RESEARCH ARTICLE

A new species of *Troglobius* (Collembola, Paronellidae, Cyphoderinae) from a Brazilian iron cave

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Abstract

The genus *Troglobius* (Collembola: Paronellidae: Cyphoderinae) is known only from the Southern Hemisphere, the two species found so far were described from caves in Madagascar and Brazil. In this paper we describe a new Brazilian species and complement the descriptions of the previously known species. The new species is a troglomorph and was collected in a single cave, in iron formations in the state of Minas Gerais. Brazil. The original diagnosis of the genus is supplemented with new characters based on observations of the new species.

Keywords

Cave Fauna, Iron Ore, Iron Caves, Taxonomy, Southern Hemisphere

Introduction

The genus *Troglobius* Palacios-Vargas & Wilson, 1990 comprises two species, both from caves in Southern Hemisphere, the type species *T. coprophagus* from Madagascar and *T. brasiliensis* Palacios-Vargas & Zeppelini, 1995 from Brazil. The genus belongs

to the Collembola family Paronellidae Börner, 1913 (Entomobryomorpha) and has been assigned to the subfamily Paronellinae since its original description and in recent contributions (Soto-Adames et al. 2008).

The distribution of the genus in Brazil ranges from caves in the Amazon rain forest in Northern region, to caves in the subtropical Atlantic Forest domains in Southeastern region. Sampling in caves in iron formations are bringing new records to the distribution of the fauna in Brazilian iron caves, and important information on the generic characters of the genus. Unpublished material is still waiting for formal descriptions suggesting that the diversity of the genus in Brazil is likely to be much greater than the single described species.

The gap of information about cave fauna and cave Collembola from Africa make for the moment difficult to propose biogeographic hypothesis to explain the origin and distribution of the genus. Considering that the two new species (*T. coprophagus* and *T. brasiliensis*) are confined to caves (as it is known up to date) and isolated from one another with disjoint distribution, we can suppose that this is a relict genus with Gondwanian distribution. However, the answer will depend on the distribution and better knowledge of the African species.

This study presents a new species of *Troglobius* from iron-caves, which is described and compared with the two already described species. Furthermore, complementary morphological data is given to the description of *T. coprophagus* and *T. brasiliensis*. A new diagnosis is proposed to the genus, the taxonomy was revised and comparative analysis of the morphology of the species, with the diagnosis of subfamilies in Paronellidae resulted in new taxonomic arrangement for the genus in the Cyphoderinae subfamily.

Methodology

Specimens were collected with pitfall traps, fixed in ethanol 70% and slide mounted in Hoyer' solution. Two paratypes were dissected. Head and body chaetotaxic systems followed Soto-Adames et al. (2008) and Szeptycki (1979). Abbreviations used in the text: Ant I-IV, antennal segments I-IV; Th II-III, thoracic segments II-III; Abd I-VI, abdominal segments I-VI.

Redefinition of the genus *Troglobius* Palacios-Vargas & Wilson, 1990

Taxonomic position:
Family Paronellidae Börner, 1913
Subfamily Cyphoderinae Börner, 1913 (sensu Soto-Adames et al., 2008) comb. n.

Genus *Troglobius* Palacios-Vargas & Wilson, 1990

Type species. *Troglobius coprophagus* Palacios-Vargas & Wilson, 1990

Diagnosis. Habitus as Entomobryomorpha, eyeless, without pigment. Body with ciliated chaetae and hyaline ovoid scales. Mesothorax and metathorax not humped or bent. Ant. IV not subdivided. Labial chaetotaxy with chaetae: M1, M2, R, E and L1 present, L2 absent. Head bearing two pairs of trichobothria. Mucro elongate, four times as long, or more, than its greatest width, with many serrations along one or both dorsal lamellae. Dens without spines, anterior side covered with scales. A couple of large scale, about the same size as mucro, present on apex of anterior surface of dens. Posterior side of dens with, at least, three rows of chaetae and scales. Some (or most) scales, or thick chaetae, of internal row deeply fringed. In other rows only some distal chaetae modified. Unguis and unguiculus with winged teeth.

Remarks. The original description places the genus in the family Paronellidae Börner, 1913. Soto-Adames et al. (2008) define the subfamilies Paronellinae and Cyphoderinae as follows:

Separation between Paronellinae and Cyphoderinae is based on the presence of fringed dental scales in the latter and their absence in the former. All cyphoderines are eyeless, whereas the majority of paronellines have eyes. In addition, all cyphoderines have scales, whereas many paronellines lack these scales.

The morphology of the dental scales, the absence of eyes, the shape of mucro and the presence of large scales at the tip of dens, about the same size as mucro, indicate that the genus *Troglobius* belongs to the subfamily Cyphoderinae (with 128 species distributed among 13 genera of blind Collembola) instead of Paronellinae (with 384 species in 27 genera) as proposed by Bellinger et al. (2013).

In the new proposed diagnosis for the genus, we conclude that the labial chaeta "R" is present, normal shaped, and variable in size, while chaeta L2 is always absent. An additional lateroventral trichobothria, close to the basis of antennae is present in each side of head, this feature is also seen in *Cyphoderus*, another genus of Cyphoderinae. The genus *Troglobius* can be differentiated from other Cyphoderinae by the shape of the mucro, which has, at least one lamella serrated.
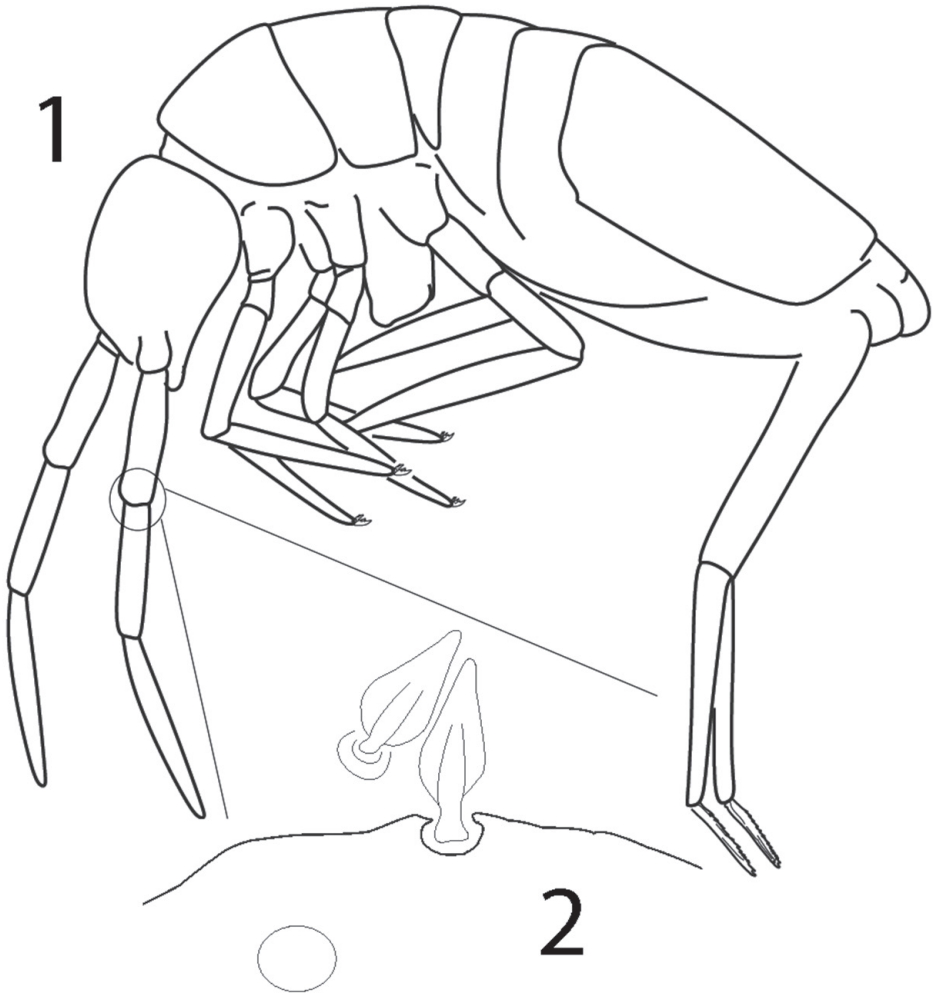
Description of the new species

Troglobius ferroicus sp. n.
http://zoobank.org/C04718C0-B0D7-42F8-9304-96D66BEDFA11
Figs 1–13

Type locality. Brazil, Minas Gerais, Itabirito, Várzea do Lopes. Cave VL29/30, 20°20'05"S, 43°56'18"W. Aphotic zone on water film over the substrate and organic matter, 8 individuals collected.

Figures 1–2. 1 Habitus, *Troglobius ferroicus* sp. n. **2** Antennal sensilla of apex of Ant. II, same type of sensilla seen on apex of Ant. III. *T. ferroicus* sp. n.

Holotype. Slide mounted in Hoyer's solution. Deposited at "Coleção de Referência de Fauna de Solo da Paraíba – Universidade Estadual da Paraíba" (CRFS/UEPB), #2766. Várzea do Lopes, Itabirito, MG, Brazil. Cave VL29/30. 02-06/x/2011. Leg. Speleology team of Carste consultants.

Paratypes. One paratype deposited at Laboratorio de Ecología y Sistemática de Microartrópodos - UNAM (slide #2837 donated from CRFS/UEPB). Várzea do Lopes, Itabitito, MG, Brazil. Cave VL 29/30. Andrade Col. 03-20/XI/2007.

One paratype deposited at Muzeu Nacional – Universidade Federal do Rio de Janeiro - MN/UFRJ (slide #2765 donated from CRFS/UEPB) Várzea do Lopes, Itabitito, MG, Brazil. Cave VL 29/30. 03/IV/2012. Other two paratypes at CRFS/UEPB, under numbers #2838 and 3098, from the same locality as the holotype.
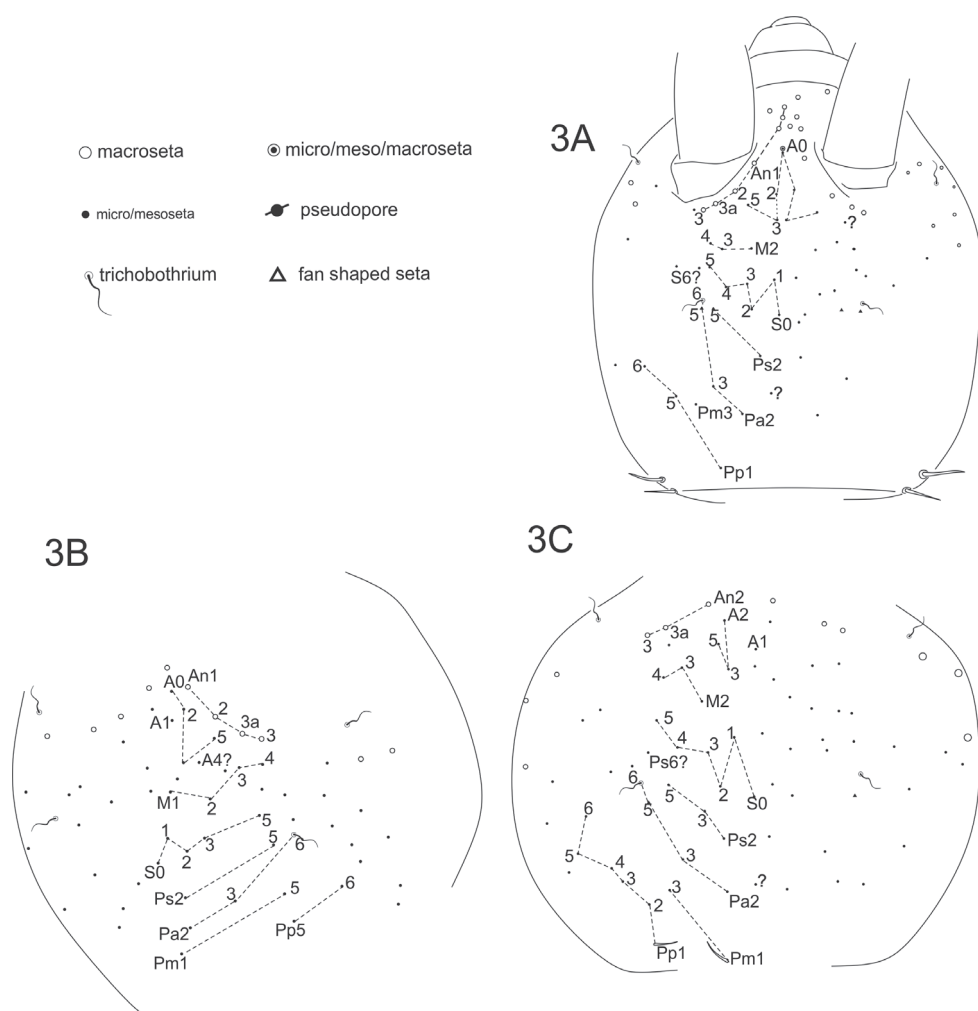
Figure 3. Cephalic chaetotaxy A (*T. ferroicus* sp. n.), B (*T. brasiliensis*), C (*T. coprophagus*).

Description. Habitus of entomobryomorphs (Fig. 1). Length (n = 4) 2.3 mm in average (1.9-2.7 mm). Color pearly white. Body with smooth and ciliated chaetae of different sizes, oval scales hyaline, trichobothria long and ciliated.

Ratio head:antenna 1:2.5, ratio antenna:body length 1:1.9. Ant. I-II with scales. Ratio of Ant. I-IV as 1: 2.6; 2.5; 3.7. Apex of Ant. II with three candle-shaped sensilla, two close to each other and a third laterally displaced (Fig. 2). Apical sensory organ of Ant. III with two candle-shaped sensilla, apical guard chaetae thicker than others. Ant. IV undivided, with rows of chaetae and sensilla or finely ciliated microchaetae, without apical bulb.

Eyes and pigment absent, head with two pairs of trichobothria (Fig. 3A), chaetae Ps5 and Pa5 ciliated. Pre-labral and labral chaetae formula 4/5, 5,4; all chaetae
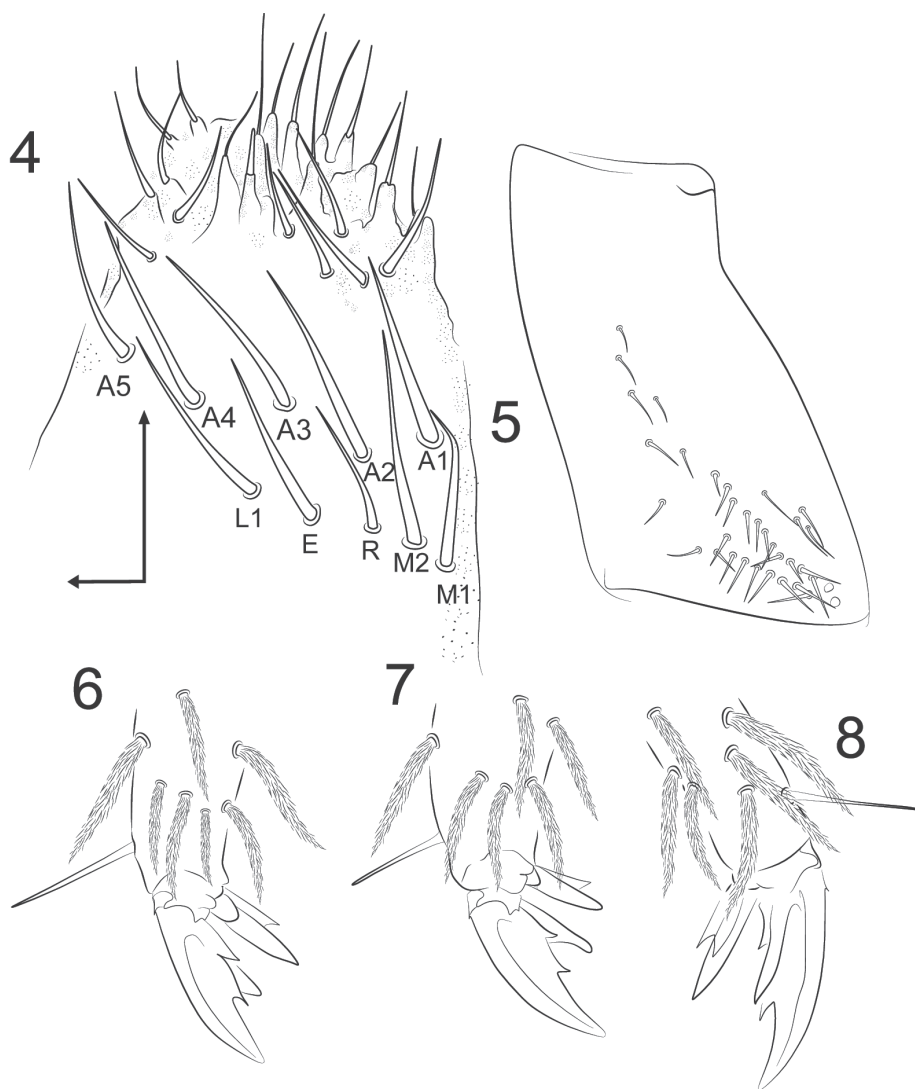
Figures 4–8. 4 Labial triangle, *T. ferroicus* sp. n. **5** Metatrochanteral organ, *T. ferroicus* sp. n. **6** Unguis I, *T. ferroicus* sp. n. **7** Unguis II, *T. ferroicus* sp. n. **8** Unguis III, *T. ferroicus* sp. n.

smooth. Labial triangle with anterior row (A1-5) smooth. All labial triangle chaetae smooth, labial seta R well developed, L2 absent (Fig. 4).

Legs with ciliated setae. Coxa of leg I with one chaeta. Metatrochanteral organ with 21 chaetae forming a "V" and 13 additional short chaetae (Fig. 5). Tenent hairs acuminate, shorter than ventral lamella of unguis (Fig. 6-8); ratio unguis: tenent hair = 1: 0.65. All unguis with two inner basal winged teeth of different sizes; one median inner tooth and one very small tooth at the base of the outer lamella. All unguiculi with well-developed outer lamella and normal sized inner lamella (Figs 6–8).
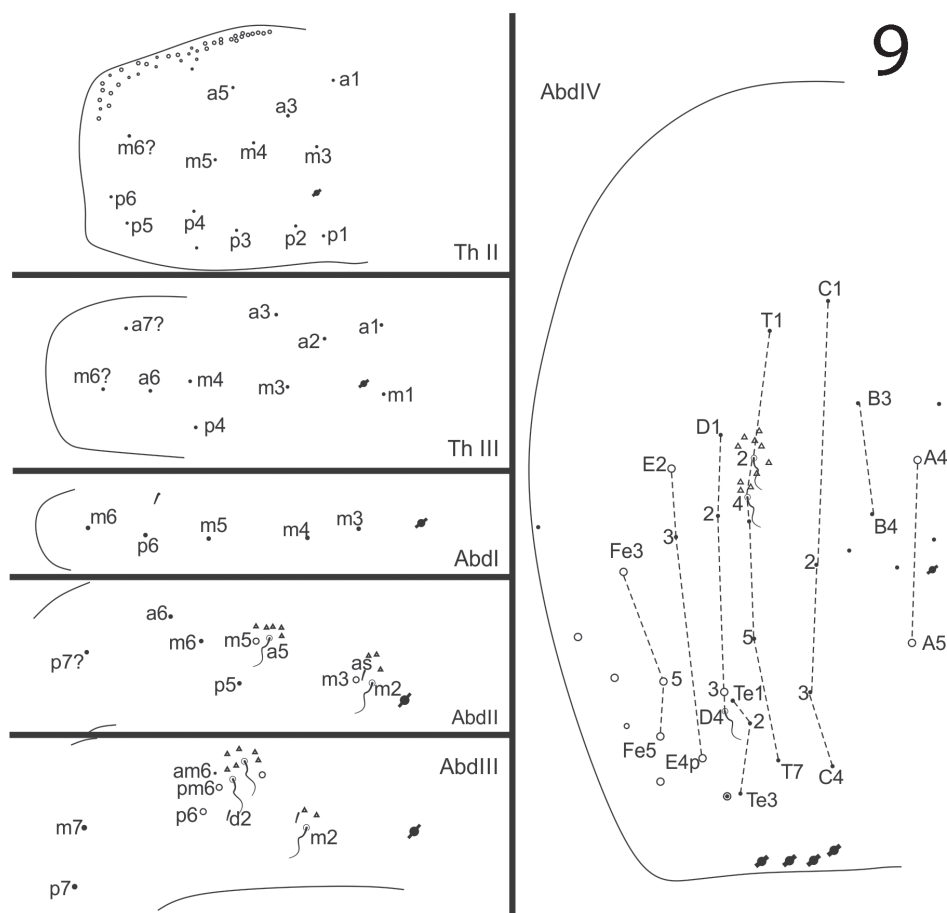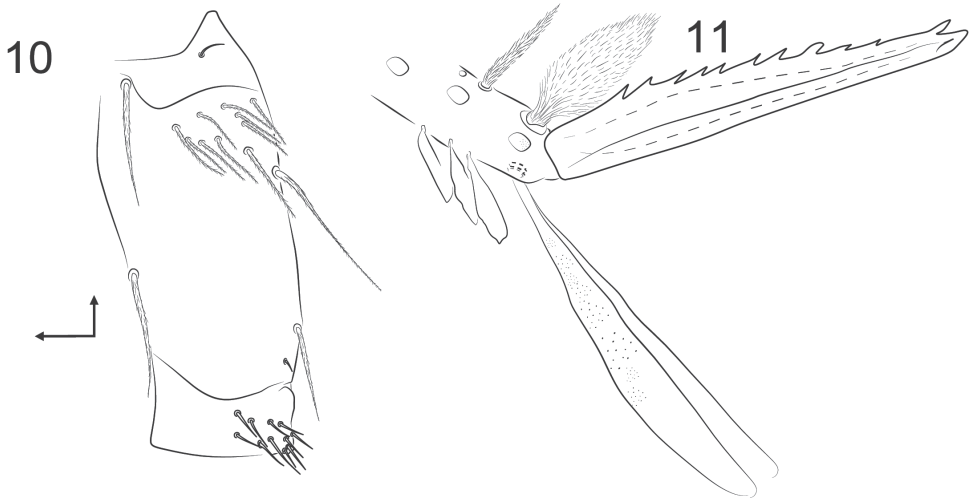
Figure 9. Body chaetotaxy *T. ferroicus* sp. n.

Abd II with two trichobothria, Abd III-IV with three trichobothria each; Abd. IV with 4+4 lenticular organs on posterior margin (Fig. 9). Posterior surface of ventral tube with 2 long macrochaetae, scales on proximal end, 10 proximal pairs of ciliated chaetae and 11 distal pairs of small smooth chaetae; anterior face with only 2 pairs of ciliated macrochaeta (Fig. 10).

Furcula with scales and ciliated chaetae; mucro, dens and manubrium ratio 1.0:3.8;4.2. The distal end of dens with a pair of large scales, about the same size of mucro (Fig. 11). Dens without spines, normal scales on anterior side, internal row of posterior face with 21 fringed scales (Fig. 12A), median row with one fringed scale (Fig. 13A). Other rows with finely ciliated chaetae in irregular pattern. Mucro elongated, bearing 10–15 teeth on outer lamella, inner lamella smooth with one tooth at the tip.

Etymology. The name of the new species *T. ferroicus* sp. n. comes from Latin *ferrum* (iron) and Greek *oikos* (house). This makes allusion to the iron caves where the new species was found.

Figures 10–11. 10 Ventral tube chaetotaxy, lateral view, *T. ferroicus* sp. n. **11** Distal end of dens and mucro, *T. ferroicus* sp. n.

Distribution. Good's biogeographic zone 27, northeastern and central Brazil (Good 1974, Culik and Zeppelini 2003). The climate is defined by dry winters and wet summers, with average temperatures of 18 °C during winter and 22 °C in summer.

Habitat. *T. ferroicus* sp. n. is found in small (less than 100 m deep) iron-caves, dwelling on water film over the rocks and organic debris in the aphotic zone. The species was found in a single cave, and it is likely to be a troglobite which lives in the in the Mesovoid Shallow Substratum (MSS) (Juberthie et al. 1980) and the adjacent cave.

Conservation status. This cave is placed in a mining area with iron ores, which represents a putative threat to the species if the cave and surrounding environments, including the MSS, are not preserved. The species was included in the recent evaluation of the Brazilian Fauna Red List, and must be published in the next issue of the Brazilian Red List as Critically endangered (CR).

Remarks. *T. ferroicus* sp. n. is the largest species in the genus, adults are 2.3 mm long in average (2-2.8 mm), while other species are about 1.5 mm long (Table 1). It has the longest antennae and ratio of head:antennae (Table 1). Ungual internal unpaired teeth are lacking in *T. coprophagus*, there are two teeth in *T. brasiliensis* and a single tooth in *T. ferroicus* sp. n. Mucronal serration is different in *T. brasiliensis*, where both edges are serrated, in *T. coprophagus* and *T. ferroicus* sp. n. the inner edge is smooth. One important diagnostic feature to differentiate the species is the relative size of mucro and dens, the ratio mucro/dens in *T. ferroicus* sp. n. is 1:4.0, in *T. brasiliensis* is 1:3.0 and *T. coprophagus* 1:2.5. Number and shape of the dental scales can, also, easily differentiate the species (Figs 12–13). Chaeta m2 on Abd. I is lacking in *T. ferroicus* sp. n., and present in the other two species, and chaeta T1 on Abd. IV is present in *T. ferroicus* sp. n., but absent in the other two species.
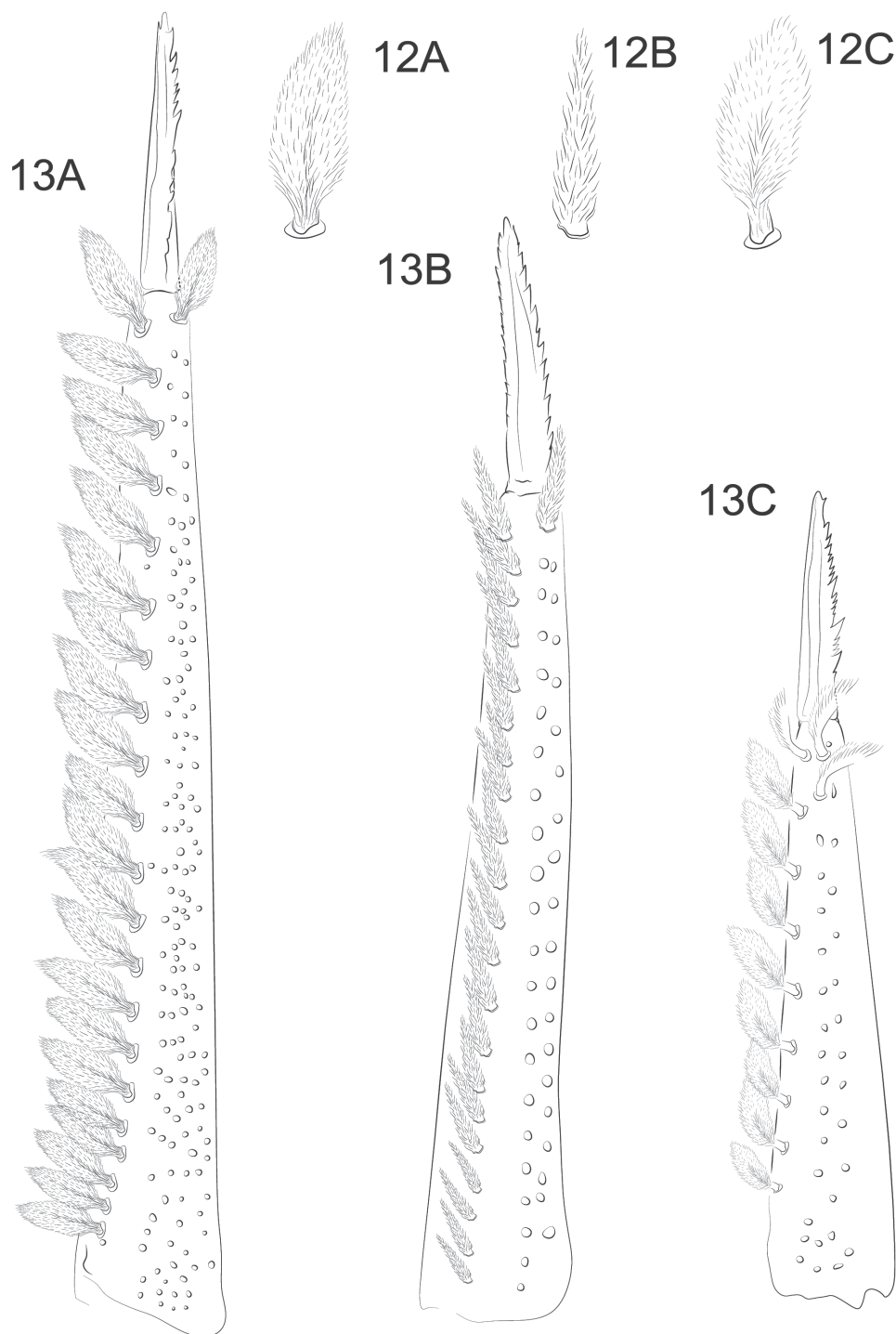
Figures 12–13. 12 Dental chaetae A (*T. ferroicus* sp. n.), B (*T. brasiliensis*), C (*T. coprophagus*) **13** Dens and mucro A (*T. ferroicus* sp. n.), B (*T. brasiliensis*), C (*T. coprophagus*).

