species ranges is also shown. N, number of species in group. Scolopenromorpha and Geophilomorpha combined as Chilopoda, Polydesmida and Polyxenida combined as Diplopoda.

	Median range km²	N	Species ranges km²
Pseudoscorpiones	22	22	1–145994
Palpigradida	345	4	1–35642
Schizomida	5.4	29	1–55
Araneae	3.7	18	1–1413
Opiliones	1.2	2	1–2.4
Chilopoda	30	10	1–2166
Diplopoda	16	6	1–353159
Pauropoda	34	6	1–7148
Symphyla	8.3	22	1–1368
Isopoda	2.5	30	1–1462
Diplura	16	15	1–12282
Thysanura	11	22	1–1845
Blattodea	29	19	1–2166
Hemiptera	3646	6	1–43501
Coleoptera	60	18	1–17772
Diptera	19725	2	1–39448

widespread species included a ubiquitous polyxenid millipede Lophoproctidae sp. B01 found both in the Pilbara and Yilgarn, the pseudoscorpion *Tyrannochthonius aridus* found on the surface and below ground, the hemipteran Meenoplidae sp. B03 of which adults have remnant eyes (eyeless species had ranges of 461 and 10 km²), and the dipteran Sciaridae sp. B01.

Discussion

Richness of troglofauna in Pilbara

While the main purpose of this paper is to highlight the value of scraping as a technique for collecting troglofauna from the vadose zone, the results of the sampling reported here also show that the Pilbara region of Western Australia supports a significant troglofauna community and complements the results of other subterranean surveys showing the Pilbara is rich in stygofauna (Eberhard et al. 2009, Halse et al. 2014). Sampling of 59 mostly small areas in the Pilbara collected 549 species and morphospecies considered to be troglofauna. Other environmental impact assessments in the Pilbara have collected many additional troglofauna species (e.g. Harvey et al. 2008, Baehr et al. 2012, Smith et al. 2012).

Culver et al. (2013) recently pointed out the difficulties of comparing species richness between different regions of the world, especially when there is an element of

extrapolation involved in species estimates. While nearly all the troglofauna species collected from the Pilbara are undescribed, most are represented by voucher specimens in the Western Australian Museum and many have been defined by DNA analysis as well as morphological study. Working morphological diagnoses for 130 undescribed species are available on the Western Australian Museum's website (http://www.museum.wa.gov.au/catalogues/waminals). Thus, it is unlikely that additional taxonomic study will substantially change the number of troglofauna species we have identtified.

A much more significant issue is that <1% of the Pilbara has been sampled. While most of the areas sampled are iron formations, which current information suggests support more troglofauna than other geologies of the region (EPA 2007, 2013), even iron formations are very poorly sampled. This, together with the fact that other environmental assessment surveys are known to have collected additional species, suggests the current list of 549 taxa from the Pilbara substantially underestimates the actual number of species in the region, even if a small proportion of the taxa considered to be troglofauna are surface species. It is therefore likely that Guzik et al.'s (2010) estimate that 960 species of troglofauna occur in the western half of Australia, mostly in the Pilbara and Yilgarn, will prove to be too low.

Perhaps the most interesting points to note in relation to the richness of troglofauna in the Pilbara are that firstly it is a fauna of small spaces in the landscape matrix of the vadose zone rather than a fauna of caves and, secondly, it occurs in a very arid setting. Average annual rainfall in the Pilbara is 250-400 mm and average maximum January temperature is 39–41 °C (http://www.bom.gov.au/climate/averages/tables/ca_wa_names.shtml, see Fig. 3 for locations). Annual pan evaporation is approximately 3500 mm (Luke et al. 2003).

Troglofauna v. soil fauna

We believe our recognition of species boundaries was mostly sound but we excluded a potentially significant number of species from our troglofauna list by ignoring mites, collembolans and oligochaetes. On the other hand, some of the species we recorded as troglofauna may be soil fauna. The possible inclusion of some soil species reflects the difficulty of assigning animals to ecological categories on the basis of morphological information (Sket 2008). The process was made more difficult by the preponderance of surface species in scrape and trap samples, including some species of specialised soil fauna that lacked eyes and pigmentation. While soil fauna usually have smaller, more compact bodies and shorter appendages than cave troglobites (Juberthie and Decu 1994), it is unclear how well these characters distinguish between soil fauna and troglofauna for species of diplurans, atelurinid thysanurans, pauropods, palpigradids, symphylans and geophilomorph or *Cryptops* centipedes.

The difficulties of categorising species is illustrated by paligradids and pauropods. Both groups were collected almost entirely by scraping. Although Western Australian palpigradid species appear to lack the elongated appendages typical of most cave pal-

pigradids, Barranco and Harvey (2008) considered *Eukoenenia guzikae*, which was collected from a well in the Yilgarn, to be troglofauna. While the wide range of the morphologically similar Palpigradi sp. B01 (35,642 km^2) suggests it may be a surface species or trogloxene, two other similar-looking species have known ranges of 36 and <1 km^2, which suggest they are more likely to be troglobites. Furthermore, some palpigradid specimens brought live into the laboratory desiccated rapidly at room humidity and temperature (unpublished observation), suggesting they are unlikely to be surface species.

After morphological examination, Ulf Scheller (personal communication) concluded that Pilbara pauropods are predominantly, if not entirely, surface fauna. However, two lines of evidence suggest the morphology and ecology of pauropods may not be matched. First, it seems surprising for osmoregulatory reasons that pauropods occur in surface soils of an area as hot and arid as the Pilbara, although they have been collected from arid surface habitats in Israel (Scheller and Broza 1999). Second, while two species have moderately large ranges (ranges of 7148 and 4072 km^2), the other four species collected at ≥3 drill holes had ranges more likely to be associated with troglobites (1–47 km^2).

There is also evidence from Cape Range, just south of the Pilbara, that species lacking troglomorphies may use subterranean environments as refugia from arid surface conditions. Humphreys (1993) found that many of the beetles considered to be surface species occupy caves without being present in the surrounding surface environments and this may also apply to groups such as pauropods and palpigradids.

Comparison of sampling methods

The non-karstic vadose zone appears to have been relatively little sampled for troglofauna anywhere other than Australia, despite its potential to harbour very significant biodiversity. Consequently, the yields of trapping and scraping cannot readily be compared to sampling methods used elsewhere. However, the traps used in this study were baited with wet leaf litter. Other studies have shown that the addition of water and leaf litter to an arid zone cave attracted troglobitic species, provided a site for their reproduction (Humphreys 1991) and improved the yield of pitfall traps (Weinsten and Slaney 1995). More elaborate versions of the traps used here, incorporating propylene glycol as a preservative, have been used successfully by Lopez and Oromi (2010) and others to sample troglofauna in true MSS. Thus, it is considered reasonable to treat the trapping undertaken here as representative of existing best practice when evaluating scraping results.

In the rocky iron ore formations of the Pilbara, scraping yielded approximately three-quarters more troglofaunal animals and twice as many species per sample as recorded by trapping (Table 1). Nearly all groups of troglofauna were collected more efficiently by scraping, so that use of that technique provided a more complete picture of the troglofauna community than if trapping alone was used (Figs 5, 6). Fewer samples were collected in the rocky iron ore and gold bearing formations sampled in the Yigarn

and capture rates were lower than in the Pilbara. These lower capture rates were probably the result of logistical issues associated with sampling inclined holes and the fact that fewer troglofauna occur in Yilgarn habitats other than calcrete (see Bennelongia 2009, Guzik 2010). The pattern of bias among different orders in the Yilgarn was similar to the Pilbara (Fig. 6) but scraping did not collect greater numbers of animals and species per sample (Table 1). This probably reflected the greater proportion of isopods and lower proportion of arachnids and hemipterans in the Yilgarn samples and the catch biases associated with these groups (Fig. 5).

Implications for assessment

The low yields of trapping and scraping have implications for the adequacy of environmental impact assessments in regions where troglofauna occur. In such regions, the identification and protection of areas that are particularly rich in troglofauna should be a conservation priority. Using trapping alone, only about a third of the troglofauna species present in Areas 1 and 2 would be collected by the 60 samples recommended for impact assessment by EPA (2007). Use of both scraping and trapping would result in 60 combination samples collecting about half the species present (Fig. 6).

The relatively small proportion of species collected by troglofauna sampling using currently recommended levels of effort is probably the result of two factors. First, the number of species in a sample and the degree to which the sample collects all species from an area is dependent on the number of animals collected (Fisher et al. 1943). Individual troglofauna samples collect very few animals and, therefore, large sample efforts are needed to document a high proportion of the fauna. Second, it has been shown for cave troglofauna that, depending on environmental conditions, often not all species in an area are accessible to sampling at any one time (Krejca and Weckerly 2007); in the case of the non-karstic vadose zone there may be times when species will not enter drill holes. Sampling on multiple occasions will increase the likelihood that species are present during sampling. The current assessment guidelines recommend sampling occurs in two seasons (EPA 2007).

Conclusions

Three broad conclusions are drawn from this study.

First, the non-karstic vadose zone of the Pilbara is rich in troglofauna. This is likely to be the case in many other parts of the world and more surveys should be conducted to identify the general importance of the non-karstic habitats globally. Holes drilled for geological exploration associated with mine development provide easy access to the deeper vadose zone.

Second, scraping appears to be a useful technique for sampling troglofauna in the vadose zone. Under at least some circumstances, it yields more fauna and provides results

faster than trapping because there is no colonisation period required. However, because of the relatively low yields of all sampling methods for troglofauna trialled to date, we suggest scraping and trapping should usually be used in combination to maximise yields.

Third, the low yields obtained from troglofauna sampling highlight the importance of identifying, prior to sampling, the proportion of the fauna that needs to be collected to adequately characterise a troglofauna community for the purposes of environmental impact assessment. Analyses should be conducted during assessment to evaluate whether this target has been met.

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RESEARCH ARTICLE

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DNA sequences of troglobitic nicoletiid insects support Sierra de El Abra and the Sierra de Guatemala as a single biogeographical area: Implications for *Astyanax*

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Abstract

The blind Mexican tetra fish, *Astyanax mexicanus*, has become the most influential model for research of cave adapted organisms. Many authors assume that the Sierra de Guatemala populations and the Sierra de El Abra populations are derived from two independent colonizations. This assumption arises in part from biogeography. The 100 m high, 100 m wide Servilleta Canyon of the Boquillas River separates both mountain ranges and is an apparent barrier for troglobite dispersion. *Anelpistina quinterensis* (Nicoletiidae, Zygentoma, Insecta) is one of the most troglomorphic nicoletiid silverfish insects ever described. 16S rRNA sequences support that this species migrated underground to reach both mountain ranges within less than 12,000 years. Furthermore, literature shows a plethora of aquatic and terrestrial cave restricted species that inhabit both mountain ranges. Thus, the Servilleta canyon has not been an effective biological barrier that prevented underground migration of troglobites between the Sierra de Guatemala and the Sierra de El Abra. The Boquillas River has changed its course throughout time. Caves that in the past connected the two Sierras were only recently geologically truncated by the erosion of the new river course. It is likely that, with the geological changes of the area and throughout the 2-8 million years of evolutionary history of cave *Astyanax,* there have been opportunities to migrate across the Servilleta canyon.

Keywords

Anelpistina quinterensis, Neonicoletia, Cubacubaninae, Nicoletiidae, Zygentoma, Insecta, Thysanura, Silverfish, *Astyanax,* blind tetra, Characidae, Sierra de El Abra, Sierra de Guatemala,16S rRNA, Molecular clock, Colonization

Introduction

In recent years, the blind Mexican tetra fish *Astyanax mexicanus* (De Filippi, 1853) has become the most influential model for genomic and evolutionary research of cave adapted organisms. Regrettably, there is great confusion regarding the origin of the 29 populations that inhabit the Sierra de El Abra, Sierra de Guatemala, and Micos mountain ranges in Northeastern Mexico and, also, if the populations derived from a single or from multiple colonizations. A plethora of publications has accumulated over time with terms such as phylogenetically old/new populations, lineages A/B, phylogenetically old/new clusters, and old/new epigean stocks, with individual cave fish populations having been assigned contradictorily to one or to another set (see for example figure 1 in Gross 2012).

Many current authors embrace the hypothesis that Sierra de Guatemala populations derived from a new epigean stock and that Sierra de El Abra populations derived from an old stock. This is complicated by some El Abra populations, such as the Pachón cave population, having subsequently hybridized with the new stock (Bradic et al. 2012). It is seldom assumed that the Guatemala populations could also have an old stock origin which has then been obscured by extensive hybridization with the new stock, and much less that populations from both mountain ranges could have a single underground cave adapted ancestor. One reason derives from biogeography and an apparent barrier between the two mountain ranges. The Cañon de la Servilleta (Napkin canyon) of the River Boquillas separates both mountain ranges (Figure 1). Reddell (1981) subdivided the two mountain ranges into separate biogeographical areas and authors working with *Astyanax*, such as Gross (2012), have assign populations to either region based on geography, regardless of there being no genetic studies (ex. Jineo, Bee and Vasquez caves). Intrinsically, it has been assumed that this 100 m high, 100 m wide canyon has been a biological barrier that prevented underground migration of troglobites between the two karstic areas, and therefore colonization had to occur independently on both mountain ranges.

The purpose of this paper is not to resolve if troglobitic *Astyanax* derived from single or multiple origins. What we will address is if the Servilleta canyon has been an effective barrier for migration of troglobites in general, and thus if the Sierra de Guatemala and the Sierra de El Abra should be considered two separate cave biogeographic areas. For this, the DNA sequences of troglobitic nicoletiid insects (Zygentoma, also known as silverfish or Thysanura) of genus *Anelpistina* from populations inhabiting both Sierras were analyzed and a phylogeny was obtained. Our results will help to establish if these troglobites are a single or multiple species, and thus support if they are the product of a single colonization followed by underground migrations, or derived from multiple colonizations.

Anelpistina quinterensis (= *Neonicoletia quinterensis* Paclt, 1979) is a rather large troglobite (8.5 cm long, antennae and terminal filaments or caudal appendages included), which was first described from Grutas de Quintero, in Sierra de El Abra. When re-describing the species, Espinasa et al. (2007) reported its presence in Pachón

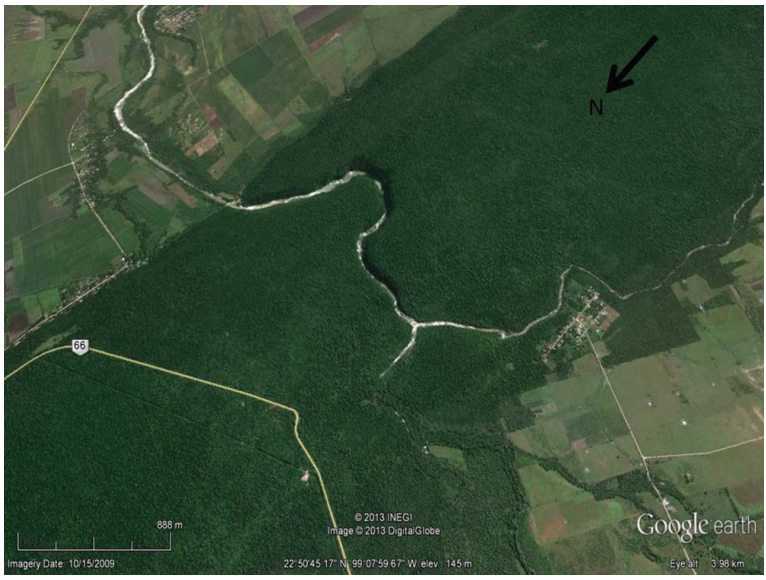

Figure 1. The Cañon de la Servilleta of the River Boquillas separates the contiguous Sierra de Guatemala, to the north, from the Sierra de El Abra, in the south. Limestone is restricted to the green forested hills. This study tested if this 100 m high, 100 m wide canyon was an effective biological barrier that prevented underground migration of troglobites between the two karstic areas.

and Yerbaniz Caves, also within the Sierra de El Abra. They mentioned that "It is likely that *Anelpistina quinterensis* is restricted to the caves of Sierra de El Abra". With their highly elongated legs, antennae (almost thrice as long as the body), and caudal appendages (twice as long as the body), it is one of the most troglomorphic nicoletiids ever described (Figure 2). They can be found walking on mud banks and they are probably restricted to highly humid environments. In caves where they are abundant, they are never found near the entrance or in drier passages. As a very highly adapted troglobite, it is very unlikely that it can survive on the surface and its habitat must be restricted to underground passages. Its range probably reflects connectivity within a karstic area throughout geologic times and during the evolutionary history of the species.

Methods

Three caves near the town of Gómez Farías in the Sierra de Guatemala are inhabited by *Anelpistina* populations whose taxonomic identity has not previously been defined: Sótano de los Mangos, Sótano del Plan, and Sótano de Jineo. Two specimens per cave were studied and their DNA extracted. For this study, the 16S rRNA sequences of two *A. quinterensis* from Grutas de Quintero were already available in GeneBank (DQ280127.1). Also from Sierra de El Abra, two new specimens of *A. quinterensis* from Sabinos cave were obtained (3/20/13). For reference, the caves of Sabinos, Pachón and Sótano de Jineo can

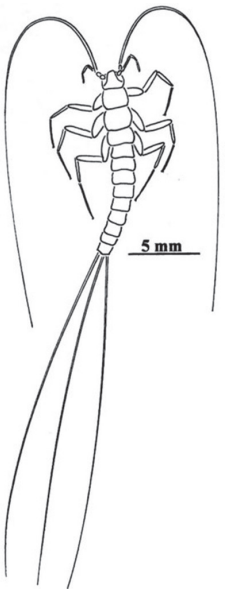

Figure 2. *Anelpistina quinterensis* is one of the most troglomorphic described species of nicoletiids. This relatively large eyeless insect is albino and has extremely elongated appendages. Its habitat is restricted to very humid portions of the caves such as mud banks. It is doubtful that it can survive in an epigean environment. Its habitat probably reflects connectivity within a karstic area throughout geologic times and during the evolutionary history of the species.

be found in figure 1 of Gross (2012) and described in Mitchell et al. (1977). Sótano de los Mangos and Sótano del Plan are in the neighboring area of Sótano de Jineo. Grutas de Quintero is near Pachón cave, but on the eastern side of the Sierra de El Abra.

Genomic DNA was extracted using Qiagen's DNEasy® Tissue Kit by digesting a leg in lysis buffer. Amplification and sequencing of the 16S rRNA fragment followed standard protocols and primers for the 16S rRNA fragment used in the past for nicoletiids (Espinasa and Giribet 2009). Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software SequencherTM 3.0. External primers were excluded from the analyses. Sequences from the new *Anelpistina* populations (GenBank# KF917530-KF917534) and sequences of all other nicoletiid species available in GeneBank were aligned and neighbor joining analysis was performed using ClustalW2.

