

A review of the diversity, adaptations and groundwater colonization pathways in Cladocera and Calanoida (Crustacea), two rare and contrasting groups of stygobionts

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With 4 figures and 2 tables

Abstract: Cladocera and calanoid Copepoda are ubiquitous in surface freshwater, each with about 500–600 species and subspecies distributed worldwide. Both groups also contain some subterranean species, which occur in karst and porous aquifers. The first stygobiotic calanoid, *Microdiaptomus cokeri*, was described in 1942, and the first cladoceran, *Alona smirmovi*, in 1973. Currently, seven species of Cladocera are confirmed and three more are possible stygobionts. In Calanoida, nine species are recognized stygobionts and three more are possible. In the last few years the number of described stygobiotic taxa has increased and may continue to do so, as caves and gravel-beds are more intensively studied. Stygobionts are probably derived from a Miocene fauna that, under the constraint of a worsening climate, took advantage of the development of caves and found subterranean refugia. The Pleistocene glaciations accelerated this colonization process, which persists to the present. Both groups do not contribute much to the overall subterranean biodiversity, but they exhibit adaptations that may help to reveal the mechanisms of colonization of the subterranean realm. Some of their characteristics are related to feeding and to maintaining their position during cave floods, and are shared with other stygobionts. In addition, stygobiotic Cladocera and Calanoida have distinct life-histories and morphological adaptations. In karst-dwelling Cladocera, parthenogenesis supports fragmented populations, whereas karst-dwelling Calanoida are planktonic, a unique way of life among freshwater stygobionts.

Key words: Porous aquifer, Karstic aquifer, Ecology, Biogeography, Adaptations, Calanoida, Cladocera.

Introduction

Un-frozen subterranean freshwater environments can be divided into waters in consolidated rocks and in unconsolidated sediments and in karstic, fissured, and porous aquifers (Galassi 2001, Gibert & Derhaveng 2002). In consolidated rocks, the most important aquifer is the karstic system, producing caves on a macro-scale and fractures on a micro- or macro-scale. In unconsolidated sediments the most important porous

aquifer is the hyporheic zone of rivers and phreatic groundwater. These systems differ by the dimensions of the interstices filled with water that provide living space for organisms and determine their size. Groundwater is populated by a specialised fauna called stygobionts, and by accidental taxa (stygoxenes) temporarily imported from the surface. Stygophiles are intermediate between stygobionts and stygoxenes: they spend part of their life belowground, and may even be more common in groundwater than in surface waters.

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Stygobionts most likely originated from epigeal (= surface) ancestors that became isolated below-ground, and adapted to life in a habitat characterized by darkness, a relatively constant temperature and limited food. Such a fauna requires morphological (Christiansen 2004), physiological (Hervant & Malard 2005, Simčič et al. 2005), behavioural (Romero 2004, Parzefall 2005) and ecological (Aden 2005, Hüppop 2005) adaptations.

Subterranean aquatics, whether karstic or porous, contain representatives of many taxonomic groups found in epigeal fresh or marine waters. Some have only a few groundwater species (e. g. Cnidaria – *Velkovrhia enygmatica*; Bivalvia – *Congeria kusceri*; Polychaeta – *Marifugia cavatica*) while others have many, sometimes even more than surface waters (e. g. Gastropoda, Amphipoda, Isopoda, Harpacticoida, Cyclopoida, Sincarida) (Botosaneanu 1986, Hobbs III 2005). Several groups (e. g. Anostraca, Notostraca and Conchostraca) have no subterranean representatives at all.

In recent decades, several variants of a gradualistic model of colonization of the subterranean environment have been proposed (Coineau & Boutin 1992, Holsinger 1994), the recent model by Stoch (2004) stating that the first step consists of a few initial invaders that adjust to a stygobiotic way of life. Some of these pioneers (e. g. in the crustacean genera *Niphargus*, *Diacyclops*, *Elaphoidella*) may later develop impressive adaptive radiations.

Here, we examine the Cladocera and Calanoida (Copepoda), two crustacean groups similar in species richness, each with an estimated 500–600 species distributed worldwide. They inhabit lakes, ponds and pools, and extreme habitats such as ephemeral, high-mountain, and saline inland lakes. Some Cladocera (but not Calanoida) are even semi-terrestrial and climb moss-covered trees in wet forests (Frey 1980). Only a few members occur in the subterranean environment. Stygobiotic Calanoida are only known from karstic aquifers (caves and springs), whereas Cladocera live in both porous and karstic aquifers, with species ranging from stygophile to strict stygobionts.

This article discusses the subterranean representatives of both groups and their adaptations, the mechanisms that may have facilitated their penetration to the subterranean environment, and formulates hypotheses about their geographical distribution.