Table 1. Comparative measurements and ratios of the three species of the genus *Troglobius*.

	Size	Ratio head/Ant	Ratio body/Ant	Ratio Ant segments	Ungual teeth	Ratio mucro/dens:man
T. brasiliensis	1.5	1:1.8	1:1.7	1:2.5; 2.5; 3.0	2	1:3.5;4.2
T. coprophagus	1.4	1:1.7	1:2.3	1:1.2; 5.6; 13.1	0	1:3.4;5.7
T. ferroicus sp.n.	2.3	1:2.5	1:1.9	1:2.6; 2.5; 3.7	1	1:3.8;4.2

Finally, *T. coprophagus* was found in La grotte d'Andrafiabe (25°C and 100% of humidity) in Ankarana, which is a small limestome massif in the extreme North of Madagascar. *T. brasiliensis* was found in Cave Limoeiro at Pará, North Brazil and Gruta dos Paiva at Sao Paulo, Southeastern Brazil, both in limestone massifs. The new species *T. ferroicus* sp. n. was found in one iron cave in the state of Minas Gerais, with very different climate and environmental conditions.

Complements to the description of previous species

Troglobius brasiliensis Palacios-Vargas & Zeppelini, 1995

Remarks. Head chaetotaxy as Fig. 3B. Apex of Ant. II and Ant. III with small candle shaped sensillae. Dens anterior side with hyaline scales; posterior side with three rows of modified thick chaetae (Fig. 12B), internal row with 21 fringed chaetae, the distal chaeta of median row is also heavily fringed, external row with about 20 barbulate chaetae (Fig. 13B). A large chaeta, about as long as mucro, is present at the distal tip of the dens. Mucro with 12–14 teeth on inner lamella and 14 in outer lamella. Body chaetotaxy as in Fig. 14.

Material examined. Paratype, Brasil, Sao Paulo, Iporanga. Cave Gruta dos Paiva. col. Trajano, E. 20/II/1989.

It is important to mention that the species *T. brasiliensis* was described from specimens collected in caves in Northern Brazil. The type locality of this species is Cave Limoeiro (7-X-1988, Brasil, Pará, Medicilandia, Caverna Limoeiro. Col. E. Trajano), a small municipality in Para state in the Amazonic Region. A single specimen, assigned as paratype, comes from a different locality in Southeastern Brazil, far away from the type locality. This is the specimen studied here. Some differences were observed in specimens from both localities, as mentioned in the original description (Palacios-Vargas and Zeppelini 1995) and as shown in Fig. 13B in this work and Fig. 9 in the original description. The specimens from the type locality have many fringed chaetae and two fringed distal scales on posterior side of dens. The specimen from Iporanga has only fringed chaetae. This suggests that the Holotype and Paratype may represent different species, therefore, a detailed revision of the holotype of *T. brasiliensis* is needed.
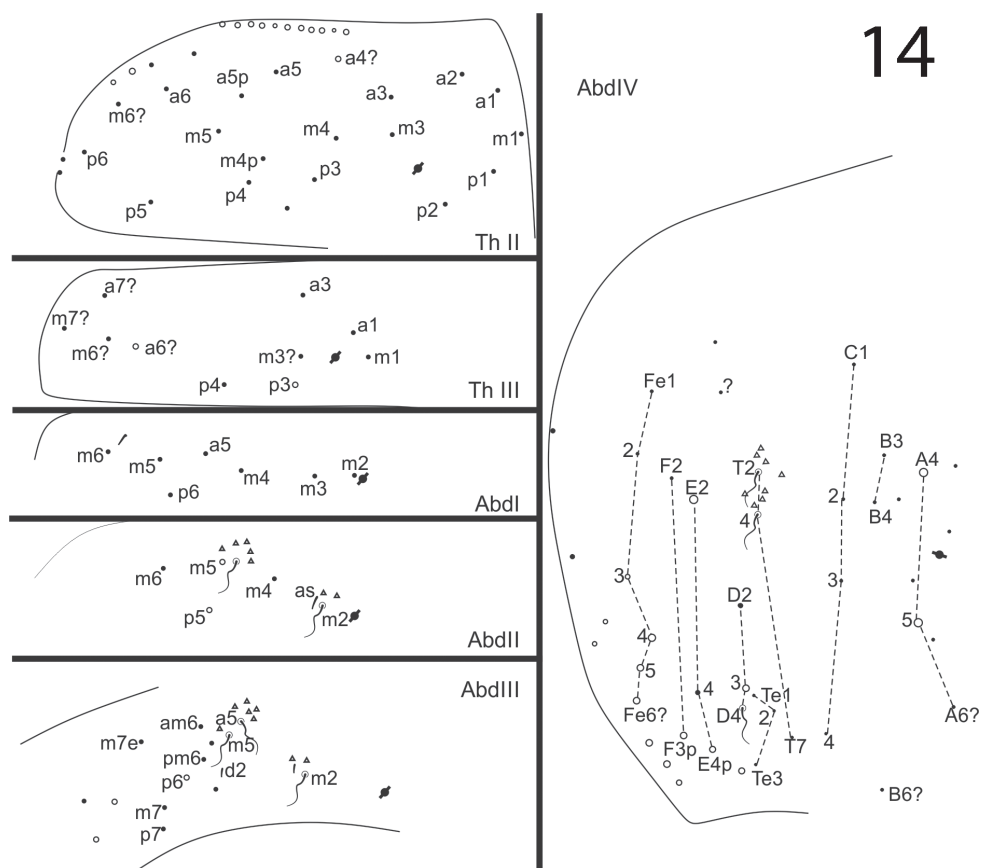
Figure 14. Body chaetotaxy *T. brasiliensis*.

Troglobius coprophagus **Palacios-Vargas & Wilson, 1990**

Remarks. Head chaetotaxy as in Fig. 3C. Abd IV with 3+3 lenticular organs posterior margin. Dens anterior side with hyaline scales; three rows of modified thick fringed scales (Fig. 12C) present on posterior side, internal row with 9–10 fringed scales, median row with 2 distal fringed scales, other median chaetae thick and ciliated, external row with about 11 barbulate chaetae (Fig. 13C). A large chaetae, about as long as mucro is present at the distal tip of the dens. Mucro with about 19 teeth on outer lamella, inner lamella smooth. Body chaetotaxy as in Fig. 15.

One of the species author, Wilson, was not able to maintain specimens alive once they were taken outside the cave, which indicates that they are very sensitive to temperature changes (Palacios-Vargas and Wilson 1990).

Material examined. Two paratypes, Madagascar, Grote d'Andrafiabe. 08/IX/1986.
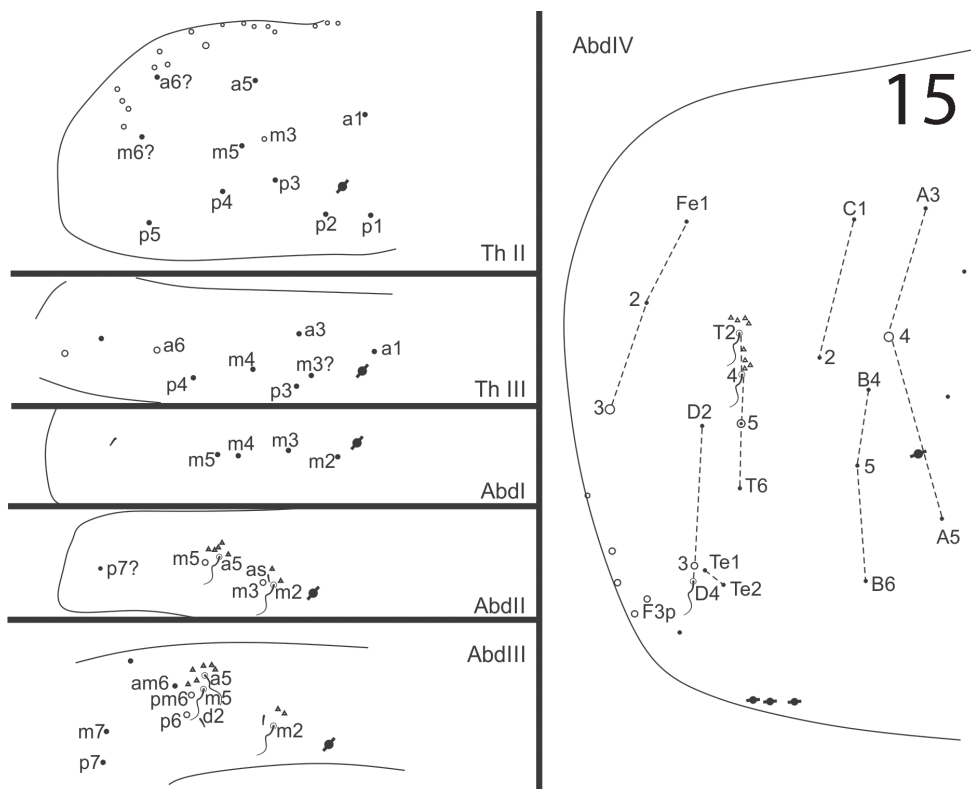
Figure 15. Body chaetotaxy *T. coprophagus.*

Ackowledments

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REVIEW ARTICLES

Subterranean
Biology

Stygobitic oligochaetes (Annelida, Clitellata) in Poland with remarks on their distribution in Central Europe

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Abstract

In Poland eleven stygobitic species have been found, whereas in Central Europe their number reached 23 species. Lumbriculidae are represented by 10 species, Enchytraeidae by seven, Naididae by three, whereas Dorydrilidae and Lumbricidae by two and singular species, respectively. Some species are known from Central Europe exclusively, but majority of them have wider distribution.

Keywords

Subterranean waters, invertebrate fauna

To the memory of my friend dr. Beatrice Sambugar, who encouraged me to prepare this lecture for the symposium entitled "Subterranean Aquatic Annelida", which took place during the 20th International Conference on Subterranean Biology in Postojna (2010).

Introduction

Taking into account their morphological features stygobitic and stygoxenic oligochaete species are indistinguishable. Therefore for this taxonomical group the attribution to stygobionts is based on species distribution. Species found exclusively in subterranean waters (including shallow groundwater sensu Schmidt and Hahn (2012)) are treated as

stygobionts. Sometimes they could be found in benthic samples also, mainly in springs and occasionally in running waters during low water level periods when rivers are fed directly by underground waters (Allan 1995). In such a situation other stygobionts, for example *Niphargus tatrensis* can be also found (Dumnicka 2005). Among oligochaetes there are many stygophilic species that live and reproduce successfully in surface and subterranean waters whereas stygoxenic species could survive for some time in underground waters but do not reproduce effectively in this environment.

The studies on oligochaetous annelids in subterranean waters of Poland as well as other countries of Central Europe: Czechoslovakia (now Czech Republik and Slovakia), Germany and Hungary started many years ago (Vejdovsky 1882, Lengersdorf 1929, Mohr 1929, Dudich 1932, Stammer 1936, Hrabě 1937, 1942), but generally they were done sporadically and in restricted areas. Nevertheless successive studies allowed to add subsequent species to the list of stygobitic oligochaetes or to find new localities of their occurrence. The state of knowledge about stygobitic oligochaetes was first summarized by Juget and Dumnicka (1986) and recently by Des Châtelliers et al. (2009). Moreover information that accumulated in the last years and some data not considered by Des Châtelliers et al. (2009) was taken into account in the present study: for Austria – paper by Danielopol and Pospisil (2001), for Czech Republik - by Hrabě (1981) and Schenkova et al. (2010), for Germany – by Stein et al. (2012), for Slovakia – by Šporka (1992) and Bitušik et al. (2010), for Poland – by Dumnicka (1998, 2000, 2005, 2009a,b) and Dumnicka and Koszałka (2005).

The current list of stygobitic oligochaete species from Poland and Central Europe is presented and the origin of species known exclusively from Central European countries is discussed. The probable ways of recolonisation after the last glaciation period by some species known from southern Europe is also discussed.

Results

Species distribution in Poland

At present eleven stygobitic species are known from Polish territory (Table 1), the majority of them was found in one geographical region. Some of them were collected from a very restricted area e.g. in one or two closely situated localities and in a very small number of specimens, for example *Trichodrilus spelaeus* – one individual in a pool situated on the bottom of an adit in Klecienko (Sudetes) (Moszyński 1936); *T. pragensis* – a few individuals in two caves in Sudetes (Dumnicka 2000), *Haber zavreli* - two individuals in two wells in Beskidy Mts (Kasprzak 1973) and *Fridericia dissimilis* - seven mature individuals in an intermittent spring in Sudetes (Dumnicka 1998).

The others, known from a limited area in Poland formed significant part of oligochaete taxocens, e.g. *Gianius aquaedulcis* in three closely situated springs located in Warta drainage area (Dumnicka 2009a) and *Enchytraeus polonicus* in a few caves of Kraków Częstochowa Upland (Dumnicka 1981). Among species known from singular

Table 1. Distribution of stygobitic oligochaetes in Poland.

Species	Localities	Habitats	References
Family Lumbriculidae			
Trichodrilus cernosvitovi Hrabě	Tatras, Pieniny Mts., K-Cz Up.	interstitial waters, springs	Kasprzak 1979, Dumnicka 2000, 2005
Trichodrilus moravicus Hrabě	Tatras, Sudetes, Pieniny Mts., Beskidy Mts.	interstitial and cave waters	Kasprzak 1979, Dumnicka 2000, 2009b
Trichodrilus pragensis Vejdovský	Sudetes	cave waters	Dumnicka 2000
Trichodrilus spelaeus Moszyński	Sudetes	locus typicus only (an adit)	Moszyński 1936
Family Naididae			
Rhyacodrilus subterraneus Hrabě	Mazurskie Lakeland	interstitial waters	Dumnicka and Koszałka 2005
Gianius aquaedulcis (Hrabě)	K-Cz. Up.	springs	Dumnicka 2009a
Haber zavreli (Hrabě)	Beskidy Mts.	phreatic waters	Kasprzak 1973
Family Enchytraeidae			
Cernosvitoviella parviseta Gadzińska	Tatras, Sudetes, Beskidy Mts., Pd-W. Lowland	cave and phreatic waters	Dumnicka 2000, 2009b
Enchytraeus dominicae Dumnicka	Tatras, Sudetes, K-Cz Up. Święt. Mts Pd-W. Low.	cave, phreatic, interstitial waters and springs	Dumnicka 2000
Enchytraeus polonicus Dumnicka	K-Cz Up.	wet sediments and cave waters	Dumnicka 1981
Fridericia dissimilis Dumnicka	Sudetes	springs	Dumnicka 1998

Abbreviations: K-Cz Up.: Kraków –Częstochowa Upland; Pd-W. Low.: Południowowielkopolska Lowland; Święt. Mts.: Świętokrzyskie Mts.

localization only *Rhyacodrilus subterraneus* was stated in northern Poland (Mazurian Lakeland), in interstitial waters of a small stream (Dumnicka and Koszałka 2005). In contrast, some other species, such as *Enchytraeus dominicae*, *Cernosvitoviella parviseta*, *Trichodrilus moravicus* and *T. cernosvitovi* are widely distributed in underground water bodies of various categories (Dumnicka 2000). Up to now all enchytraeids (4 species) and *Trichodrilus spelaeus* were found only in Poland.

Species distribution in Central Europe

The number of stygobitic oligochaete species known from separate countries of Central Europe varying from two in Austria to eleven in Poland (Fig. 1). This number likely reflects research effort rather than the true diversity of stygobitic species in each country.

The list of stygobitic oligochaetes found at present in Central Europe is not long – it contains 23 species (Table 2) among them five (mainly enchytraeids) are known from Poland exclusively. Family Lumbriculidae is represented in these countries by 10 species and Enchytraeidae by seven, moreover Naididae, Dorydrilidae and Lumbricidae are represented by a few or singular species.
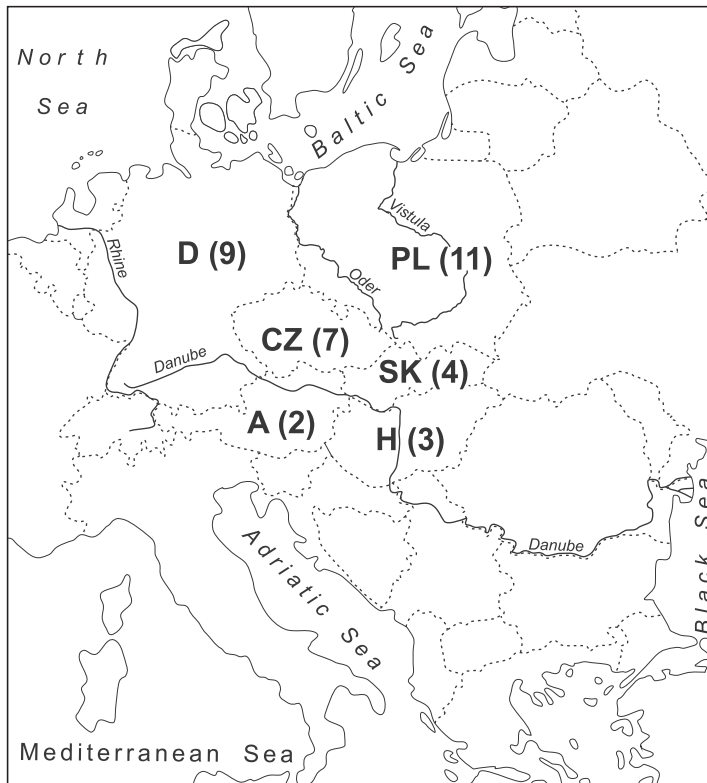
Figure 1. Number of stygobitic oligochaete species known from the countries of Central Europe.

Among stygobitic oligochaete fauna studied in all Central European countries some endemic species (e.g. known exclusively from one geographic region in one country) have been encountered. They are represented by: the above-mentioned *Trichodrilus spelaeus* (from Poland)*, Helodrilus mozsaryorum* (Baradla cave, Hungary) (Zicsi et al. 1999) and *Stylodrilus absoloni* (Moravian Karst, Czech Republik) (Hrabě 1981). It seems that *Dorydrilus tetrathecus*, and *Trichodrilus medius*, both known from a few closely situated localities in Germany (Hrabě 1960) could be also treated as endemics. Moreover, up to now, two *Trichodrilus* species (*T. moravicus* and *T. tatrensis*) (Table 2) are known from a few countries of Central Europe solely. *T. moravicus* is known from various subterranean habitats in three neighboring countries (Table 2), whereas *T. tatrensis* was found mainly in bottom samples of some streams flowing below the caves in Slovakian part of Tatra Mts. (Hrabě 1981) and in subterranean waters in Germany (Hrabě 1960). The remaining Lumbriculidae and all Naididae species known from Central Europe have wider distribution: Lumbriculidae and *Gianius aquaedulcis* are known mainly from southern Europe whereas *Haber zavreli* and *Dorydrilus michaelseni* from Great Britain also (Table 2). The distribution of *Rhyacodrilus subterraneus* is dissimilar – this species is known from subterranean waters of Central Europe and Belgium (Martin et al. 2009), and from cold surface waters of the northern part of this

continent (Erséus et al. 2005). It was also found in mountain streams of France and Spain (see Dumnicka and Koszałka 2005).

The studies on Enchytraeidae from the discussed environment were only done in three central European countries: Hungary, Poland and recently in Germany (Stein et al. 2012). Stygobitic species found in these countries belong to aquatic (*Cernosvitoviella*) or semi-aquatic, as well as terrestrial genera (*Enchytraeus, Enchytronia, Fridericia*) and they have limited distribution (Table 2).

Table 2. Distribution of stygobitic oligochaetes known from Central Europe. References concerning species distribution in Poland are in Table 1.

Species	Found in Europe		Habitat	References
	central	**western**		
Family Lumbriculidae				
Trichodrilus cernosvitovi Hrabě	PL	B, F, RO, SLO	int., spr., ben.	Des Châtelliers et al. 2009, Giani et al. 2011
- *medius* Hrabě	D		int., phr.	Hrabě 1960
- *moravicus* Hrabě	PL, CZ, SK		cav., int., phr.	Hrabě 1981
- *pragensis* Vejdovský	PL, D, CZ	RO, F, E, SLO	cav. int., phr.	Dumnicka 2000, Giani et al. 2011
- *strandi* Hrabě	CZ, SK	I,F,SLO,HR,E	cav. int. spr. ben.	Schenkova et al. 2010, Achurra and Rodriguez 2008
- *tatrensis* Hrabě	D, SK		int., phr., ben.	Šporka 1992, Bitušik et al. 2010
- *tenuis* Hrabě	D, A	F, E, SLO	int. phr.	Des Châtelliers et al. 2009, Giani et al. 2011
- *spelaeus* Moszynski	PL		art. c.	
Stylodrilus absoloni (Hrabě)	CZ		cav. int.	Hrabě 1981
Guestphalinus wiardi (Michaelsen)	D	SLO	cav. int.	Des Châtelliers et al. 2009
Family Dorydrilidae				
Dorydrilus tetrathecus Hrabě	D		phr.	Hrabě 1960
- *michaelseni* Piguet	D, A	F,GB, I,CH	int. cav., ben.	Juget and Dumnicka 1986
Family Naididae				
Gianius aquaedulcis Hrabě	D, PL	F, I, E	int., phr. spr., ben.	Des Châtelliers et al. 2009
Haber zavreli Hrabě	PL, CZ, SK	I, SLO	int. cav. phr.	Hrabě 1981, Giani et al. 2011
Rhyacodrilus subterraneus Hrabě	PL, D, CZ	N, F, E, S, B	int. phr. ben.	Dumnicka and Koszałka 2005
Family Lumbricidae				
Helodrilus mozsaryorum (Zicsi)	H		cav.	Zicsi et al. 1999
Family Enchytraeidae				
Cernosvitoviella aggtelekiensis Dozsa-Farkas	H		cav.	Zicsi et al. 1999
C. parviseta Gadzińska	PL		cav. phr.	
Enchytraeus dominicae Dumnicka	PL		cav. phr. int. spr.	
- *polonicus* Dumnicka	PL		cav.	
- *bohemicus* Dumnicka	CZ		art. c..	Dumnicka 1996
Enchytronia christenseni Dozsa-Farkas	H		cav.	Zicsi et al. 1999
Fridericia dissimilis Dumnicka	PL		spr.	

Abbreviations: ben. – benthos; cav. – cave waters; int. – interstitial waters; phr. – phreatic waters (wells); art. c. - artificial cavities; spr. - springs.

Discussion

The state of knowledge on stygobitic oligochaete fauna in each of the studied countries of Central Europe varies. Aquatic oligochaete fauna was not studied in Hungary whereas there is plenty of information concerning distribution of such fauna in subterranean waters of Germany, Czech Republic, Slovakia and southern part of Poland. Representatives of Enchytraeidae family were studied only in Poland, Hungary and recently in Germany.

Although the diversity of aquatic subterranean oligochaete fauna in Central Europe is distinctly smaller than in Mediterranean countries (Giani et al. 2001, Des Châtelliers et al. 2009, Giani et al. 2011, Achurra and Rodriguez 2008) there are some stygobitic species, known exclusively from this region. Perhaps some of them are true endemics or they have not been found yet in other geographic regions. The widening of the distribution area as a result of intensive studies can be seen in the case of *Guestphalinus wiardi*, known for many years exclusively from Germany and then found in Slovenia by Hrabě (1973). Stygobitic oligochaete species from family Dorydrilidae (*D. tetrathecus*) and Lumbriculidae such as *Trichodrilus moravicus, T. medius*, *T. tatrensis, T. spelaeus* and *Stylodrilus absoloni*, known only from subterranean waters of Central Europe probably diversified in this region before the Tertiary or during the warm Pleistocene periods, when that area was not glaciated, and they survived in singular or several refugia.

Within the genus *Trichodrilus* some of the species have high similarity of various anatomical structures (including genital organs) demonstrating their close relationship to other species living both in epigean and hypogean waters of small, separated areas. Molecular studies should allow to establish the degree of their separation (autonomous or sister species, or same species with some morphological and anatomical differences).

The rarity of stygobitic oligochaete species occurrence makes all studies on their distribution, speciation and ecology very difficult. They are usually found in an extremely small number of samples taken from subterranean waters and even the confirmation of species occurrence and findings of new localities are very difficult. During the studies of 261 localities in Slovenia (Giani et al. 2011) among 19 stygobitic oligochaete species known earlier from this country, the occurrence of only three of them was confirmed. Moreover, ten other stygobionts were found on one to eight sampling stations and *Trichodrilus pragensis* - the most frequently found species - was observed at ten sampling stations (Giani et al. 2011). During large-scale studies on subterranean invertebrate fauna from various geographical regions in Germany, only *Dorydrilus michaelseni* was found (Stein et al. 2012) beside epigean oligochaete species.

The majority of stygobitic species found in Central Europe (except for Enchytraeidae) is known from southern Europe. They could have recolonized subterranean waters of Central Europe after glacial/interglacial periods along water courses - especially along the Danube River. Its catchment area covers large parts of Czech Republic, Slovakia, Germany and Austria. Such migration towards the north part of

Germany along the Rhine River was also possible. Some species could have migrated by underground connections between catchments area of Danube – Oder and Danube –Vistula River in Poland. The presence of such connection is well documented for the Danube – Oder tributaries (Sudetes, Śnieżnik Massive). Kleśnica stream (Poland) is connected by a few kilometers long underground flow with Morava stream (Czech Republic) (Ciężkowski et al. 1996). In the Tatra Mts. such a connection was not confirmed by water-tracing tests, but the existence of such a flow is assumed (Gradziński et al. 2009). The recharge area was located on the southern part of the higher part of the mountains in Slovakia where there is a small sinkhole in the Cicha Valley (catchment of the Danube River). The discharge is probably through resurgences located at the foot of the northern slopes of the mountains in the Bystra Valley in Poland (catchment of the Vistula River). Migration along water courses facilitates wide spreading of species living in hyporheic waters, which usually have wider distribution than those living in phreatic waters. Such a model of species distribution was also mentioned for the genus *Niphargus* (Karaman and Ruffo 1986). Probably the migration of stygobionts of marine origin, mainly known from southern Europe (Sambugar et al. 1999) took place by latitudinal ice – marginal river valleys. The distribution of *Gianius aquaedulcis* in northern and central parts of Germany and Poland could be the example of such a migration.

It seems that the occurrence of some oligochaete species in epigean or hypogean habitats depends on water temperature. For example, *Rhyacodrilus subterraneus* was not found in epigean waters in Poland despite many studies done on their oligochaete fauna, but it lives in epigean streams of northern Europe and mountain streams of France and Spain (see Dumnicka and Koszałka 2005). *Trichodrilus tatrensis* is also known from subterranean (Hrabě 1960) or cold epigean waters (Šporka 1992, Bitušik et al. 2010). Such a species could be named "local" stygobionts in regions where they occur in subterranean waters only.

All the enchytraeid species described and found up to now in the subterranean environment of Central Europe are listed in Table 2, but due to the taxonomic revision began by Schmeltz and Collado (2010) the determination of their exact number is not possible for the moment.

Conclusions

Stygobitic oligochaete fauna of Central Europe is not rich, comprising only 23 species. They were found in various subterranean habitats, but the most frequently in interstitial waters. Some species (14, half of them represents family Enchytraeidae) are known only from Central Europe what suggests their speciation *in situ* and survival during the glacial/interglacial periods in refugia. The species having a wide distribution could have recolonised subterranean waters of Central Europe along water courses from non-glaciated regions of southern Europe.