Results

The 16S rRNA fragment from the six specimens from the three caves of Sierra de Guatemala was identical and 499 bp long. The two Sabinos Cave specimens differed among themselves by two bp (0.4%) and were 498 bp. The Quintero specimens were

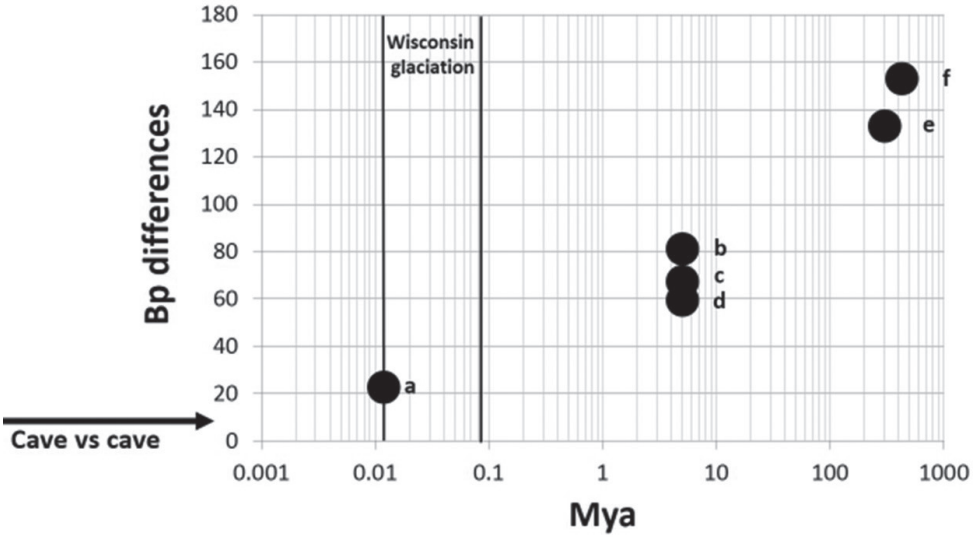

Figure 3. Base pair differences versus estimates of divergence in nicoletiids. Base pair differences in the 16S rRNA fragment is plotted against estimates of divergence times millions of years ago (Mya). Molecular clock calibrating points were extracted from: **a** populations of *Anelpistina musticensis* that got separated into different islands when the sea level rose after glacial times 12,000 years ago (Espinasa et al. 2011) **b** and **c** species of *Prosthecina* and **d** species of *Anelpistina* from Baja California that got separated from the mainland species when the Gulf of Cortes formed 5 mya (Espinasa et al. 2009) **e** time when nicoletiids arose from a common ancestor with Lepismatids 302 mya (Regier et al. 2010), and **f** time when insects arose from a common ancestor with anostraca in the Silurian-Ordovician boundary 427 mya (Gaunt and Miles 2002). The lower arrow indicates the 11–12 bp differences between the Sierra de Guatemala and the Sierra de El Abra *Anelpistina* populations. Such sequence difference is consistent with a common origin very recently, less than 12,000 years ago, and therefore after the environmental disturbances of the ice age.

identical and 498 bp. Within the Sierra de El Abra, specimens from Quintero and Sabinos differed among each other by 5 bp (1%). The Sierra de Guatemala specimens differed from the Quintero specimens by 11 bp (2.2%) and from the Sabinos specimens by 12 bp (2.4%). The neighbor joining analysis showed all to be monophyletic and very distant from any other nicoletiid insect that has had their 16S sequenced, including surface specimens from the neighboring areas.

A comparison of the DNA differences among the *Anelpistina* of Sierra de El Abra and Sierra de Guatemala was made against other nicoletiid species with dated speciation events (Figure 3). When the molecular clock was originally calibrated for nicoletiids (Espinasa et al. 2011), one point in particular was used for the end of the ice age. During glacial times when the sea level was lower, the islands of Mustique and Union Island (Grenadine islands in the Caribbean) formed a single land mass. The nicoletiid populations of *Anelpistina musticensis* separated and were isolated 12,000 years ago when the sea levels started to rise. These isolated populations now have 16S rRNA fragments that differ by 21 bp (Espinasa et al. 2011). The 11-12 bp difference

between the Sierra de El Abra and Sierra de Guatemala populations implies that these cave populations shared a common ancestor fairly recently, about 5,000 years ago, and certainly less than 12,000 years ago when the ice age ended. Such a recent origin supports that the *Anelpistina* populations belong within the same species. This is also in agreement with data from the 16S rRNA fragment sequences of nicoletiid species across the subfamily Cubacubaninae (Espinasa and Giribet 2009), where the 11-12 bp difference is within the range of 22 different populations that belong to the same species. Furthermore, morphologic analyses failed to find any discriminative character between the Guatemala and the El Abra populations. It is therefore supported that all populations from both Sierras belong to *A. quinterensis*.

Discussion

Our results support that troglobitic *A. quinterensis* from both Sierras had a common ancestor less than 12,000 years ago. We believe that this fairly recent common ancestor of the *A. quinterensis* population was a cave adapted organism which, through systems of caves and microcaves, migrated underground to reach and establish the current cave populations. As mentioned above, *A. quinterensis* may not survive on the surface and is one of the most troglomorphic nicoletiid insects. With such a recent common ancestor, it is unlikely that a surface ancestor would have had enough evolutionary time to independently colonize the caves of both mountain ranges, and then convergently develop such an advanced degree of troglomorphy. It would also be extremely unlikely that this independent evolution would yield indistinguishable morphologies in the two derived populations. Finally, since this surface ancestor would have been present long after the disturbances of the ice age had ended and, therefore, when environmental conditions have remained relatively stable, it would be expected that the surface species would still be present. Search for nicoletiids on the surface has successfully resulted in collecting other species, but never a surface specimen of *A. quinterensis.* In conclusion, it appears that *A. quinterensis* has been able to migrate between the two sierras and, therefore, the Servilleta canyon has not been an effective barrier to its underground dispersal.

Anelpistina quinterensis is not alone in having been able to disperse between both mountain ranges. There are at least four aquatic troglobites shared between the Sierra de El Abra and the Sierra de Guatemala; "the entocytherid ostracod *Sphaeromicola cirolanae* Rioja, the cirolanid isopods *Speocirolana bolivari* (Rioja) and *S. pelaezi* (Bolivar), and the mysid *Spelaeomysis quinterensis* (Villalobos)" (Reddell 1981). At least six species of terrestrial troglobites are also found in both Sierras; "the squamiferid isopod *Spherarmadillo cavernicola* Mulaik, the trichoniscid isopod *Brackenridgia bridgesi* (Van Name), the amblypygid *Paraphrynus baeops* Mullinex, the opilionid *Hoplobunus boneti* (Goodnight and Goodnight), the centipede *Newportia sabina* Chamberlin, and the collembolan *Pseudosinella petrustrinatii* Christiansen" (Reddell 1981). As can be seen from this certainly incomplete list, there are many instances of troglobites inhabiting both areas. This plethora of shared troglobites indicates that in the evolution of cave organ-

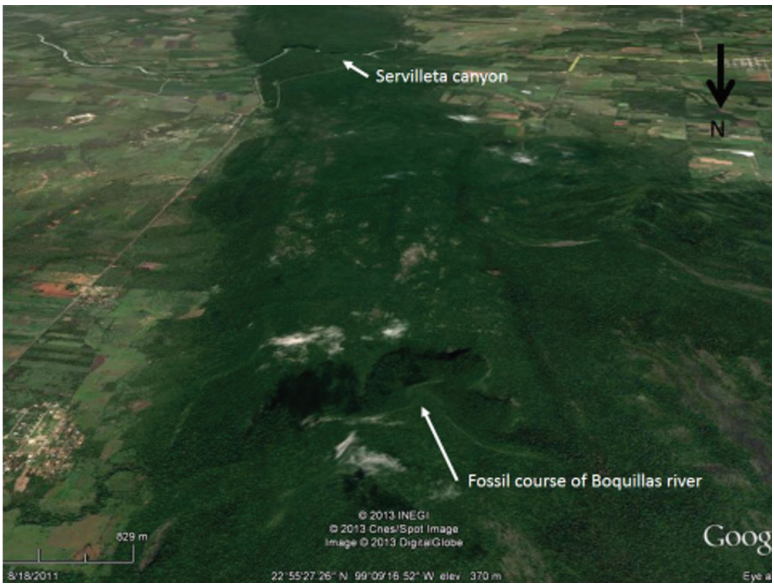

Figure 4. The Boquillas River has changed its course throughout time. The Boquillas River currently separates the karstic areas of Sierra de Guatemala from the Sierra the El Abra. In the upper part of the figure, the Boquillas River is seen crossing the sierras through the Servilleta canyon. On the bottom part of the figure, a fossil canyon indicates the river's ancient course. Caves that in the past connected the Sierra de El Abra in the south to the Sierra de Guatemala in the north were only recently geologically truncated by the erosion of the new river course. Limestone is restricted to the green forested hills.

isms, the Servilleta canyon has not been an effective biological barrier that prevented underground migration of troglobites between the Sierra de Guatemala and the Sierra de El Abra. Both karstic areas can therefore be considered a single biogeographical area.

Regarding its geologic history, Sierra de El Abra has been "emerging" as limestone is exposed by erosion, following the progressive lowering of the base level to the current elevation of the present coastal plain. Throughout this process, the river Boquillas, which currently divides Sierra de El Abra and Sierra de Guatemala, has vastly changed its course. As can be seen in Figure 4, there are the remains of a fossil river course further north of its current path. The Servilleta canyon was formed in relatively recent geological times when the Boquillas River changed its course to a more southern location and started cutting through the karstic layers. Exploration of the Servilleta canyon has revealed the presence of caves on one side of the canyon, and exactly on the other side of the canyon, with the same angle, complementary caves. This implies that there were caves connecting both mountain ranges which have recently been cut by the erosion of the Boquillas River. Biological dispersal of troglobites could have used these ancient caves. They could also use the connecting cavities that must exist below the current river level that have yet to be eroded by the Boquillas River. Alternatively, somehow they may have managed to survive the minor 100m "jump" between caves on either side of the canyon.

Conclusion

Regardless of the means used by troglobites to successfully migrate between the two mountain ranges, the main conclusion of this work is that the Servilleta canyon does not appear to be an effective biological barrier between the Sierra de Guatemala and the Sierra de El Abra. Troglobites of sizes comparable to the blind *Astyanax*, both aquatic and terrestrial, are found in both Sierras. *Astyanax* colonized the cave environment 2–8 million years ago (Gross 2012). Since nicoletiids have been able to migrate in between southern El Abra and the Sierra de Guatemala in less than the last 12,000 years, it is likely that, with the geological changes of the area throughout the evolutionary history of *Astyanax,* there have been opportunities to migrate across the current Servilleta canyon.

Undoubtedly the *Astyanax* populations of Sierra de El Abra and Sierra de Guatemala have been sufficiently isolated from each other so as to have, to a certain extent, independent evolutionary histories. This is reflected by microsatellite markers (Bradic et al. 2012) and the independent and parallel evolution of multiple troglomorphic characters such as albinism (Protas et al. 2006), brown phenotype (Gross et al. 2009), and the genetic basis of eye regression (Borowsky 2008), to give some examples. But as mentioned before, there is the possibility that the Guatemala populations may also have had an origin from the same old stock as the El Abra populations, but the genetic evidence has been obscured by extensive hybridization with the new stock.

Initial sequencing of mitochondrial DNA placed the Sierra de El Abra Pachón population within the new stock. Only subsequent studies showed its old stock origin having been obscured by hybridization. Some genetic markers also support that Sierra de Guatemala populations may as well have an old stock origin, but more intensely obscured by hybridization. For example, while most microsatellite markers (Bradic et al. 2012) of the Guatemala populations are shared with surface specimens, there is one allele (a6 f1-256) only present in the Sierra de Guatemala and Sierra de El Abra caves, but absent in surface populations. If the two sierras had actually been in separate biogeographic areas and the Servilleta canyon had been an effective barrier, the different *Astyanax* populations would undoubtedly be the result of independent colonization. Recognizing that there is no effective barrier for troglobite migration between the two areas, reduces this certainty. Genomic studies will resolve if a fraction of the genome of the cave fish from both sierras is shared at the exclusion of all surface populations.

Acknowledgments

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RESEARCH ARTICLE

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Measurements of the diet in two species of *Troglophilus* Krauss, 1879 cave crickets from Italian subterranean habitats (Orthoptera, Rhaphidophoridae)

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Abstract

The diet of two populations of cave crickets, *Troglophilus cavicola* from Veneto, northern Italy and *Troglophilus andreinii* from Apulia, southern Italy, were studied by analyzing faecal and gut contents. The results obtained document different food preferences in these two species. In the *Troglophilus cavicola* population arthropod remains were dominant in the diet, whereas in the *T. andreinii* population vegetables (green and fibres) were the more abundant food category. Furthermore, study of the overlap of food resource exploitation among age and sex sub-samples seems to indicate a separation of diet among the young instars and other age classes of the populations. Differences in diet between males and females were observed only in the population of *T. andreinii*.

Keywords

Troglophilus, Troglophilinae, Rhaphidophoridae, trophic niche, cave crickets, feeding habits, Italy

Introduction

In general, the feeding habits of Rhaphidophoridae cave crickets are considered to be omnivorous–saprophagous (Chopard 1938). Early observations on the genus *Dolichopoda* Bolivar, 1880 indicated the exploitation of vegetable matter and arthropod remains (Monti 1902). A similar diet has also been described for other Rhaphidophoridae

(Macropathinae),such as species of the Australian genera *Gymnoplectron* Hutton, 1897, *Pallidoplectron* Richards, 1958, *Micropathus* Richards, 1964 and *Pallidotettix* Richards, 1964 (Richards 1962; 1968; 1970), *Spelaeiacris tabulae* Peringuey, 1916 from Wynberg cave, Capetown, SouthAfrica (Carchini et al. 1991) and of the Patagonian species *Heteromallus cavicola* Ander, 1932 (Di Russo et al. 1996). Furthermore, other data indicating a omnivorous diet are available for some species of the North American genera *Hadenoecus* Scudder, 1863 and *Ceuthophilus* Provancher, 1876 (Hubbel and Norton 1978, Lavoie et al. 2007).

An example of predatory habits was reported by Chopard (1959) for *Rhaphidophora oophaga* Chopard, 1959, of which some individuals were observed to eat eggs of the Black-nest Swiftlet *Collocalia maxima* Hume, 1878 in the Subis cave, Sarawak, Malaysia.

In a more recent study, conducted on some Italian populations of the genus *Dolichopoda*, the trophic niche has been quantitatively described (De Pasquale et al. 1995). In this case, differences in the exploitation of resources in relation to the age of the cave, climatic stability of the habitat, type and amount of trophic resources and the diversity of the biotic community were established. Concerning species and populations of the genus *Troglophilus* Krauss, 1879, very little is known about their feeding habits. Early, fragmentary observations suggested an omnivorous diet for *Troglophilus* (Remy 1931, Avesani et al. 2009). Semi-quantitative analysis confirming this type of diet are available only for some populations of *Troglophilus cavicola* (Kollar, 1833) from Slovenian cave habitats (Novak and Kustor 1983).

The present study is part of a more extensive research project investigating the population ecology of the Italian species of *Troglophilus* cave crickets (Di Russo et al. 2008). In particular, the results on trophic resource exploitation of two populations belonging to *Troglophilus cavicola* and *T. andreinii* Capra, 1927 are reported and discussed in relation to their population ecology.

Systematics and ecological background

The genus *Troglophilus* has a wide east-mediterranean distribution, with 15 species occurring from Austria and Italian pre-Alps to the Balkan-Anatolian region (Eades et al. 2013). The large number of species present in the latter area suggests a primary centre of dispersal located in the eastern paleo-Mediterranean (Ruffo 1955). Recently, some studies on the population ecology of *Troglophilus* have been carried out, but information on this topic is very fragmentary and insufficient. In particular, these studies, conducted on some populations of *T. cavicola* and *T. neglectus* Krauss, 1879 from Slovenia caves, allowed for only a partial description of their life cycle as completed in two years. Furthermore, the occurrence of two seasonal ecophases was established: one hypogean in winter and one epigean in summer. According to these observations, the crickets use the caves only as winter shelter, and defect the caves in summer to feed and to reproduce in the epigean habitat (Novak and Kustor 1983; Pehani et al. 1997). Beginning in 2000, similar studies were also carried out in some Italian populations of *T.*

cavicola and *T. neglectus* from the Lessini Mountains (Latella et al. 2002; Avesani et al. 2009) and of *T. andreinii* from Apulia (Di Russo and Rampini 2005). In these studies, strong summer reductions of population sizes in caves were reported, confirming the existence of the two ecophases in the Italian populations just as in those from Slovenia. However, although summer population reductions occur in *T. andreinii,* these appear to be less marked and involve a very short time span.

Materials and methods

This study is based on the analysis of 225 faecal and gut content samples collected periodically from August 2004 to October 2005. In *T. cavicola* we analysed 76 samples (56 feculae and 20 guts) from Covoli di Velo cave in the Lessini Mountains (Grotta Inferiore dei covoli di Velo 44 V/VR, altitude 878 m a.s.l., Velo Veronese, Verona). For *T. andreinii*, an endemic species of Apulia, we analysed 149 samples (98 faeces and 51 guts) from Tranese cave [Grotta della Masseria Tranese 486 Pu/BA, altitude 330 m a.s.l., Contrada Tranese, Putignano, Bari]. Sub-samples from individuals of different sex and developmental stages (adult, nymphs and young) were analyzed. In particular for *T. cavicola*, we utilized 24 adults, 26 nymphs and 26 young instars, while for *T. andreinii* 52 adults, 47 nymphs and 50 young instars have been used. The distinction in these three age classes is based on the maturation of sexual characters for the adults and the hind tibia lenght for the other two classes (Nymph = hind tibia included between 15 and 21 mm; Young instar = hind tibia < 15 mm). Fresh faeces were collected from individuals put in a plastic box separately for 24 hours without any food supply. Gut samples were obtained by dissection of fresh individuals. Both faecal and gut pellets were conserved in alchool 70%. The comparison among gut and faecal samples does not reveal any differences in their content, permitting us to pool data obtained by these two types of source.

Feacal and gut contents were spread in Euparal on 24×24 mm slides and examined with an optical microscope (Leica M.Z.12.5). The material analysed was classified into three categories: green vegetables, vegetable fibres and arthropod remains. In order to obtain quantitative estimates, we used a 576 mm^2 grid and the food items scored were recorded as proportion of the total area observed. The niche breadth was cumulatively evaluated, for each periodical sub-sample, by the Gini-Simpson index (Gini 1912):

$$B = \mathbf{1- \sum p_i^2}$$

where p_i = the proportion of the ith item in the faecal or in the gut content.

The overlap in the resource exploitation among age and sex sub-samples was investigated by the Morisita index (1959) as modified by Horn (1966):

$$M_o = 2 \mathbf{\sum p_{ij} p_{ik} / \sum p^2_{ij} + \sum p^2_{ik}}$$

where p_{ij} and p_{ik} are the proportions of ith item utilization by the jth and kth populations.

The examined specimens and slides are deposited in the collections of the Museo Civico di Storia Naturale of Verona and of the Dipartimento di Biologia e Biotecnologie "Charles Darwin", Università degli Studi di Roma "Sapienza.

Results

Annual diet comparison between the two species

The percentages of utilization of the food resources are compared in the two species over the entire year. For the *Troglophilus cavicola* population (Covoli di Velo), we observed the dominance of arthropod remains in the diet (69.44%); by contrast, in the *T. andreinii* population (Tranese cave), vegetables (green and fibres) were the more abundant food category, reaching about 92%.