Material and methods

To study morphological adaptations in Cladocera, papers with high-quality illustrations of thoracic limbs (especially P1 and

P2) were studied (Brancelj 1990, 1992, 1997, Alonso 1996), and original specimens were examined. To prepare line drawings of P2, specimens of five taxa (*Alona hercegovinae* Brancelj, 1990, *Alona protzi* Hartwig, 1900, *Alona affinis* (Leydig, 1860), *Alona guttata* G. O. Sars, 1862 and *Alona phreatica* Dumont, 1983) were dissected under an Olympus SZH2 stereomicroscope in a drop of glycerol. Limbs were mounted on permanent slides in glycerol, sealed with nail polish. Examination was done under oil immersion, at a magnification of 1000×. Drawings of all five species were made using a drawing tube mounted on an Olympus microscope (BHS40).

Material for SEM was examined under a JEOL JSM-840. Specimens were critical-point dried, gold-coated and mounted on stubs after removing the valves and P1 to expose the P2 for examination. All material examined is in the collection of A. Brancelj.

Results

Cladocera

Cladocera were long considered a group without stygobiotic representatives (Dumont 1987), although epigeal species had sporadically been recorded from subterranean environments as early as the late 19th century (for a review, see Dumont & Negrea 1996). About 100 out of a total of about 600 species of Cladocera have currently been recorded from subterranean habitats (Dumont & Negrea 1996), but less than 2% of them are true stygobionts. Almost all belong to the anomopod family Chydoridae (suborder Radopoda), with a strong dominance of the genus *Alona* s. l.

The first stygobiotic species, *Alona smirnovi* Petkovski & Flöbner, 1972, was described by Petkovski & Flöbner (1972) from a karstic spring on the shore of Lake Ohrid (an ancient lake on the border of Macedonia and Albania). Later, Dumont (1983) described *Alona phreatica* Dumont, 1983 from the hyporheic zone of a small river (Couze Pavin) in the French Massif Central. Dumont (1983) also described *Alona bessei* Dumont, 1983 another possible inhabitant of groundwater in the Rhône basin, but this might be a morphological form of the plastic *Alona guttata* (Dumont, pers. obs.). *Karualona alsafadii* Dumont & Brancelj, 1994 from Yemen (Dumont & Brancelj 1994) and *Alona elegans* Kurz, 1875 are to date the only described stygobiotic cladocerans from a porous aquifer outside Europe.

Some *Alona* s. l., now recognised as stygobionts, were originally discovered as isolated specimens in the outflow of springs or in spring-fed running water connected to groundwater. This is the case of *Alona protzi* Hartwig, 1900, which was found in or outside of groundwater in England, the Netherlands, Ger-

Table 1. List of stygobiotic Cladocera and Calanoida with estimated total number of specimens collected so far (according to different authors). (?) indicates two taxa with uncertain ecological status; (*) indicates stygophilic populations only; juv. = juveniles; ephipp. = ephippia.

Taxon	Author	N	females	males	juv.	ephipp.
CLADOCERA						
<i>Alona bessei</i>	Dumont, 1983	2	X			
<i>Alona elegans</i> *	Kurz, 1875	≈ 300	X	X	X	
<i>Alona hercegovinae</i>	Brancelj, 1990	≈ 100	X	X	X	
<i>Alona phreatica</i>	Dumont, 1983	≈ 1000	X	X	X	X
<i>Alona protzi</i>	Hartwig, 1900	≈ 100	X	X	X	
<i>Alona sketi</i>	Brancelj, 1992	12	X		X	
<i>Alona smirnovi</i>	Petkovski & Flößner, 1972	several	X			
<i>Alona stochi</i>	Brancelj, 1997	10	X			
<i>Karualona alsafadii</i>	Dumont & Brancelj, 1994	80	X		X	X
? <i>Macrothrix bialatus</i>	Motas & Orghidan, 1948	several	X			
? <i>Pleuroxus pigroides</i>	(Lilljeborg, 1900)	57	X			
CALANOIDA						
<i>Argyrodiaptomus cavernicolax</i>	Shen & Tai, 1965	few (?)	X	X	(?)	
<i>Hadodiaptomus dumonti</i>	Brancelj, 2005	62	X	X	X	
<i>Microdiaptomus cookeri</i>	Osorio-Tafall, 1942	≈ 30	X	X		
<i>Spelaeodiaptomus rouchi</i>	Dussart, 1970	few (?)	X	X		
<i>Speodiaptomus birsteini</i>	Borutzky, 1962	9	X	X		
<i>Stygodiaptomus ferus</i>	Karanovic, 1999	1		X		
<i>Stygodiaptomus kieferi</i>	Petkovski, 1981	17	X	X	X	
<i>Stygodiaptomus petkovski</i>	Brancelj, 1991	95	X	X	X	
<i>Troglodiaptomus sketi</i>	Petkovski, 1978	≈ 1000	X	X	X	

many, (Hartwig 1900), Romania (Negrea 1983), the Alps (Italy, Stoch pers. comm.), Turkey (Güher 2001) and southern Finland (Sarmaja-Korjonen pers. com.). The fact that few of these specimens were found outside groundwater, (mis)led Dumont & Negrea (1996) to classify it as rare and stygophile.