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RESEARCH ARTICLE

A proposal for a groundwater habitat classification at local scale

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Abstract

Distribution of groundwater invertebrate communities in porous aquifers (and their habitats) varies on spatial scales and many attempts have been made to classify these on various scales. The new data-based approach, presented here, classifies the complex distribution of groundwater habitats on a local scale (i.e. along transects of < 100 m) and merges the latest classification approaches at this scale. Data from a regional (i.e. approximately 100 km²) biogeographic groundwater survey was analysed in terms of stability of: community structure, different intensities of surface water influence, and occurrence, together with the distribution of stygobites within those groundwater ecosystems. On the investigated local scale, the faunistic communities' composition is mainly depending on surface water influence, coupled with immission of dissolved oxygen and organic matter. Derived from this finding, five types of faunistic habitats are proposed: (I) Stressed groundwater habitats, (II) Stable groundwater habitats, (III) Rain fed groundwater habitats , (IV) Surface water fed groundwater habitats, and (V) Hyporheic habitats.

Keywords

Introduction

According to their occurrence in groundwater or surface water, invertebrates can basically be classified as stygobites, stygophiles and stygoxenes (Thienemann 1926). Stygobites spend their whole life-cycle completely in groundwater and are well-adapted to low food and oxygen supply (Culver 1982, Hervant et al. 1997, Malard and Hervant 1999, Mösslacher and Hahn 2003). In contrast to stygobites, stygophilic invertebrates commonly occur in well food- and oxygen supplied surface waters. However, they can immigrate into groundwater actively, if the conditions there are favourable (Malard et al. 1996, Malard and Hervant 1999, Sket 1999). Invertebrates that are carried passively into groundwater (for example by pulse-like surface water intrusion into groundwater) are called stygoxenes. Stygoxenes cannot endure the specific living conditions in groundwater for long periods of time. This classification, based on autecological data of species is well known. However does not represent a description of groundwater habitats.

In this paper "habitat" as the living space of a particular species (sensu Abercrombie et al. 1966) is used synonymously with "biotope" as the living space of a community (Schaefer 2012).

Groundwater invertebrate communities and the habitats in which they occur, show different patterns depending on the spatial scale regarded. Many independent attempts have been made in the past to classify groundwater communities and habitats, each focusing on different combinations of scales. With the exception of the classification based on faunal communities from groundwater wells in the federal state of Baden-Wuerttemberg (South-Western Germany) by Hahn and Fuchs (2009), most classifications in the past were based purely on theoretical considerations. Galassi et al. (2009a) did not intend to develop a new classification of groundwater habitats, yet they described the occurrence of species and faunistic composition in the investigated area. Other studies were mainly focussing on certain sub-classes, like copepods (Galassi et al. 2009b), or disregarded areas besides alluvial plains (Datry et al. 2008) where different faunistic composition patterns and habitats were dominating, or dealt with biodiversity patterns among different types of aquifers (Malard et al. 2009). Because of those restrictions, the findings of these studies are not easily transferable to other regions. Here, we develop a multi-scale classification scheme that is based on data from a ca. 100 km^2 region in Southern Germany, which also considers previous theoretical attempts.

For clarification purposes, we summarise the most important classification attempts to date:

(i)　　Husmann (1966, 1967) proposed a typological system which was closely linked to the latitudinal zonation of running waters, based on the hydrological understanding of surface water flow, ignoring that fundamentally different flow patterns below the surface.

(ii)　　Illies (1967) explained the distribution and diversity of surface-water species by connecting species to a list of habitats and regions, which he called bioregions. Since it has recently been shown that the surface bioregions do not mirror

groundwater regions (Hahn and Fuchs 2009, Stein et al. 2012), this classification does not offer much help or assistance in classifying groundwater habitats.

(iii)   Illies' (1967) concept was modified by Botoşăneanu (1986) by adding groundwater habitats which were affiliated to different types of subterranean biotopes or by the hydraulic conductivity of the aquifers.

(iv)   Ecological issues, the hydraulic conductivity as well as the type of aquifer, were the basis of the typology by Gibert et al. (1997) and Gibert (2001).

(v)   Schmidt and Hahn (2012) stressed the heterogeneity within groundwater habitats and suggested a classification of groundwater habitats accordingto the degree with which they were characterised by surface water ingressions.

(vi)   Another attempt to classify groundwater habitats on different spatial scales (i.e. macroscale, continent; landscape scale, km; local scale; dm to m) was proposed by Hahn (2009).

(vii)   Stein et al. (2012) developed a data-based approach for classifying groundwater habitats into stygoregions at large scales.

(viii)   Based on heterogeneous data, Gutjahr et al. (2013a) classified groundwater habitat types on a local scale, depending on the degree to which they were stressed by diffuse effects, such as hypoxia, silt or iron ochre.

On a local scale (i.e. from decimetres to metres), another recent classification approach for groundwater habitats was suggested by Hahn (2006). It is based on the estimation of alimony (availability of organic nutrients) and oxygen supply, expressed by the groundwater-fauna-index (GFI). Except for the last three, none of the aforementioned typologies were tested using faunistic datasets. Moreover, only the classifications (vii) and (viii) are based on the identified faunistic assemblage. The approach by Stein et al. (2012) is on an almost continental scale and differs from the approach by Hahn and Fuchs (2009), which was developed only on samples from South-Western Germany on a landscape scale.

Gutjahr et al. (2013a) identified three groups of habitats according to the level of resilience to stress: (1) stressed habitats with low numbers of taxa and individuals, (2) intermediate habitats with highest numbers of taxa and individuals, and (3) stable habitats with intermediate numbers of taxa and individuals. This classification enables the evaluation of faunistic stability of communities and the evaluation of sampling efficiency at a sampling site (e.g. how many samples are needed to catch 95% of the species occurring at the investigated site).

The degree of surface water intrusion drives faunistic composition to a great extent. In order to assess the influence of surface water on groundwater environments at landscape level, the GFI was proposed by Hahn (2006). It distinguishes three groups of ecological groundwater habitats, based on the availability of organic nutrients. He classified oligo-alimonic habitats as those with weak hydrological exchange, a poor food and oxygen supply and often an absence of groundwater invertebrates. Meso-alimonic habitats have a moderate hydrological exchange and food supply with moderate to high oxygen concentrations and are dominated by a stygobiotic fauna. A strong influ-

ence of surface water accompanied by moderate to high oxygen and nutrient supply is considered to be characteristic for eu-alimonic habitats. Those habitats often harbour an abundant and species-rich fauna which is a mixture of stygoxenes and stygophiles.

Schmidt and Hahn's (2012) theoretical approach clearly distinguishes between rain fed groundwater ecosystems and surface water fed groundwater ecosystems. The former are recharged by precipitation infiltrating into groundwater through the soil and the latter are recharged by water infiltrating into groundwater from surface water bodies. Local conditions have varying degrees of groundwater and surface water interfusion and the scale might range from dm in low conducive sediments to km in highly conducive sediments.

For the classification of groundwater habitats within a landscape, a characterisation of groundwater invertebrate communities is required (Tomlinson and Boulton 2010, Larned 2012) at regional and local scale. In order to reach this goal, the latest regional / local concepts are used (Hahn 2006, Schmidt and Hahn 2012, Gutjahr et al. 2013a) and tested on a dataset which comprised different spatial scales but was mainly focussed on the local scale (i.e. from decimetres to metres). With this background, data from 30 traps in groundwater wells situated in four catchments in Rhineland-Palatinate (Germany) were analysed and the results were compared with findings of the aforementioned concepts (Hahn 2006, Schmidt and Hahn 2012, Gutjahr et al. 2013). This study aims to check an empiric groundwater faunistic dataset on the background of previously available classification approaches (Hahn 2006, Schmidt and Hahn 2012, Gutjahr et al. 2013a) and to merge them, where appropriate.

Material and methods

Study area

The study area is situated in the federal state Rhineland-Palatinate, South-Western Germany. It comprises three different natural geographic regions, the Pfälzerwald Mountains, the Haardtrand and the Upper Rhine Plateau. Groundwater wells were located in transects in four alluvial floodplains, the Kolbental [KT], Klammtal [HB] (both in the Pfälzerwald Mountains), the Modenbachtal [MB] (Haardtrand) and the Offenbacher Wald [OW] (Upper Rhine Plateau) (Table 1, Fig. 1).

The Pfälzerwald Mountains have unfertile and sandy soils in conjunction with a high rate of groundwater recharge (~ 25% of precipitation, i.e. 200–300 mm y^{-1}) (LUWG 2005). The groundwater is characterised by high oxygen concentrations and low conductivity values (mean ~ 103 µS cm^{-1}). The Haardtrand is the Western fault belt of the Upper Rhine Plateau, which encompasses a fractured geology, intensive steep slope viticulture and intermediate conductive groundwater (mean ~250 µS cm^{-1}). Soils in the Upper Rhine Plateau are fertile in most instances, thus intensive agricultural use dominates (Geiger et al. 2008) and groundwater is featured by high conductivities (mean ~ 590 µS cm^{-1}).
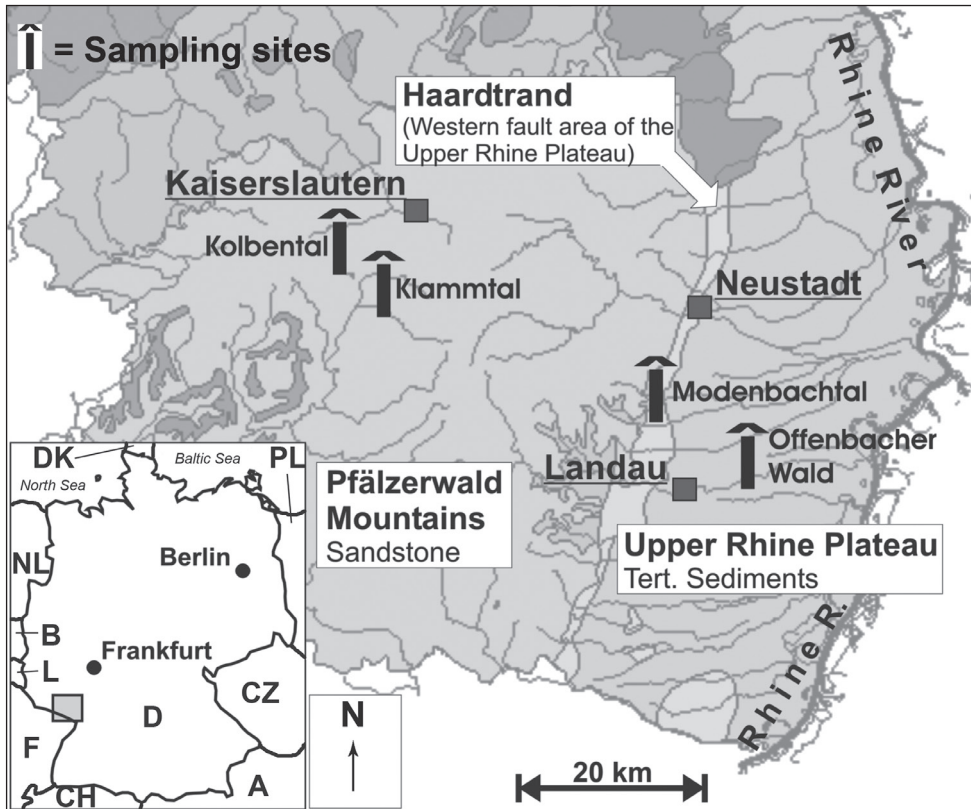
Figure 1. Map of the study area (from Hahn 2006, modified). All sites were equipped with 4–5 transsectional groundwater wells. Boxes: The respective natural regions [Pfälzerwald Mountains = Central Uplands; Haardtrand and the Upper Rhine Plateau = South-Western Uplands (according to Stein et al. 2012)]. Abbreviations on overview map: A = Austria, B = Belgium, CH = Switzerland, CZ = Czech Republic, D = Germany, DK = Denmark, F = France, L = Luxembourg, NL = Netherlands, PL = Poland.

The sandy and unfertile soils of the Offenbacher Wald are completely covered by forests.

Annual mean temperatures decrease from the Offenbacher Wald (10.1 °C) over the Haardtrand (9.7 °C) to the Pfälzerwald Mountains with 8.6 °C by mean (for details see Hahn 2005, 2006).

Sampling methods

To sample invertebrates, unbaited stratified trap systems (Table 1) were installed in wells (Hahn 2005, 2006, Bork et al. 2008). The wells were arranged in transects from the slope of the hill towards the brook in intervals of approximately 50 meters, ending up with the last well directly next to the brook. Thus 13 wells including stratified trap systems in four regions and additionally two hyporheic traps were sampled (Table 1).

Table 1. Sampling sites.

Landscape	Site	Trap	Valley	Lat. (N.)	Lon. (E.)	altitude of terrain surface above sea level [m]	depth [m] below terrain surface	Recharge from	groundwater-fauna-index (GFI) (mean)
Pfälzerwald	KT1	KT1/A		49°23'57.2"	7°40'50.61"	289.0	1.70	lateral groundwater instream from the adjacent fractured rock sandstone aquifer	2.13
Pfälzerwald	KT1	KT1/B		49°23'57.2"	7°40'50.61"		2.90		1.54
Pfälzerwald	KT1	KT1/C		49°23'57.2"	7°40'50.61"		6.90		1.43
Pfälzerwald	KT2	KT2/A		49°23'55.94"	7°40'51.73"	288.5	1.61	deep alluvial groundwater	2.38
Pfälzerwald	KT2	KT2/B	Kolbental	49°23'55.94"	7°40'51.73"	296.0	2.81		2.09
Pfälzerwald	KT2	KT2/C		49°23'55.94"	7°40'51.73"	296.0	6.81		0.47
Pfälzerwald	KT3	KT3/A		49°23'55.21"	7°40'50.56"	288.0	1.19	lateral groundwater instream from the adjacent fractured rock sandstone aquifer	1.62
Pfälzerwald	KT4	KT4/A		49°23'55.4"	7°40'49.52"	288.0	1.51		5.11
Pfälzerwald	KT4	KT4/B		49°23'55.4"	7°40'49.52"	288.3	3.81		4.02
Pfälzerwald	HB1	HB1/A		49°20'8.95"	7°40'33.97"	270.0	1.52	lateral groundwater instream from the adjacent fractured rock sandstone aquifer	3.72
Pfälzerwald	HB1	HB1/B		49°20'8.95"	7°40'33.97"	291.0	2.72		2.74
Pfälzerwald	HB1	HB1/C		49°20'8.95"	7°40'33.97"	291.0	3.42		2.27
Pfälzerwald	HB2/HZ	HB2/HZ		49°20'9.23"	7°40'33.27"	268.7	0.72	deep alluvial groundwater	3.58
Pfälzerwald	HB2	HB2/A	Klammtal	49°20'9.20"	7°40'33.27"	269.0	1.46		1.69
Pfälzerwald	HB3	HB3/A		49°20'9.64"	7°40'32.42"	270.0	1.62	lateral groundwater instream from the adjacent fractured rock sandstone aquifer	7.72
Pfälzerwald	HB3	HB3/B		49°20'9.64"	7°40'32.42"		2.82		4.06
Pfälzerwald	HB3	HB3/C		49°20'9.64"	7°40'32.42"		3.82		2.52
Haardrand	MB3	MB3/B		49°15'29.48"	8°4'54.79"	197.0	3.75	surface water / brook	3.52
Haardrand	MB4	MB4/A		49°15'29.74"	8°4'54.64"		2.20		8.77
Haardrand	MB4	MB4/B	Modenbachtal	49°15'29.74"	8°4'54.64"		3.40		3.58
Haardrand	MB4	MB4/C		49°15'29.74"	8°4'54.64"	197.0	7.40	mainly deep groundwater / little surface water influence	1.83
Upper Rhine Plateau	OW1	OW1/A		49°12'52.50"	8°11'45.41"	128.0	2.09	surface water impact from gully	2.12
Upper Rhine Plateau	OW1	OW1/B		49°12'52.50"	8°11'45.41"		3.29		0.20
Upper Rhine Plateau	OW2	OW2/A	Offenbacher Wald	49°12'50.63"	8°11'45.49"	127.5	1.49	alluvial forest, periodically flooded	3.94
Upper Rhine Plateau	OW2	OW2/B		49°12'50.63"	8°11'45.49"		2.69	occasional surface water impact	1.48
Upper Rhine Plateau	OW3/HZ	OW3/HZ		49°12'47.88"	8°11'45.98"	127.0	0.87	surface water impact from brook	11.93

Landscape	Site	Trap	Valley	Lat. (N.)	Lon. (E.)	altitude of terrain surface above sea level [m]	depth [m] below terrain surface	Recharge from	groundwater-fauna-index (GFI) (mean)
Upper Rhine Plateau	OW3	OW3/A		49°12'47.85"	8°11'45.98"	127.5	2.20		1.36
Upper Rhine Plateau	OW4	OW4/A		49°12'47.33"	8°11'46.33"		1.54	occasional surface water impact, otherwise alluvial groundwater	1.15
Upper Rhine Plateau	OW4	OW4/B		49°12'47.33"	8°11'46.33"	127.5	2.74		0.79
Upper Rhine Plateau	OW4	OW4/C		49°12'47.33"	8°11'46.33"		6.74		0.35

To sample the fauna, the contents of the traps (0.9 L) were pumped and an additional 2 L were sampled afterwards for hydro-chemical analyses. Physical and chemical properties of groundwater, such as temperature, dissolved oxygen (DO), pH-value and electric conductivity (EC) were measured using a WTW multi-meter Multiline P4 directly after pumping out the water. Total dissolved iron was measured using a Merck Reflectoquant reflectometer. Organic matter (OM) was estimated and scaled into four categories: absent, little, much, very much according to Hahn (2006).

The mean depths (below surface) of the investigated groundwater wells were 5.0 m (1 well) and 7.5 m (12 wells) and all tapped into the shallow local aquifer. The traps were installed in triplicates: the first trap (A) was always installed just below groundwater table, the second trap (B) in the middle of the water column and the third trap (C) at 0.5 m above the bottom of the wells (Table 1). The two hyporheic wells [HZ] (Table 1) contained one trap each, which was installed 0.3 m below the sediment surface. Faunistic samples were taken and then brought back to the laboratory within 24 hours and sorted alive. For more detailed information on the processing of the samples see Hahn (2005).

Fauna was determined to species level and ecological information given on species was derived from Einsle (1993), Janetzky et al. (1996), Meisch (2000), Wägele (2007) and Schminke (2007 a–c).

For this study, data were analysed from 30 traps containing fauna and which had been sampled on 13-15 occasions over an eighteen month period (2001–2002) (Tables 1 and 2). Due to seasonal fluctuations in water levels, one trap fell dry repeatedly and was not taken into account for this analysis.

Statistical analysis

Faunal data were not normally distributed (Shapiro-Wilk-test) even after log (x+1) transformation (p < 0.05) and non-parametric tests were performed. SIMPER analyses were applied to taxonomic data, which was available at species level (Clarke 1993). This process was developed specifically for the comparison of faunal samples in which typical or dominant species are identified, and pairs of species that do occur together more frequently are considered. Based on a matrix of presence-absence of taxa (or pairs of species) a similarity percentage can be calculated to express faunistic similarities between samples of a certain sampling site or sampled trap. False-negative rates were calculated for each trap in order to draw conclusions on the aspects of sampling efficiency (Eberhard et al. 2009, Gutjahr et al. 2013a). The GFI was calculated according to Hahn (2006).

Faunal communities' abundances were fourth rooted after incorporating a dummy variable to overcome bias from extremely heterogeneous faunistic data among traps. Bray-Curtis dissimilarities among traps were then calculated based on faunistic data at species level. This dissimilarity matrix was plotted in the multi-dimensional scaling method (MDS). Vectors of physical and chemical characteristics of groundwater,

explaining the distribution of the traps by multiple correlation, were integrated to the MDS to indicate possible influences of the physical and chemical characteristics of groundwater to the faunistic assemblages. To test whether scaled groups were statistically distinguishable, a one-way ANOSIM analysis (Clarke 1993) and a distribution-free discriminance analysis (DA; 10,000 permutations) were performed. The MDS, ANOSIM and DA were carried out by PRIMER v6 with PERMANOVA+ -Addon (Primer E Ltd.). All other processing was performed using Excel 2007 (Microsoft Corporation) or SPSS 15.0 (SPSS Inc.).

Results

Five distinct ecological groups were identified, mainly based on a MDS (Fig. 2), but also on a cluster analysis (single linkage, see Suppl. material 1) as well as the site particularities (Table 1):

(I)   stressed groundwater habitats [Stressed]
(II)   habitats where groundwater was secluded from all surface water influences, thus was comparably stable in faunistic composition [GWstable]
(III)   rainwaterfed groundwater habitats [GWrainfed]
(IV)   surface waterfed groundwater habitats [GWswb], and
(V)   Hyporheic habitats [Hyporheic].

The KT1/A and KT2/A traps (Table 2) were defined to be "GWstable" and "GWrainfed", according to the MDS plot (Fig. 2), though the cluster analysis would have categorised them as "Stressed". The separation of all groups was significantly and strongly distinct (one way ANOSIM: global R = 0.608, p=0.001). The pair-wise comparisons for the groups within this ANOSIM GWstable to GWrainfed (R = 0.006, p = 0.4) as well as GWstable to Stressed (R = 0.02, p = 0.438) were not significant. Using a DA, a total of 90% (p = 0.0001) of the samples were correctly classified to one of the identified habitats.

The MDS ordination (Fig. 2) revealed a separation at landscape level on the y-axis with sampling sites from the Pfälzerwald Mountains at the higher end, the Upper Rhine sampling sites at the lower end and the Haardtrand region with an intermediary position. The distribution of the sampling sites on the x-axis shows an increase in (from the left to the right) surface water influence. This is indicated by faunistic communities' composition as the proportions of stygobiotic fauna decrease and GFI (Fig. 2) values rise.

Stressed habitats (I) were found in all natural investigated regions and constituted a heterogeneous group (about 50% of all the traps). This type of habitats was characterised by a low average of standard deviation of temperature, DO and OM and a high amplitude of measured values (Fig. 3). Stressed habitats always harboured populations of low abundances (mean = 1.08 individuals/sample) and a broad fluctuation of
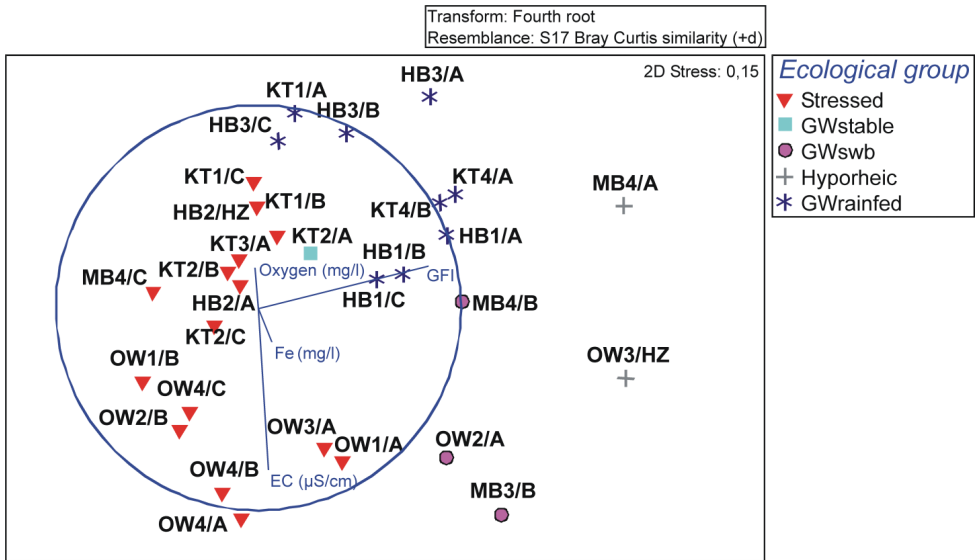
Figure 2. MDS (Multi-dimensional scaling) ordination of invertebrate assemblages of each trap (faunal data aggregated by mean for traps having 13–15 samplings). Vectors show physical and chemical parameters of groundwater explaining the distribution of traps within the MDS best (Fe = Total dissolved iron [mg l^{-1}]). Naming of the traps in accordance with Table 1.

proportions of stygobites (mean = 52.18%) accompanied by low SIMPER similarity values (Table 3 and Fig. 4d). The average group SIMPER similarity was 6.76%, with the cyclopoids *Diacyclops* cf. *languidoides* and *Paracyclops fimbriatus* (Fischer, 1853) being the characteristic species.

Table 2 and Figure 4c show different distributions of abundances between the scaled groups. Derived from this data low abundances are less than 12 individuals/sample, intermediate abundances are 12 to 79 individuals/sample and high abundances are more than 80 individuals/sample.

One trap (KT2/A), situated in the Kolbental (Pfälzerwald Mountains), was classified as GWstable (II). This trap was featured by groundwater, which was well-shielded from surface water and characterized by very low standard deviation of temperature (mean = 1.45 °C) and low GFI-values (mean = 2.38). KT2/A displayed the highest percentages of stygobites (mean = 99.49%) in low abundances (mean = 11.6 individuals/sample), stable faunal communities (Fig. 4b-d) and low standard deviations of temperature (Fig. 3a). GFI values were low, indicating oligo-alimonic conditions (lower dashed line in Fig. 4a). The SIMPER test (as there was only one site in the group, a SIMPER test was performed for all the sampling occasions) generated *Diacyclops* cf. *languidoides* as a characteristic species at this habitat. The similarity of the 15 samples was 78.44% by SIMPER, indicating very stable communities (Table 3).

GWrainfed (III) habitats were situated mainly at the edges of valleys and only in the Pfälzerwald Mountains. They were characterised by groundwater from adjacent fractured rock aquifers. The standard deviation of temperature of 1.52 °C (Fig. 3a)

Table 2. Taxa vs. site matrix for the samples of the traps investigated (faunal data aggregated by sum over 13 – 15 samples). Marked taxa (grey) proved to be most important in differentiating between habitats by SIMPER analysis (Table 3).