Fig. 1 reports comparisons among periodical samples of the two populations. In this case, differences in the exploitation of food resources in different periods of the year are evident. Particularly in the *T. cavicola* population, there is a clear increase of vegetable fibres in summer samples and a complete shift to the exploitation of arthropods remains (over 98%) in winter. By contrast, in the *T. andreinii* population, the diet is mainly based throughout the year of vegetables (never below 80%), with a small increase of fibres and arthropods in autumn and winter.

These findings are confirmed by the trophic niche breadth analysis conducted on the periodical samples of the two populations. In Fig. 2 seasonal values of the Gini- Simpson index for the two population are compared, showing an opposite trend of variation. In particular, in *T. cavicola*, which has a mean value of the index of 0.44, the highest values of the niche breadth are found in summer and spring, where all the three food categories were exploited. The lowest values occur in winter, where mainly arthropod remains were used. By contrast, in *T. andreinii* the mean values of the Gini-Simpson index were significantly lower (0.16), with a moderate peak in winter corresponding to a more balanced exploitation of the three resources.

Overlap of food resource exploitation among age and sex sub-samples

Fig. 3 compares the annual diet of different age sub-samples. In both populations young individuals show a significantly different exploitation of resources in comparison to those of nymphs and adults. In particular, these differences are greater in the *Troglophilus cavicola* population than in that of *Troglophilus andreinii*. The diet of young instars of *T. cavicola* is mainly based on vegetables (fibres+vegetable matter = 69%), whereas arthropods are dominant in nymphs and adults. In the *T. andreinii* population the diet of the three age classes appears more balanced, with a little difference in the young instars where a moderate increase of arthropods occurs. These results can be described by the overlap analysis using the Morisita index (Fig. 4). The dendrograms performed using Euclidean distances based on matrices of this index clearly show a separation of the diet of the young instars from that of nymphs and adults.

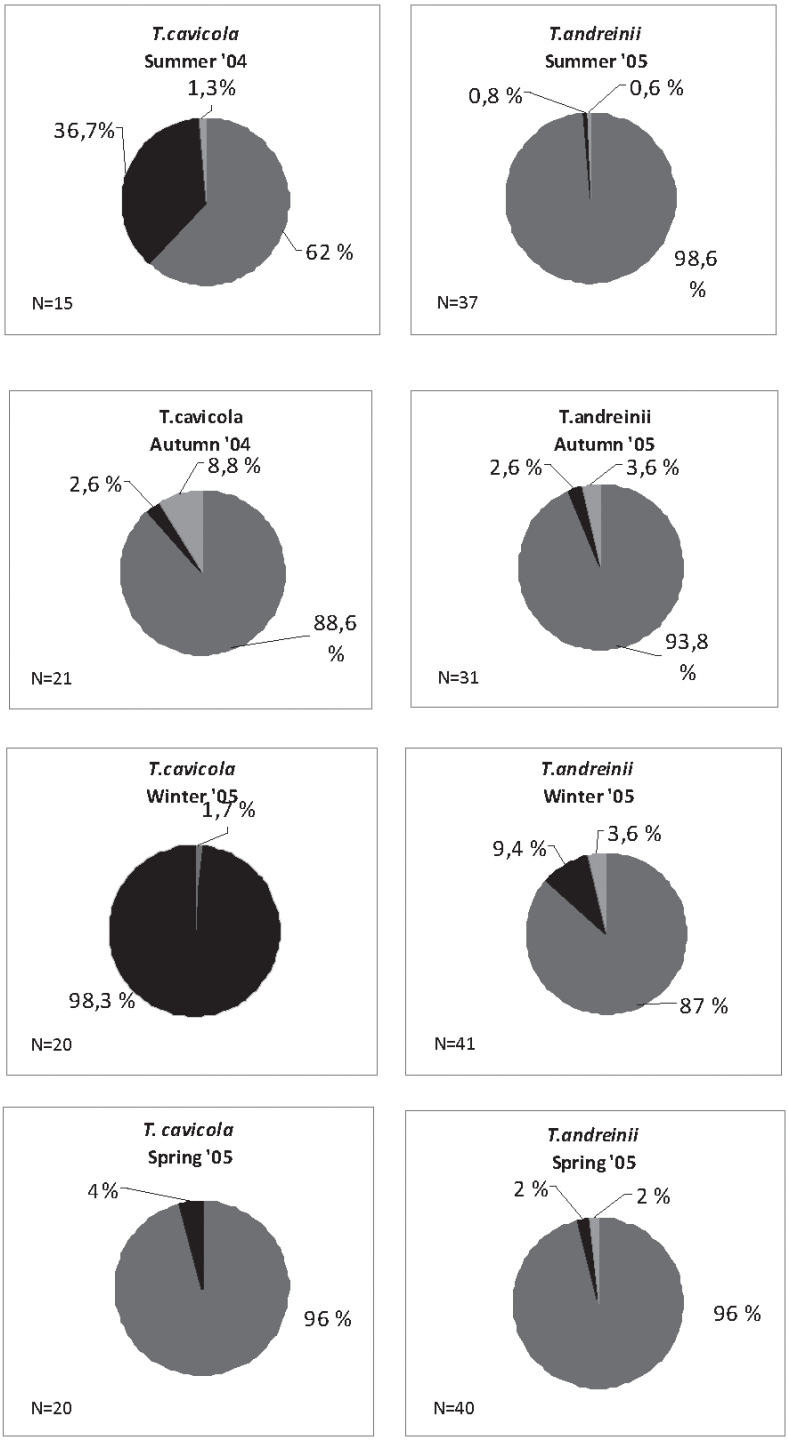

Figure 1. Seasonal comparison of food resource exploitation among cumulate samples of *T. cavicola* and *T. andreinii*. Grey: green vegetables; light grey: fibres; black: arthropod remains.

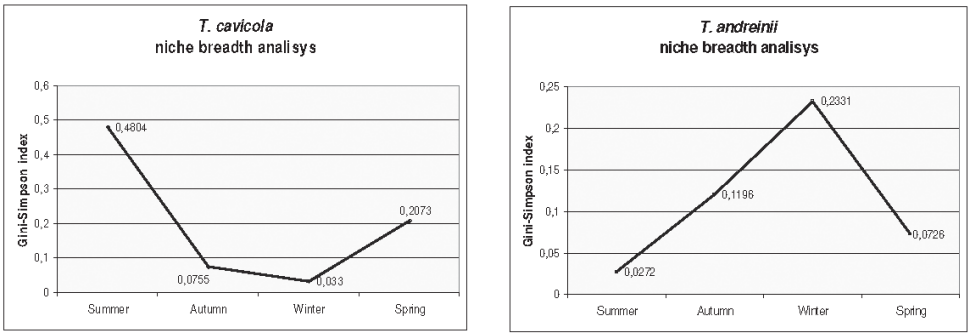

Figure 2. Comparison of seasonal niche breadth in *T. cavicola* and *T. andreinii* populations

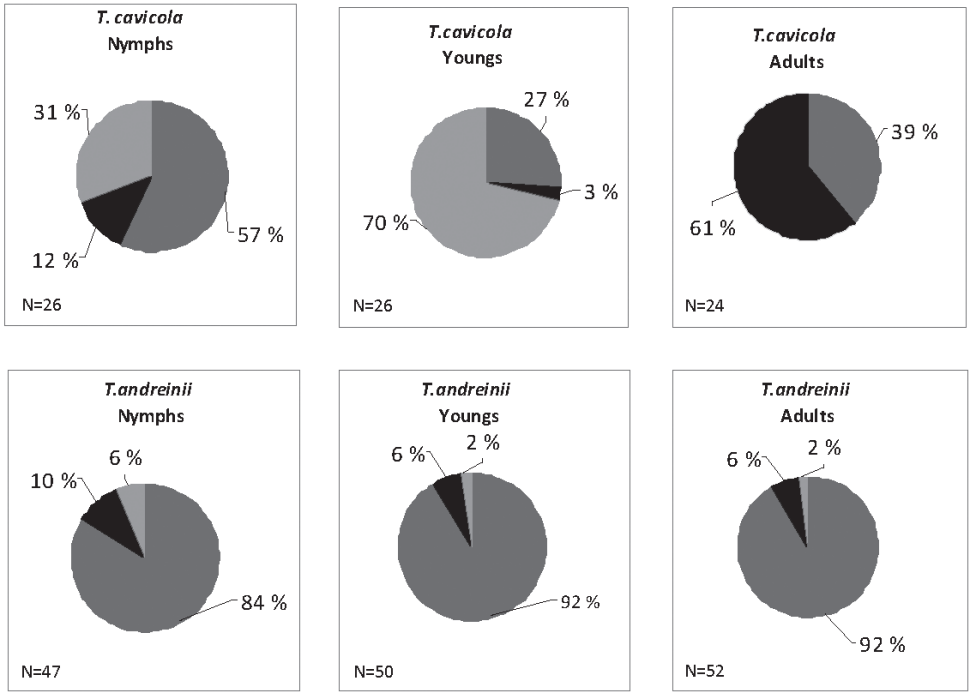

Figure 3. Comparison of the diet among age sub-samples (Young instars, Nymphs and Adults) of *T. cavicola* and T. *andreinii*. Grey: green vegetables; light grey: fibres; black: arthropod remains.

Differences in diet between males and females have been observed only in the Tranese population and only in a periodical sample. In this case, female niche breadth in autumn has a value of the Gini-Simpson index higher in comparison with that of males (i.e. 0.336 vs 0.04). This result seems related to the exploitation in this period of a consistent percentage of arthropod remains by females (Fig.5).

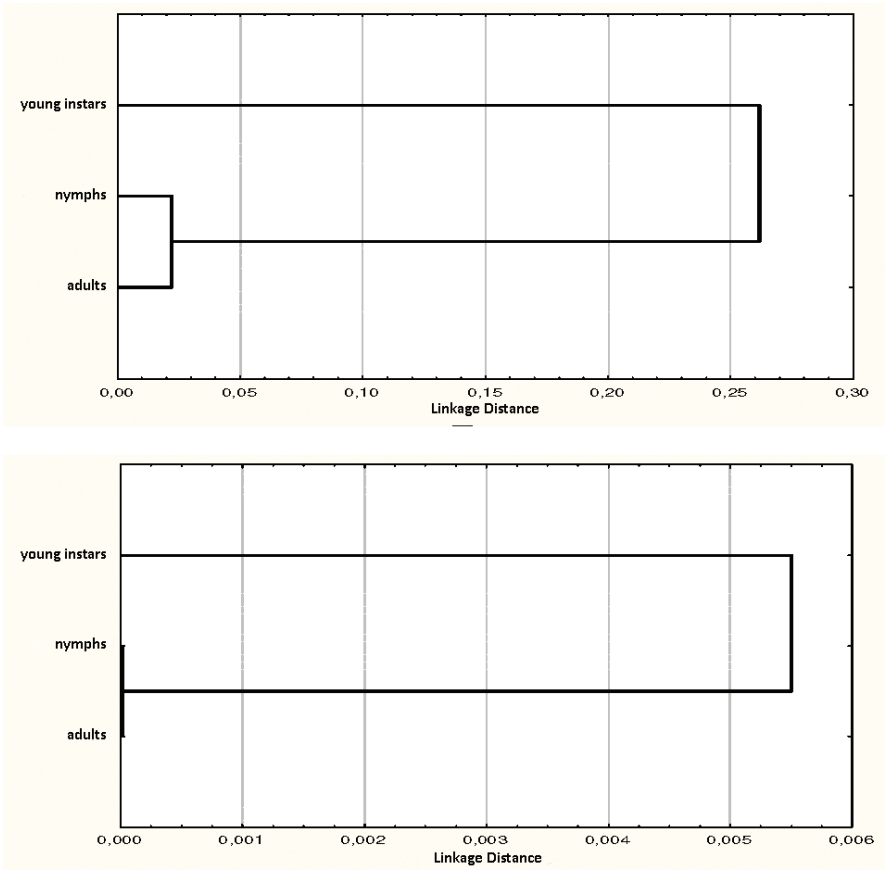

Figure 4. Overlap analysis of food resource exploitation conducted in individuals of different age (young instars, nymphs and adults). The dendrograms were performed using euclidean distances based on the Morisita-Horn index matrices. (a: *T. cavicola*, b: *T. andreinii*).

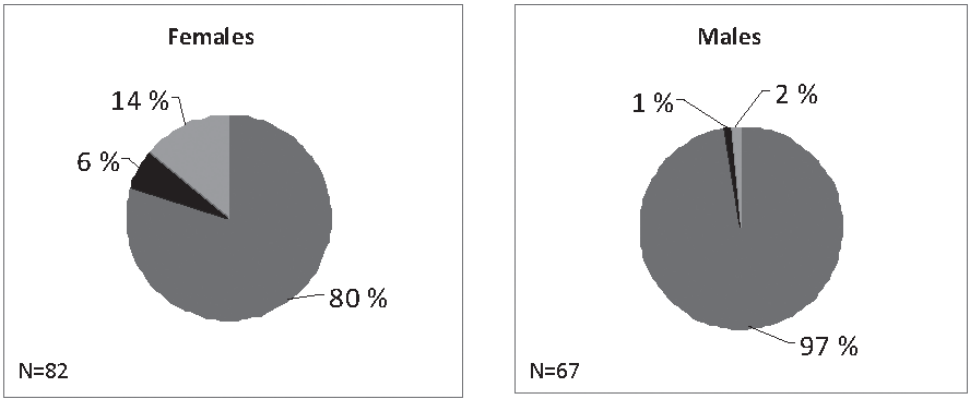

Figure 5. Comparison of the autumnal diet between female and male sub-samples of *T. andreinii*. Grey: green vegetables; light grey: fibres; black: arthropod remains.

Discussion

Remarkable differences in exploitation of these resources can be observed comparing the diet of the two populations herein studied. In particular, in the *T. cavicola* population we observed a diet mainly based on arthropod remains (69%). This finding is chiefly due to the exploitation inside the cave of this type of resource in winter during the strictly hypogean ecophase. In this case, individuals at Covoli di Velo were forced into the cave by the hard climate typical of pre-alpine winter. The large amount of arthropods in this unfavourable winter period can be related to the high availability of this resource inside the Covoli di Velo, where a complex cave biocenosis is present (Caoduro et al. 1994, Zorzin et al. 2000). By contrast, in the *T. andreinii* population, located in southern Italy, the abundance of vegetable matter (green and fibres) appears constant throughout the year suggesting a continuous exploitation of vegetables outside the cave habitats. In the Tranese cave, due to the typical dry climate of the Mediterranean regions, all phases of the life cycle are completed in the hypogean habitat, as suggested by the observations of mating and hatching of eggs in the cave, with crickets moving outside the cave only for daily food. This result is in agreement with the reduction in the Tranese population of a strict division of the population phenology in two ecophases as shown by the occurrence in summer of a remarkable number of individuals in the cave.

The analysis of niche breadth seems to confirm this result: lower values of Gini-Simpson index in *T. cavicola* indicating a dominance of a single type of resource (arthropods), are observed only in winter; in the same period, the Tranese population shows an higher value of this index, suggesting a diet more balanced among the available resources outside and inside the cave.

Conclusions

On the whole, as described for other Rhaphidophoridae species (Di Russo and Sbordoni 1998), we can confirm for *Troglophilus* populations an omnivorous diet based on vegetable matter and arthropod remains.

The study of overlap resource exploitation seems to indicate a separation of diet among young instars and the other age classes of the populations (nymphs and adults). This finding is confirmed by the Morisita–Horn index and is more evident in the *T. cavicola* populations. As found in the Italian *Dolichopoda* populations (De Pasquale et al. 1995), differences in niche during the life cycle can affect population dynamics and reduce competition for food among individuals of different ages (Polis 1984, Ebenman 1987). Differences in diet between males and females have been observed only in the Tranese population. In this case, the increase of female niche breadth in autumn is related to the exploitation of a consistent percentage of arthropod remains. This period of the year corresponds to the time of reproduction of the population (Di Russo et al. 2008); therefore, as found in other arthropods (Coll and Guershon 2002), increasing the animal component in the diet could be interpreted as a protein requirement for maturation of eggs.

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The scientific contribution of Guy Magniez (1935–2014)

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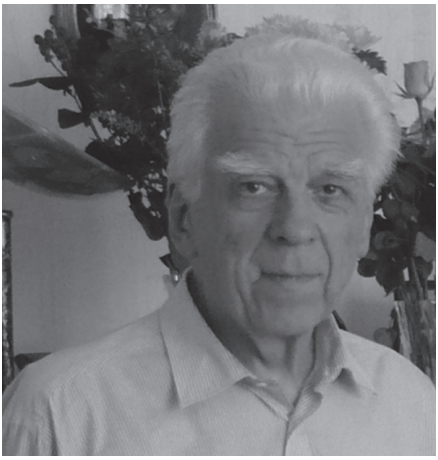

Photo 1. Photography of Guy Magniez.

His career at the laboratory of animal and general biology in Dijon, France

Guy Magniez was born on 23 August 1935 at Marey-sur-Tille, a small village in Côte-d'Or (France). He followed high school studies in Dijon and obtained his high school diploma in 1953. He was an elementary school teacher during one year before joining the University of Dijon where he passed a Bachelor's degree in Natural Sciences in 1958. In 1959, he involved in research at the Laboratory of Geology and obtained a Master degree by submitting a research report on the microfacies of crinoidal Bajocian limestones. Once he successfully passed the Aggregation for secondary education in Natural Sciences in 1960, he integrated the Research Laboratory of Animal and General Biology at the University of Dijon under the direction of Professor Husson. He began with the organization of practical classes for first-year students and a few years later he became responsible for organizing practical classes in general biology and genetics for bachelors. At this time, Guy was in charge of breeding fruit flies for teaching purpose in

addition to breeding stenasellids for his research activity (see below). Then, he was offered a teaching assistantship and took the lead of the bachelor program in Natural Sciences. After submitting his state doctoral Thesis in 1976, he delivered lectures to under-graduate and graduate students. He became an associate professor in 1985 and was responsible for preparing graduate students to become teachers. Guy was an exceptional pedagogue: he supervised numerous master students (including the first author of this memorial) and led many students to go into teaching natural sciences. In addition to his heavy teaching duties, Guy also invested much time into social and administrative tasks at the University of Dijon. As a committed educator, Guy was both extremely modest and discrete: he has always been greatly appreciated by his colleagues and students. He was appointed knight of the Order of Academic Palms as soon as 1979, and officer in 1986. His research began in 1960 and he was retired in 1999, but he was still contributing to national and European research projects in the 2010's (Deharveng et al. 2009, Morvan et al. 2013).