Sampling sites	Number of samples	*Niphargus aquilex* SCHIOEDTE, 1855	*Acanthocyclops robustus* (SARS, 1863)	*Acanthocyclops vernalis* (FISCHER, 1853)	*Diacyclops bisetosus* (REHBERG, 1880)	*Diacyclops crassicaudis* (SARS, 1863)	*Diacyclops cf. languidoides*	*Diacyclops languidus* (SARS, 1863)	*Eucyclops serrulatus* (FISCHER, 1851)	*Graeteriella unisetigera* (GRAETER, 1908)	*Macrocyclops albidus* (JURINE, 1820)	*Megacyclops viridis* (JURINE, 1820)	*Paracyclops fimbriatus* (FISCHER, 1853)	*Thermocyclops dybowskii* (LANDE, 1890)	*Tropocyclops prasinus* (FISCHER, 1860)	*Atheyella crassa* (SARS, 1863)	*Bryocamptus minutus* (CLAUS, 1863)	*Bryocamptus pygmaeus* (SARS, 1863)	*Bryocamptus typhlops* (MRAZEK, 1893)	*Canthocamptus staphylinus* (JURINE, 1820)	*Moraria (Moraria) brevipes* (SARS, 1863)	*Parastenocaris brevipes* KESSLER, 1913	*Parastenocaris germanica* KIEFER, 1936	*Parastenocaris fontinalis borea* KIEFER 1960	*Parastenocaris psammica* SONGEUR 1961	*Candona candida* (MÜLLER, 1776)	*Candonopsis kingsleii* BRADY & ROBERTSON, 1870	*Cryptocandona reducta* (ALM, 1914)	*Cryptocandona vavrai* KAUFMANN, 1900	*Fabaeformiscandona latens* (KLIE, 1940)	*Fabaeformiscandona wegelini* (PETKOVSKI, 1962)	*Heterocypris reptans* (KAUFMANN, 1900)	*Pseudocandona albicans* (BRADY, 1864)	*Troglochaetus beranecki* DELACHAUX 1921	*Anthrobathynella stammeri* (JAKOBI, 1954)	Type of habitat
KT1/B	15						18					1	4								1															Stressed
KT1/C	15						8					1	30								1		2													Stressed
KT2/B	15						8																1													Stressed
KT2/C	15						1												1																	Stressed
KT3/A	15						21																													Stressed
HB2/HZ	15	1					5						34																							Stressed
HB2/A	15						1						2																							Stressed
MB4/C	14					1							3																							Stressed
OW1/A	15				12			1																10									1			Stressed
OW1/B	15																															1				Stressed
OW2/B	15							21																				1								Stressed
OW3/A	15				9																	1		1												Stressed
OW4/A	15							1														6	4	19	2											Stressed
OW4/B	15																					1		3	1											Stressed

Species	OW4/C	KT2/A	KT1/A	KT4/A	KT4/B	HB1/A	HB1/B	HB1/C	HB3/A	HB3/B	HB3/C	MB3/B	MB4/B	OW2/A	MB4/A	OW3/HZ
Type of habitat	Stressed	GWstable	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWrainfed	GWswb	GWswb	GWswb	Hyporheic	Hyporheic
Anthrobathynella stammeri (JAKOBI, 1954)									22	9	27					
Troglochaetus beranecki DELACHAUX, 1921			4					1	4							
Pseudocandona albicans (BRADY, 1864)														1	347	
Heterocypris reptans (KAUFMANN, 1900)																
Fabaeformiscandona wegelini (PETKOVSKI, 1962)																89
Fabaeformiscandona latens (KLIE, 1940)		1	45													
Cryptocandona vavrai KAUFMANN, 1900						2										
Cryptocandona reducta (ALM, 1914)							1									
Candonopsis kingsleii BRADY & ROBERTSON, 1870																7
Candona candida (MÜLLER, 1776)														5		
Parastenocaris psammica SONGEUR, 1961																
Parastenocaris fontinalis borea KIEFER, 1960									12			1806				
Parastenocaris germanica KIEFER, 1936				1					194	5	1					
Parastenocaris brevipes KESSLER, 1913																
Moraria (Moraria) brevipes (SARS, 1863)				2					607	24	10					
Canthocamptus staphylinus (JURINE, 1820)														1		110
Bryocamptus typhlops (MRÁZEK, 1893)										2	2					
Bryocamptus pygmaeus (SARS, 1863)									10	1	2					
Bryocamptus minutus (CLAUS, 1863)														5	32	
Attheyella crassa (SARS, 1863)														4	93	87
Tropocyclops prasinus (FISCHER, 1860)															1	1
Thermocyclops dybowskii (LANDE, 1890)															1	
Paracyclops fimbriatus (FISCHER, 1853)		1	44	2	3				80	3	1			12	1529	9
Megacyclops viridis (JURINE, 1820)			33													
Macrocyclops albidus (JURINE, 1820)																10
Graeteriella unisetigera (GRAETER, 1908)												20		2		
Eucyclops serrulatus (FISCHER, 1851)				5	4										84	
Diacyclops languidus (SARS, 1863)	1	7												698		2
Diacyclops cf. *languidoides*		165	147	2206	1077	669	52	82	922	339	57			4	40	
Diacyclops crassicaudis (SARS, 1863)												39	44	80		
Diacyclops bisetosus (REHBERG, 1880)				220	103	521	35	53	13	1		20	70	106	3301	164
Acanthocyclops vernalis (FISCHER, 1853)									10							
Acanthocyclops robustus (SARS, 1863)												10			198	714
Niphargus aquilex SCHIÖDTE, 1855			99	186	8	33	1									
Number of samples	15	15	15	15	15	15	15	15	15	15	15	14	14	15	13	13
Sampling sites	OW4/C	KT2/A	KT1/A	KT4/A	KT4/B	HB1/A	HB1/B	HB1/C	HB3/A	HB3/B	HB3/C	MB3/B	MB4/B	OW2/A	MB4/A	OW3/HZ

Table 3. Results of a SIMPER similarity test (for aggregated data of 13-15 sampling events per trap). SIMPER similarity for stressed sites calculated from all data of trap KT2/A. Key species cumulating to inner group similarity to > 60%.

Ecological group	Average faunistic similarity per sampling site	Index species	Contribution to groups inner similarity [%]
Stressed	6.76	*Diacyclops* cf. *languidoides*	47.1
		Paracyclops fimbriatus (FISCHER, 1853)	18.7
GWstable	78.44	*Diacyclops* cf. *languidoides*	98.8
		None	-
GWrainfed	58.02	*Diacyclops* cf. *languidoides*	55.3
		Diacyclops bisetosus (REHBERG, 1880)	19.8
GWswb	29.48	*Diacyclops bisetosus* (REHBERG, 1880)	68.1
		Diacyclops crassicaudis (SARS, 1863)	20.9
Hyporheic	43.69	*Acanthocyclops robustus* (SARS, 1863)	28.6
		Diacyclops bisetosus (REHBERG, 1880)	27.3
		Attheyella crassa (SARS, 1863)	23.3
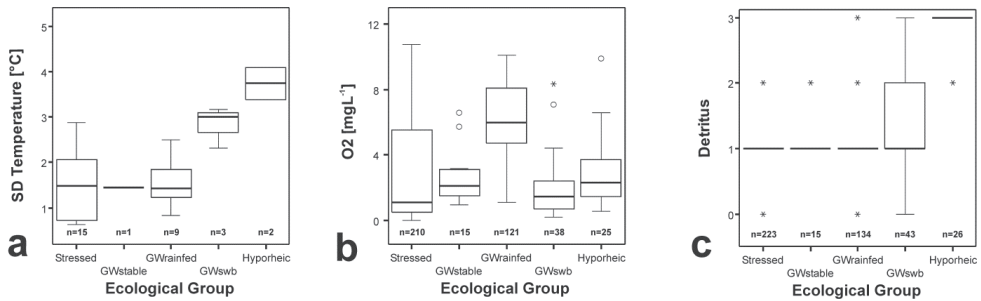
Figure 3. Standard deviations of environmental factors for each of the ecological groups. **a** Temperature [I =Stressed, II = GWstable, III = GWrainfed (recharged by precipitation), IV = GWswb (surface water body-recharged), V = Hyporheic] **b** DO-concentration, and c) detritus contents (estimated). Box = Interquartile range, vertical black bar = median; whiskers showing the lowest and highest non-outlier. Circles showing outliers and stars extreme outliers.

was in the same low range as groups (I) and (II). The percentage of stygobites was high (mean = 77.23%), with intermediate abundances (mean = 59.2 individuals/sample; Fig. 4b, c). The average SIMPER similarity was 58.02% and *Diacyclops* cf. *languidoides* was again the characteristic species, contributing with 55.31% to the inner similarity of this group. Only in this group – but not in all traps - amphipods (Table 2) were found in appreciable numbers.

GWswb habitats (IV) were found in the Haardtrand and in the Upper-Rhine-Plateau. These habitats were situated within only a few meters from a brook. This special vicinity was reflected by higher variances in GFI values (mean = 3.68, SD GFI = 3.52) and declining proportions of stygobites (mean = 61.14%; Fig. 4a, b) in comparison to groups (II) and (III) abundances that were found to be lower (mean = 66.4 individuals/sample) (median, Fig. 4c) than for group (III). The average SIMPER similarity (Table 3, Fig. 4d) for faunistic communities was 29.48% and the taxon contributing
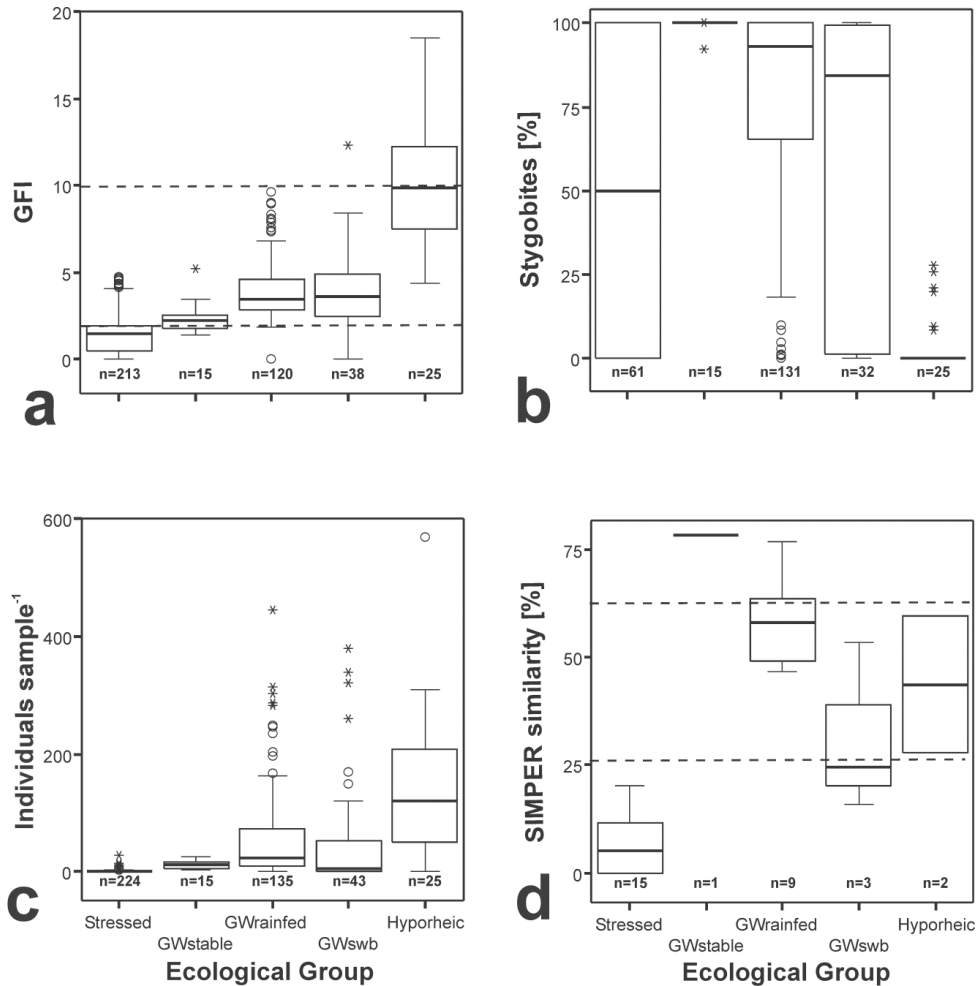
Figure 4. Boxplots on **a** Groundwater-Fauna-Index-values **b** percentage of stygobiotic species **c** Individuals per sample (one outlier omitted each in the groups GWrainfed, GWswb and Hyporheic) and **d** similarity [%] of faunistic communities in scaled ecological groups. Thresholds for alimony are marked by dashed lines (after Hahn 2006) in Fig. 4a and for faunistic stability (according to Gutjahr et al. 2013a) in Fig. 4d; n = number of samples, box = Interquartile range, vertical black bar = median; whiskers showing the lowest and highest non-outlier; circles showing outliers and stars extreme outliers.

most to the group's inner similarity was the cyclopoid *Diacyclops bisetosus* (Rehberg, 1880) (68.05%). The previous two types of habitats (III and IV) were characterised by a higher alimony (Fig. 4a).

Hyporheic habitats (V) were identified only in the Haardtrand and the Upper-Rhine-Plateau. The sampling sites were situated in the hyporheic zone of the brook. The influence of the flowing waters was reflected by the highest GFI values (mean = 10.41; Fig. 4a), by the highest numbers of individuals (mean = 265.5 individuals/sample) as well as by the lowest proportions of stygobites in all groups (mean = 4.49%;

Fig. 4b, c). The average SIMPER similarity here was 43.69% (Fig. 4d) and the most formative species for this group was the stygophilic cyclopoid *Acanthocyclops robustus* (Sars, 1863) with 28.60% contribution to the group's inner similarity (Table 3).

Discussion

Aquifers are an assemblage of habitats, where life is limited mainly by the supply of oxygen and organic carbon (Schwoerbel 1961, Husmann 1966, Williams and Hynes 1974, Strayer et al. 1997, Pospisil 1999, Datry et al. 2005, Schmidt and Hahn 2012). These characteristics could be reflected by the subterranean invertebrate communities, well described by the GFI and the SIMPER similarity in this survey. The SIMPER similarity calculated over samples per site or trap describes, in the broadest sense, the variability of faunal communities. High variability may be an indicator for stressors within a habitat (Gutjahr et al. 2013a). To simplify matters, other potentially important factors such as community assembly rules, biotic interactions and stochastic effects, are not considered here.

The distribution on the MDS ordination corresponds to the altitude of the sampling sites with the Pfälzerwald Mountains on top, and by the faunistic communities' composition as the proportions of stygobiotic fauna decrease and GFI values rise. Furthermore, GWrainfed habitats were found predominantly in the Pfälzerwald Mountains and GWswb habitats in the Upper Rhine Plateau. Species like *Niphargus aquilex* (Schioedte, 1855) do not indicate GWrainfed, but groundwater from adjacent fractured rock. *Diacyclops* cf. *languidoides* and *Diacyclops languidoides* (Lilljeborg, 1901) are considered to be typical of the Pfälzerwald Mountains and the Upper Rhine Plateau respectively (Hahn 2004, 2006), though the taxonomy of this group is still questionable. An overlapping of species, some of which typical for the Pfälzerwald and others which are typical for the Upper Rhine Plateau at the sampling sites of the Haardtrand, pointed towards a transition zone between two stygoregions: the Central Uplands and the South-Western Uplands (Stein et al. 2012). This posed the question as to whether a landscape effect or an aquifer-related effect is observed here, rather than general ecological patterns.

At the Haardtrand, all habitat types described in this study, with the exception of the rare GWstable, were found. However, GWrainfed site MB1 was not considered here due to low sampling frequency (four sampling occasions only). This site however, situated at the edge of the valley, was similar with respect to invertebrate assemblage to the GWrainfed habitats, situated 50 km apart.

Stressed habitats were observed all over the study area, which negated the assumption of an effect caused only by a transition between two stygoregions. Aquifer-related effects could be neglected, as all traps were situated in porous aquifers. However, the GWrainfed habitats of the Pfälzerwald were influenced by groundwater from the adjacent fractured rock aquifers (Hahn 2004). At the Haardtrand [MB] most types of habitats - excluding GWstable and GWsoilrech - were represented within an area of

one hectare. This area constituted a small-scale example of the variability across all types of groundwater habitats of the whole investigated area. Having most types of habitats within the transition zone between two stygoregions (as delineated by Stein et al. 2012) indicated rather ecological and functional patterns than bioregional ones.

The five types of habitats identified here are considered distinct groundwater habitats. It was challenging to assess the ecological appartenance to a definite category for some of the traps. The MDS ordination clustered some of the traps as being "Stressed", whereas actually did comprise of stressed traps and GWswb traps (i.e. OW1/A and OW3/A). Since those traps were characterised by depleted faunal assemblages, with low SIMPER similarities (Gutjahr et al. 2013a) and surface water recharge (Hahn 2006) most seemed to take place as occasional pulse-like water intrusion from the surface. These two traps were also categorised as "Stressed". The proposed classification implies definitive parameters and may be categorised as an organisational indicator, but it cannot be assumed that each trap fits into one definitive category; there are gradients that could in fact delineate cryptic transitions between the idealised ecological groups characterised here. In accordance with former classification systems, a habitat synopsis is proposed here (Table 4).

Stressed habitats (I) were found in all natural investigated regions and constituted a heterogeneous group, comprising about 50 percent of all traps and were characterised by very harsh living conditions. Low oxygen concentrations and small pore spaces (compact aquifers), caused by iron ochre (e.g. KT3/A) or silt (e.g. MB4/C), as well as low amounts of OM acted as natural stressors in these traps (Fišer et al. 2012). The heterogeneity of this group was expressed by faunal communities as well as by abiotic parameters. Stressed habitats comprised two sub-types, indicating two different stressors. The first subtype was characterised by low OM availability at well-shielded locations mainly in the Pfälzerwald Mountains with very constant conditions, and secluded oligo-alimonic groundwater with low impact from the surface. However, there were sufficient DO-concentrations of > 1 mg L^{-1} for invertebrates, but there was virtually no stygobiotic fauna (e.g. KT2/B). This was indicated for example by the presence of species like *Diacyclops* cf. *languidoides*

Table 4. Proposal of a classification scheme of groundwater habitats by integrating former classifications on a local scale.

New classification approach	Stressed	GWstable	GWrainfed	GWswb	Hyporheic
Hahn (2006)	Oligoalimonic		Mesoalimonic		Eualimonic
GFI-value	< 2		2–10		> 10
Schmidt & Hahn (2012)	Old groundwater recharged by precipitation or surface water		Rainfed recharged by precipitation	Surface water recharged	
				Groundwater / surfacewater ecotone	
Gutjahr et al. (2013a)	Stressed sites	Stable sites	Intermediate sites		
Samples needed to catch 95% of occurring species	11.55	4.27	5.79		
SIMPER-similarity [%] (mean)	10.50	71.05	46.23		

or, especially for compact aquifers, by species of the genus *Parastenocaris* in low abundances. The second sub-type comprised traps which were almost permanently hypoxic (e.g. OW4/A) or affected by silt or iron ochre. Presumably, only occasional pulse-like surface water ingresses promoted significant supplies with DO, as for OM (e.g. OW1/A, situated directly at a gully) and ubiquistic invertebrates (e.g. the stygophilic cyclopoid *Diacyclops bisetosus*). Apart from these events, the impoverished fauna was dominated by tolerant *Parastenocaris* species (Hahn 1996, Matzke 2006). This statement was supported by the fluctuating proportions of stygobites and ubiquists at these sampling sites, as well as fluctuating standard deviations of temperature, low numbers of species and individuals. For such habitats Gutjahr et al. (2013a) proposed the term "Stressed", as they often exhibited low pore spaces, caused by ochry or silty sediments and harboured a depleted invertebrate fauna [see Hahn (2009), who named these "compact" sites]. These habitats required a high sampling effort (18.46 samplings) to collect 95% of the occurring species. Furthermore, the SIMPER similarities were comparatively low, indicating heavily fluctuating communities and a very high potential for stress. Sampling sites with GFI values lower than 2 are oligo-alimonic, according to Hahn (2006). A part of the stressed habitats would be interpreted as being "old groundwater", according to Schmidt and Hahn (2012), as they exhibited only low amounts of DO and/or OM, indicated by the lowest GFI within the study. In contrast to Gutjahr et al. (2013a) the latter two approaches did not distinguish stressed from stable habitats.

Gutjahr et al. (2013a) found faunistically and hydrologically stable habitats with little surface water influence at various depths all over the study area in Baden-Wuerttemberg, South-Western Germany. Within this study one stable trap was identified (KT2/A) in the group GWstable (II). For this habitat, the highest SIMPER similarity (78.4%), indicated very stable faunistic communities and it is assumed, that abiotic conditions are also stable here. Constantly low GFI-values (~2) indicated the upper boundary of the oligo-alimonium category. The stability was confirmed by a constantly low population and low numbers of species, but characterised by the highest percentages of stygobites. Stable habitats were characterised by a poor supply with OM and intermediate OM-values. However, they still offered enough OM and DO for the uncompetitive though tolerant stygobites, but not for ubiquists (Schmidt & Hahn 2012). DO concentrations were uniformly above the critical threshold for a permanent invertebrate colonisation (1 mg L^{-1}, Hahn 2006) and OM amounts are low, but still high enough to maintain stable invertebrate communities (Hahn 2006). These parameters indicate well-shielded groundwater with little influence from the surface. After Hahn (2006), it is believed that OM quality is lowest within GWstable habitats (with the exception of oligo-alimonic stressed habitats). Within the sampling period of fifteen months, a moderate surface water impact occurred only once. This statement was supported by one individual of the ubiquistic *Paracyclops fimbriatus* (Fischer, 1853) found in the trap. According to Schmidt and Hahn (2012), GWstable habitats (II) correspond to the "old groundwater ecosystem" with a very low influence of surface water. These ecosystems are secluded in depth and have low or almost no connectivity to the epigean environment.

While they were different in terms of the origin of the infiltratis water, the next two described habitat types (III and IV) shared some characteristics: since both were moderately influenced by surface water, well-supplied with OM and intermediately supplied with DO. The groups GWrainfed (III) and GWswb (IV) were both characterised by medium GFI values, with quite high proportions of stygobites and intermediate invertebrate abundances. With increasing surface water influence, the faunistic communities were replaced by ubiquitous species. One species characteristic of both groups and in all landscapes investigated was the ubiquistic *Diacyclops bisetosus*.

Habitats recharged by precipitation and water percolation through the soil profile (Schmidt and Hahn 2012) were grouped as GWrainfed (III), which comprised only of habitats recharged by groundwater from adjacent fractured rock, situated at the edge of the valleys and with moderate influence of intruding surface water. This was indicated by high amounts of DO (mean = 6.20 mg L^{-1}, which are typical for the Pfälzerwald Mountains) and typical species, such as the stygobiotic *Niphargus aquilex*. This species is a typical representative of the fractured aquifers in sandstones recharged by percolation water (Hahn 2006). GFI values between two and ten indicate meso-alimonic conditions (Hahn 2006). This group featured intermediate to high species richness and densities, intermediately stable habitats (in terms of DO and nutrient supply) and the strong dominance of stygobites. In terms of faunistic stability (i.e. SIMPER similarity, 58%) those habitats were found to be intermediately stable (Gutjahr et al. 2013a). This was also reflected by a high sampling efficiency of 4.13 samples necessary to collect 95% of the occurring species (Gutjahr et al. 2013a). Due to this, it is assumed that there are very few ingressions of surface water containing ubiquistic fauna, and subsequently no influence on the occurring faunal communities.

Unlike the abiotic conditions characteristic to GWrainfed, the group GWswb (IV) suggested surface water inputs from brooks, gullies and transient ponds, but not of soil water. The intrusion of surface water could take place much faster at these habitats than at GWrainfed, as higher standard deviations of temperature (mean = 1.52 °C for GWrainfed, mean = 2.84 °C for GWswb) and showed a subsequent immigration of epigean invertebrates. These habitats were less stable faunistically than those in group GWrainfed. The characteristic species to this group were the stygobiotic *Graeteriella unisetigera* (Graeter, 1910) and the ubiquistic *Diacyclops crassicaudis* (Sars, 1863), the latter known to be a riparian species (Dole-Olivier et al. 2000). However, *G. unisetigera* is not to be considered as an indicator of GWswb, but as a typical species of the Upper Rhine Valley (unpublished data). With GFI values from two to five GWswb, habitats have to be regarded as meso-alimonic habitats according to Hahn (2006). As GWswb habitats were all situated in the vicinity of surface waters, they were heavily influenced by epigean ecosystems (Schmidt and Hahn 2012). Sampling efficiency was, compared to GWstable and GWrainfed, relatively low (7.9 sampling events necessary to collect 95% of the occurring species, Gutjahr et al. 2013a). According to Gutjahr et al. (2013a) GWswb habitats should be classified as intermediately stable habitats with SIMPER similarities of roughly 29.5%.

The hyporheic (V) habitats exhibited the highest surface water influence and were regarded as being well-supplied with OM and intermediate DO-values (suggested by the highest registered scores of GFI, mean = 10.41) in combination with highest abundances (mean = 265.5 individuals /sample) and species diversity (Griebler and Mösslacher 2003). Despite being supplied in sufficient amounts of OM from the surface water, DO values were very intermediate here (as well as in GWswb), presumably due to respiration processes. Highest standard deviations of temperature (mean = 3.74 °C) indicated vast influences of surface waters. This epigean vicinity was also reflected by the faunal assemblages, which displayed the highest percentage of ubiquistic invertebrates (mean = 95.51%). Key species for this group were the ubiquistic epigean *Canthocamptus staphylinus* (Jurine, 1820)*, Acanthocyclops robustus* (Sars, 1863)*, Attheyella crassa* (Sars, 1863)*, P. fimbriatus* and *Fabaeformiscandona wegelini* (Petkovski, 1962). With regard to alimonic conditions, ecotonal habitats have to be classified eu-alimonic according to Hahn (2006) with GFI values higher or equal to ten. According to Schmidt and Hahn (2012) these habitats have to be considered surface water recharged with an ecotonal character. As no drying out or sediment runoff events took place, there were intermediately stable faunistic communities in the hyporheos (SIMPER similarity 43.7%). 5.4 sampling occasions would have been necessary to collect 95% of the occurring species within this type of habitat. Although the trap HB2/HZ was situated in the hyporheic zone, it was supplied by deep, upwelling groundwater of the valley (Hahn 2005), similar to the nearby HB2/A (distance < 1m).

Different habitats can occur in a vertical stratification within wells. This is true for MB4, where the upper trap MB4/A was classified as hyporheic (V) due to surface water influence by the nearby brook. MB4/B in the middle was influenced faunistically by both groundwater and surface water and it was thus classified as GWswb. MB4/C was influenced by deep and predominantly hypoxic groundwater with sporadic pulses of surface water. This was indicated by low numbers of individuals and the occurance of the ubiquistic *P. fimbriatus*.

Vertical stratification is also true for KT1 and KT2. KT1/A was classified as GWrainfed, faunistically indicating the underground runoff from the hills slopes. KT2/A was found to be a trap with very constant abiotic conditions and very stable faunistic communities. KT1/B /C and KT2/B /C were traps with constant conditions, well DO supply but OM was lacking. These traps were classified as stressed habitats and due to the absence of food only low numbers of species and individuals could be found.

From these findings abiotic characteristics and community traits of the habitat types are proposed (Table 5).

Previous classification schemes of GW habitats by Hahn (2006), Schmidt and Hahn (2012) as well as by Gutjahr et al. (2013a) are convergent and can be combined to reflect a wider range of characteristics within the described habitats. Compared to other classification approaches, the approach presented here includes alimony as well as surface water connection and stress related parameters. At a landscape scale, the type of aquifer seems to drive the groundwater habitats (Gibert et al. 1997, Hahn and Fuchs 2009). However, all traps analyzed here are situated within porous aquifers, so that consequently statements on the aquifers influence on these communities are not

Table 5. Characteristics of the groundwater habitats proposed (- = low/little; o = intermediate; + = high/much); SIMPER similarity for stable sites calculated from all data of trap KT2/A. The table has orientational character; there may be gradients and smooth transitions. The columns are in accordance to those in Table 4.

	Type of habitat				
Traits	**Stressed**	**GWstable**	**GWrainfed**	**GWswb**	**Hyporheic**
metazoan abundance	-	-	o	o	+
number of species	-	-	+	+	+
% stygobites	variable, depending on site	+	o	o	-
alimony	variable, depending on site	-	o	o	+
oxygen	variable, depending on site	o	o	o	+
OM	variable, depending on site	-	o	o	+
stability	-	+	o	-	o
Samples needed to catch 95% of occurring species	18.46	5.4	4.13	7.9	5.4
SIMPER similarity [%] (mean)	15.75	78.44	46.73	28.84	40.45

possible – with one exception: the influence of fractured rock aquifers in the Pfälzerwald Mountains. Differences between two of the stygoregions described by Stein et al. (2012) were detected, but could not explain the groundwater invertebrates' distribution all over the investigated area. In particular, samples from the Haardtrand, the transition zone between the stygoregions Central Uplands and South-Western Uplands (Stein et al. 2012), harboured (with the exception of GWstable) all groups identified within this classification concept.

The groundwater habitats derived from faunistic data yielded in a five-class system provided valuable information for understanding patterns in the sampled region of the Palatinate, South-Western Germany and allowed the incorporation of former classification approaches. Gutjahr et al. (2013b) transferred the findings of this study to another local scale groundwater survey in Baden-Wuerttemberg, South-Western Germany, where four of the five habitats proposed here were identified. While limited to a comparatively moderate dataset from a specific region, we propose that this concept describes general patterns. Hence, it will be exciting to see whether this approach can be supported by further studies and study areas, including karstic regions.

Acknowledgements

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Supplementary material 1

Graphic output of a hierarchical cluster analysis (single linkage) of faunistic data.
Authors: Simon Gutjahr, Susanne I. Schmidt, Hans Jürgen Hahn
Data type: Statistical data.
Explanation note: Cluster analysis of the ecological goups' faunistic data based on a Bray-Curtis-similarity-matrix (calculated using a dummy variable).
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Supplementary material 2

Output of the one-way ANOSIMS' pairwise test.
Authors: Simon Gutjahr, Susanne I. Schmidt, Hans Jürgen Hahn
Data type: Statistical data.
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Supplementary material 3

Output of a Canonical Analysis of Principal Coordinates (CAP).
Authors: Simon Gutjahr, Susanne I. Schmidt, Hans Jürgen Hahn
Data type: Statistical data.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

RESEARCH ARTICLE

Distribution and conservation status of *Speleonycta ozarkensis* (Insecta, Zygentoma, Nicoletiidae) from caves of the Ozark Highlands of Arkansas and Oklahoma, USA

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Abstract

The "thysanuran" (Zygentoma: Nicoletiidae) *Speleonycta ozarkensis* is the only troglobiotic nicoletiid from the Ozark Highlands. It was originally described with only four specimens from four different cave systems in Arkansas and Oklahoma. The scarcity of available specimens has made it difficult to determine whether morphological variation among populations represents intraspecific or interspecific variation. We examined molecular (16S rRNA) variation among populations and found no evidence that they represent a species complex. Because of its limited distribution and lack of ecological and life history data, *S. ozarkensis* may be a species of conservation concern. We therefore conducted a conservation status assessment. We bioinventoried 44 caves in Arkansas and Oklahoma to determine the distribution of *S. ozarkensis*. A new locality in Adair Co., Oklahoma, was discovered and new specimens were collected to better assess morphological variation among populations. Data on ecology and life history was gathered. We determined the conservation status of the species and identified potential threats to existing populations. Despite being known from a few localities, *S. ozarkensis* has a broad distribution approaching 10,000 km^2. Molecular

data suggest *S. ozarkensis* is capable of considerable dispersal and is primarily an epikarstic species, perhaps explaining why it has been infrequently collected from caves. Conservation assessments revealed that *S. ozarkensis* is at a slight risk of extinction. We identified seven threats impacting populations that vary in scope and severity, but only recreational caving (three caves) and development associated with urbanization (one cave) have the greatest potential to immediately impact populations.

Introduction

A new genus and species of troglobiotic nicoletiid ("thysanurans", bristletails or silverfish), *Speleonycta ozarkensis* Espinasa et al., 2010 (Insecta: Zygentoma: Nicoletiidae: Cubacubaninae), was recently described from specimens collected from four localities in the Ozarks Highlands of northeastern Oklahoma and northwestern Arkansas. The four localities are Bear Hollow Cave and Chambers Hollow (=Uno) Cave in Benton Co., Arkansas, Single Barrel Cave in Cherokee Co., Oklahoma, and Black Hollow Cave in Delaware Co., Oklahoma. At the time of the description, nicoletiid silverfishes were also known from two additional localities in Arkansas (Tweet's Cave and Wolf Creek Cave in Newton Co.) and one locality in Oklahoma (McGee's Cave in Delaware Co.), but specimens were not available for examination. Espinasa et al. (2010) assumed that all seven populations belonged to the same species, and that it was endemic to cave systems within the Ozark Plateau region, spanning a distribution approaching 10,000 km^2 (Figure 1).

Despite several collecting trips, these caves only yielded a single specimen of *S. ozarkensis* from each locality (Espinasa et al. 2010). The four available specimens at the time of description varied with respect to stage of postembryonic development and gender (2 males and 2 females). The scarcity of specimens has made it difficult to determine whether morphological variation among populations represents intraspecific or interspecific variation. There is the possibility that the reported sexual secondary differences (Espinasa et al. 2010) among specimens of the same gender but from different caves actually reflect that the species is a complex of closely related species. For example, multiple cave populations of nicoletiids of the genus *Texoreddellia* spanning over 300 km of Texan karst were thought to be the same species (*T. texensis*; Wygodzinsky 1973), until molecular analyses revealed this group actually represents a complex of at least six closely related species (Espinasa and Giribet 2009).

Because of the species' limited distribution and lack of information on ecology and life history, *S. ozarkensis* may be a species of conservation concern. However, the conservation status of this nicoletiid has not yet been assessed under NatureServe and IUCN Red List criteria, because of the recency of its description. In addition, genetic information (16S rRNA gene) is only available for a single specimen collected from Chambers Hollow (=Uno) Cave, Benton Co., Arkansas. The objectives of the present study were to: (1) determine the distribution of *S. ozarkensis* within the Ozark Highlands of northwestern
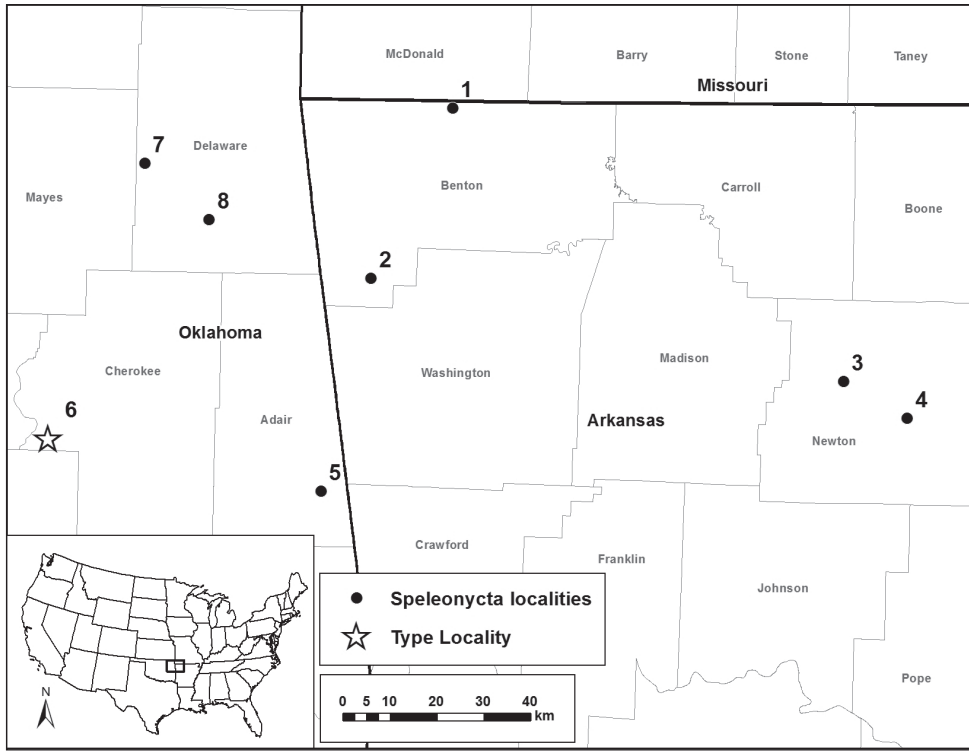
Figure 1. Distribution of *Speleonycta ozarkensis*. Numbered localities correspond to those listed in Table 1.

Arkansas and northeastern Oklahoma; (2) compare newly collected specimens to better assess morphological variation among populations; (3) generate novel DNA sequences for new populations; (4) collect information on ecology and life history, such as microhabitat within caves; and (5) determine the conservation status of *S. ozarkensis* and identify potential threats to existing and newly discovered populations.

Materials and methods

Cave surveys. We surveyed 44 caves in northwestern Arkansas and northeastern Oklahoma within the suspected range of *S. ozarkensis* between January 2010 and May 2014. Sampling effort varied among caves due to variation in cave length and amount of available habitat. Nonetheless, visual encounter surveys (VESs) were conducted by a minimum of two observers for at least two person-hours in all caves and consisted of visually searching terrestrial habitat (cave floor, cave walls, and underneath rocks and other debris) in human-accessible passages. Specimens encountered were collected by hand and deposited into 95% ethanol for preservation. Any rocks, logs and other debris were returned to their original positions to minimize habitat disturbance.

Morphological analysis. Observations of body parts were made with a Motic K series stereo microscope. Specimens in ethanol were observed directly in petri dishes so as to preserve the integrity of the specimens.

Molecular methods. Genomic DNA from two specimens was extracted using Qiagen's DNEasy® Tissue Kit by digesting a leg in lysis buffer. PCR amplification and sequencing of a 501 bp portion of the 16S rRNA mitochondrial gene followed standard protocols and primers used previously for nicoletiids (Espinasa and Giribet 2009). Sequencing reactions were performed using original PCR primers and run on an ABI PRISM 3100 (Applied Biosystems) sequencer. Forward and reverse sequences for each sample were aligned and edited in Sequencher 3.0 with ambiguous base calls verified manually by examining the electropherogram for each sequence. These sequences were aligned to each other into contigs and then a Blast analysis was performed to find which sequence in GenBank had highest similarity.

Conservation assessments. We conducted conservation assessments under both NatureServe and IUCN Red List criteria. NatureServe's system of assessing conservation status uses ten primary factors grouped into three main categories: rarity, trends, and threats (Master et al. 2009). Rarity factors include range extent, area of occupancy (AOO), number of occurrences, number of occurrences with good viability or ecological integrity, population size, and environmental specificity. Trend factors include both short-term and long-term trends in population size, extent of occurrence (EOO), AOO, number of occurrences, and viability or ecological integrity of occurrences. Finally, threat factors include threat impact and intrinsic vulnerability to threats. Other information is often used in addition to the ten conservation status factors to assess conservation status, including the number of protected or managed occurrences, rescue effect, and other considerations. NatureServe conservation global status assessments for each lineage were calculated using default points and weights with the NatureServe Rank Calculator worksheet available in Microsoft Excel (Faber-Langendoen et al. 2009). All *Speleonycta* populations are known from caves, so we considered the species to have a 'very narrow' environmental specificity.

Both NatureServe and IUCN Red List assessments use two different measurements of geographic range size: extent of occurrence (EOO; referred to as range extent by NatureServe) and area of occupancy (AOO). We calculated EOO and AOO using the web-based program GeoCAT (Bachman et al. 2011). EOO was calculated as a convex hull, which is the smallest polygon that contains all the sites of occurrence and no interval angles exceeding 180°. We followed NatureServe (Faber-Langendoen et al. 2009) and IUCN (2010) guidelines and used a grid size of 2 km (4 km²) to estimate AOO. Accurate estimates of population size are difficult for most subterranean species due to the inaccessibility of and difficulty associated with surveying in cave habitats. Short-term and long-term trends were not used in conservation status assessments because of the lack of historical data for most occurrences. The IUCN-Conservation Measures Partnership Classification of Threats (Salafsky et al. 2008) following the threat assessment protocol outlined in Master et al. (2009) was employed to evaluate the scope, severity and timing of specific threats that are either observed, inferred or suspected to impact *Speleonycta* populations.

A species may be classified as critically endangered (CR), endangered (EN), or vulnerable (VU) on the IUCN Red List if it meets specific conditions under any one of these five criteria (IUCN 2001): (A) past, present, or projected reduction in population size over three generations; (B) small geographic range in combination with fragmentation, population decline or fluctuations; (C) small population size in combination with decline or fluctuations; (D) very small population or very restricted distribution; and (E) a quantitative analysis of extinction risk. Criteria for threat classification under categories A, C, and E require evidence of declining trends in population size. Unfortunately, such data are lacking for *Speleonycta* populations at present. Consequently, our assessments focused on criteria under categories B and D.

Results

Since 2010, we surveyed 14 caves in northwestern Arkansas, including 5 in Benton, 7 in Carroll, one in Newton, and one in Washington counties, and 30 caves in northeastern Oklahoma, including five in Adair, one in Sequoyah, and 24 in Delaware counties. Apart from the seven caves where nicoletiids had previously been reported, at only a new single locality were nicoletiids found. Three nicoletiids were observed at a cave (AD85) in Adair County, Oklahoma, on 18 November 2012 (Fig. 2). The cave consists largely of a maze of largely dry, joint-controlled passages with a few isolated drip pools. All three individuals were observed underneath rocks in a ca. 50 m section of passage. The cave was very dry due to prolonged drought conditions in the region. Presumably, these specimens were seeking refuge underneath larger rocks that retained some moisture. Two specimens were collected for morphological and genetic studies. In addition to *S. ozarkensis*, other species were observed in the same section of the cave, including diplurans (*Litocampa* sp.), harvestmen (*Crosbyella* sp.), and fungus beetles (*Ptomaphagus cavernicola* and *P. shapardi*).

The two specimens collected were adult males measuring 7.3 and 8 mm in body length and were identified as *S. ozarkensis*. Despite considerably smaller than the two males used to described the species by Espinasa et al. (2010), which measured 11 mm, the males collected from AD85 also exhibit adult characters, including: (a) pedicellus with clusters of unicellular glands, and a bladelike spine not very sclerotized and with more unicellular on the outer border; (b) tibia of first leg very stout (two times longer than wide) with a large bulge with three distinctly long, sclerotized, and curved macrochaetae; (c) paramera attain apex of stylets IX, are stout with a distal semi-eversible vesicle and with long specialized macrochaetae; and (d) cerci with sensory pegs.

The 16S rRNA sequences of both specimens collected from AD85 were identical. Moreover, the sequences were also identical to the 16S rRNA sequence from the *S. ozarkensis* specimen previously collected from Chambers Hollow (=Uno) Cave, Benton Co., Arkansas (GenBank no. KJ128288).

Conservation assessment. *Speleonycta ozarkensis* has an EOO of 9,833.69 km² and an AOO of just 32 km² in the Ozark Highlands of northeast Oklahoma and

Figure 2. *Speleonycta ozarkensis* from AD85, Adair Co., Oklahoma.

northwest Arkansas (Fig. 1). The species has only been documented from eight caves in five counties. Estimates of population size are unknown but believed to be small. The maximum number of individuals observed during a single survey is three from AD85; a single individual has been observed at the other seven localities. This species was unknown to science until 2010. Consequently, inferences on trends in population sizes, quality of habitat and range size are extremely limited. Quality of habitat at the eight known localities has not changed significantly in the last five years.

We identified seven threats documented or suspected to impact populations of *S. ozarkensis* at the present or in the future, including residential and commercial development, agriculture (livestock farming and ranching), energy production and mining, amateur and scientific collecting, recreational caving, dams and water management, and pollution. These threats vary in scope (negligible to restricted), severity (negligible to serious) and overall impact (negligible to low) among populations. The most significant threats include recreational caving at localities with unrestricted access and the potential for urbanization within the recharge zone of Bear Hollow Cave in Benton Co., Arkansas. Of the eight known occurrences of *S. ozarkensis*, the main entrances of at least five caves occur on public lands or are managed for their cave and karst resources. Two caves are located on U.S. Forest Service land in Arkansas and are gated or have restricted access due to the presence of endangered bats (Chambers Hollow and Wolf Creek caves). One cave is located on public land owned by the state of Oklahoma (Black Hollow Cave). Additionally, two caves are owned by The Nature Conservancy and are gated (Bear Hollow and McGee's caves). Scientific and amateur

Table 1. Known localities and specimens of *Speleonycta ozarkensis* in the Ozark Highlands of Arkansas and Oklahoma.

Locality No.	Cave	County	State	Ownership /Management	No. Specimens
1	Bear Hollow Cave	Benton	AR	The Nature Conservancy	1
2	Chambers Hollow Cave	Benton	AR	U.S. Forest Service	1
3	Tweet's Cave	Newton	AR	Private	1
4	Wolf Creek Cave	Newton	AR	U.S. Forest Service	1
5	Wady Cave (AD85)	Adair	OK	Private	3[a]
6*	Single Barrel Cave	Cherokee	OK	Private	1
7	Black Hollow Cave	Delaware	OK	State of Oklahoma	1
8	McGee's Cave	Delaware	OK	The Nature Conservancy	1

[*] type locality
[a] two specimens vouchered.

collections are believed to have had a very minimal affect on any single population, as only six specimens have been collected to date. We assigned an overall threat impact of "Medium" based on a NatureServe conservation threat assessment.

We assigned a NatureServe conservation global rank of G3 (Vulnerable) to *S. ozarkensis* and state ranks of S2 (Imperiled) and S1S2 (Critically Imperiled to Imperiled) for Arkansas and Oklahoma populations, respectively. We classified *S. ozarkensis* as "Near Threatened" under the IUCN Red List classification. While *S. ozarkensis* classifies as "Vulnerable" under Criterion 1a (EOO < 20,000 km^2 and severely fragmented range or known to exist at no more than 10 locations), there is insufficient evidence at the present time to infer if a continual observed or projected decline (Criterion 1b) and/or extreme fluctuations (Criterion 1c) in EOO, AOO, extent or quality of habitat, number of populations or number of mature individuals exists.

Discussion

Few nicolettid are known from caves in the US despite the family being common in neotropical caves (Espinasa and Giribet 2009). In temperate regions, cave nicoletiids have a limited distribution and are known only from California (Espinasa and Botelho in press), Arizona (*S. anachoretes*; Espinasa et al. 2012), Texas (at least six species in the genus *Texoreddellia*, Espinasa and Giribet 2009) and the Ozark Highlands of Arkansas and Oklahoma (*S. ozarkensis*). *Speleonycta ozarkensis* is the only described nicoletiid thysanuran (silverfish) from the Ozark Highlands.

Despite only being collected from eight caves in five counties, *S. ozarkensis* apparently has a broad distribution approaching 10,000 km^2 and includes three ecoregions (Dissected Springfield Plateau, Lower Boston Mountains and Upper Boston Mountains). Ecoregions are defined by general similarity of surface ecosystems, including similarity in both biotic and abiotic characteristics (such as geology, physiography and hydrology that are important constraints in cave development). While previous studies have shown that

ecoregions often delimit range boundaries for many subterranean organisms (Niemiller and Zigler 2013, Niemiller et al. 2013), this was not the case for *S. ozarkensis* as its broad distribution spans three different ecoregions (Woods et al. 2004; Woods et al. 2005).

More important, molecular data indicate that this species may not be comprised of significant fragmented and isolated populations as distributional data might otherwise suggest. DNA sequences of the 16S rRNA gene were identical between specimens from Chambers Hollow Cave in Benton Co., Arkansas, and AD85 Cave in Adair Co., Oklahoma. These two caves are separated by 48.8 km (straight-line distance). Within the subfamily Cubacubaninae, intrapopulation variation averages 1.7 nucleotide differences (range 0–7; n = 29) while interpopulation variation averages 3.4 nucleotide differences (range 0–13; n = 22) within species at the 16S rRNA locus (Espinasa and Giribet 2009). Among sister species, genetic variation averages 31.2 nucleotide differences (range 10–64; n = 14). Identical gene sequences between the specimens located ca. 50 km away suggests that *S. ozarkensis* is capable of considerable dispersal and is primarily an epikarstic species exploiting the extensive network of cracks, fissures and smaller cavities nearer the surface than larger subterranean voids that are human accessible. This may explain why *S. ozarkensis* has been infrequently collected from caves.

A broad distribution despite few known localities has obvious implications with regards to conservation and management of *S. ozarkensis*. Our conservation status ranks, G3 (Vulnerable) for NatureServe and "Near Threatened" for IUCN Red List, reflect a broad distribution as well as a medium threat impact. While only known from eight cave systems, five *S. ozarkensis* populations are afforded some protection. Four caves are gated and one occurs on public land and is managed for its biological and other resources. Restricted access limits potential negative impacts to the populations from recreational caving. The three other localities occur on private lands and may be at a greater risk from recreational caving. The population at Bear Hollow Cave in Benton Co., Arkansas, faces the greatest risk of impacts associated with urbanization. Although the risk is currently low, we recommend that residential and commercial development within the recharge zone and the potential impacts to cave life within this cave system be monitored over the next 10 years. In addition, another recommendation is to resurvey and monitor known localities while also searching for additional cave systems that may harbor *S. ozarkensis*. The establishment of long-term monitoring programs is needed to provide data on population sizes and threats to individual populations over time. Additional surveys are also warranted to document new localities but also assess whether the distribution of *S. ozarkensis* is greater than currently known. The species likely also occurs within cave systems and epikarst of the Ozark Highlands of southwestern Missouri.

The two specimens collected from AD85 Cave in Adair Co., Oklahoma, have increased our understanding of the postembryonic development of the *S. ozarkensis*. A typical adult morphology and presumably sexual maturity has been attained by 7.3 mm, indicating that *S. ozarkensis* reaches sexual maturity at a length comparable to troglobiotic nicoletiids in the sister genus *Texoreddellia* (Espinasa and Giribet 2009). In addition, when reviewing the morphology of the new specimens from AD85, a
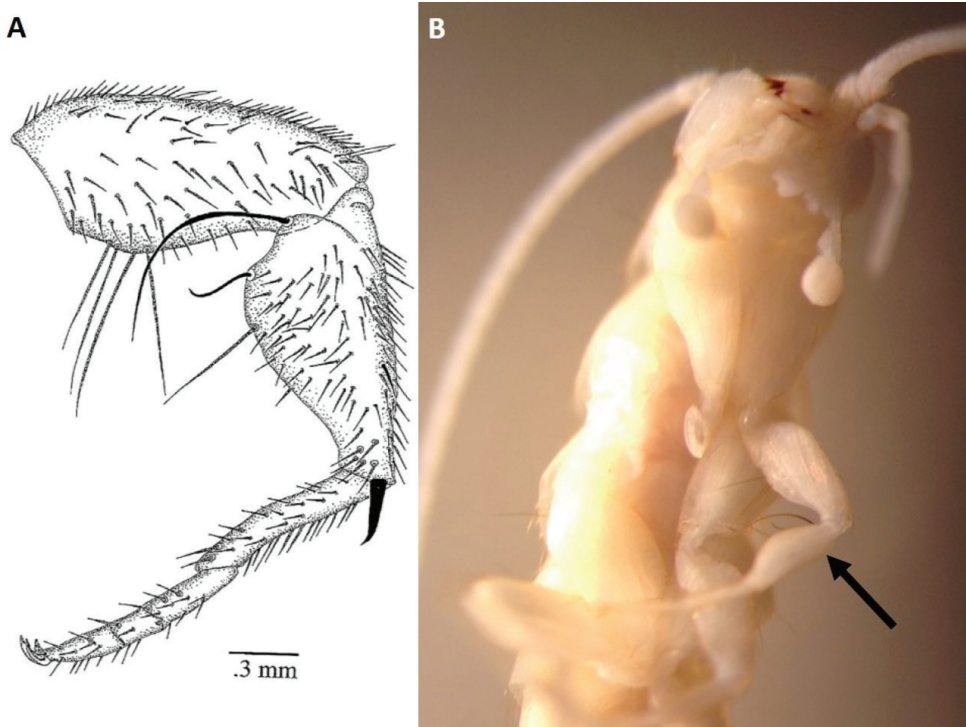
Figure 3. When *Speleonycta ozarkensis* was originally described (Espinasa et al. 2010), the diagnosis states that "tibia of second leg very stout (2 times longer than wide) with a large bulge with 3 distinctly long, sclerotized, and curved macrochaetae", as shown in (**A**). This description was based on a single specimen that had already been dissected and mounted in a fixed slide, with the legs isolated from each other. Examination of new specimens (**B**) shows that the order of legs was misidentified and that the modification is actually on the first leg pair.

mistake in the original description of the species became evident. In the original description it reads, "On male holotype, tibia of *second* leg very stout (two times longer than wide) with a large bulge with three distinctly long, sclerotized, and curved macrochaetae" (Fig. 3A). These structures were described based on a single individual because "the male from Uno Cave had neither (first or) second legs…so it is unknown if the three distinct macrochaetae are present in the legs." The two new specimens from AD85 have these diagnostic modifications, but on the *first* leg (Fig. 3B). Regrettably, when the holotype from Single Barrel Cave in Cherokee Co., Oklahoma, was provided for examination, the specimen had already been dissected and mounted in a fixed slide, with the individual legs separated from each other. The order of the legs was assumed by their position in the slide and their proportional size. It is now evident that the enlarged bulge makes the first leg appear larger than the second leg in this species, thus contributing to its incorrect identification. Diagnosis of the species should now say that modifications of male legs are on the first pair, not on the second.
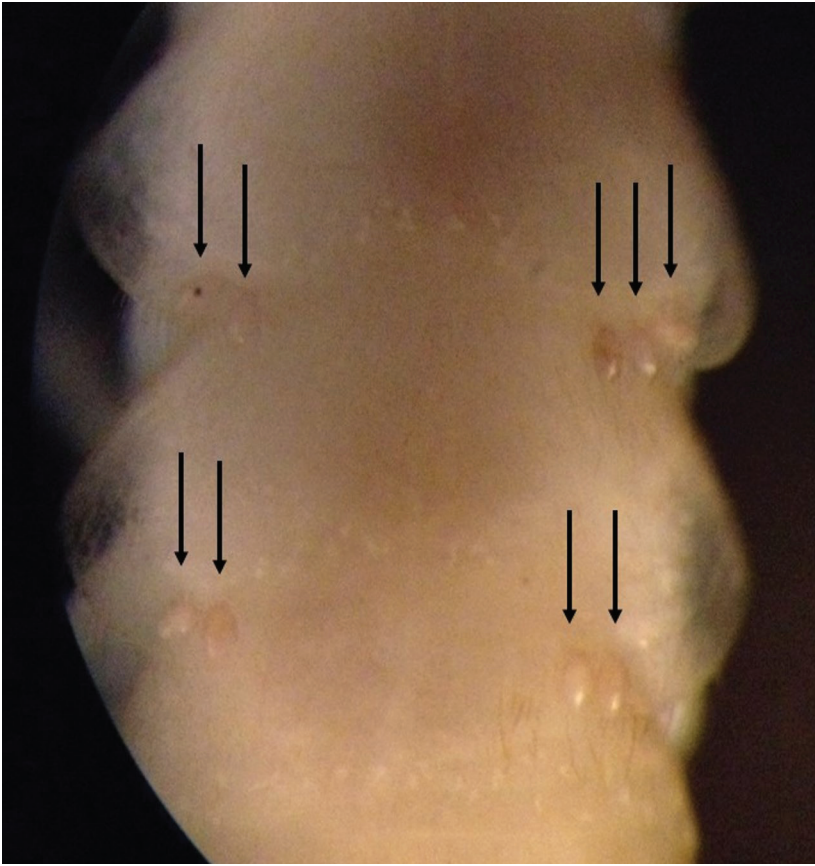
Figure 4. Parasitic acari on thoracic notas of *Speleonycta ozarkensis* from AD85 Cave. They await taxonomic description.

An interesting final observation is that the specimens from AD85 in Adair Co., Oklahoma, are host to parasitic mites (Acari; Fig. 4). Parasitic mites in nicoletiids have also been observed in troglobiotic *Anelpistina mexicana* Espinasa, 1991, but never reported in the literature. These parasitic acari await taxonomic description.

Conclusion

Speleonycta ozarkensis is the only described troglobiotic thysanuran from the Ozark Highlands. New biological inventories since 2010 in Arkansas and Oklahoma yielded only one new locality, AD85 in Adair Co., Oklahoma, bringing the total number of occurrences to eight caves in five counties. Despite being known from a few localities, *S. ozarkensis* has a broad distribution approaching 10,000 km². In addition, molecular evidence suggests that *S. ozarkensis* is capable of considerable dispersal and may be primarily an

epikarstic species, perhaps explaining why it has been infrequently collected from caves. Our conservation assessments showed that *S. ozarkensis* is at a slight risk of extinction. We identified seven threats impacting populations that vary in scope and severity, although only recreational caving (three caves) and development associated with urbanization (one cave) have the greatest potential to impact populations in the short term.

Acknowledgments

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REVIEW ARTICLES

Subterranean
Biology

Sulfide Shrimp? Observations on the concealed life history of the Thermosbaenacea (Crustacea)

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Abstract

The discovery and subsequent observation over various years of a massive population of the thermosbaenacean *Tethysbaena ophelicola* Wagner in the subterranean karstic sulfide pool of Ayyalon (Israel) enabled us to reach conclusions about the previously unknown life strategy of this crustacean super-order. These are preferably monophagous sulfur-bacteria-eating pelagic shrimps of stratified subterranean pools, adapted to microaerobic-anaerobic conditions, by among others ovoviviparity and the probable help of sulfide detoxifying bacterial endosymbiosis.

Keywords

Thermosbaenacea, Chemoautotrophy, Sulphobacteria, Biospeleology, Ayyalon, Ophel

The frequency of the Thermosbaenacea

The Thermosbaenacea is a super-order of small, blind subterranean crustaceans are routinely mentioned in the same breath with the Speleogriphacea, Mictacea and a host of strange underworld creatures (Olesen et al. 2014). Giere (2009) calls them "rare meiobenthic malacostracans". Iliffe and Kornicker (2008) include them among "living fossil animals", although *Tulumella*, one of their genera, is widespread and common in some West-Indian anchialine cave waters. These crustaceans count today 36 species and are circumtropically distributed.