The biology of *Stenasellus virei*

Most of the information on the life cycle of *Stenasellus virei* Dolfus, 1897 (Stenasellidae) are from dedicated studies conducted by Guy during the sixties and seventies. From 1960 to 1976, he collected several populations of *Stenasellus* in the Pyrenees and Cantabria and reared them in the Moulis Cave (Pyrenees), Antheuil Cave (Côte-d'Or) and in thermostatic rooms at the University of Dijon. Rearing was a necessary step to document the life cycle of *Stenasellus* because the body size distribution of cave populations was truncated, with almost no juveniles due to a strong cannibalism (Magniez 1973). Collecting *S. virei* alive and rearing them for more than 15 years was obviously a challenging task. Collecting in cave yielded a few individuals with no juveniles and sampling in the hyporheic zone did not provide many more individuals as many of them were killed during pumping. Reproduction events were rare even in controlled conditions and larvae born in aquaria were preyed by adults when they were not rapidly isolated. Moreover, rearing care had to be interrupted for 17 months when Guy had to perform his military service (1961–1963). Despite these difficulties, Guy reported in 1975 his detailed findings on the biology of *S. virei* in a 250-page long article published in International Journal of Speleology (Magniez 1975). The intramarsupial development of *S. virei* lasts 9–10 months but the female keeps an empty marsupium for several months after releasing its larvae because the reproductive intermolt lasts 15–16 months. A reproductive intermolt is always followed by at least one genital-rest intermolt (lasting 9–11 months), so that the shortest interval between successive egg-laying periods of a single female is at least 2 years. Post marsupial larval development is about 11-month long and an additional 5–6 years are needed before the female reaches its age at first reproduction (i.e. 6–7 years or more). Life span in males and females is about 12 and 15 years, respectively. As reproduction events occurs at best every 2 years and the number of eggs per reproduction event is on average 32 (range: 15–60 eggs), a

single female would at most produce 150 eggs during its life. Detailed information on the life cycle and reproduction biology of groundwater organisms are scarce. However, biological features such as generation time and number of offsprings per individual are essential for understanding evolution in the subterranean environment. Younger generations of scientists are increasingly becoming aware of the value of detailed biological information on subterranean species provided by tedious studies conducted during the second half of the 20th century.

Systematics of Asellidae and Stenasellidae

Perhaps, the greatest contribution of Guy consisted in clarifying the systematics of Stenasellidae and Asellidae. He described or co-described a total of 109 taxa among which 6 genera (*Bragasellus*, *Gallasellus*, *Metastenasellus*, *Neostenetroides*, *Parastenasellus*, and *Sibirasellus*), 3 species of *Asellus*, 13 species of *Bragasellus*, 48 species of *Proasellus*, 5 species of *Synasellus* and 31 species and subspecies of Stenasellidae (Table 1). As his taxonomic knowledge of the Aselloidea and meticulous morphological descriptions were rapidly recognized by the scientific community, Guy received biological material from all over the world and described species in Africa, America, Asia and Europe (Figure 1). Although Guy never published a single cladogram, his approach of the systematics of Aselloidea was all about finding the relationships among species through time. Relationship between taxa was essentially inferred from the shape of male copulatory organs (second pleopods), the detailed structure of which was revealed by means of scanning electron microscopy as early as the seventies (Henry and Magniez 1969).

Following the seminal work of Racovitza (1919), Henry and Magniez (1968, 1970) initiated the modern systematics of Asellidae by distributing into 8 genera the heterogeneous set of taxa that had long been attributed to the genus *Asellus* E.L. Geoffroy, 1762. Their motivation stood from the necessity to distinguish between distinct "natural groups", the members of which shared more evolutionary history with each other, than they did with members of other groups. Fifty years later, the foundations of the asellid systematics as described by Henry and Magniez (1968) are still valid, even though the family contains many more genera and species. Many of the evolutionary inferences made by the authors on the basis of morphological characters were corroborated by recent phylogenetic studies using molecular markers (Morvan et al. 2013). The systematics of Asellidae has been continuously refined by Guy and Jean-Paul Henry for the last 20 years (Henry and Magniez 1993, 1995, Magniez 1996, Magniez and Henry 2001). The family is now represented by three distinct lineages. The *Asellus* pattern lineage with its diversification center in the north-Pacific area contains species belonging to the two *Asellus* subgenera *Asellus* (*Asellus*) and *Asellus* (*Arctasellus*) Geoffroy (Boreal Eurasia and Alaska) and the genera *Mesoasellus* Birstein (Baïkal), *Phreatoasellus* Matsumoto (Japan, Korea), *Nipponasellus* Matsumoto (Japan), *Columbasellus* Lewis, Martin & Wetzer (Washington, see Lewis et al. 2003), *Uenasellus* Matsumoto (Japan), *Sibirasellus* Henry & Magniez (Primorye) and *Calasellus* Bowman (West of North-

Table 1. List of genera, species and subspecies described or co-described by Guy Magniez. Numbers refer to the location of species and subspecies as indicated in Figure 1.

Asellidae

1. *Asellus (Asellus) levanidovorum* Henry & Magniez, 1995
2. *Asellus (Asellus) primoryensis* Henry & Magniez, 1993
3. *Asellus (Phreatoasellus) joianus* Henry & Magniez, 1991

Bragasellus Henry & Magniez, 1968

4. *Bragasellus afonsoae* Henry & Magniez, 1988
5. *Bragasellus aireyi* Henry & Magniez, 1980
6. *Bragasellus bragai* Henry & Magniez, 1988
7. *Bragasellus comasi* Henry & Magniez, 1976
8. *Bragasellus comasioides* Magniez & Brehier, 2004
9. *Bragasellus escolai* Henry & Magniez, 1978
10. *Bragasellus lagari* Henry & Magniez, 1973
11. *Bragasellus lagarioides* Henry & Magniez, 1996
12. *Bragasellus meijersae* Henry & Magniez, 1988
13. *Bragasellus molinai* Henry & Magniez, 1988
14. *Bragasellus notenboomi* Henry & Magniez, 1988
15. *Bragasellus rouchi* Henry & Magniez, 1988
16. *Bragasellus stocki* Henry & Magniez, 1988

Gallasellus Henry & Magniez, 1981

17. *Proasellus alavensis* Henry & Magniez, 2003
18. *Proasellus albigensis* (Magniez, 1965)
19. *Proasellus aragonensis* Henry & Magniez, 1992
20. *Proasellus bagradicus* Henry & Magniez, 1972
21. *Proasellus bardaunii* Alouf, Henry & Magniez, 1982
22. *Proasellus bellesi* Henry & Magniez, 1982
23. *Proasellus beroni* Henry & Magniez, 1968
24. *Proasellus beticus* Henry & Magniez, 1992
25. *Proasellus boui* Henry & Magniez, 1969
26. *Proasellus bouianus* (Henry & Magniez, 1974)
27. *Proasellus burgundus* Henry & Magniez, 1969
28. *Proasellus cantabricus* Henry & Magniez, 1968
29. *Proasellus chappuisi* Henry & Magniez, 1968
30. *Proasellus chauvini* Henry & Magniez, 1978
31. *Proasellus claudei* Henry & Magniez, 1996
67. *Synasellus hurki* Henry & Magniez, 1995
68. *Synasellus leysi* Henry & Magniez, 1995
69. *Synasellus meijersae* Henry & Magniez, 1987
70. *Synasellus notenboomi* Henry & Magniez, 1987

Stenasellidae

71. *Magniezia gardei* Magniez, 1978

Metastenasellus Magniez, 1966

72. *Metastenasellus leysi* Magniez, 1986
73. *Metastenasellus powelli* Magniez, 1979
74. *Metastenasellus tarrissei* Magniez, 1979
75. *Mexistenasellus parzefalli* Magniez, 1972
76. *Mexistenasellus wilkensi* Magniez, 1972

Parastenasellus Magniez, 1966

77. *Stenasellus bedosae* Magniez, 1991
78. *Stenasellus boutini* Magniez, 1991
79. *Stenasellus bragai* Magniez, 1976
80. *Stenasellus cambodianus* Boutin & Magniez, 1985
81. *Stenasellus chapmani* Magniez, 1982
82. *Stenasellus covillae* Magniez, 1987
83. *Stenasellus deharvengi* Magniez, 1991
84. *Stenasellus escolai* Magniez, 1977
85. *Stenasellus foresti* Magniez, 2002
86. *Stenasellus grafi* Magniez & Stock, 2000
87. *Stenasellus henryi* Magniez & Stock, 2000
88. *Stenasellus javanicus* Magniez & Rahmadi, 2006
89. *Stenasellus kenyensis* Magniez, 1975
90. *Stenasellus messanai* Magnez & Stock, 2000
91. *Stenasellus mongnatei* Magniez & Panitvong, 2005
92. *Stenasellus monodi* Magniez, 2000
93. *Stenasellus rigali* Magniez, 1991
94. *Stenasellus stocki* Magniez, 2001
95. *Stenasellus strinatii* Magniez, 1991
96. *Stenasellus vermeuleni* Magniez & Stock, 2000
97. *Stenasellus virei angelieri* Magniez, 1968
98. *Stenasellus virei boui* Magniez, 1968
99. *Stenasellus virei hussoni* Magniez, 1968
100. *Stenasellus virei margalefi* Magniez, 1996
101. *Stenasellus virei rouchi* Magniez, 1996

Gnathostenetroidae

Neostenetroides Carpenter & Magniez, 1982

102. *Neostenetroides stocki* Carpenter & Magniez, 1982

Janiridae

103. *Mackinia birstein*i Henry & Magniez, 1991

America). Magniez (1996) suggested that this lineage could be attributed the rank of a subfamily. The Atlantic lineage is represented in North America by the genera *Caecidotea* Packard, *Lirceus* Rafinesque, *Lirceolus* Bowman & Longley, *Remasellus* Bowman & Sket and *Salmasellus* Bowman and in Europe by the genera *Bragasellus* Henry & Magniez (Portugal and Spain), *Gallasellus* Henry & Magniez (France) and *Synasellus* Braga (Portugal and Spain). Hidding et al. (2003) brought molecular evidence that the genus *Baicalasellus* Stammer (Baïkal Lake) belonged to this Atlantic lineage and the same could hold true for the genus *Stygasellus* Chappuis (Romania). The Mediterranean lineage is represented by the species-rich genus *Proasellus* Dudich (including the genus *Chthonasellus* Argano & Messana [see Morvan et al. 2013]), which extends

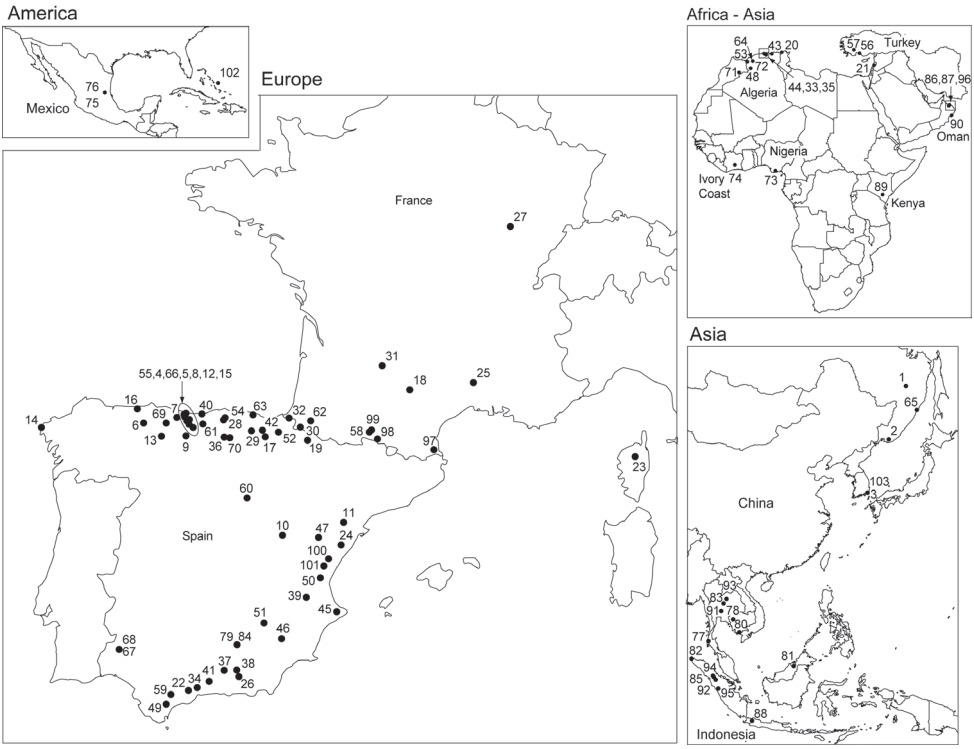

Figure 1. Maps showing the location of the 103 species and subspecies of aquatic isopods described or co-described by Guy Magniez. Numbers indicate taxa, the names of which are provided in Table 1.

longitudinally from Iran to Spain and latitudinally from Algeria to Sweden. This genus was finely dissected into several lineages by Guy and Jean-Paul Henry, many of which were corroborated by recent molecular studies (Morvan et al. 2013).

In his last poster presented at the 19th International Symposium of Subterranean Biology in Fremantle, Australia (21-26 September 2008), Magniez (2008) had a note reminding the thoughts of Armand Viré (1902) when he captured the first stenasellid in Padirac Cave (Lot, France): "he thought *Stenasellus virei* was a relict and he had captured the last specimens!". Today, the Stenasellidae comprises more than 75 taxa belonging to 10 genera. Almost half of these taxa were described by Guy. We provide in Figure 2 the chronology of the discovery of stenasellids as portrayed by Guy in his last poster as well as his own interpretation of the worldwide distribution of the family (see below).

Subterranean biogeography

Guy has voluntary remained unprecise as to when the colonization of groundwater by marine ancestors and diversification of groundwater lineages occur. This is because he

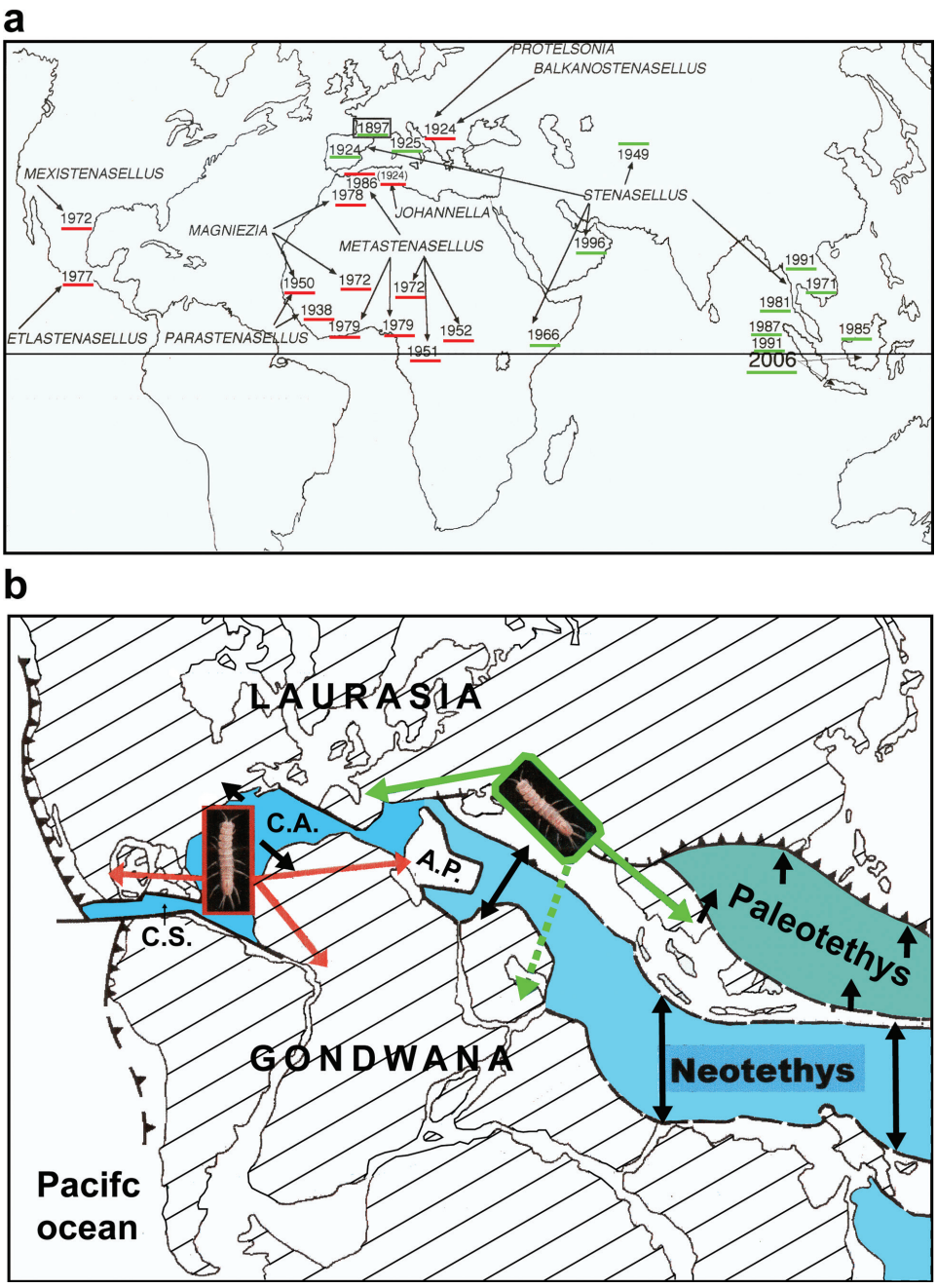

Figure 2. a. Chronology of the discovery of stenasellids. Red and green lines refer to as the "Atlantic" and "Mesogean" stocks as indicated in the lower panel. The genus *Acanthastenasellus* Chelazzi & Messana 1985 (Somalia) was not mapped by Guy **b** Hypothetic colonization of groundwater by ancestor of the stenasellids. C.S. Caribbean sea C.A. Central Atlantic Ocean A.P. Apulian plate. Panels a & b are modified after Magniez (2008). The paleogeographical map in panel b was modified after Monod (2005), Encyclopaedia Universalis.

has always been much skeptical about the assumptions of the climatic relict hypothesis. In particular, he never associated inland groundwater colonization to any marine transgression because colonization was necessarily an active process to him. Moreover, he was convinced that some species of asellids and stenasellids had experienced considerable range expansion by dispersing into extensive alluvial systems (Magniez 1999). Again, Magniez'view of diversification and species range dynamics in groundwater aselloids recently received support from molecular studies. Morvan et al. (2013) documented a constant diversification rate during most of the course of Aselloidea evolution, thereby challenging the view that species diversification in temperate groundwater has been primarily driven by continental-scale perturbations in the physical environment. Then, Eme et al. (2013) provided evidence that the large geographic range of *Proasellus cavaticus* (Leydig, 1871) in northern Europe reflected recent, presumably postglacial, dispersal.

To end up this memorial, we provide below two biogeographic scenarios elaborated by Guy that would warrant further testing by today's generation of subterranean phylogeographers. The first scenario is an attempt to explain the distribution of stenasellids at global scale (Figure 2). It was a part of his poster presented at the 19th International Symposium of Subterranean Biology but Guy did not publish this work. Guy hypothesized that the ancestors of stenasellids were anophtalmous thermophilic burrowers living in coastal unconsolidated sediments of the Neo-Tethys Sea, the sediments of which accumulated from Trias to Eocene. He further suggested on the basis of morphological taxonomy that the initial colonization of groundwater gave rise to two distinct groups of stenasellids. The first one, to which he referred as the "Atlantic stock", includes species from the New World, West Africa and the Balkans (red lines in Figure 2). He speculated that the drift of the Apulian block (a fragment of Gondwana) and its fusion with Eurasia might account for the presence of *Protelsonia* Méhely and *Balkanostenasellus* Cvetkov in the Balkans. The second group, to which he referred as the "Mesogean stock" corresponds to the genus *Stenasellus* Dolfus which extends from southern Europe (Iberian Peninsula, southern France, and Italy) to the eastern horn of Africa and Asia. According to Guy' predictions , a phylogenetic tree of Stenasellidae would exhibit a clear basal dichotomy with all genera except *Stenasellus* clustering into a monophyletic group and all *Stenasellus* species clustering into another monophyletic group.

The second scenario attempts to explain the evolutionary history of the Iberian stenasellids (Magniez 1999; Figure 3). Guy suggested that the '*breuili'* and the '*virei*' groups might have evolved independently during Cretaceous on two separate emerged continental blocks to which he referred as the "Iberian Meseta" and the "Catalona-Corbières-Corsica-Sardinia block" (the "Thyrrhenian continent" in Figure 3) (but see Dercourt et al. 1993 for detailed paleogeographical maps). Then, the fragmentation of the Thyrrhenian block and south-eastern migration of the Corsica-Sardinia plate (from 30 to 6 my BP) isolated *Stenasellus racovitzai* Razzauti, 1925 from the '*virei*' group. Finally, each group experienced a more recent geographical expansion as some eurytopic and eurytherm species dispersed along alluvial corridors of the Guadalquivir, Duero, Ebro and Garonne river systems (Figure 3).

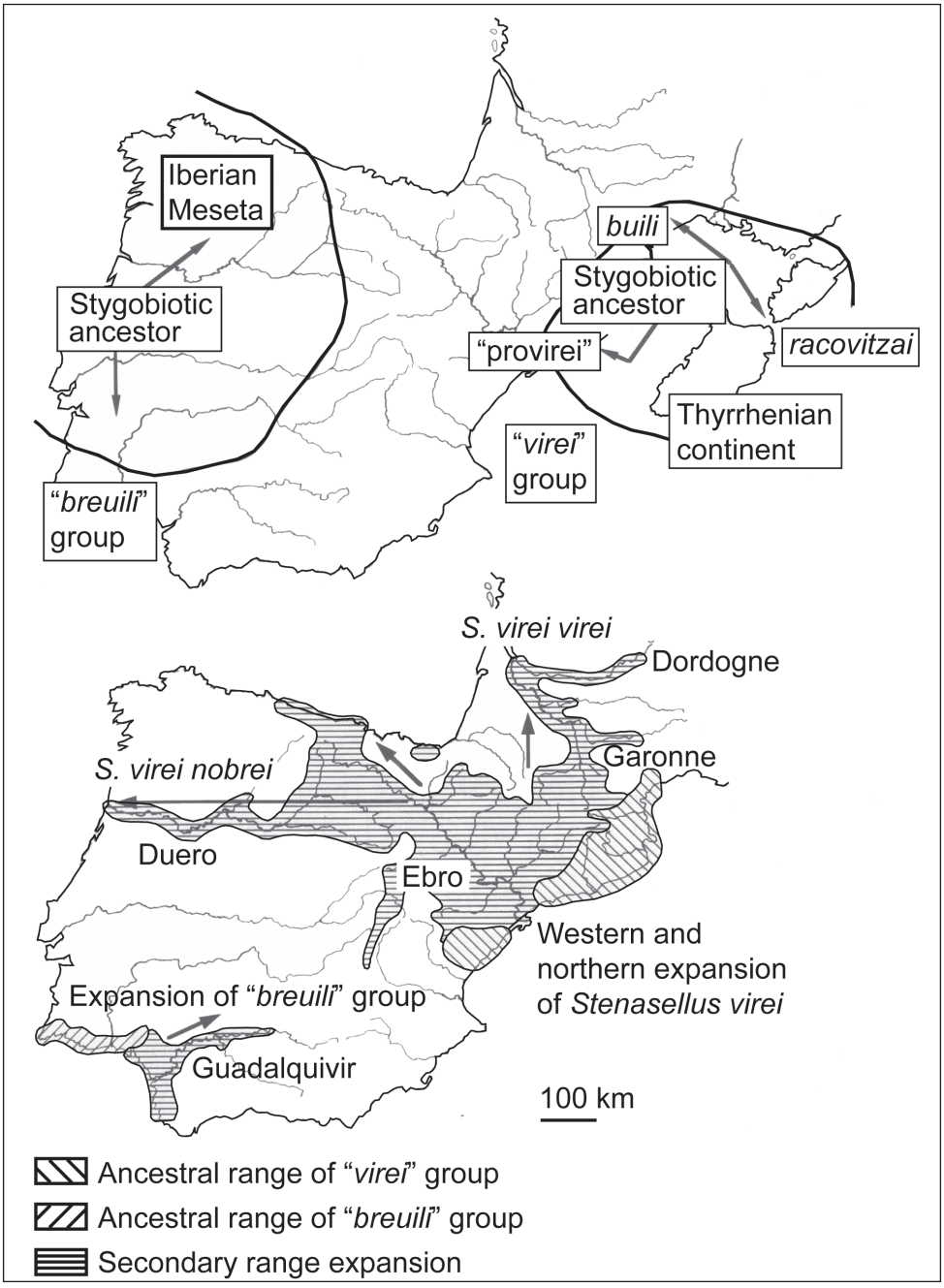

Figure 3. Paleobiogeography of the Ibero-Aquitanian stenasellids. Upper panel. Palaeogeographic continental areas attributed to the stygobiotic ancestors of the "*breuili*" and "*virei*" groups before migration of the Corsica-Sardinia plate. Limits are very approximate. Lower panel. Recent expansion of geographic ranges of the "*breuili*" and "*virei*" groups due to dispersal along large Ibero-Aquitanian river systems. Modified after Magniez (1999).

Guy Magniez devoted much of his life to the taxonomy of the Aselloidea and described, named and classified more than 100 taxa. Yet, his scientific contribution goes well beyond the taxonomy of the Aselloidea. All along his career, he attempted to merge information from biology, ecology, and evolutionary biology to explain the geographic distribution of groundwater species through geological time. His name would be forever associated to the modern systematics of the Aselloidea but he has also left us many predictive biogeographic scenarios that warrant further testing.

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Supplementary material 1

Publications by Guy Magniez

Authors: Florian Malard, Jean-Paul Henry, Christophe J. Douady

Data type: References list.

Explanation note: A list of 153 articles authored or co-authored by Guy Magniez.

Link: doi: 10.3897/subtbiol.13.7412.app1

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RESEARCH ARTICLE

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Niphargus plurispinosus sp. n. (Crustacea, Amphipoda), a stygophile and hypotelminorheic representative from Central Europe

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Abstract

The detailed description of the morphology of *Niphargus plurispinosus* **sp. n.** from Slovakia is presented. Over 300 specimens were collected from a permanent seepage spring on repeated visits between May 2011 and May 2013. The type locality is located in the foothills of the Zemplínske vrchy mountains in the East Slovakian Lowland (NE part of Pannonian Lowland) - small, low and isolated hills formed during Neogene volcanic activity. Volcanic rocks draw together fragments of massives of Palaezoic and Mesozoic age as same as Neogene sediments. The new species can be classified as stygophile, living in the shallow subterranean habitat. The species has small subequal gnathopods, sexually dimorphic uropod III, sexually non-dimorphic uropod I in juveniles, dimorphic uropod 1 in adults. They are extremely different in the post-reproductive stage, when they have 2-4 dorsal spines (arranged in a transverse row) on the telson and supporting dorsal spines.

Keywords

Niphargus plurispinosus sp. n., Crustacea, Amphipoda, Carpathian region, Slovakia, morphology, variability, phenology, ecology

Introduction

There are over 300 species and subspecies described in the genus *Niphargus* Schiödte, 1849, distributed mainly in ground waters of Europe (Boxhall and Fišer 2004, Väinöla et al. 2008, http://niphargus.info). Most species have a poor ability to disperse (Trontelj et al. 2009) and many of the taxa are known only from their type locality. The identification of large number of species remains problematic (Fišer et al. 2008b, 2009a, 2009b), and species descriptions are of varying quality. The taxonomy of *Niphargus* has been complicated by regional differences in practice, which are nearly as diverse as the genus itself (Fišer et al. 2009b).

The situation in Slovakia is no different. Eight species of the genus *Niphargus* are currently known from Slovakia (Košel 2012). Four of them are relatively common, with rather large and well defined distribution areas, and can be relatively easily identified (*N. tatrensis* Wrześniowski, 1888, *N. aggtelekiensis* Dudich, 1932, *N. hrabei* S. Karaman, 1932 and *N. valachicus* (Dobreanu & Manolache, 1933). Two species, *N. bajuvaricus* Schellenberg, 1932 and *N. inopinatus* Schellenberg, 1932, are found in areas of tectonic dislocations with numerous mineral springs (Hudec and Mock 2012). The remaining species are the enigmatic *N. carsicus* Straškraba, 1956 (sole type sample from the Zádiel Gorge in Slovak Karst) and *N. dudichi* Hankó, 1924, known from groundwater of SW Slovakia.

Three years of intensive study of superficial subterranean habitats (sensu Meštrov 1962, Culver et al. 2006, Culver and Pipan 2009a, 2009b) of various volcanic regions in eastern Slovakia resulted in the discovery of an undescribed species that was, in early analysis, misidentified as *N. baloghi* Dudich, 1940 (Hudec and Mock 2012). In contrast to previously described species, characterized with the most one dorsal spine, the species described here possess several dorsal spines on the telson. More detailed analysis revealed this is a new species.

The aim of this paper is to present detailed morphology of this species and to provide some data on its autoecology.

Material and methods

All material was collected at the same locality: South-east Slovakia, south-east foothill of the Zemplínske vrchy Mts. (different to nearby Zemplén Mts. in Hungary), in Hatfa in the village Viničky (48°25'11"N, 21°44'59"E). The mountain chain Zemplínske vrchy Mts. is a small part of the Carpathians (covering an area of about 6 × 15 km, with the highest top at 465,3 m.a.s.l.). The complicated geology includes Paleozoic (non-carbonate), Mesozoic (carbonate) and Tertiary (Sarmatian volcanism, sediments) deposits (Karniš and Kvitkovič 1970). The mountains are close to the eastern slopes of the volcanic Slanské vrchy Mts. which have different *Niphargus* fauna (unpublished).

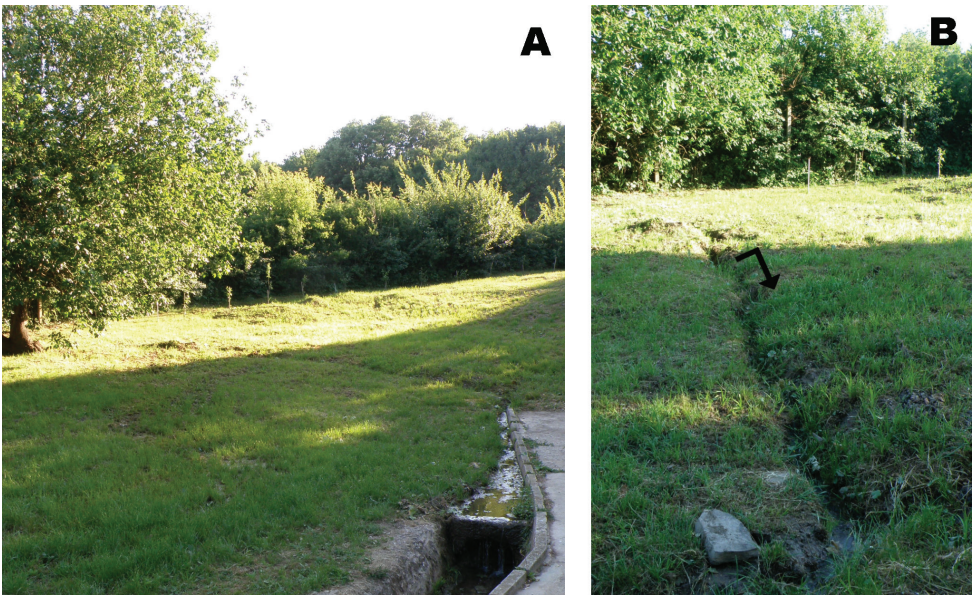

Figure 1. Locality (Viničky-Hatfa) of *N. plurispinosus* sp. n. in Slovakia: **A** general view **B** detail of location where the specimens were collected (Photo: A. Mock).

The locality Hatfa near the village Viničky is situated on the boundary of areas with different history and bedrock (limestone and volcanic pyroclastics), on the south-east foothills of the hills, close to the limestone area. The type locality is a small, permanent spring close to a private reservoir of groundwater (164 m.a.s.l.), with artificial drainage to the meadow at the east foothill (Fig. 1). Water temperature in the spring is relatively high all year round (10–13°C). Chemical parameters were pH = 7.08, conductivity = 1310 μS, concentration of Ca ions 18.6 mg.L-1 and concentration of Mg ions 32.1 mg.L-1. The stream bed varies from 5–10 cm in width and 1-3 cm in depth. All specimens were collected from a drainage ditch under the stones, leaf litter and grass using a tea-strainer (leg. A. Mock).

Most specimens were preserved in 75% ethanol, with a few samples preserved in 96% ethanol for future DNA-analysis. One to three specimens from numerous samples were morphologically studied.

From all samples we took the smallest and the largest specimens. Adult females were distinguished by the presence of eggs whereas adult males were identified on the basis of having elongated exopodites of uropods III. All other stages were assumed to be correlated with body size (see in Variability chapter).

Specimens were boiled in KOH and chlorophenol; later they were dissected and mounted on slides using SWANN-medium. Fine details were examined using a Leica microscope with magnifications 100–400× using black field or phase contrast. All pen-drawing were made using camera lucida. Digital photos were taken with an Olympus DP10 camera mounted on a Leica microscope or stereomicroscope.

Symbols and abbreviation used in the text:

(⬅) very important taxonomic character;
(⬅) useful supporting taxonomic character.

All other abbreviations of morphological terms used in the text correspond with Fišer at al. (2009b). L = length, BL = body length, TL = telson length.

Systematics

Order Amphipoda Latreille, 1816
Suborder Gammaridea Latreille, 1802
Family Niphargidae G. Karaman, 1962
Genus *Niphargus* Schiödte, 1849

***Niphargus plurispinosus* sp. n.**
http://zoobank.org/9900B10D-7230-44DD-86CB-F904F45EF08A
http://species-id.net/wiki/Niphargus_plurispinosus
Figs 2–9

Etymology. The species name was derived from the Latin words: *plus, pluris* (= more) *spina* (=spine, thorn); *Niphargus* with more then 1 dorsal spine (thorns) on the telson.

Type material. The type series was collected in the locality Viničky - Hatfa.

Holotype. Viničky - Hatfa 11 May 2013: one adult male (16 mm) in vial preserved in ethanol, (NHMUK 2014.381 Natural History Museum, London, Great Britain).

Allotype. Viničky - Hatfa 11 May 2013: one adult female with eggs (14 mm) in vial preserved in ethanol (NHMUK 2014.382NHM, London).

Paratypes. Viničky - Hatfa 11 May 2013: one large male (17 mm) in vial preserved in ethanol (NHMUK 2014.383 NHM, London); 1 dissected large male (20 mm) mounted in Swann-medium (NHMUK 2014.384 NHM, London); 1 dissected largest female (17 mm) without eggs mounted in Swann-medium (NHMUK 2014.385 NHM, London) and 1 dissected large male (20 mm) (in the authors collection).

Paratype serie. Viničky - Hatfa 19 June 2012 50 specimens of different stages in one vial preserved in ethanol (NHMUK 2014.386-395 NHM, London).

Paratypes. More than a dozen specimens deposited in Ljubljana, Slovenia (Collection of C. Fišer), the remaining samples in the authors collection.

Other examined material. 2011: 26 May: 5 specimens (3 adult ♂, 2 adult ♀ with eggs); 14 October: 30 specimens (25 subadult ♀ and 5 subadult ♂); 23 November: 6 specimens (4 subadult ♀ and 2 subadult ♂);

2012: 12 January: 10 specimens (6 subadult ♀ and 4 ♂); 23 February: 32 specimens (22 juveniles, 4 subadult ♀ 6 subadult ♂); 21 March: 35 specimens (2 adult ♀ with eggs, 33 juveniles); 3 May: 19 specimens (1 adult ♀ with embryos, 1 adult

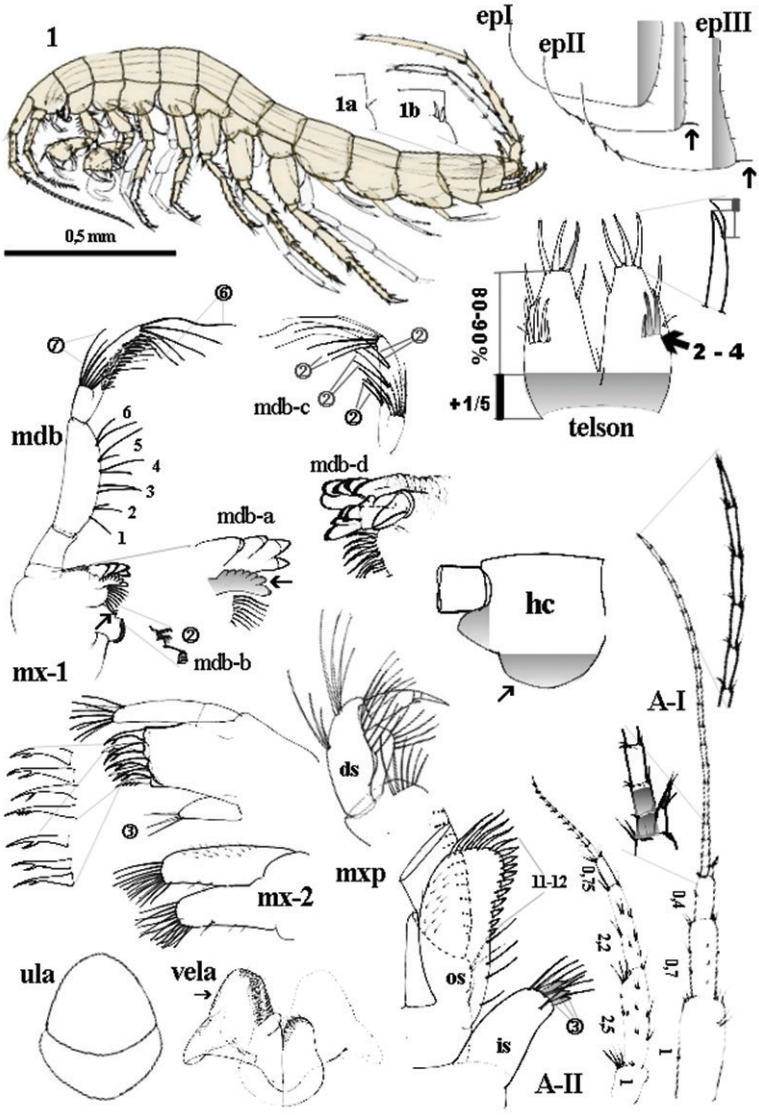

Figure 2. *N. plurispinosus* sp. n.: **1** male, general view; **1a-1b** dorso-later thorns; **mdb** - mandibula and details of **mdb-a** left incisor and lacina mobilis; mdb-b) two setae between bisserated thorns; **mdb-c** setae pattern on distal segment of mdb-palp; mdb-d, right incisor and lacina mobilis; **mx-1** 1st maxilla; **mx-2** 2nd maxilla; **ula** upper lip; **vela** ventral labium; **mxp** maxilliped: **in** inner segment **os** outer segment; **ds** distal segment of palp; **epI-epIII** epimeral plate I-III; **A-I** 1st antenna; **A-II** antenna; **hc** head capsula, left lateral view; **telson**, dorsal view. Not scaled, except of the general view of the male.