The specific biotope of the continental thermosbaenaceans is unknown, or in better terms unreachable and their life history equally hidden. Rich populations of the nominal species, *Thermosbaena mirabilis* Monod resettle the hot bath basins they live in after every disinfection of them, coming from the "interstitial" (see discussion by Barker 1959). *Tethysbaena texana* (Maguire), reported in small numbers in several wells and pumps, is expected to inhabit (Wagner 1994) the whole area of the extensive Edwards Aquifer of Texas, hundreds of meters below ground. *Halosbaena acanthura* was discovered by Stock (1976) in inshore coral rubble in Curaçao and in later samplings, some of which by means of a biophreatic pump, over a wide area extending from Cuba to Colombia in a diversity of habitats. Classical localities of thermosbaenaceans were cave lakes, but it was already early evident that these lakes were not their primary habitat. *Tethysbaena vinabayesi* Wagner, 1994 was encountered during several years in immense numbers in a cave lake in Cuba. After suffering pollution, the crustaceans almost disappeared into the groundwater and only a few individuals could be collected from the detritus on the bottom (Wagner 1994). Several thermosbaenacean species, on the contrary, have been described only from few and damaged specimens spilled-out at spring heads. Such was for instance the case of *Tethysbaena relicta*, which I found in fragments (Por 1962) in the hot spring Hamei Zohar by the Dead Sea in Israel. When later, scattered specimens of the same species were collected in a drift net fixed to the outflow of a major thermo-haline spring, a few hundred kilometers to the north, it became clear that *T. relicta* inhabits the whole groundwater system of the Dead Sea-Jordan Rift Valley aquifer (Dimentman and Por 1991).

With the exception of three or four species which live in marine anchialine caves where they could be reached only by submarine cave divers, thermosbaenaceans were rarely collected and observed in their natural environment (Fryer 1965). They appear in wells, are swept-out in mostly thermal springs, pumped-out from aquifers and interstitial by various devices and occur in spring-fed subterranean lakes. Consequently, species of Thermosbaenacea have mostly been described on the basis of single, often damaged specimens, or in the best of the cases from marginal non-reproducing populations. It was more or less generally accepted that the core populations of these crustaceans are found deep underground in the inaccessible phreatic waters (e.g. Schram 1986). Therefore, even though the external morphology of the Thermosbaenacea has been exceptionally well described, many aspects of their life history of the animals remain hidden. Still, as it will be shown, the Thermosbaenacea are mainly very specialized and probably extremely frequent inhabitants of a very typical kind of subterranean waters, the sulfidic pools.

Only by observing the live animals in their natural environment, the natural history of the Thermosbaenacea can be sketched out. In this review paper I supplement older fragmentary natural history data on preserved or laboratory kept animals with new observations, and propose a consistent life strategy of these unique extremophilic crustaceans (Fig. 1).
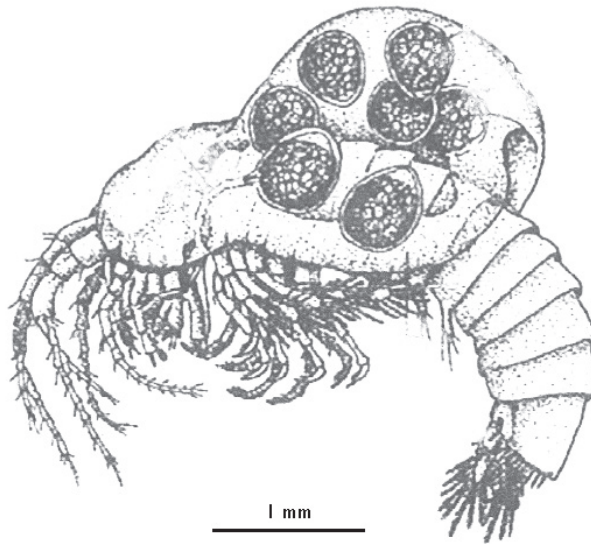
Figure 1. The extremophilic *Thermosbaena mirabilis* Monod from, El Hamma, Tunisia, a little known illustration (Barker 1962).

The core habitat at Ayyalon

The sulfidic and bacteria-rich waters of a subterranean phreatic pool in Israel (Por 2007) which houses a large population of a thermosbaenacean living in its natural environment offered us the opportunity to study the behavior and to understand the functional significance of many characteristic features of these crustaceans. This became possible, when by chance, a system of karstic ducts in the Israeli inner coastal plain became accessible at the bottom of a hundred meter deep limestone quarry (Por 2007, Naaman 2011). It led to a phreatic void containing a deep pool, called in literature the Ayyalon Cave (Fig. 2). This pristine groundwater pool was found to be inhabited among others by an extremely numerous and thriving population of thermosbaenaceans (Por 2007, 2011, 2012), later described as *Tethysbaena ophelicola* Wagner, 2012 (Fig. 3). The fauna of the practically anaerobic, sulfidic and hot pool (Naaman 2011) is probably exclusively based on the chemoautotrophic production of sulfide oxidizing bacteria (Por 2007, Por et al. 2013), even though, in the abundant bacterial mats only *Beggiatoa* sp. has been identified till now. The specimens of *T. ophelicola*, measured slightly more than two mm, well within the average of the super-order. The population of thermosbaenaceans, floating freely in the pond and collected by hand net could reach by my estimate several thousands of individuals by square meter. No particular concentration around the margin of the pond or attraction to the light was noted.

Figure 2. The sulfidic pool of Ayyalon (photo I. Naaman).
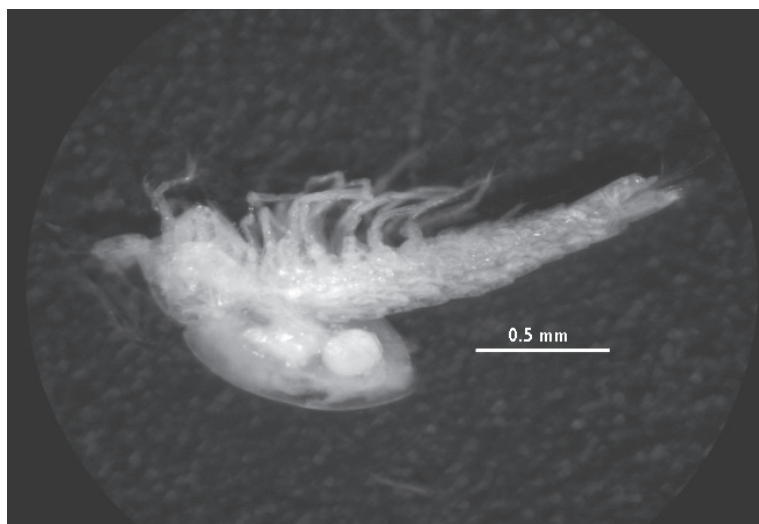
Figure 3. *Tethysbaena ophelicola* Wagner from the sulfidic pool of Ayyalon.

Due to the possibility of observing live populations of *T. ophelicola* in their own aquatic medium over a period of several years many natural history observations could be made, which, I propose can be extrapolated to Thermosbaenacea in general. Most importantly, sulfide bacteria-based chemoautotrophic groundwater ecosystem, called the Ophel biome (Por 2007), is suggested to be much looked for core habitat of the continental thermosbaenaceans.

Hypogene and sulfidic cave systems

The majority of the karstic duct and cave systems are generated by carbonic acid dissolution of horizontally flowing calcium carbonate-carrying phreatic water. However, according to Palmer (2007), some 10% of the caves worldwide are formed by sulfuric corrosion by artesian ascending hydrogen sulfide-carrying phreatic water. The ophelic ecosystems are generated in pools where the ascending anaerobic sulfidic water is contained in the chimneys of hypogene origin (Klimchouk 2007) until reaching oxygene-carrying phreatics or air voids where bacteria oxidize sulfide to sulfate. Sulfidic and hypogene speleogenesis have only recently attracted the attention of the speleologists. For example, Vaxevanopoulos (2009) calculated that 20% of the accessible Greek caves contain hypogenic features. These are ducts which once must have contained aggressive sulfidic water, but are now dried out because of changes that occurred in the water table. It is reasonable to assume that many "active" ducts containing sites like the Ayyalon pool exist deep in the inaccessible aquifers. Some of these are most probably inhabited by *Tethysbaena aiakos* which has been described from various localities in Greece by Wagner (1994), from several damaged specimens swept out to the surface.

Subterranean lacustrine crustaceans

The massive *Tethysbaena ophelicola* population in Ayyalon pool is swimming or floating in the open standing waters of the little lake (Por 2007). Stella (1951) already considered that *Tethysbaena argentarii*, the second species of the super-order to be discovered, is planktonic, but Fryer (1965) declared this observation to be "definitely misleading". Thereafter, in literature the characterization of the thermosbaenaceans prevailed as benthonic animals. Evidently, Stella saw the animals in their Italian subterranean cave, very probably near their native environment. Fryer on the contrary, observed them in Petri dishes after being sent to England. The capacity for occasional swimming of the captured animals though was mentioned by Fryer (1965). The picture would clarify as follows: The Thermosbaenacea are active and gliding swimmers, which despite their millimetric sizes, classify as micronecton rather than plankton. The animals, as shown, clearly prefer floating and swimming to walking. *Ipso facto*, the Ayyalon pool becomes into the first known example of a subterranean lacustrine lenitic environment with biota adapted to life in standing groundwater bodies, a "spelaeopelagial".

The Thermosbaenaceans are able to crawl also awkwardly on the bottom with their pereiopods. This happens normally when the liquid volume is limited or otherwise inadequate. This capacity had to be evidently maintained in their subterranean world in which the lenitic habitat is evidently unstable or when there is a need for the active diffusion of the species.
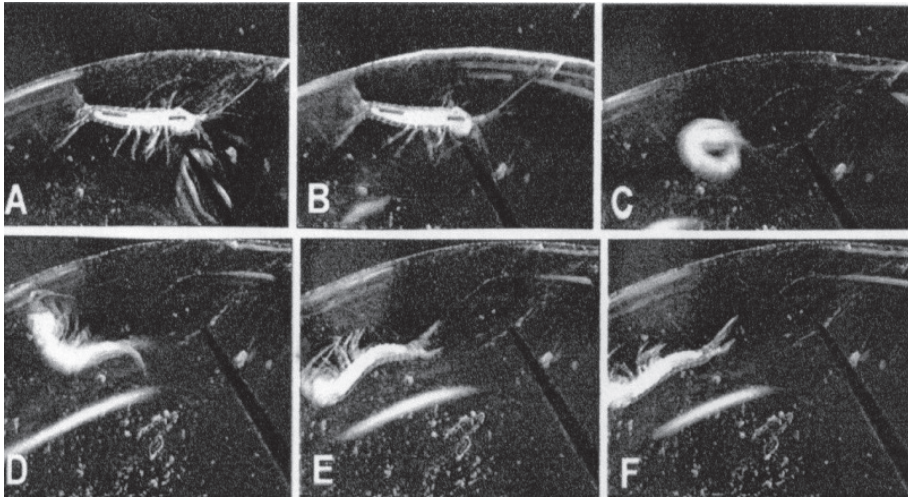
Figure 4. *Tethysbaena argentarii* Stella. Backdown swimming recovery after disturbance (filmed sequence by Olesen et al. 2006).

The meaning of back swimming

Barker (1962) was first to mention that *Thermosbaena mirabilis* swims in an upside-down, notonectic position. Fryer (1965) observed that *Tethysbaena argentarii* swims in inverted position which "recalls an anostracan" often "hanging" motionless on the water surface. The observations in the Ayyalon populations of *T. ophelica* confirmed this type of behavior. The animals were customarily gliding-hovering below the water surface and or on the bacterial crust. While studying the escape behavior of *T. argentarii*, Olesen et al. (2006) observed that the animals returned to the basic notonectic swimming stance after each disturbance (Fig. 4). This is reminiscent of the hyponeustonic life style of some open water crustaceans.

Such a hyponeuston-like behavior permits the thermosbaenacean to maintain itself in the density layer of the redox interphase where the bacteria concentrate. This can be either the surface of the sulfidic pool itself or the pycnocline between the aerobic and anaerobic phreatics. By hovering-floating they can probably adjust to the microlevel where all the composing factors are found in the optimal possible combination for them. Yaeger (1988) observed that the populations of the anchialine *Tulumella grandis* are concentrated below, or just above and below, the density interface, while Bowman and Iliffe (1988) found *T. unidens* just above the halocline. Possibly though, in the anchialine media, it all depended probably, on the level of the maximum bacterial concentration in the pycnocline of the sulfur and other types bacteria.

The possibility that this super-order uses the mechanical support of water density to glide-on is most probably a considerable energy saving for the animals in the anoxic or microxic, chemoautotrophic media in which they live.

The sulfide shrimp

As they live in a limnetic environment, thermosbaenaceans are characteristically soft-bodied and weakly chitinized. Blood-filled lacunae accompany the integument of the body and of the limbs, providing a diffuse respiratory lacunar system that to a large extent replaces the well-defined vascular respiratory system of other crustaceans (Wirkner and Richter 2009). They can probably relay on gas exchange through the whole body surface, not unlike what meiobenthic animals with similar body mass do in in oxygen poor sediments (Giere 2009). The blood-filled carapace, the pereiopodal exopodites, and the maxillipedes have an especially important respiratory function (Siewing 1958, Barker 1962). Fryer (1965) observed that when the animals are at rest, the maxillipedal epipodites continue beating and with increasing rhythm when oxygen decreases in the water. These mouthparts are especially important in creating a respiratory current which bathes the cavity of the dorsal brood pouch (Zilch 1976).

Observations on the Ayyalon population could not add anything specific and new concerning the physiological aspects of thermosbaenacean anaerobism. There is little doubt that the super-order possesses the arthropodan haemocyanin as its respiratory pigment, like most of the crustaceans. They have a very strong affinity to low oxygen concentrations and are resistant to high temperatures and presence of hydrogen sulfide (Hochachka and Sommers 2000, Grieshaber and Völkel 1998). A West Indian *Tulumella* species is known to use the so called "malate dismutation" of the anaerobic respiration cycle, which produces up to five times more energy than the normal glycolysis (Bishop et al. 2004). It is reasonable to assume that the continental thermosbaenaceans also make use of the malate-improved anaerobiotic respiration which liberates them to a considerable extent from the "oxygen debt".

Hydrogen sulfide is strongly toxic to metazoans (Grieshaber and Völkel 1998). In contradiction, many, if not most of the Thermosbaenacea have been reported from waters where hydrogen sulfide is abundant. Sket (1988, 1996) dedicated much attention to the relationship between the Dalmatian subterranean fauna and sulfidic waters and particularly to *Tethysbaena halophila* (S. L. Karaman, 1953). Wagner (2012) mentioned that anoxic conditions are favorable to Thermosbaenacea and feeding on sulfur bacteria is widespread among them. The massive population of *Tethysbaena ophelicola* at Ayyalon, considered here as the outstanding biotope of the super-order, was found to have H_2S values up to 4.7 mg/l (Naaman 2013). This was the reason for calling them "sulfide shrimp" (Por 2011).

A strong possibility for the way in which the animals cope with the sulfide toxicity is the fact that the specimens of *Tethysbaena* at Ayyalon have their intestinal tract clogged with live bacteria, even in the young instars newly cast from the brooding pouches of the females (Fig. 5). In literature, there are several instances in which thermosbaenaceans are drawn or photographed with dark, full intestines (e.g. *Halosbaena acanthura* in Stock 1976) (Fig. 6). Fryer (1965), when observing live specimens of *Tethysbaena argentarii*, found their gut usually filled from end to end with "unrecognizable material of brownish hinge broken down by the time it became available".
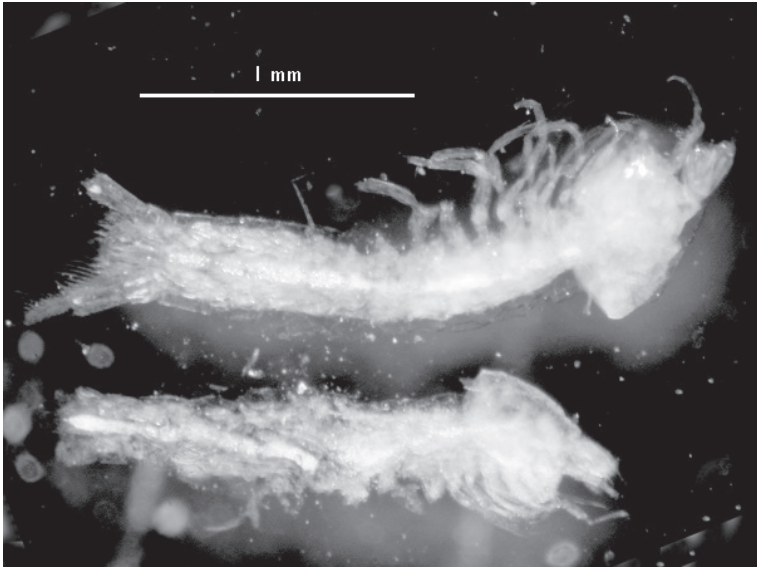
Figure 5. Juvenile *Tethysbaena ophelicola* Wagner (intestine filled with bacteria (?)) (photo N. Ben Eliahu).
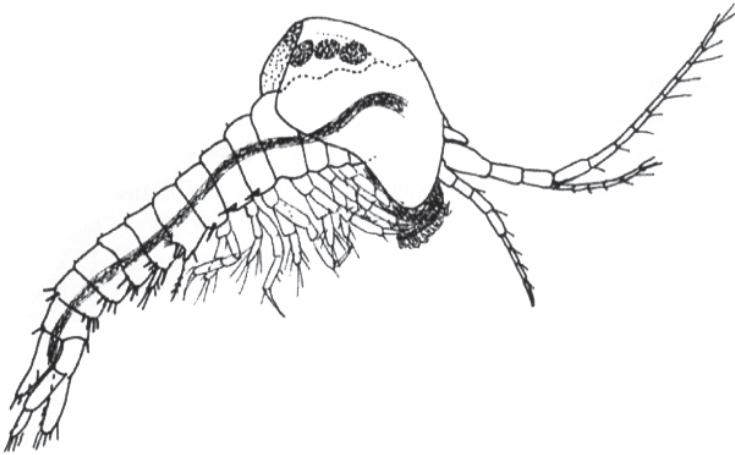

Figure 6. *Halosbaena acanthura* Stock with full intestine (Stock 1976).

Fryer found it strange that this material remained in the intestine in some cases for more than two days. Schram (1986) makes the general statement that the mid gut of the Thermosbaenacea is apparently always loaded with "detritus…the animals dine on". It is possible that we are not dealing with detritus in these pelagic animals, but with bacterial biomass which became unrecognizable in preserved specimens and even in specimens which are encountered or not kept in their natural medium. Only by observing the intestinal filling of live specimens *T. ophelicola* of Ayyalon in their aquatic medium under the appropriate magnification it became clearly visible that it consists of densely packed rounded cells that can represent bacterias.

The affirmation of the symbiotic role of the bacterial filling of the intestinal tract is at this stage purely circumstantial. It is probably similar to what happens in the oceanic alvinellid hot vent worms, where bacteria stored in special trophosomes serve both as food and detoxify sulfide. An even closer comparison would be with certain oligochete and nematode species of the oxygen-deficient and hydrogen sulfide-infested littoral environments which present various degrees of ecto- and endo-symbiosis with sulfur bacteria. In the sulfurous cave lake of Frasassi, Baurmeister et al. (2012) found that the legs of the amphipod *Niphargus itus* Karaman are thickly covered by symbiotic sulfur bacteria. Future microbiological investigations will have to prove the nature of this permanent relationship between *Tethysbaena ophelica* and its bacteria. If positive, it will be the first case of endosymbiosis in the subterranean aquatic medium.

Ovoviviparity: the crucial synapomorphy

There is surprisingly little mentioning of the thermosbaenacean dorsal brood pouch, its different stages and that of its content in the more recent literature.

The source material at our disposal is in the older works by Stella (1959), and Fryer (1965) on *Tethysbaena argentarii* and by Barker (1962) and Zilch (1972) on *Thermosbaena mirabilis*. The sustained observations of live specimens of *Tethysbaena ophelicola*, in their environment at Ayyalon, and especially the discovery of their supposed bacterial endosymbionts allowed us to use what is known and present an interpretation of the function of the brood pouch, probably the key innovation of the Thermosbaenacea.

The dorsal brood pouch formed by an extension of the carapace of the reproducing female, is no doubt the most singular characteristic of the Thermosbaenacea. It is evident though that in the life history of the sulfide shrimp in its extremely adverse environment, the ovoviviparous development provided by the dorsal breeding pouch, a real marsupium, is a key adaptation for survival.

Dorsal breeding, exceptional among the higher crustaceans, is a consequence of the notonectic positioning of the pelagic Thermosbaenacea in the density interphase. The marsupium with the developing eggs and embryos is protruding-hanging into the anaerobic level, protected from eventual predation. It is however, ventilated with a permanent stream of oxygenated water by the beating maxilliped and pereiopods.

Stella (1955) and Barker (1962) thought that the early embryos reach the pouch freely, being swept in by the aeration current of the thoracal limbs. Zilch (1972), in a detailed work on *Thermosbaena mirabilis*, described how the eggs are transferred to the brood pouch in batches, protected by a membrane (Fig. 7). The membrane is dissolved upon arrival in the pouch. This mode of translocation could perhaps be specific only to this single species in its extremely hot environment.

Several authors mentioned that the eggs and developing juveniles are free in the pouch and "constantly agitated by the inhalant respiratory current" within the liquid content of the pouch (Barker 1962). This observation was confirmed on the Ayyalon species.
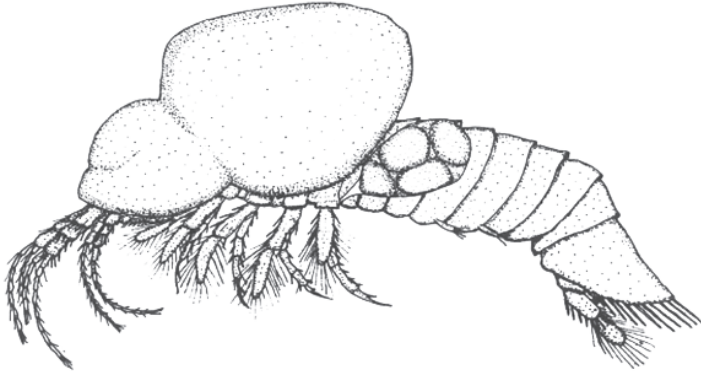
Figure 7. *Thermosbaena mirabilis* Monod. Female, translocating a membrane-protected batch of developing eggs to the brooding pouch (drawing by Zilch 1972).

Figure 8. *Tulumella* sp. from the Bahamas with fullgrown progeny (photo Culver and White 2006).

It seems from the few ovigerous specimens we know that the eggs are large-sized and rich in vitelline matter. Zilch mentions up to 12 embryos in a pouch in *Thermosbaena*. The maximum we saw in *Tethysbaena ophelica* was of 8. As the embryos develop and grow inside the marsupium, their number decreases, most probably due to abortion. A maximum of ovoviviparity was figured in a Caribbean anchialine species of *Tulumella* (Fig. 8), where the marsupium contained an almost ready to be hatched youngster. At least in the assumed typical environment of the Ayyalon pool no seasonality was observed.

The hemolymph lacunae of the cuticular breeding pouch walls were described by Siewing (1958) and by Barker (1962) as especially spacious and in *T. mirabilis* Zilch (1972) describes also some accessory cuticular vesiculated trabeculae emerging into the brooding cavity. The short heart of the thermosbaenaceans is placed immediately below the brood pouch, obviously in the super-order to better serve marsupial circulation

(Wirkner and Richter 2009). Water current is being streamed into the pouch by the beating of the maxillipeds and the anterior thoracopods. The maxillopods themselves are lacune-filled respiratory organs. A favorable micro-environment is probably maintained in the brood chamber by the suction-like current of more oxygenated water from above and by a rich supply of respiratory pigment in the marsupium walls. These are propitious conditions for the development of the embryos of the down-hanging females in the pycnocline. Hydrogen sulfide from the surrounding water is therefore, probably, not much present in the brooding sack medium, since otherwise it would inhibit the development of the youngsters and eventually kill them. The youngsters pass through developmental stages similar to the manca's of the peracarid crustaceans. They leave the brood pouch when they have already functional digestive tract openings. This was evident in the large population of *Tethysbaena ophelicola* of Ayyalon, where the smallest and young hatched specimens already have the intestine filled with bacteria. These accumulated food cells serve most probably also as symbionts which protect the animals against the surrounding hydrogen sulfide toxicity. Without ignoring the other adaptations, the acquisition of this ovoviviparity was decisive in permitting life for the super-order Thermosbaenacea in the pycnoclines of the subterranean waters of the world.

Heat tolerant extremophiles

Monod's *Thermosbaena mirabilis*, "the Marvelous Heat Walker" of Tunisia which lives and reproduces in 42 °C thermal springs (Barker 1962) gave its name and fame to the whole super-order. It is likely the most heat-adapted aquatic metazoan known. The name of the super-order is however somewhat misleading. The animals, as shown, prefer floating and swimming to walking. Schram (1986) indeed wrote that the thermosbaenaceans seem to prefer thermal groundwater. The heuristic example of the Ayyalon population indicates that it would be better to speak of a thermo-tolerance of these crustaceans, and not of a preference.

The rich thermosbaenacean population of the sulfidic pool of Ayyalon lives at a medium temperature of 29.6 °C, which is 5 °C higher than that of the surrounding fresh aquifer (Naaman 2011). The hypogenic artesian groundwaters that are ultimately providing food for these thermosbaenaceans are naturally a few degrees warmer, owing to geothermal heating. Regional tectonism, or exothermic chemical reactions can further contribute to the geothermic warming of the sulfidic deep water. In any case, the pycnocline can have also a sharp thermocline component.

T. relicta (Por) from the Jordan-Dead Sea Valley has also been found at 31 °C, *T. somala* (Chelazzi & Messana) from Somalia at 31 °C and *T. halophila* from Dalmatia is also a thermobiont. The dense populations of *Thethysbaena* found in the often neglected and heavily polluted wells of some Caribbean islands like, as for instance *T. haitiensis* (Wagner 1994) live on "secondary" sulfur bacterial accumulations and do not have to contend with elevated temperatures.

Finally, the thermosbaenaceans can be characterized as limno-euryhaline adapted. They have been reported from water sources which have mineral loads shifting from limnic to mesohaline levels (Wagner 1994). It is evident that in spring outflows or in the pumping devices the salinity interface or halocline, which is another component of the pycnocline, is destroyed and the two originally superposed phreatic waters mix. In the microcosm of the bacteria-rich pycnocline the crustaceans have, however, to face and adapt to radical local changes and fluctuations in dissolved mineral content.

Conclusions

More than half a century has passed since I found in December 1960 my first thermosbaenacean species (Por 1962), and later the paper in which I identified the new subterranean biome Ophel at Ayyalon (Por 2007). Here I propose it to represent the core environment of the Thermosbaenacea. For now I have neither the support, nor the time for further factual and experimental testing of my hypotheses. However, there must be countless aquatic environments like that discovered in Ayyalon, since they supported the evolution of this taxon.

In my opinion, decisive in the evolutionary history of the Thermosbaenacea was their monophagy of sulfur bacteria. From this starting point the entire natural history of the group can be derived as sketched-out above and resumed in the following (Fig. 9).

Thermosbaenaceans are blind, specialized pelagic, bacterial consumers in subterranean stratified standing waters, particularly of sulfur bacteria. Since bacterial chemoautotrophy takes place chiefly in the thin redox and density discontinuity layers, thermosbaenaceans tend to accompany these levels like a kind of upside-down swimming neuston. In response to the radical hydrochemical changes and fluctuations that take place in the thin discontinuity layer (pycnocline), the thermosbaenaceans have developed survival mechanisms to anaerobism, resistance to hydrogen sulfide poisoning, to elevated water temperatures as well as to fluctuating dissolved mineral content. Sulfide is most probably detoxified by intestinal bacteria. All the Thermosbaenacea are ovoviviparus, breeding the offspring in the dorsal marsupium until it acquires its own battery of extremophilic capacities.