♂, 17 juveniles); 19 June: 17 specimens (1 subadult ♀, 1 subadult ♂ (both about 12 mm long), 10 juveniles (up to 8 mm), 5 neonates (not longer than 4 mm); 9 July: 16 specimens (15 juveniles and 1 neonate); 28 September: 10 specimens (all juveniles); 22 November: 33 specimens (32 juveniles, 1 subadult ♀);

2013: 11 May: 21 specimens (3 ♀ with embryos, 7♀ larger but without embryos (post adults), 7 ♂ more than 20 mm long (post adults), 25 juveniles), preserved in clear ethanol; part of material is in Ljubljana in C. Fišer's collection.

All remaining material is retained in the authors´ collection.

Diagnosis. *Niphargus plurispinosus*: Middle-sized, oblong species with small gnathopods, sexually dimorphic uropod III and sexually non-dimorphic uropod I in juveniles, but different in adults, and extremely different in postreproductive stage. Coxal plates as follows a) cx-1 rhomboid; b) cx-7 reduced, trapezoid-oval plate with elongate posterior corner and one seta close to posterior corner; other coxal plates (cx-2 to cx-6) without species-specific features. Epimeral plates - first two vaulted in posterior-ventral corner; 3rd angular (juveniles and adult males). Telson: lobes slightly narrowing distally, with even end, deep cleft: 70–80%; 2-3(4) dorsal spines arranged in a transversal row; c) terminal spines do not exceed 35–40% of telson length, and decrease with age down to 20%. Pereopods V-VII bases - elongate-oval, length ratios = 1.0/1.2/1.25; dactyli with long nail (30–40% of dactylus length) and one tiny spiniform thorn near nail base. Mouthparts: maxillae I inner lobe with 3 setae: 2 terminal and 1 subterminal; maxillipeds inner lobe with 3 lancet-like stout setae.

Description of adults (paratypes). If it is not specified the characters are the same for both sexes.

Body shape. Longish and narrow (Fig. 2: 1). Body length: up to 20 mm (male), 17 mm (female). Colour: white-yellowish (living specimens); shortly after preservation in alcohol the body turns white.

Head (Fig. 2: 1; hc). Short angular, without rostrum; anterior margin deep sinusoid (deep incision at the joint of AI and long, lobe); ventral (cheek) margin expressive vaulted. Surface of head capsule is smooth. Minute yellow spot on each side of head between AI and AII present in living specimens, but these quickly fade on preservation.

Antennae: 1st antenna (Fig. 2: A–I). length 35–40 % of BL; ratio between the three pedunclar segments is 1 : 0.7 : 0.4; flagellum with 20–21 segments of different sizes expressed as ratio to 1st peduncular segment: 0.17 (1st -2nd) - 0.18 (3rd-4th) - 0.10 (5th- 6th) - 0.20 (up to 21st); each segment with a few minute sensillae and one elongate bi-segmented aesthetasc along distal margin; accessory flagellum bi-articulated and short (shorter than 1st two segments of flagellum) with 4 sensillae on tip. 2nd antenna (Fig. 2: A-II): length 50 % of A1; peduncule segments ratio =1 : 2.5 : 2.2. Flagellum bears basal longer segment (0.7 of 1st segment) and ≤10 short segments of moderate size to 1st segment: 0.5 (1st -2nd) - 0.38 (3rd–4th) - 0.25 (up to 10th).

Upper lip (labrum) (Fig. 2: ula; Fig. 3: 1). Sclerotised, oval-quadrangular, 2-articulated: basal segment narrow, distal segment sub-oval; whole surface is smooth or with long submarginal suture in distal portion.

Mandibles (Fig. 2: mdb). Right mandible (Fig. 2: mdb-a): incisor with 4 sclerotised teeth, lacina mobilis with 7 small, vaulted teeth arranged in short fan; the first one is larger (Fig. 2: mdb-a ⬅). Left mandible (Fig. 2: mdb-d): incisor with 5 sclerotised teeth; lacina mobilis with 4 sclerotised teeth. Both mandibles: between lacinia and molar a row of 8 longer and 1 shorter of thick, bi-serrated setae; 2 minute setae on middle

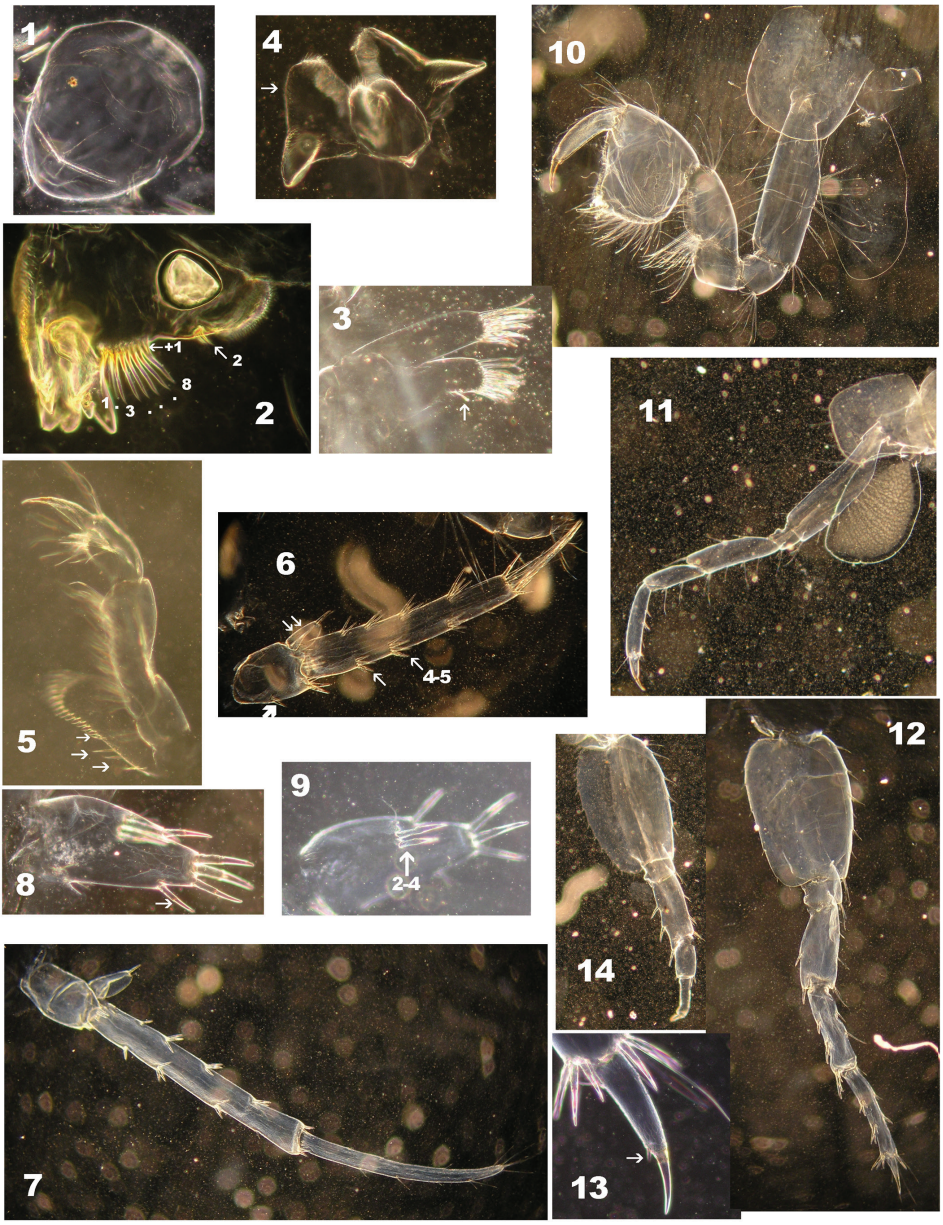

Figure 3. *N. plurispinosus* sp. n.: **1** upper lip **2** right mandible **3** second maxilla **4** labium **5** half maxilliped (without inner portion) **6** 3rd uropod (female) **7** 3rd uropod (juvenile male) **8–9** telson (one lobe of male) **10** 2nd gnathopod **11** 4nd pereopod **12** 7th pereopod **13** distal segment and 6th pereopod **14** 6th pereopod: regeneration of segments behind basis (Photo: I. Hudec). Not drawn to scale.

position of the outer molar margin (Fig. 2: mdb-b; Fig. 3: 2 ⬅); molar plate sclerotised and one longer seta at the inner base of molar. Mandibular palp three segmented: the shortest basal segment without setae; the two distal articles are of equal lengths; mid-

dle segment with 6 transverse bunches of setae (each with 2 setae) along inner margin; distal segment with 1 (basal) transversal row of 7 setae (A-setae sensu Karaman 1985) close to inner margin; four rows (each with 2 setae) on outer-central portion (B setae sensu Karaman, 1985); comb of 20–25 setae (distally increased) along inner margin (D setae sensu Karaman 1985) and 8 (10) elongate setae on distal end (E setae sensu Karaman 1985).

Maxillae I (Fig. 2: mx-1). Palpus 2-segmented, distal segment asymmetric vaulted with 14 longer terminal setae and 1 smaller seta on outer margin (near the tip); outer lobe 7 denticulated spines arranged in two rows (inner row 4 - outer row 3 teeth); spines with secondary structures (denticles) as follows: 6 uni-, 1 two-denticled; inner lobe with 3 setae (2 on tip and third one in subterminal position) (Fig. 4: 5).

Maxillae II (Fig. 2: mx-2; Fig. 3: 3). Both lobes sub-equal in size, with apical setae (inner and outer lobe) and one seta in ventral margin of ventral lobe. Dorsal margins of both lobes with fine hairs.

Labium (Fig. 2: vela; Fig. 3: 4). Larger inner lobes trapezoid-like with sub-triangular flat posterior protruding on each portion; its outer distal (⬅) and anterior-inner carries fine feather-like setae. Smaller outer lobes compact and of subovoid shape are fine serrated on distal portion.

Maxillipeds (Fig. 2: mxp; Fig. 3: 5). Inner lobe short with 7 long setae, (6 marginal- plus 1 submarginal seta) and 3 flattened spine-like setae on apical part (⬅); outer lobe reach up to ½ of 2nd segment of maxilliped palp, with 4 isolated longer setae near base and crest of 9–11 flattened spine-like setae which increase distally along inner margin and are followed by 5 longer denticulated setae along distal arc. Outer surface finely setuled. Palp 4-segmented: 1st basal, subtriangular segment with one bunch of 4–5 setae on inner side; 2nd segment, (the longest one) with 14 transversally oriented rows of setae along inner margin and bunch of 7–10 setae close to distal end (outer side) and one bunch of setae near base of inner margin; 3rd segment small sub-oval with two long setae in the middle of dorsal margin and two rows of numerous setae around dactylus; 4th – dactylus with 1 short, bent denticle and 1 short spiniform seta on 2/3 length of ventral margin and one seta in 1/3 of dorsal margin; terminal nail about 1/3 of whole dactylus.

Coxal plates (Fig.5: cx) flattened with isolated setae along ventral margin. CxI rhomboid-like, antero-ventral corner broadly subrounded; cxII–IV rectangular, angles rounded; anterior, ventral and posterior margins vaulted; cxV–VI similar with well developed anterior lobe ventrally with 4 setae along distal end (cxV) or 1–2 setae (cxVI); posterior part elongated posteriorly with two setae close to posterior ventral angle (cx V) or 4 setae along posterior margin (cxVI); cxVII reduced, trapezoid-oval plate with elongated posterior corner and one seta close to posterior end (⬅).

Gills uniformly broad, asymmetric sub-oval, reaching from 4/5 (basis II) to 1/3 (basisVI) of basis.

Pereon appendages (Figs 3, 5, 6).

Gnathopods I (Fig. 5: gp-I; Fig. 6: G). Basis – trapezoid broad (L/W ±50%), laterally flattened; a group of longer setae close to anterior ventral angle; two groups

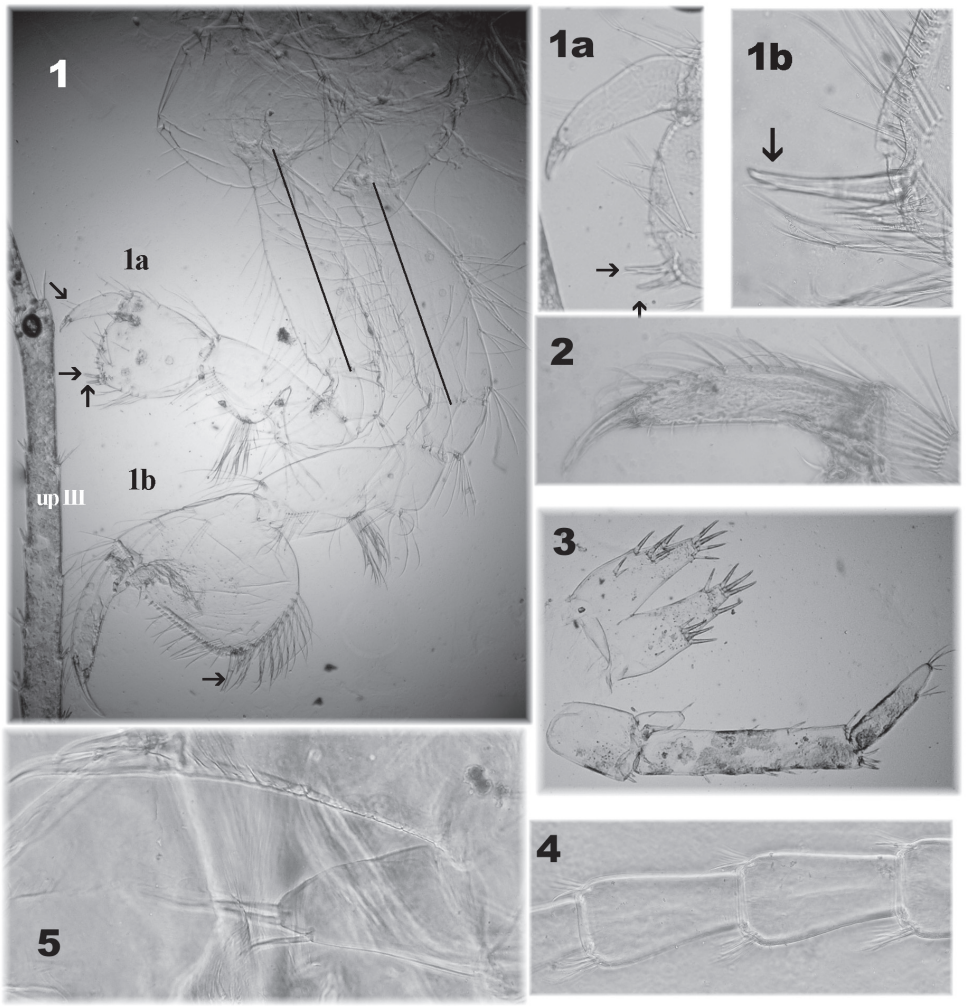

Figure 4. *N. plurispinosus* sp. n. – "postreproductive" male: **1** gnathopods with detail of deformed gpI (1a) and normal developed gpII (1a) **2** gpII -setae on dactylus **3** telson and regenerated (?) upIII **4** segment setae on flagellum of AI **5** maxillae I: position of three setae on inner portion (Photo: I. Hudec). Not drawn to scale.

of setae. Ischium – sub-quadrangular, bears 7–9 setae on posterior-distal angle. Merus – sub-angular (almost identical with ischium) bears transverse row of setae and a short row of submarginal setae near anterior-distal corner. Carpus – elongate sub-trapezoid with the longest dorsal margin; dorsal margin with 1 group of long setae almost on anterior-distal corner; ventral margin with expressive bulb in ventral base covered with expressive large setae on surface; a submarginal row of setae follows posterior margin; carpus length 60–65% of basis length and 110% of propodit length. Propodus – sub-quadrate; anterior margin with 3 transverse oriented rows of long setae (two rows along margin and third one almost on anterior-distal corner); distal margin (palm) is

convex or almost even with 4 long setae each interrupted with 2–4 minute (thorn-like) setae. Palmar corner with the bunch of 4–5 longer setae close to strong palmar spine; one long, blunt pointed, thick seta; it is followed by 3 shorter, stronger and serrated, spiniform setae on outer side and one supporting minute stout spine on inner surface. Along posterior margin 6–7 transversal rows of numerous setae are present. Two groups of tiny, setae are present on outer surface (close to ventral corner). Dactylus – long (as maximal height of propodit); along anterior margin 6-7 longer, single setae (⬅); along inner margin a row of sparse minute setae.

Gnathopods II (Fig. 3: 10; Fig. 5: gp-II). Basis – trapezoid narrow (L/W ±30%), sub-oval in transsection; sparse row of long, sub-equal setae along anterior margin and three bunches of setae on posterior margin: a) numerous, long setae on basal angle; b) few setae almost in middle position; c) few setae near posterior-distal angle. Ischium – sub-quadrangular, bears 7–9 setae on posterior-distal angle. Merus – sub-angular (almost identical with ischium), bears transverse row of setae and a short row of submarginal setae near anterior-distal corner. Carpus – elongate sub-trapezoid, its dorsal margin the longest; dorsal margin with 2 groups of setae near anterior-distal corner; ventral margin with expressive bulge in ventral base covered with expressive large setae on surface; a submarginal row of setae follows posterior margin; carpus length 70–75% of basis and 120% of propodit. Propodit– subquadrate; anterior margin with 3 transversely oriented rows of long setae (two rows along margin and the third one almost on anterior-distal corner); distal margin (palm) convex with 3–4 longer setae each interrupted with two to four minute (thorn-like) setae. Palmar corner with the bunch of 4–5 longer setae close to base of strong palmar spine; one long, blunt pointed and thick seta and 3 stronger-spiniform serrated setae on palmar corner; one supporting minute stout spine on inner surface. Along posterior margin 6 –7 transversal rows of numerous setae are present. Four groups of doubled or triplet, tiny, spiniform setae are present on outer surface. Dactylus – long (as maximal height of propodit); along anterior margin 6–7 longer, single setae (⬅); along inner margin a row of sparse minute setae.

Propodits of both gnathopods sub-equal in size, the second one slightly larger. Compared to body size, gnathopods are small.

Pereopods III-IV (Fig. 3: 11; Fig. 5: ppIII, ppIV). Both subequal in morphology and size. Secondary spines of each segment have unknown taxonomic value. Distal ends of each propodus with 2 expressive long and 4 shorter seta-like thorns on anterior corner; 2 shorter seta-like thorns and 2 stout thorns on ventral corner correspond with pereopods V. Dactyli III-IV each with long nails (up to 30-40% of dactylus length) with dorsal plumose seta in the proximal third of anterior margin of the article, and one tiny spiniform thorn near the nail base. Spiniform spine is slightly bent to dactylus.