With their very specialized life style and its unique morphology, the Thermosbaenacea represent, without doubt, a taxon separate from the Peracarida as considered by the majority of the carcinologists. The dorsal breeding pouch would justify this separation from Peracarida as already suggested by Siewing (1963). Anyhow, as mentioned by Schram (1986) the *lacinia mobilis* of the mandible can no longer be considered as a feature exclusive to the peracarids. Many cladistics analyses also place the Thermosbaenacea as the sistergroup to other peracarid orders (Siewing 1963), which justify its separation from them. However, I suggest not to return to Siewing's "Pancarida", but to maintain Thermosbaenacea for the sulfide shrimp as being a more suggestive name for the group. Besides the Thermosbaenacea, only the
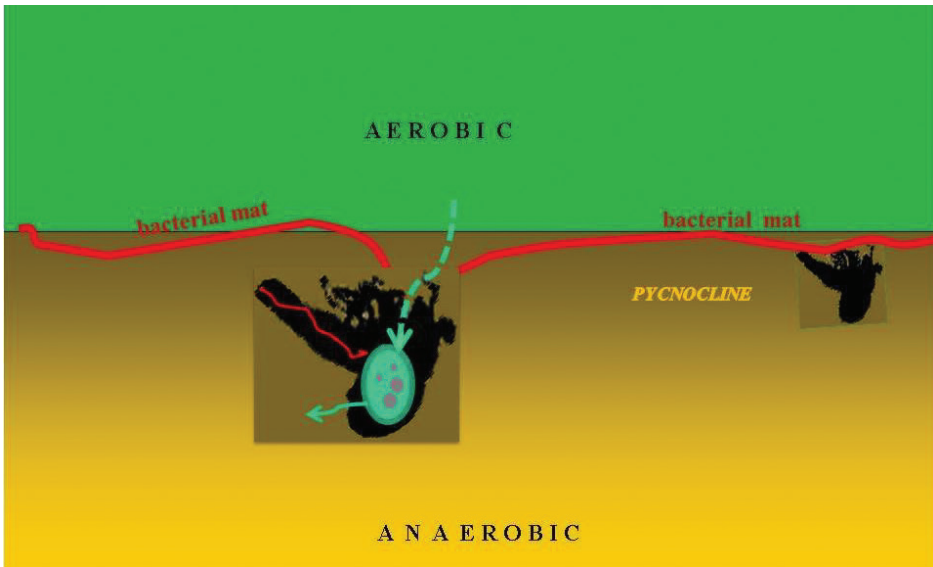
Figure 9. Schematic presentation of the suggested life strategy of a thermosbaenacean (for explanations see text).

copepod *Metacyclops longimaxillis* Defaye & Por is known to live and reproduce in the sulfidic chemosynthetic productive water of the ophiric biome. We do not know anything about their specific adaptation of this cyclopoid to this extreme environment. Only the elongated and setose maxillae which gave the name to the species indicate that it is a primary subterranean bacterioplankton consumer (Defaye and Por 2010).

Biospeleology has been a merely descriptive discipline until recent, since the objects of its study have been only isolated members of allochthonous and truncated ecosystems (Gibert and Deharveng 2002). The ophelic chemoautotrophic system as it appears now in Ayyalon is a firmly established autarchic subterranean ecosystem with its own energy budget and with all the specialized levels, bacterial primary producers and primary consumers like the sulfide shrimp. This is independent from the sunlit biosphere with as yet unfathomed consequences (Por 2011, 2012). Wagner (2012) in his study of *Tethysbaena ophelicola* indeed presents a first hypothetic subterranean chemoautotrophic aquatic food web.

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RESEARCH ARTICLE

Epigean and hypogean *Palaemonetes* sp. (Decapoda, Palaemonidae) from Edwards Aquifer: An examination of trophic structure and metabolism

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Abstract

This study addresses the causes of the metabolic depression observed when examining the metabolism of hypogean versus epigean organisms. We examined the two current hypotheses regarding the cause of metabolic cave adaptation, a paucity of food and low oxygen availability, both necessary for ATP production, by first determining if the hypogean environment examined, Edwards Aquifer, was resource limited. Stable isotope analyses indicate that there is extensive microbial chemolithoautotrophic production providing resources for the hypogean organisms. $\delta^{13}C$ values (≤30‰) were well below that of terrestrial biome indicating that C in the aquifer originates from chemolithoautotrophic inorganic carbon fixation, not photosynthetically derived material resulting from terrigenous sources. Data suggest the artesian system is a complex geochemical ecosystem providing inorganic energy sources from both methane and sulfates. Metabolism, examined via key aerobic and anaerobic proxies, and organismal proximate composition indicated there was no difference between metabolic rates and energy storage of *Palaemonetes antrorum* (stygobitic) and *Palaemonetes kadiakensis* (epigean). This indicates that resources within the oxic aquifer are not limited. We demonstrate that it is necessary for one, or both, of these selective pressures to be present for metabolic cave adaptation to occur.

Introduction

Scientists have long been intrigued by the adaptations of subterranean organisms to their stygian environment. Like the deep sea, the subterranean environment remains in total darkness and generally has low energy to sustain life (Poulson 2001). Subterranean environments have been perceived as extreme environments to which it is difficult for organisms to adapt, a view that is not unchallenged (Seibel and Drazen 2007). Research on subterranean life has progressed a great deal over the last several decades, and the research focus has shifted from species adaptation to an ecosystem approach (Gibert et al. 1994). It is now recognized that subterranean ecosystems support substantial diversity of both prokaryotes and eukaryotes (Culver and Sket 2000, Holmes et al. 2001, Humphreys 2008, Guzik et al. 2011) much of which may become imperiled (Van Beynen and Townsend 2005) before they are recognized taxonomically (Niemiller et al. 2013).

Subterranean aquatic environments are considered extreme on several accounts. Such habitats are in continuous total darkness and so lack direct photosynthetic energy input being mostly dependent on a slow flux of allochthonous energy input in the form of organic carbon derived from the surface, else, as is increasingly being reported, in the form of inorganic molecules derived by chemoautotrophy. Additionally, subterranean aquatic systems may be dysoxic or anoxic with regions of toxic hydrogen sulfide. Some systems, such as anchialine systems and the saline parts of the Edwards aquifer, have distinct haloclines which mark the clines conducive to microbial chemolithoautotrophic production (Hutchins et al. 2011). The structural diversity of such systems (Pohlman 2011) and the contained microbial and metazoan communities (Sarbu 2000, Seymour et al. 2007) are still poorly understood although, as noted by Humphreys (1999), they may be sought by analogy with adaptations to anoxic sediments (Fenchel and Finlay 1995).

It is widely recognized that a low metabolic rate is one of the adaptations to the low energy subterranean milieu, one that occurs in both air living and aquatic members of the subterranean fauna. However, Seibel and Drazen (2007) posit that the metabolic rates found in deep sea (and by analogy in subterranean) species are the 'normal' rates but that the higher metabolic rates found in surface species are actually elevated rates, being an adaptation to the faster pace of predator threatened epigean life. This argument cannot be sustained on first principles for subterranean species owing to the polarity in the argument; comprehensive phylogenetic studies routinely support that subterranean species are derived from epigean species (e.g., Leys et al. 2003) with few tentative claims of reversals to surface living (Domes et al. 2007, Kornicker et al. 2010, Prendini et al. 2010).

Regardless of the mechanism that resulted in colonization of the subterranean environment, stygobites tend to have convergent physical and physiological characteristics, termed troglomorphies. These include reduced body size, regressed or absent eyes, enhanced sensilla, loss of pigmentation and reduced metabolic rates when compared to their closest phylogenetic epigean counterparts. Metabolic rates were first measured on troglobitic amphipods (Gal 1903) and numerous studies followed (Poulson 1963, Vandel 1965, Barr 1968, Caine 1978, Culver 1982, Hüppop 1986, Hervant and Mathieu 1995, Gannon et al. 1999, Hervant and Renault 2002, Bishop et al. 2004), yet few researchers reported having found no reduction in metabolic rates in cave populations (Culver and Poulson 1971). The cause of the metabolic depression reported in stygobitic crustaceans and fishes has been a subject of debate for decades. One hypothesis is that depleted environmental oxygen levels limit the rate of ATP production in cave organisms. A comparison of oxygen consumption rates of congener amphipods from both an oxic environment and an anoxic to dysoxic system, resulted in a significantly greater organism mass and metabolic rate in the *Spelaeonicippe buchi* (Andres, 1975) from the oxic conditions of Túnel de la Atlántida in Lanzarote compared to *S. provo* Stock & Vermeulen, 1982 from Bahamian stygobitic systems (Bishop and Iliffe 2009). Additionally, anchialine shrimp, *Barbouria cubensis* (von Martens, 1872), collected from dysoxic to oxic cave systems in the Bahamas demonstrated significantly greater metabolic rates measured as oxygen consumption and activities of key enzymes in the intermediary metabolic pathways than *B. cubensis* collected from an anoxic environment in the Yucatan (Bishop and Iliffe 2012).

Theoretically, low environmental oxygen levels in the cave or subterranean habitat reduces the rate at which food is converted to energy, making the impacts of oxygen availability on the physiology of aquatic organisms extensive but, as mentioned above, metabolic depression can be observed even in oxynormal atmosphere. So, oxygen partial pressure cannot be the only factor leading to reduced metabolic rates in cave organisms. Gannon et al. (1999) found cave crayfish of the genera *Procambarus* Ortmann, 1905 and *Troglocambarus* Hobbs, 1942 had significantly reduced oxygen consumption yet the cave environment ranged from dysoxic to oxic and dissolved oxygen content of the cave did not vary significantly from that of the surface pools outside the cave. In addition, terrestrial subterranean animals also have lower metabolic rates and reduced respiratory surfaces than their epigean relatives although cave atmospheres are rarely very depleted in oxygen (Kuntner et al. 1999).

A second theory is that low food availability in the cave environment favors organisms with lower metabolic requirements. However, Culver and Poulson (1971) found no reduction in metabolic rate in cave populations of *Gammarus minus* Say, 1818 and speculated that energy was not limiting for the population studied and thus would not have impacted the metabolism. It is notable that this species is also widely present in epigean environments.

The hypotheses outlined above have not changed for decades yet our knowledge of groundwater fauna has increased profoundly over the same time period (Gibert 1994, Wilkens et al. 2000, White and Culver 2012). Although some subterranean environments are characterized by a reduced chemical environment and some re-

gions are dysoxic or even anoxic, not all cave systems are resource limited or oxygen depleted. Here, we investigate whether a species of stygobiont shrimp inhabiting a cave that contains abundant dissolved oxygen and adequate energy has a metabolic rate which differs from that found in its epigean relatives, both associated with the Edwards Aquifer, Texas. This study examines the existence of metabolic cave adaptations in stygobitic organisms when the organisms reside in an oxic environment. We examine metabolism, and proximate compositional differences between epigean and stygobiotic *Palaemonetes* sp. residing in Edwards aquifer. Additionally, we examine potential food sources in the subterranean environment, primarily microbial chemolithoautotrophic production with the intent to address the assumption of resource limitation in the stygobitic environment. The unique environment provided by the sample site, Edwards Aquifer, allows us to examine stygobitic and epigean congeners from an oxygenated environment. We posit that if food energy resources are adequate and the oxygen needed for ATP production is not limiting, the metabolism of hypogen organisms should not differ significantly from their epigean relatives. Additionally, we anticipate that stable isotope analyses will indicate that the two closely related species are consuming very different resources.

Edwards Aquifer and *Palaemonetes*

The Edwards Aquifer is formed in marine carbonates of Cretaceous age, ranging from 100 – 230 m in thickness, which were subsequently exposed, eroded by solution (kartsification) and overlaid by further sediments in places forming an artesian aquifer. Extensive faulting in the Edwards Aquifer region resulted in the formation of the Balcones fault zone and subsequent limestone dissolution increased porosity, resulting in large caverns and creating new subterranean habitat. The faulting also altered the ground water movement creating new entry points for freshwater organisms (Longley 1986). During the Pleistocene ice ages and at times of severe drought, the subterranean environment of the aquifer provided constant temperature and suitable environment to sustain subterranean organisms (Holsinger 1992).

Like the extensive thermomineral Movile system in Romania (Sarbu et al. 1996), the Frasassi Caves in Italy (Porter et al. 2009), and more recently, in the Ayyalon cave in Israel (Por 2007), there are indications that Edwards Aquifer may provide chemosynthetic energy sources. A study by Birdwell and Engel (2009) observed persistent signatures of microbial CDOM in the aquifer and questioned the assumed dependence of karst aquifer ecosystems on terrigenous carbon.

The oxic environment (>3 mg L^{-1} O_2) of the Edwards Aquifer supports one of the richest subterranean communities explored to date, with approximately 91 animal species of which 44 species are endemic stygobionts (Longley 1981). An abundant resident of the artesian part of the Edwards aquifer is the Balcones cave shrimp, *Palaemonetes antrorum* Benedict, 1896, whereas the Mississippi grass shrimp, *P. kadiakensis* Rathburn, 1902, is found in the surface pools of the same karstic region.

Palaemonetes Heller,1869 (Decapoda: Palaemonidae) comprise an important part of the temperate and tropical aquatic food webs (Lowe and Provenzano 1990) and about 14 species are known from North America. There has long been poor resolution of the systematic relationships of the group on account of morphological homogeneity and poor character definition (Strenth 1976), but recent molecular analysis indicates that it is paraphyletic with *Palaemon* Weber, 1795 but that those North American species included in the analysis formed a monophyletic clade (Cuesta et al. 2012). From a detailed morphological study, Collins (1998) concluded that the genus is primarily marine and that *P. kadiakensis* is not a recent invader of freshwater from the marine realm. He established that *P. antrorum* is a proper member of the genus *Palaemonetes* but is highly derived from a surface freshwater clade that includes *P. kadiakensis* and is possibly even the sister species of *P. kadiakensis* (Collins 1998, Fig 3.19).

Materials and methods

Specimen collection and sample preparation

Specimens of two species were collected at the Edwards Aquifer Research and Data Center (EARDC), San Marcos, Texas. *Palaemonetes antrorum* were collected from a well discharge pipe (ID 30.4 cm) in a 500 μm mesh net. *Palaemonetes kadiakensis* were collected from the surface pool at the EARDC, using a hand held net. Specimens were stored in a -80°C freezer until they were shipped on dry ice to Penn State University. At the time of collection, the dissolved oxygen ranged from 6.1–6.3 mgL^{-1} in the aquifer and was 8.3 mgL^{-1} in the surface pool. The temperature for both the aquifer and surface pool was 21°C.

Individual specimens were weighed to the nearest milligram (WM). Specimen size permitting, two subsamples of abdominal muscle approximately 0.1 g each were introduced frozen into the homogenizing medium, ice-cold dionized water, at dilutions of 1:10 mass: volume. For small specimens where size did not permit the removal of two subsamples, one set of samples was used for stable isotope analyses and the other was reserved for enzyme activity and proximate compositional analyses. Best efforts were made to obtain a complete suite of sizes for each assay. Tissue samples were homogenized at 0–4°C using a sonic dismembrator.

Stable isotope analyses

Following homogenization, the samples were placed in vials and dried for 72 hours at 60°C. The samples were acid fumed to remove any carbonate and then analyzed to derive δ^{13}C, δ^{15}N, C and N by the Stable Isotope Facility at University of California at Davis following their standard protocols for analysis of solids by a PDZ Europa ANCA-GSL elemental analyzer interfaced to a continuous flow PDZ Europa 20-20isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Standards were interspersed with

the sample runs and give a long term standard deviation of 0.2 ‰ (permil) for ^{13}C and 0.3‰ for [15]N. Sulfur and δ [34]S were derived by the same facility using a SerCon elemental analyzer and custom cryo-focussing system interfaced to a SerCon 20-22 IRMS (Sercon Ltd., Cheshire, UK). Standards were interspersed with the sample runs and give a long term standard deviation of 0.2 ‰ for δ [34]S. Laboratory standards were directly calibrated against IAEA S-1, S-2 and S-3 and are reported on the VCDT scale.

Stable isotope data are reported as permil (‰) deviation from a standard:

$$\delta\ (‰) = (Rsa/Rstd -1) \times 1000,$$

where R is expressed as the ratio of the heavy to the light isotope, namely, in our case, [13]C / [12]C, [15]N / [14]N and [34]S / [32]S, with the primary standards being respectively Pee Dee Belemnite, atmospheric air, and Canyon Diablo meteorite reported on the VCDT scale.

Metabolism and proximate composition

One issue that arises when measuring the metabolism of organisms is the distinct possibility that laboratory confinement may lead to over estimation of metabolism (Quetin et al. 1994, Ritz 2000). This is of particular concern when the experiments involve the comparison of physiological parameters of organisms from disparate environmental conditions, such as well-lit surface pools and the crevicular subterranean habitat. To reduce the possible artificial introduction of variability between the two species resulting from confinement, we chose to examine the metabolic potential of both species of *Palaemonetes* by measuring the activities of key enzymes of the intermediary metabolism. The activities of these enzymes have been shown to correlate with oxygen consumption rates (Childress and Somero 1979, Hochachka et al. 1988, Thuesen and Childress 1993, Lemos et al. 2003, Seibel 2007). Citrate synthase [CS, EC 4.1.3.7; citrate: oxaloacetate-lyase (CoA-acetylating)] activity was assayed following the methods of Torres and Somero (1988). L-Lactate dehydrogenase (LDH, EC 1.1.1.27; lactate: NAD+ oxidoreductase) activity was assayed in the pyruvate reductase direction also using methods described by Torres and Somero (1988). All enzyme activities were assayed in triplicate on a spectrophotometer at 20 ± 0.1°C. Means of the replicates are reported in μmol of substrate converted to product per minute.

Specimens were also assayed for protein and lipid content following the methods described in Donnelly et al. (1993). Proximate composition is reported as concentration, a percent of wet mass, and is the component's proportion of the organism's total mass.

Statistical analyses

All analyses were conducted with significance at $p < 0.05$. F-tests were used to determine equality of variances. As a result of heteroscedasticity, all statistics on were performed on

log transformed enzyme data but means and standard errors are reported on back-transformed data. Two sample t-tests (two-tailed) were conducted to determine if differences in CS and LDH activities, as well as protein and lipid concentrations, existed between the epigean and hypogean species. All regressions were generated using the least-squares method. A two-tailed Student's t test was used to test for differences between the mass-specific enzyme activities of the two species using log of wet mass and log of the mass-specific enzyme activities. Stable isotope and elemental data were tested for differences using factorial analysis of variance with species as factors (StatView 512+).

Results

Epigean *Palaemonetes kadiakensis* were significantly larger (P =<.001, $\bar{x}$ = 0.261 ± 0.0282 g WM, n = 20) than *Palaemonetes antrorum* collected from with in the aquifer ($\bar{x}$= 0.098± 0.0067 g WM, n = 25), accordant with previous studies in which hypogean and epigean organisms were compared (Caine 1978, Issartel et al. 2005, Bishop and Iliffe 2009).

Stable isotopes

Ten samples each of *P. kadiakensis* and *P. antrorum* were assayed for the various elemental and stable isotope variables. Sampled weights assayed did not differ significantly in any analysis (Table 1). Compared to *P. kadiakensis*, the stygobitic *P. antrorum* had less N and S per sample but a greater ratio of C:N. In addition, the stygobitic species had highly significant lighter values of δ^{13}C, δ^{15}N and δ^{34}S (Table 1) and the scatter and magnitude of these differences is shown in Figure 1. The two outlying δ^{13}C values denote a different energy source for these individuals and their exclusion emphasizes the magnitude of the differences between the two species' trophic biology. The 16‰ difference in ^{34}S between *P. kadiakensis* and *P. antrorum* (cf 4.8‰ for ^{13}C and 3.4‰ for ^{15}N) provides a high signal-to-noise ratio of the sources for ^{34}S and so this measure will be an especially useful addition to any multiple isotope study of the Edwards Aquifer system, and probably others, as it has also proved useful at the producer level (Connolly et al. 2004).

Enzyme activity and proximate composition

No significant difference was observed between the individual activities of the enzymes of the two *Palaemonetes* species (CS, P = 0.304; LDH, P = 0.076) (Figures 2a and b). Citrate synthase activity for *P. antrorum* ranged from 0.742–6.363 (mean 3.171±0.3517 µmol min $^{-1}$, n = 23) while the larger *P. kadiakensis* CS activities ranged 1.428–4.264 (mean 2.359±0.1560 µmol min^{-1}, n = 20) (Figure 2a). Stygobitic
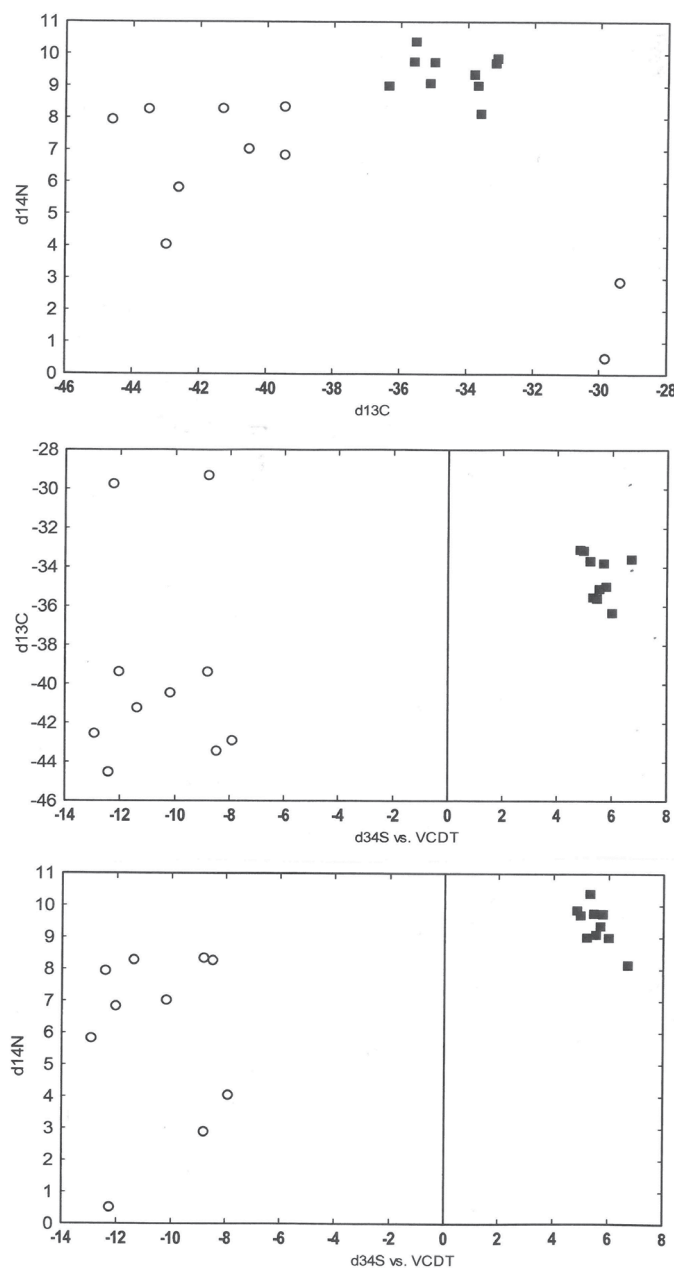
Figure 1. Stable isotope ratio plots, upper to lower: a) $\delta^{15}N$ on $\delta^{13}C$; b) $\delta^{13}C$ on $\delta^{34}S$; c) $\delta^{15}N$ on $\delta^{34}S$. Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

organisms demonstrated only a slightly greater range of LDH activity than observed in the epigean organisms (Figure 2b). Mean LDH activities were 0.642±0.0392 μmol min $^{-1}$ and 0.632±0.0390 μmol min $^{-1}$ for *P. antrorum* and *P. kadiakensis*, respectively.

Table 1. Mean and variation in stable isotope and elemental statistics for *Palaemonetes kadiakensis* (epigean) and *P. antrorum* (stygobiont). N=10 in each case.

	$\delta^{13}C$	C (µg)	$\delta^{15}N$	N (µg)	Sample (µg)	C : N	$\delta^{34}S$ vs. VCDT	S (µg)	Sample (µg)	%S
P. kadiakensis										
Mean	-34.49	248.7	9.41	73.30	0.60	3.41	5.56	7.29	720.8	1.009
St. dev	1.16	34.4	0.63	11.37	0.02	0.12	0.54	1.56	118.8	0.117
P. antrorum										
Mean	-39.33	245.7	5.97	42.12	0.60	5.96	-10.48	4.30	582.5	0.805
St. dev.	5.42	40.2	2.69	5.00	0.01	1.46	1.90	1.91	264.7	0.199
P. kadiakensis vs *P. antrorum*										
$F_{s\,1,18}$	7.658	0.033	15.463	62.982	0.038	30.473	661.9	14.686	2.976	7.768
P	0.0127	>0.05	0.001	0.0001	>0.05	0.0001	0.0001	0.0012	>0.05	0.0122
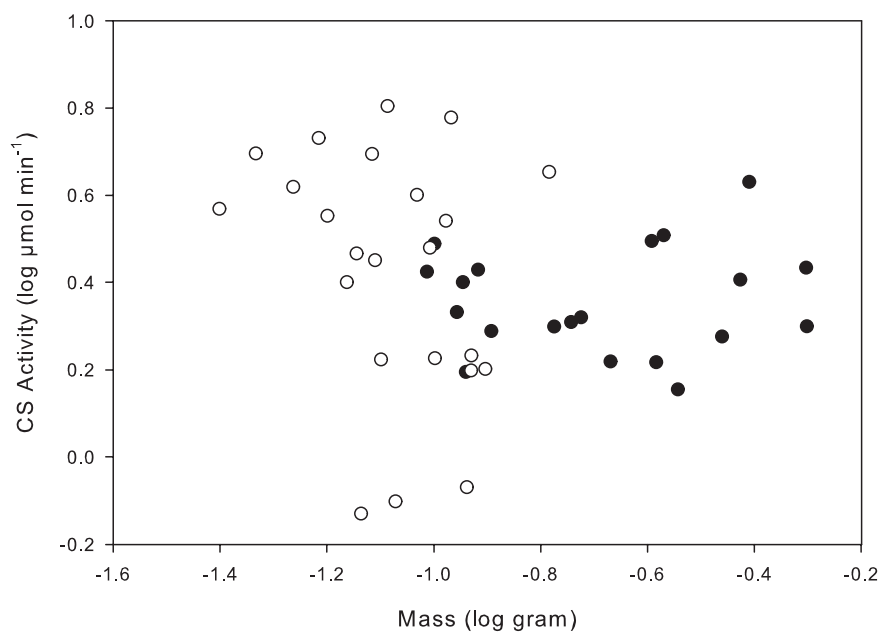
Figure 2a. Individual CS activities (log µmol min^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

As can be observed in Figure 2, neither *P. antrorum* nor *P. kadiakensis*, demonstrated a significant increase in enzyme activity (µmol substrate converted to product min $^{-1}$) with increasing individual mass (CS, P = 0.304; LDH, P = 0.076. However, when mass- specific (µmol min $^{-1}$ g WM^{-1}) enzyme activities were examined, there was a significant difference in the slopes of the least squares regressions. The slope of
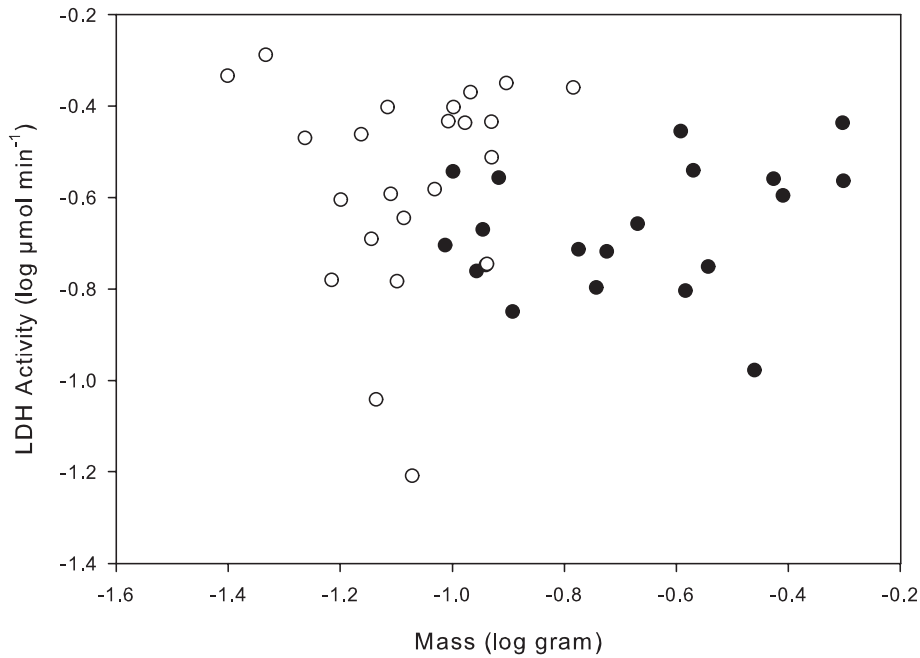
Figure 2b. Individual LDH activities (log μmol min^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).
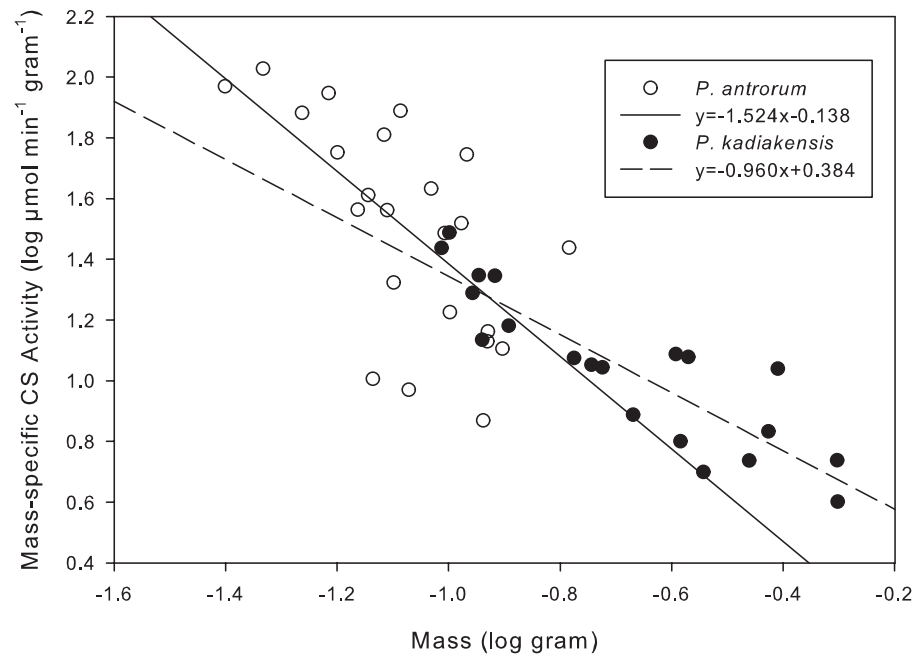
Figure 3a. Mass-specific CS activities (μmol min^{-1} g^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).
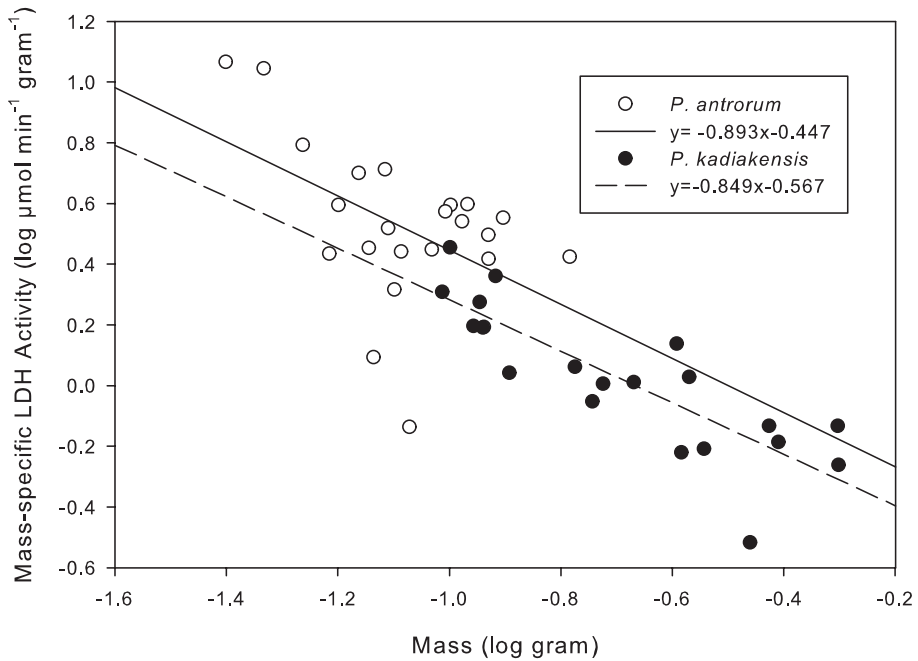
Figure 3b. Mass-specific LDH activities (log μmol min^{-1} g^{-1}) on Mass (log gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).
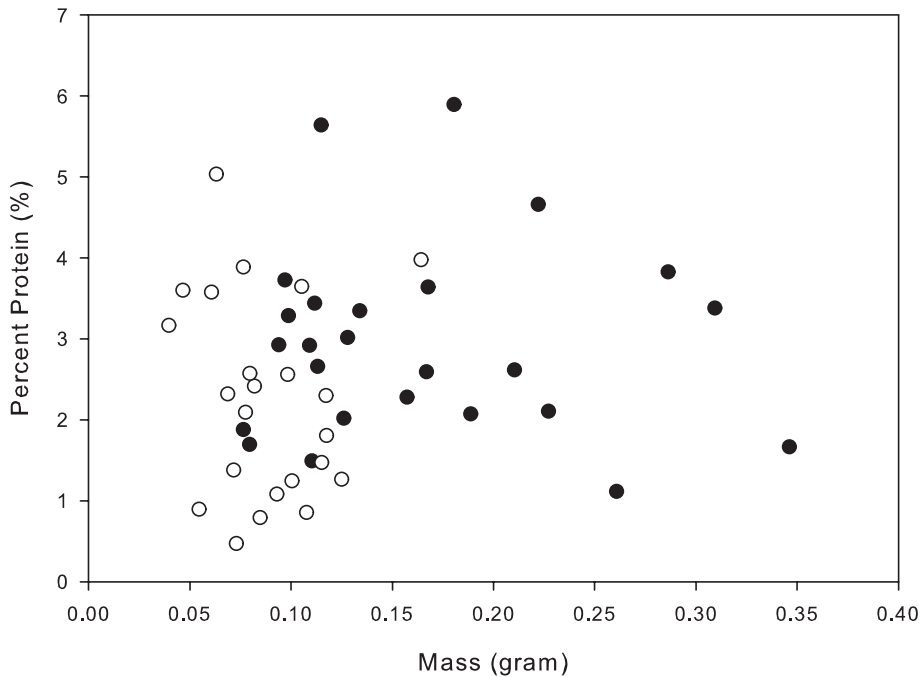
Figure 4a. Percent protein (%) on Mass (gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).
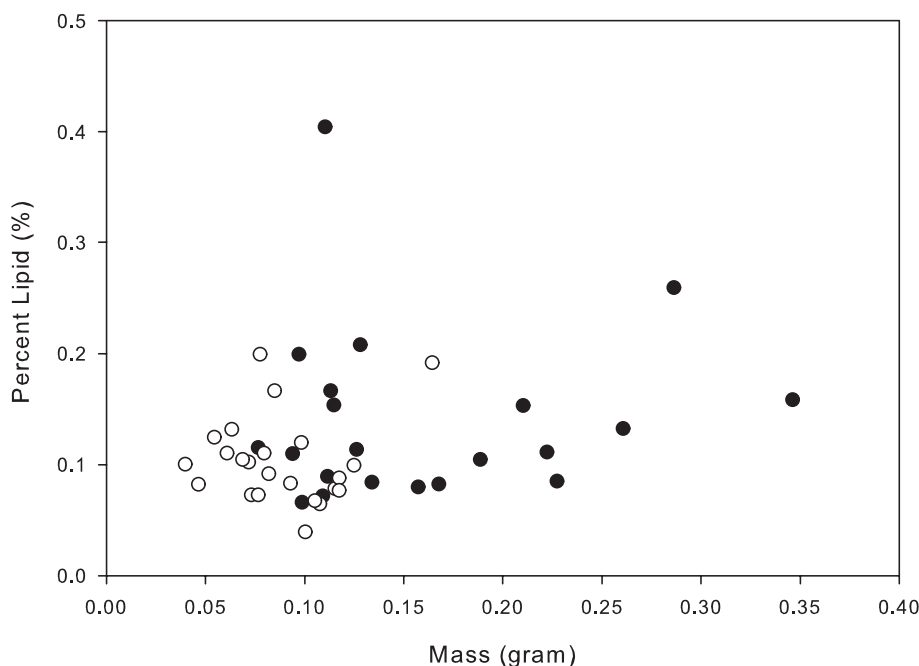
Figure 4b. Percent lipid (%) on Mass (gram). Closed symbols denote *Palaemonetes kadiakensis* (epigean); open symbols denote *P. antrorum* (stygobitic).

mass-specific CS activity on mass for *P. antrorum* ($y = -1.524x - 0.138$, $n = 20$, $R^2 = 0.41$) was significantly greater (Student's t, $t_{0.05(2), 39} = 2.023$, $|t| = 7.054$) than for *P. kadiakensis* ($y = -0.960x + 0.384$, $n = 23$, $R^2 = 0.78$). Figures 3a and b show the log transformed data with regression data. A corresponding difference was not observed in LDH activities, where the slope and intercept of the two mass-specific regressions did not differ significantly between the two species (*P. antrorum*: $y = -0.893x–0.447$, $n = 20$, $R^2 = 0.25$; *P. kadiakensis*: $y = -0.849x - 0.567$, $n = 20$, $R^2 = 0.70$).

As with individual enzyme activities, no significant difference was observed between the epigean and hypogean protein or lipid concentrations (Figure 4a and b) although both were slightly higher in the epigean species. Mean protein concentration for *P. kadiakensis* was 2.953%±0.1403% and for *P. antrorum* was 2.276%±0.2591; P = 0.061. Mean lipid concentration was 0.14%±0.0171% and 0.103±0.0082%; P = 0.052 for *P. kadiakensis* and *P. antrorum*, respectively.

Discussion

The $\delta^{13}C$ and $\delta^{15}N$ values for *Palaemonetes kadiakensis* (epigean) were well grouped whereas the values for *P. antrorum* (stygobitic) has a tight group plus two outliers indicating two distinct food sources. The outliers denote a principally terrestrial input of C from C3 photosynthetic source with $\delta^{13}C$ values similar to surface aquatic amphipods

reported in the vicinity of Movile Cave (Sarbu 2000) but with much lower $\delta^{15}N$ values (ca 5 vs 10‰). The main cluster of *P. antrorum* data showed exceptionally light $\delta^{13}C$ values, mean $\delta^{13}C$ -41.78‰; as $\delta^{13}C$ values ≤30‰ are well below that of terrestrial biome, demonstrating that photosynthetically derived material is not generally important in the artesian ecosystem and that C in the ecosystem originates from chemolithoautotrophic inorganic carbon fixation (Engel et al. 2004). Values for *P. antrorum* are lighter than those recorded in Lower Kane Cave, Wyoming, a cave formed by sulfuric acid speleogenesis (Engel et al. 2004), or in the sulphidic based system in Frasassi Cave, Italy (Sarbu et al. 2000). The values are also lighter than those reported for any fauna by Pohlman et al. (2000) for Mayan Blue, an anchialine cave where the fauna were utilizing particulate organic matter derived from nitrifying bacteria which is expected to have a $\delta^{13}C$ = -45 to -35‰. They argued this would account for the unusually light $\delta^{13}C$ values measured in *Tulumella unidens* Bowman & Iliffe, 1988 (Thermosbaenacea) and *Typhlatya mitchelli* Hobbs & Hobbs, 1976 (Atyidae), mean $\delta^{13}C$ =-34.4 and -36.1 respectively, both biofilm grazers.

Only in Movile Cave, where methanotrophic and chemoautotrophic bacteria provide the basis for cave life, did the cave fauna show $\delta^{13}C$ values (mean $\delta^{13}C$ ca -42‰: fig. 17.10: Sarbu 2000) comparable to those of *P. antrorum* ($\delta^{13}C$ = -41.78, s.d. = 1.92‰). This suggests that the energy source for the artesian population in the Edwards Aquifer is also based on methane which has a $\delta^{13}C$ -60‰ as indicated in Fig. 5 which shows the $\delta^{13}C$ and $\delta^{15}N$ stable isotope data from the Edwards Aquifer together with the range of $\delta^{13}C$ values typical obtained from different energy sources. Figures depicting a succinct synopsis of the stable isotope structure of the fauna in the various chemoautotrophic cave ecosystems are provided by Pohlman (2011).

Opsahl and Chanton (2006) examined the ^{13}C, ^{14}C and ^{15}N isotopic composition of *Cambarus cryptodytes* Hobbs, 1941 from caves and bores in the Upper Floridan aquifer and concluded that in the deep aquifer—those parts of the aquifer remote from photosynthetic carbon sources—the cave crayfish lived at least partly on a methane based bacterial chemosynthetic pathway with the methane being derived from surface wetlands, but they found no difference in $\delta^{15}N$ between spring and bore living individuals. Note, however, that they found wide variation between samples and that the mean $\delta^{13}C$ value for the bore samples was about the same as for our spring samples for *P. kadiakensis* ($\delta^{13}C$ 34.7 vs 34.5‰), a value similar to that for the atyd shrimp *Typhlatya mitchelli* found in anchialine chemotrophic systems (Pohlman et al. 2000). The much lighter $\delta^{13}C$ values for *P. antrorum* and the large difference in $\delta^{15}N$ between spring (*P. kadiakensis*) and bore (*P. antrorum*) samples indicates that there is not a close coupling of surface and artesian systems as would be expected as the presence of an artesian system indicates the presence of an aquitard, which would intercept downward movement of surface derived carbon.

The $\delta^{15}N$ values for both *Palaemonetes* species are positive in marked contrast to the light (negative) values seen in samples taken from sulphidic caves in Romania and Italy (Sarbu 2000, Sarbu et al. 2000). C:N ratios for *P. kadiakensis* are comparable to those seen in marine crabs (Harms et al. 1994) whereas the ratio in *P. antrorum* (C:N
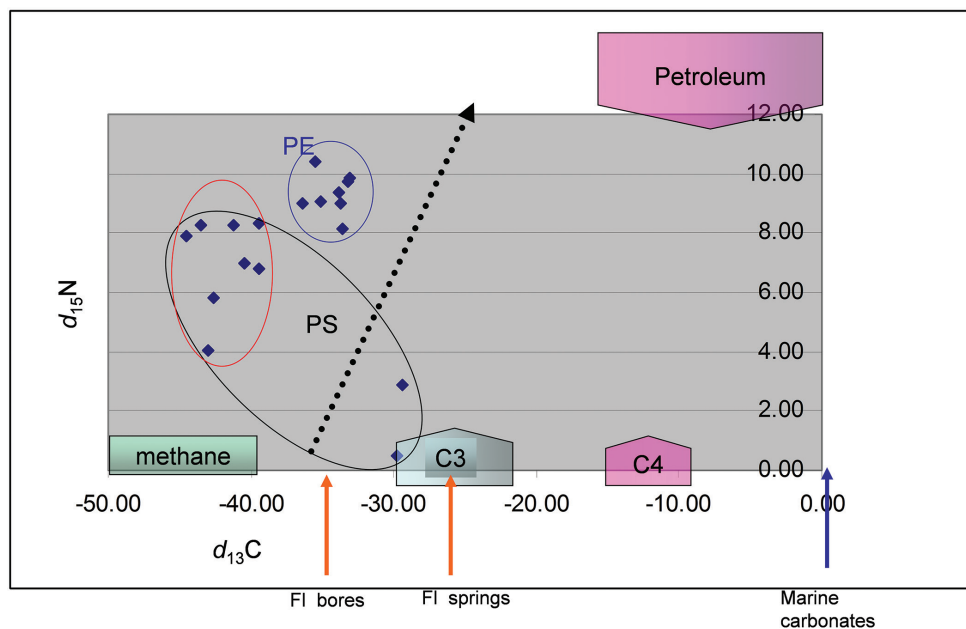
Figure 5. Plot of $\delta^{15}N$ on $\delta^{13}C$ values for the stygobiont *Palaemonetes antrorum* (PS, red oval and black oval respectively excluding and including two outliers, see text) and the epigean *P. kadiakensis* (PE, small blue oval). The range of values of $\delta^{13}C$ derived from different energy sources (see text) is indicated (methane, and photosynthesis from C3 and C4 plants) as well as values typical of marine carbonates and petroleum. The diagonal dotted line denotes a typical trajectory (not the value) of amplification of $\delta^{15}N$ values with progression through trophic levels (Vanderklift and Ponsard 2003).

5.96) is comparable to that found in the fauna of the sulphidic Movile Cave (C:N 5.7. Sarbu 2000).

The clearest isotopic separation between *P. kadiakensis* and *P. antrorum* is seen in $\delta^{34}S$ respectively +5.56 and -10.48. The strongly negative $\delta^{34}S$ values for *P. antrorum* is similar to the bivalve *Pillucina pisidium* (Dunker, 1860) in a *Zostera marina* community which harbours chemoautotrophic bacterial symbionts (Kharlamenko et al. 2001) but it is much more depleted in $\delta^{13}C$ (about -39 vs -28‰). However, the significance of this marked difference is unclear because the $\delta^{34}S$ values of microbial mats (biofilm) will reflect the $\delta^{34}S$ values of the waters from the two sources because if sulfur-oxidizing bacteria are involved they exhibit negligible sulfur isotope fractionation during the transformation of sulfide to elemental sulfur and elemental sulfur to sulfate (Toran and Harris 1989). Sulfur isotope compositions confirmed that sulfur content and sulfur speciation may not correlate to microbial metabolic processes in natural samples, thereby complicating the interpretation of sulfur records both modern and ancient (Engel et al. 2007). Kinkle and Kane's (2000) call for increased attention to be paid to the microbiology of subterranean systems has been amply justified by the subsequent investigations.

Marine waters currently have values $\delta^{34}S$ about +21‰ although they were substantially lower (17.5‰) in the Paleocene (Paytan et al. 1998). Biologically driven sulfate reduction depletes the value by -18‰ and repeated metabolic cycles can deplete $\delta^{34}S$ to -50‰ (Schlesinger 1997).

The two species of *Palaemonetes* we studied have light $\delta^{34}S$ values but those for *P. antrorum* at $\delta^{34}S$ -10.48‰ are exceptionally light compared with values reported for hydrothermal vent and seep species compiled by Conway et al. (1994) which mostly lie between $\delta^{34}S$ -5 and 0‰. Even the iconic hydrothermal vent vestimentiferan, *Riftia pachyptila* Jones, 1981, was in this category ($\delta^{34}S$ -4.7 to +4.7‰: Fry et al. 1983a) and the lightest $\delta^{34}S$ for any vent arthropod was -0.8 to -0.1‰ in a Galapagos hydrothermal vent crab *Bythograea thermydon* Williams, 1990 (Decapoda) (Fry et al. 1983b). The only values comparable to those we report for *P. antrorum* were from the lucinid clam *Pseudomiltha* sp. (Mollusca) from a Louisiana hydrocarbon seep with $\delta^{34}S$ values between -11.5 to +1.3‰ (Brooks et al. 1987) and which contain endosymbiotic chemosynthetic bacteria (Schweinemanns and Felback 1985).

The consumer assimilation effects as organic matter moves through the food chain are small for $\delta^{34}S$ in contrast to the effects on $\delta^{13}C$ and $\delta^{15}N$. The predominant biological process affecting $\delta^{34}S$ of sulphur containing compounds is dissimilatory sulfate reduction by bacteria (Conway et al. 1989, 1994).

Vetter and Fry (1998) found that thiotrophic animals relying on microbially derived reduced sulfur compounds in anoxic sediments had $\delta^{34}S$ from -30 to -20‰, whereas those relying on sulphides from seeps or hydrothermal vents had higher values of $\delta^{34}S$ -10 to +7, which nearly encompasses the values for both species of *Palaemonetes*. Animals relying on methane oxidation for their energy production may also have had low $\delta^{34}S$ from +8 to +10‰, indicating some reliance on assimilatory uptake of inorganic sulfur from seep fluids for the biosynthesis of compounds containing organic sulfur (Vetter and Fry 1998).

For *P. antrorum,* the exceptionally low $\delta^{13}C$ values coupled with low $\delta^{34}S$ suggest the artesian system is a complex geochemical system providing inorganic energy sources from both methane and sulfates, as is to be expected in a petroleum driven system. Natural oil seeps do occur along the Balcones fault zone and the hydrocarbons and oil-field brines have a major influence on the geochemistry of the 'bad water' zone in the central part of the Edwards Aquifer (Sharp and Banner 1997).

The objective of this research was to explore trophic structure, metabolism, and proximate compositional differences between the epigean *P. kadiakensis* and stygobiotic, *P. antrorum,* to address the hypotheses as to the presence and causes of metabolic adaptation to the subterranean environment. We discovered that metabolic depression was not evident in key enzymes involved in the aerobic and anaerobic metabolism of the two species, although previous studies examining oxygen consumption rates and enzyme activities in stygobitic organisms did observe a reduction in metabolic rates both as enzyme activities and respiration rates (Bishop et al. 2004, Bishop and Iliffe 2009, 2012). Although unexpected, our results are not novel. Previous studies comparing epigean and hypogean metabolic measurements, as discussed below, have

resulted in comparable rates between species, rates with no correlation with mass and even, individual rates that decline with increasing mass.

Members of the genera *Cambarus*, *Procambarus* and *Troglocambarus* are benthic dwelling crayfish from epigean and hypogean habitats. Caine (1978) examined the oxygen consumption of four species of epigean and three species of hypogean crayfish. Two of the four epigean species examined did not have significantly higher oxygen consumption rates than those observed in the hypogean *Procambarus* spp. Gannon et al. (1999), also examined cave crayfish of the genera *Procambarus* and *Troglocambarus* using Wheatly's (1989) data on the epigean crayfish *Pacifastacus leniusculus* (Dana, 1852) for comparison, and found the individual oxygen uptake rate (O_2 µmol min^{-1}) was indeed greater for the much larger epigean specimens (mean 0.291+0.407 µmol min^{-1}, range 0.002-1.309 µmol min^{-1} for specimens ranging from 0.03 – 105.4 g) but that respiration rates for the smaller hypogean species (0.18 – 3.29 g) remained within the range of the rates determined for the epigean organisms.

Crustaceans have been shown to reduce their metabolism while overwintering, potentially as a mechanism to function in a food poor environment (Quetin and Ross 1991, Meyer et al. 2002), therefore resulting in a lack of correlation of metabolism with increasing mass. Since specimens were collected in late spring early summer when food is abundant, it is unlikely that the epigean shrimp we used were conserving energy by reducing their metabolism.

A negative relationship between respiration rate and body mass was observed in *Amblyopsis rosae* (Eigenmann, 1898)*, the Ozark cavefish,* by Adams and Johnson (2001). When examining oxygen consumption throughout the year, a positive correlation between oxygen consumption and mass was observed in spring and summer but during fall and winter months there was a negative relationship. The authors attributed this result to changes in environmental conditions within the cave, particularly as a result of food availability and the presence of a bat colony in the cave from April to October.

Lack of correlation of enzyme activity with increasing mass has also been observed in crustaceans. Wilhelm et al. (2006) when examining oxygen consumption rate as a function of mass, found that the stygobitic amphipod *Gammarus acherondytes* Hubricht & Mackins, 1940 showed no increase in individual oxygen consumption with increasing mass. The authors speculated that the higher rates observed in the smaller, and therefore possibly younger amphipods was an ontogenic adaptation, providing the young amphipods with a greater ability to convert available food to energy. Activities then did not increase with increasing mass as a mechanism for the larger amphipods to conserve energy during times of low food availability. This conclusion is supported by the mass-specific enzyme activity data presented in this study. Mass-specific aerobic enzyme activities of the very small *P. antrorum* were greater than observed in small *P. kadiakensis* and then decreased to levels below the epigean species at greater sizes. The mass-specific glycolytic enzyme activities for both species were not significantly different since neither species was exposed to an oxygen limiting environment.

It is possible that metabolic potential may be uncoupled from oxygen consumption during function at normoxic conditions. The strategy of maintaining high metabolic

potential while reducing oxygen consumption would provide the hypogean organism the metabolic machinery necessary to best utilize resources when available. Selective pressures would favor a reduced oxygen consumption rate while maintaining a high metabolic potential. This is the situation observed by Simčič et al. (2005) when examining electron transport system (ETS) activity and oxygen consumption of hypogean and epigean amphipods between caves and two surface locations. ETS activities were found not to differ between animals from different locations, or between epigean and hypogean amphipods yet oxygen consumption was lower in the hypogean organisms.

Examination of the ratio of an organism's maximum aerobic potential to anaerobic potential (CS:LDH) can indicate the degree of evolutionary adaptation to environmental conditions (Childress and Somero 1990). If the ratio is >1, the organism is considered to be aerobically poised while values <1 can indicate an organism that is exposed to anaerobic conditions and must rely heavily on glycolysis. *Barbouria cubensis* collected from anchialine cave systems in the Bahamas and the Yucatan (Bishop and Iliffe 2012) reveals that metabolic potential may vary somewhat according to environmental parameters. *Barbouria cubensis* were anaerobically poised while the *Palaemonetes* in our study were all aerobically poised as anticipated since both the surface pools and the aquifer at the site of collection were both oxic.

Hervant and Renault (2002) and Ritar et al. (2003) examined prolonged fasting and utilization of energy reserves in crustaceans. Both studies showed that protein and lipids were utilized in a series of three successive phases but there was disagreement on the order of protein versus lipid utilization. Hervant and Renault (2002) state the order of utilization was 1) depletion of arginine phosphate and glycogen, 2) use of triglycerides, and 3) depletion of proteins and lipids. This strategy selects for the ability to prolong survival and resulting competitive abilities by depleting protein, i.e. muscle, as the final resort (Sánchez-Paz et al. 2006). Ritar et al. (2003) also propose three phases but the first stage of starvation is the utilization of energy rich lipid reserves, followed by protein and the final stages of starvation results in the utilization of structural lipids. Either strategy would provide energy rich reserves to be utilized should resources become limiting.

Proximate composition, in the form of lipid and protein, was not significantly different between the hypogen and epigean *Palaemonetes* sp. In fact, when compared to other epigean crustaceans, the protein and lipid concentrations for both *Palaemonetes* were within published ranges for crustaceans (Childress and Nygaard 1974, Torres et al. 1994, Bishop et al. 2004), albeit on the lower end of the ranges for both species. Previous studies comparing proximate composition of stygobitic organisms, *Barbouria cubensis,* from environments with differing oxygen concentrations showed no significant difference between two populations collected from oxic and dysoxic environments (Bishop and Iliffe 2012). Although our stable isotope analyses indicated that there was a significant difference in the food being consumed by the epigean and hypogean species of *Palaemonetes*, that difference was not translated into significant proximate compositional differences despite the large differences in C:N ratio and S content, the latter

being lower in *P. antrorum* and C:N greater owing to significantly lower N values in *P. antrorum*. We interpret this result to indicate that food resources within the aquifer were not limited. It is possible that the storage of lipids in crustaceans is a constructive feature enabling the survival of the population, but which is not necessary if resources are constantly available, as is the case with the *Palaemonetes* studied here, and therefore not selected for in the population of either the epigean or stygobitic species.

We shall now return to the initial question posed. In a cave environment containing abundant dissolved oxygen and adequate energy, will the metabolic rate of cave adapted species differ from their epigean relatives? We have demonstrated through stable isotope analyses that the aquifer is not resource limited and by comparing the proximate composition of the hypogean organisms to the epigean, we can see that the protein and lipid levels are not different, further supporting that resources are not limiting. Based upon enzyme activities, the maximum aerobic potential, or the greatest rate at which an organism can convert food into energy, and the maximum glycolytic potential, providing information on an organism's ability to function in an anaerobic environment, there was no difference in the metabolism of the two species of *Palaemonetes*. We did not observe a depressed metabolism in the stygobitic organisms indicating metabolic cave adaptation. But why were the metabolic rates of the stygobitic *Palaemonetes* not lower than in the epigean species? Why was the characteristic metabolic depression found in many cave organisms not observed in this situation? We have addressed two of the current hypotheses regarding the cause of lower metabolism in cave organisms, namely, the limitation of either/or oxygen and food. Metabolic cave adaptation is a constructive feature affected by selection; therefore, the intensity of selection may be responsible for the level of reduction of a characteristic (Hüppop 1986). Mitchell (1969) pointed out that in caves with a relatively high energy input, such as in tropical systems, where the biomass in the tropical epigean is great and production is uninterrupted, the selection pressures leading to cave adaptation could be expected to not be as strong. Because both food and oxygen are abundant in the Edwards Aquifer, the selection for a reduced metabolism is depressed. For metabolic cave adaptation to occur, selective pressures, either in the form of reduced food supply or low oxygen availability, or as in some stygobiont environments, both, must be present to exert directional selection to lower the metabolic rate in subterranean organisms.

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