Pereopods V-VII (Fig. 3: 12–13; Fig. 5: ppV – ppVII). Sub-equal in morphology but different in length. Ratio of pereopod V-VII length = 1.0/1.4/1.5, where length of 5th pereopod is almost equal to that one of ppIII and ppIV. Bases V–VII elongate-oval (⬅), with convex anterior margins and almost straight posterior margins (⬅), all almost without ventro-distal lobes; length/width ratio = 1.00/0.55–0.65; L-ratio of bases = 1.0/1.2/1.25. Along anterior margins 4–5 slender spiniform setae and one

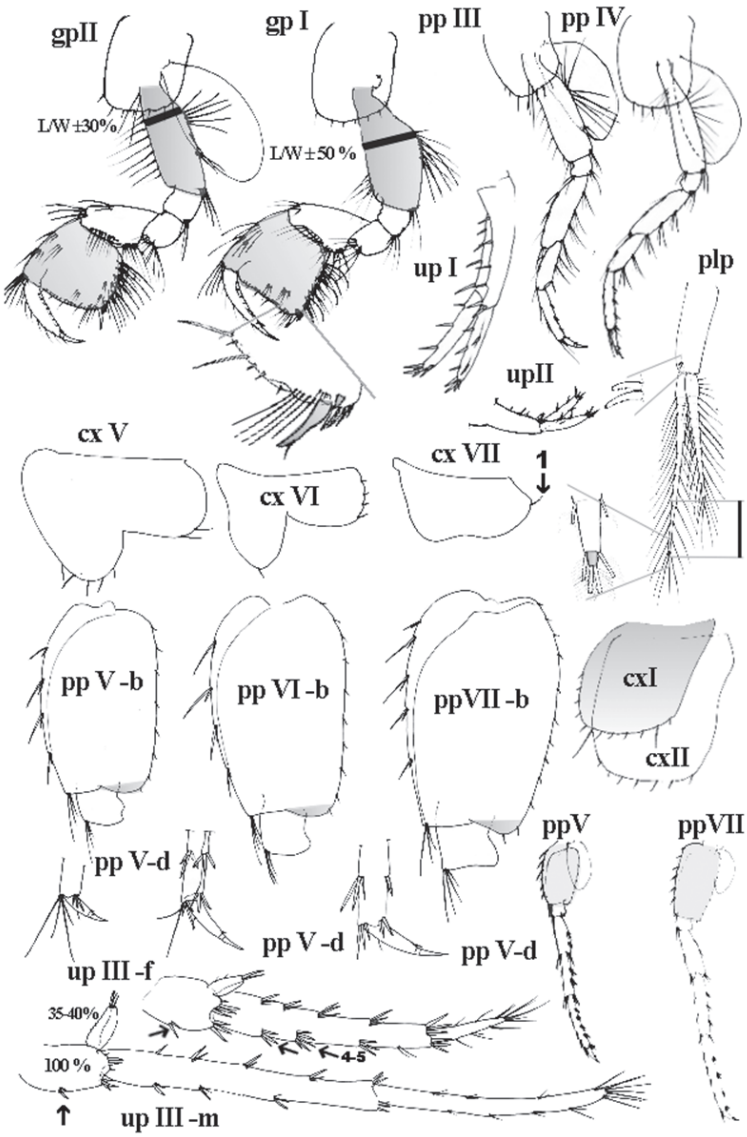

Figure 5. *N. plurispinosus* sp. n.: **gpI** 1st gnathopod; **gpII** 2nd gnathopod; **ppIII–ppIV, ppVI–ppVII** 3rd to 7th pereopods; **ppV-b – ppVII-b** bases of 5th to 7th pp; **ppV-d – ppVII-d** distal part of 5th to 7th pp; **plp** 2nd pleopod; **upI** and **up II** 1st and 2nd uropod; **upIII-f** 3rd uropod of female; **upIII-m** 3rd uropod of male; **cxI-cxVII** 1st to 7th coxal plate. Remarks: shadow colour was used to emphasise of important character. Not drawn to scale.

bunch of setae-like thorns on antero-ventral corner; along posterior margins 8–11 small setae. Distal ends of propodit V–VII with characteristic combination of long setae-like thorns and stout thorns on each pereopod: V- equal to pereopods III-IV; 6th VI-with 2 slender setae-like thorns and 2–3 stout thorns on anterior angle and 2 stout

thorns on ventral corner; 7th with 2 slender setae-like thorns and 2–3 stout thorns on anterior angle and 2 stout thorns on ventral corner. Morphology and setal patterns of dactyli V-VII are identical to those in dactyli III-IV, however the length of each nail can be variable, probably it is the result of their break or mechanical wear out.

Pleosome section (Figs 2, 5)

Pleonites I–III (Fig. 2: epI-epIII). Each composes from two different parts: dorsal and ventral part with epimeral plates on each side. Dorsal part of all pleonites with distinct anterior margin (minute hump on anterior part of distal angle); 6-8 fine setae along dorsal margin (under cover glass it is a part close to the posterior-dorsal corner). Sub-rounded, ventral epimeral plates are clearly distinguished from dorsal part. Epimeral plate I (epI) anterior-ventral corner narrow vaulted forms blunt angle with ventral margin; ventral margin convex, broadly vaulted (without thorns); posterior-ventral corner convex and broadly vaulted. Along posterior margin 5-7 setae (the first one is the longest). Epimeral plate II (epII): anterior-ventral corner broadly vaulted; ventral margin slightly convex with 2 submarginal stout thorns; posterior-ventral corner broadly vaulted. Along posterior margin 5-7 seta-like thorns (the fist one is stouter and the longest). Epimeral plate III (epIII): anterior-ventral corner broadly vaulted; ventral margin slightly convex with 3 submarginal thorns; posterior-ventral corner angular or perpendicular (⬅) with blunt tip. Along posterior margin 5-7 seta-like thorns (the first one is slightly longer).

Pleopods I-III (Fig. 5: plp). Uniform: each with smooth tubular protopod and two retinacules on distal end; two rami (longer one with 13 articles; shorter one with 15 articles). Each segment bilaterally setuled on distal end, except for the proximal segment. First basal segments 3 to 4-times longer than next segment with row of 4 to 5-times shorter setae on outer margin on shorter arm and smooth on longer segment. The most distal segment is minute and conic.

Urosome section (Figs 2, 3, 5, 7, 8).

Urosomite I (Fig. 2: 1-1a,1b) posterior-dorsal corner with 1 weak submarginal seta; ventrally 1 short, slender thorn near insertion of uropod I. Urosomite II posterior-dorsal corner with 2 submarginal spiniform thorns (one subtile and one stouter). Urosomite III without setae.

Uropods I-III: UpI and upII are morphologically similar but the first one is almost twice as long as upII; upIII is sexually dimorphic.

Uropod I (Fig. 5: upI; Fig. 7: 7). Protopodit without flap on its ventro-distal end; it is longer to both, subequally long distal rami (±1.1–1.2) protopodite bears 9 thick dorso-lateral spines, arranged in two rows (5+4). The endopodite is longer and rod-like. However with age the exopodite is gradually enlarged and transformed into a club-like structure (Fig. 4); both bear spines arranged in two rows; longer flexibile setae are on distal half; 5 thick spines of different size (two longer) on distal end. Uropod II (Fig. 5: upII): length of endopodite is 1.05–1.15 of length of exopodite and both are shorter to basipodite.

Uropod III - male (Fig. 5: upIII-m; Fig. 7: 4). Up to 35-40% of body length (all following measures are valid for adults). Base sub-oval, short (= 1/4 L of basal segment

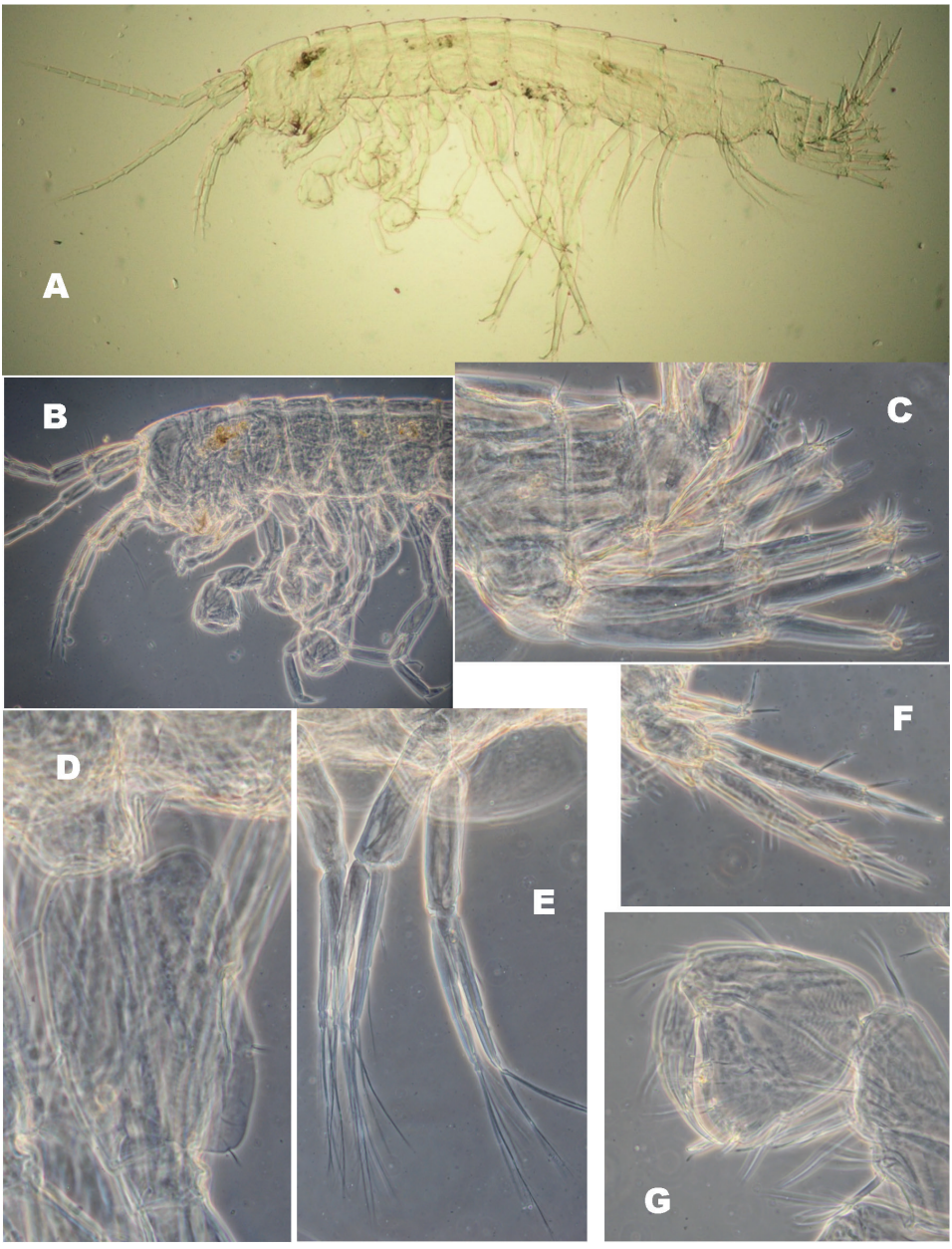

Figure 6. *N. plurispinosus* sp. n. neonate: **A** general view **B** head and anterior part **C** 1st and 2nd uropods **D** basal segment of 7th pereopod **E** 2nd pleopod **F** 3rd uropod **G** 2nd gnathopod (Photo: I. Hudec). Not drawn to scale.

of exopodite) with numerous, grouped spines along distal margin (around base of exopodite) and 2 stout spines in the middle of ventral margin (⬅). Short endopodite (up to 35-40% of base L (⬅)) bears 2–3 short thorns on distal end and 1–2 minute spines

on outer lateral margin. Two-segmented exopodite rod-shaped; basal segment slightly shorter (90-95%) to distal segment; basal segment with 4 groups of spines along ventral margin and 5 groups of spines along dorsal margin; distal segment with 4 groups of longer setae along ventral margin and 3 tiny setae along dorsal margin; distal portion with 4–6 clusters of long setae.

Uropod III - female (Fig. 3, Fig. 5: upIII-f, Fig. 8) robust, shorter then in male; base suboval (1/4 of basal exopodite segment) with two spines on ventral margin (⬅). Moderate short endopodite (up to 45% of base length) bears 2–3 short thorns on distal end and 1-2 minute spines on outer lateral margin. Two-segmented exopodite conical, narrowing distally. Basal segment 3-times longer than distal segment. Basal segment with 9 groups of spines (5 along dorsal- and 4 along ventral margin). Other group of thin longer spines on ventral margin (close to distal end). Distal segment only with groups of longer setae-like spines.

Telson - both sexes (Fig. 2: telson, Fig. 3: 8–9; Fig. 8: A–G) angular, length/width = 1.1–1.2, with deep cleft 80–90% of telson length (⬅); lobes narrowing distally, almost even in terminal part. Apical telson spines relatively short (35–40% TL) (⬅) where distal lateral flagellum protruding over the terminal end of spine. Spine position (per lobe): 3-4 terminal (apical) spines; 1(2) outer lateral spine and 1 inner lateral (mesial) spines in ¼ TL (from distal end). 2–3(4) dorsal spines are arranged in one transverse row (⬅), situated more-less in ½ TL; however the first (outer) dorsal spine is probably outer lateral spine (Fig. 8: F). Pair of plumose setae inserted mid-laterally. 2–4 thin, relative long, spines just below cleft. One slender, supporting dorsal spine which can be found before dorsal thorns was recoded in the one largest male (Fig. 8: F ⬅).

Variability. The main problem when identifying *N. plurispinosus* (and probably in all species of the *Niphargus* genus) is to distinguish principal characters sensu International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999) from "characters" which are the result of individual variability based on: a) ontogenesis and expressive heterochrony (Fišer et al. 2008a); b) individual condition, especially by subadults and adults; c) partial predation (or cannibalism? Fig. 7: 4–2) and following regeneration of body appendages; d) influence of environmental variables.

a) Ontogenesis and heterochrony. We identified expressive differences in external morphology among neonates (L up to 4 mm), first developing stage (L up to 6 mm), subadults (about 12 mm by both sexes), adults (L ≤ 15 mm in females, 16 mm in males) and the absolutely largest specimens (L >17 mm in females, 20 mm in males). Without knowledge of the comparative morphology at least of first stages, adults (described above) and the largest (postreproductive?) specimens of *N. plurispinosus* it is quite easy to identify them as 2–3 separate co-existing subspecies.

Basic difference for the neonate and (or) the first stages of *N. plurispinosus* are:

1. Head is large relative to BL (Fig. 6: A–B). It bears shorter A1 (25–30 % of BL - because of lower number (8–10) of flagellar segments); A2 reach up to 60 % of A1 and bears 4–5 longer segments in flagellum. 2. Gnathopods: both gnathopods with modified subquadrangular propodits with 2-3 transverse rows of few setae along ventral margin (Fig. 6: G); number of transverse rows increase with the individual size of

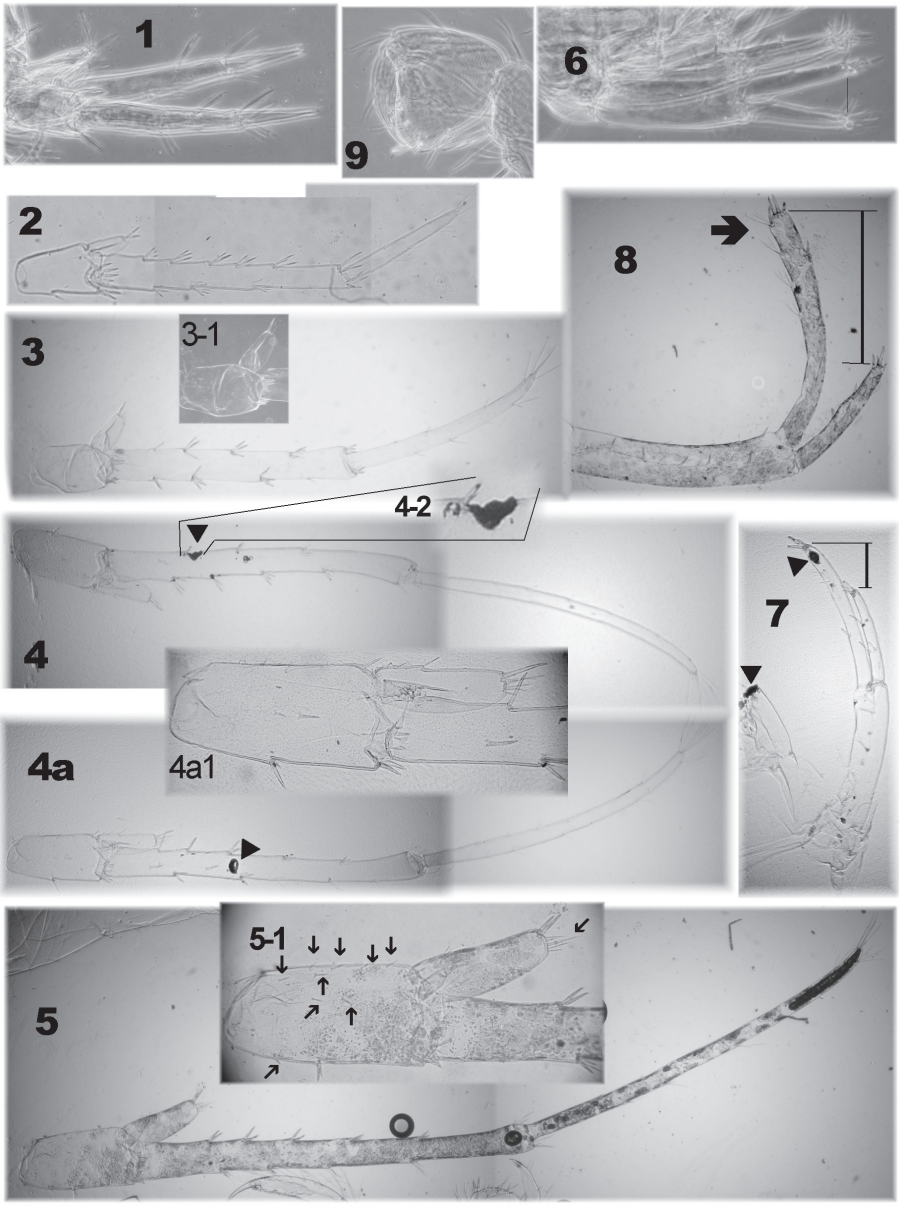

Figure 7. *N. plurispinosus* sp. n. – male: ontogenetic transformation of upI (**6–8**), upIII (**1–5**) with detail of endopodite (**3–1, 4a1, 5–1**) and presence of black-brownish callus (▼) after extraction and wounding of extremities, or after a bite (**4–2**) **1, 6** neonate **2–3** juvenile **4, 7** adult **5, 8** postreproductive male **9** gpII of neonate (Photo: I. Hudec). Not drawn to scale.

specimens (both as the number of setae in rows). Dactylus with only 2-3 setae along outer margin. (⬅) 3. Pereopods V-VII: Bases V–VII oval with expressive ventro-distal lobes, with 2 slender setae along anterior margin and 4-5 relative long setae along

posterior margin (Fig. 6: D). Size of all three bases elongated but ventro-posterior lobe decrease with age; setae along anterior and posterior margins increase in number with size. 4. Telson bears only 3 apical spines, 2 dorsal spines, and one outer lateral spine more-less in mediate distance between apical- and dorsal spines on one lobe (Fig. 8: A-B). 5. Pleopods I -III: protopods without retinacules and 4-5 elongate articles in both rami (Fig. 6E). 6. Uropod I: exopod and endopod are equal or subeqaul in size; both with small number of spine-like setae (Fig. 6: C; Fig. 7: 6). 6. Uropod III: no sexual dimorphism at this size; (⬅) all specimens resemble females with reduced number of spines (Fig. 6: F; Fig.7: 1). The sexual dimorphism is expressed in later (juveniles stages and preadults) as a continuous elongation of distal segment of exopodite (Fig. 7: 2-3) up to same length to basal segment by adults (Fig. 7: 4).

Similar patterns of postembryonic differentiation were noted also in *N. aggtelekiensis* and *N. tatrensis* (Hudec and Mock 2011). Neonates and small juveniles are often the only specimens found in sublittoral of different types of streams. This is the reason why it is important to know the morphology of juveniles.

The largests males (over 20 mm) and the largests females (17 mm), bear extreme cases of setation. Some character may change of in the following characters:

1. First antenna comparatively shorter to BL (up to 30 %); flagellum with ≤23 segments each segment with few minute sensilla but without bi-segmented aesthetascs (Fig. 4: 4). 2. Gnathopods (Fig. 4: 2): dactylus bears up to 12 single and doubled long setae (⬅) along outer margin; 3. Uropod I (Fig. 7: 8): exopod and endopod are extremely different in size (⬅); endopodite is markedly long (more than 2-times longer than exopodite) of club-like shape with reduced number of thick setae and multiplying longer setae which are restricted to the distal half of endopodite. This type of uropod resemble *N.* cf. *stygius* (Schiodte, 1847) (Fišer et al. 2009b) or *N. timavi* S. Karaman, 1954 (Karaman 1954, http://niphargus.info) and is different to adult *N. plurispinosus* (Fig. 7: 7). 4. Uropod III - male (Fig. 4: 3): up to 35% of body length. Base short tubular (> 0.14 L of basal segment of exopodite) with numerous spines on lateral surface (Fig. 7: 5–1 ⬅). Endopodite relatively long (up to 65% of base L (⬅)) bears numerous (7-9) short spines on distal end (Fig. 4: 5-1) and 2–3 minute spines on outer lateral margin. Two-segmented exopodite is rod-shaped; both segments are of the same size; basal segment armature: 5 groups of short spines along ventral margin and 6 groups of spines along dorsal margin (more like adults); distal segment with 5-6 groups of longer setae along ventral margin and 3 tiny setae along dorsal margin (more like adults); distal portion with 4-6 clusters of long setae-like spines. 4. Telson - at the largest individuals of both sexes (Fig. 8: I-J): angular, length/width = 1.1–1.2, with deep cleft ± 80% of TL (⬅); lobes narrowing distally, almost even in terminal part. Apical telson spines relatively short (20% TL) (⬅) where flagellum with lateral and more distal position is not protruding over the terminal end of spine. Spine position (per lobe): 3-4 terminal (apical) spines; 2 outer lateral spine and 1 inner lateral (mesial) spine; 3-4 dorsal spines (⬅), situated ±½ TL. Pair of plumose setae inserted mid-laterally. 1-2 spines supporting dorsal spine can be found before dorsal thorns in the largest male (Fig. 7: I-J). 2-4 thin spines just below cleft. Such plurispined telson partially resembles several

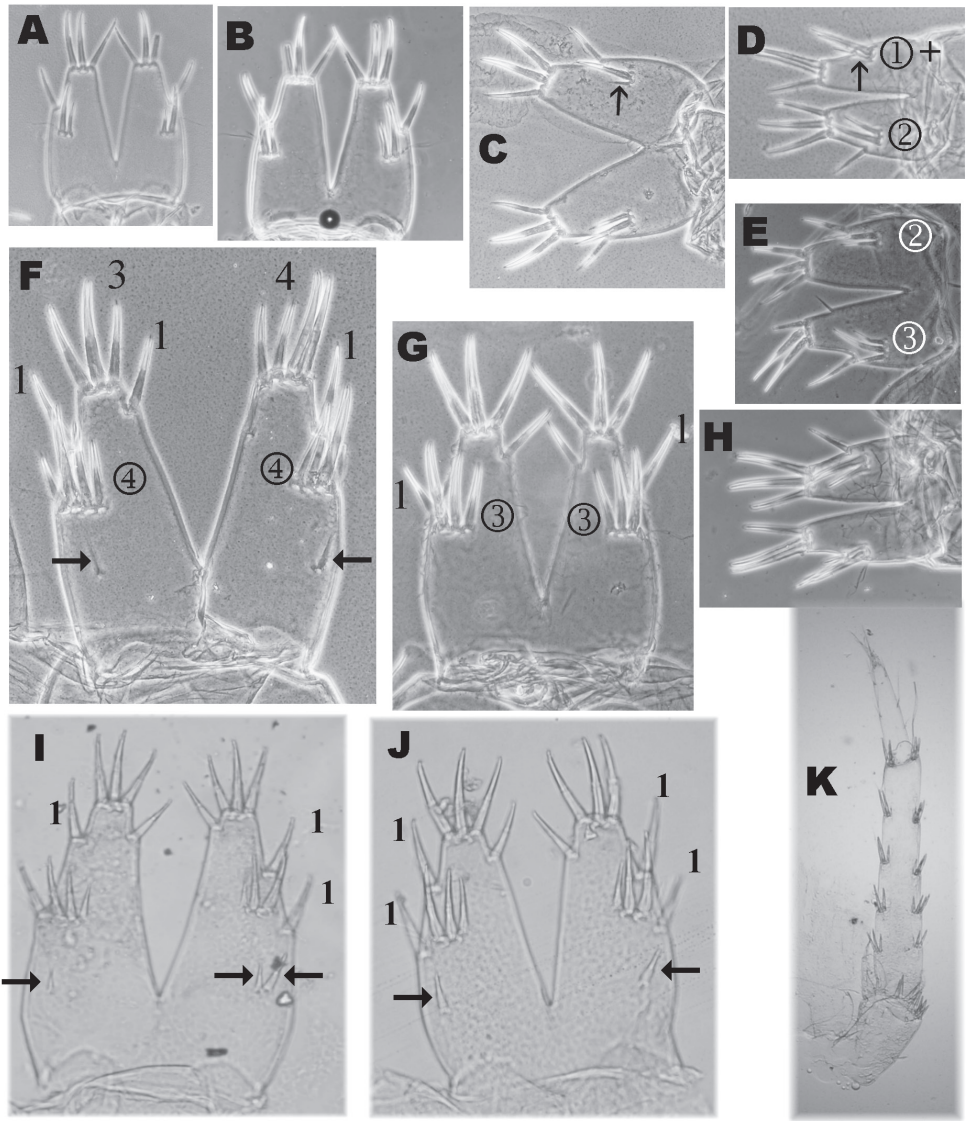

Figure 8. Variability of telson setae of *N. plurispinosus* sp. n.: **A–E, H** juveniles (males and females) **F** adult male **G** adult female **I** postreproductive male **J** postreproductive female **K** 3rd uropod of female (Photo: I. Hudec). Not scaled.

West Balkan species (*N. illidzensis* Schäferna, 1922, *N. hvarensis* S. Karaman, 1952, *N. kenki* S. Karaman, 1952) or *N. timavi* (Fišer et al. 2009b). All of the other characters correspond to that of adults.

Specimens in probably postreproductive stage were recorded only once (11 May 2013).

b) Individual condition. The number of setae on all appendages changes with ontogenesis (Fišer et al. 2008a). However the number of setae (e.g. pleopods, pereopods,

upI, upII), or of setal groups (e.g., on up III), evidently correspond to the individual fitness. Therefore they can be variable in specimens of same size (stadium). Also varying number of lateral setae on telson can account to the individual condition.

c) Regeneration or degeneration. We found that damaged extremities and body parts form blackish calluses within 12 hours of being damaged. The calluses persist after preservation and their colour is stable after the lightening process in different media (Fig. 7: 4-2, 7▼). An expressive case of regeneration was observed in a single specimen (subadult male) on the 5th pereopod (Fig. 3: 14): base normally developed, but the rest of the limb was extremely reduced. Specimens with different number of segments on AI- and AII-flagellum and males with different upIII might be probably considered as different stages of regeneration.

On the other hand the largest males (over 20 mm) were commonly found with different asymmetric gnathopods (Fig. 4:1, 1a, 1b, 2), different up III (Fig. 4: 3) damaged AI- and AII-flagellum (reduced number of segments very often without aesthetascs on AI, Fig 8: 4). However we are not sure if these damages are caused by attack, or age, or any kind of degeneration. Predatory behaviour and cannibalism were already observed in some niphargid amphipods (Fišer et al. 2010c, Luštrik et al. 2011).

Remarks on biology. The abundance (=number of animals per visit, when we collected all specimens) of *N. plurispinosus* in the surface water near the spring ranged from 6 to 68 specimens in 2012 (227 ex. for the whole year). However, less than 8 % of all specimens were adults (12 males, 2 females with eggs and one female with embryos). Ovigerous females were recorded only in spring (March - May). Neonates were reported in June (15 inds.) and July (1 ind.). Juveniles and subadults prevailed in all samples and they were recorded throughout the year (Fig. 9: A). It looks as they leave subsurface actively to search for food in surface water. They were permanently found in the surface water throughout the year without sign of disfunction in mobility or presence of dead specimens. The only exception was in May 2013 of accumulated dead and dying animals. This may have resulted from water fauna poisoning when the owner of the reservoir disinfected the drinkable water by using chemicals with chlorine. Such events seriously endangered the stygophile fauna (Fišer et al. 2010b).

Light-yellow eye spots were seen in a few living adults, but we found no rudimentary eyes. These spots were smaller than those in *Synurella ambulans* (Müller, 1846). The yellow pigment disappeared within 2 days of preservation in alcohol.

All specimens of *N. plurispinosus* were found in the shallow ditch low flow volume, up to 15 m away from the source; depending on age. Only juveniles (up to 8 mm of length) were found at the maximum distance (adults up to 5 m; subadults up to 10 m). Water temperature of the spring varied from 10.5°C (January 2012) to 13.8°C (September 2012) (Fig. 9: B). The distribution of specimens along the channel correlates with the water temperate gradient along the channel (Fig. 9: B), suggesting that water temperature over 17°C (July) may be a limiting factor for juveniles. This is in agreement with the known biology of hypotelminorheic habitats (Culver et al. 2006).

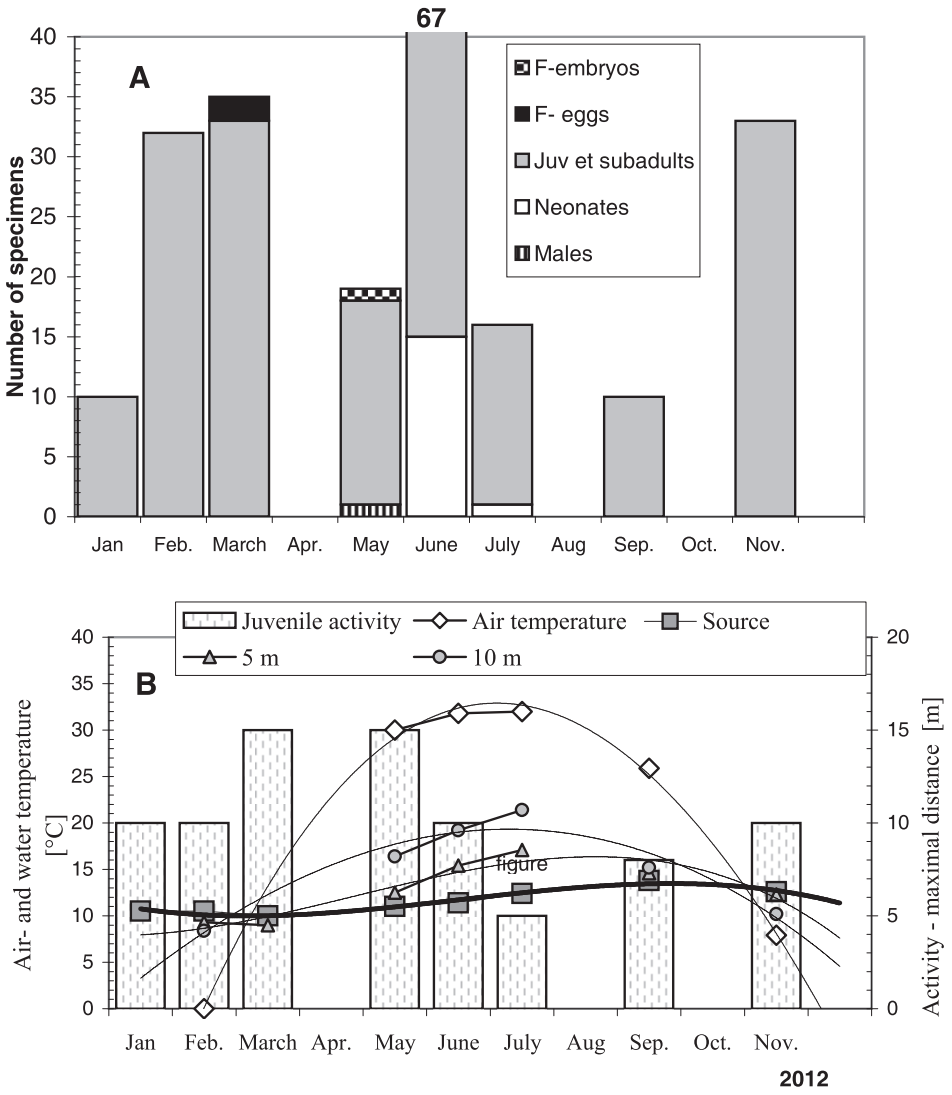

Figure 9. Relative abundance (**A**) and moving activity (**B**) of *N. plurispinosus* sp. n. in relation to temperature gradient in the artificial channel of type locality in 2012. Air and water temperatures were measured during collecting of amphipods (around 2.00pm). Notes: F-eggs = females with eggs, F-embryos = females with embryos. Source, 5 m, 10 m = water temperature at various distances from the spring (source).

N. plurispinosus was the only amphipod species found in the locality. It was collected only from a narrow (width = ± 10 cm) artificial drainage ditch, which originates from the small seep spring in the meadow (Fig. 2) and after 200 m it flows through the underground tube into a small stream with dense population of *Gammarus balcanicus* Schäferna, 1922. Such a semi-artificial locality can be considered as a unique natural laboratory for the study of biology of subterranean species that penetrate the surface.

Comparison to morphologically similar species

The high number of dorsal spines on the telson lobes is typical for the new described species. But in the determination of the species from the genus *Niphargus* at least three additional characters need to be checked. In *N. plurispinosus* these three additional characters are: small gnathopods, sexually dimorphic uropod III (adults and postreproductive stages) and sexually non-dimorphic uropod I in juveniles, but different in adults, and extremely different in postreproductive stage. For comparative purposes, we list three groups of species that share at least two out of the three traits mentioned above.

The first group consists of species with small subequal gnathopods and sexually dimorphic uropod III, but also sexually dimorphic uropod I. Several species sharing this combination have all or some of dactyls of pereopods III-VII armed with at least two spines (e.g. Karaman 1973), but there are also some species without such dactyls *N. krameri* Schellenberg, 1935 (Karaman 1984), *N. spinulifemur* S. Karaman, 1954 (Karaman 1984), *N. hadzii* Rejic, 1958 (Rejic 1958), *N. spoeckeri* Schellenberg, 1933 (Schellenberg 1933, Karaman 1993), *N. vinodolensis* Fišer, Sket & Stoch, 2006 (Fišer et al. 2006) or *N. timavi* (Karaman 1985), differing in sexually dimorphic uropod I. The presence of a single large individual with sexually dimorphic uropod I indicates some similarity with this group of species. The additional difference between these species and *N. plurispinosus* is the setal pattern on the dactylus of gnathopods I-II (single seta in *N. plurispinosus*, several setae in the above listed species).

The second group consists of species with sexually non-dimorphic uropod I, sexually dimorphic uropod III and unequal gnathopods, where the second propodus is large and its size largely exceeds the size of the first gnathopod: *N. stygius* Schiödte, 1847 (Sket 1974), *N. costozzae* Schellenberg, 1935, *N. montellianus* Stoch, 1998, *N. tridentinus* Stoch, 1998 and *N. lessiniensis* Stoch, 1998 (see Stoch 1998) and species from *N. tatrensis* complex (Fišer et al. 2010a).

Finally, a species that share all three aforementioned traits is *N. sphagnicolus* Rejic, 1958. The difference between *N. sphagnicolus* and *N. plurispinosus* includes the setal pattern on gnathopod dactyli I-II (single seta in *N. plurispinosus*, several setae in groups in *N. sphagnicolus*); number of spines at the base of uropod I (1-2 in *N. sphagnicolus*, only one in *N. plurispinosus*), lower number of submarginal ventral spines at epimeral plate III (3 in *N. plurispinosus*, 4-5 in *N. sphagnicolus*) and higher number of setae in maxilla I palpus (up to 9 in *N. sphagnicolus* and 14 in *N. plurispinosus*).

Final remarks

A detailed morphological description of *Niphargus plurispinosus* sp. n. based on large annual samples is presented. This is the first new species from Central Europe for several decades, showing that even this long-investigated territory has higher diversity

potential than was supposed; similar situation is also in some other Carpathian regions (Meleg et al. 2013). The precise systematic position of the species is unclear.

There is a very low probability (less than 10%) that all stages will be obtained in one sample. It seems that for the description of a new *Niphargus* is necessary to obtain:

1. One abundant sample (dozens specimens of different size - including juveniles and adults); such abundant and heterogenous sample is almost impossible to obtain except spring, in such cases as studied in *N. plurispinosus*.
2. One long series of samples (1 year) from one locality, collected monthly.

When describing new species it is very important beside the adult to describe also neonate and if possible also largest specimens in post-reproductive (senior) stages. The principal problem is expressive heterochrony (Fišer et al. 2008a) and how to identify adults. It seems that the key-stone in identification are the females with eggs or embryos. The presence of pleopod retinacules is typical also for subadult that can be found throughout the year. Also the length of distal exopodite segment is enlarged with age. The ratio of both exopodite segments (basal to distal) rise from 0.4 (juveniles) to 1 (adults and seniors). It is necessary to be careful especially at communities with two or more co-existing species of the same genus (cf. Angyal and Balász 2013, Karaman et al. 2010). Maybe for the better understanding of the phenology and life cycle of *Niphargus* spp. it will be useful to distinguish adult stage of females by the presence of oostegites and adultery of males by the presence of genital papillae.

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