Alona elegans is an ecologically ill-known species, rare and ranging from Western Europe to Northern Africa (Negrea 1983, Margaritora 1985, Alonso 1996). It was recently confirmed as relatively common in groundwater (PASCALIS project 2004) and it has morphological adaptations characteristic of taxa from porous aquifers (see below).

Pleuroxus pigroides (Lilljeborg, 1900) and *Macrothrix bialatus* Motas & Orghidan, 1948 (Dumont & Negrea 1996) have been found in springs and in the hyporheic zone, but their status as stygobionts remains unclear. The discovery of a population of *P. pigroides* in the weedy littoral of Lake Glubokoe, Moscow area (Smirnov 2005) suggests this taxon might not be subterranean, although it could still be ecologically similar to *A. protzi* and *A. elegans*.

A. protzi and *A. phreatica* form a natural group with *A. smirnovi* and *A. labrosa* from Lake Baikal. It is perhaps not a coincidence that this fourth species is a lake-dweller (Sinev & Kotov 2000), because Lake Baikal is also home to the epigeal *Bathynella magna*

Basicalova, 1951. Bathynellaceans too are a group otherwise restricted to subterranean waters (Kozhow 1963).

The first blind cladoceran from a karstic aquifer, *Alona hercegovinae* Brancelj, 1990, was described from the central Balkan Peninsula, followed by *Alona sketi* Brancelj, 1992 and *Alona stochi* Brancelj, 1997 from caves in Slovenia (Brancelj 1990, 1992, 1997). These species form a natural group, related to the *Alona diaphana*-complex that leads an epigeal life in the tropics-subtropics.

Calanoida

At present, 600 + taxa of Calanoida are known from temporary and permanent freshwater bodies (Bowman 1986); about ten species live in karstic aquifers. All stygobiotic Calanoida belong to the family Diaptomidae, subfamilies Speodiaptominae, Microdiaptominae, and Diaptominae. The first two subfamilies are exclusively stygobiotic, whereas the latter includes four stygobiotic taxa, beside numerous epigeal planktonic genera and species (Dussart & Defaye 2001). The first recognised stygobiotic species (*Microdiaptomus cookeri* Osorio-Tafall, 1942) lives in caves in Mexico (Elías-Gutiérrez & Suárez-Morales 1998). *Speodiaptomus birsteini* Borutzky, 1962 was

described from the Crimean Peninsula (Borutzky 1962), and *Argyrodiaptomus cavernicolax* Shen & Tai, 1965 (Shen & Song 1979) from South China. The remaining five species are European: *Spelaeodiptomus rouchi* Dussart, 1970 in France (Dussart 1970), *Troglodiptomus sketi* Petkovski 1978, *Stygodiptomus kieferi* Petkovski, 1981, *Stygodiptomus petkovski* Brancelj, 1991 and *Stygodiptomus ferus* Karanovic, 1999 in the Dinaric Balkans (Petkovski 1978, 1981, Brancelj 1991, Karanovic 1999). Only *T. sketi* is common and widespread, ranging across Slovenia, Italy and the Balkan (Stoch 1984, 1985, Brancelj 1991, 2001). *Hadodiptomus dumonti* Brancelj, 2005 was recently described from a cave in Vietnam (Brancelj 2005). More calanoids have been collected from caves in Madagascar, Cuba, and Barbados. Some have been described as new subspecies, but their status (stygothiles or stygobionts) is unclear (Petkovski 1981, Bowman 1986). The approximate total number of specimens of stygobiotic Cladocera and Calanoida collected so far is presented in Table 1.

Morphological adaptations

Obvious adaptations to the subterranean environment include a reduction of the eye(s) or loss of eye pigment, body de-pigmentation, a small size, a flattened and/or elongate body-shape, reduction of segments and number of setae, movement by crawling instead of swimming, and a neotenic (= pedomorphic) development (Bowman 1986, Dumont 1987, Dumont & Negrea 1996, Galassi 2001).

Cladocera show a gradient from stygophilic to stygobiotic in porous and karstic aquifers. Stygobiotic Cladocera from porous aquifers in Western Europe possess an eye and ocellus, although of reduced size (Dumont & Negrea 1996). In *A. smirnovi* and *K. alsafadii*, the eye has disappeared, but the ocellus remains. All taxa from karstic aquifers are blind (Brancelj 1990, 1992, 1997). The reduction of the eye and ocellus in *Spinalona anophthalma* Ciro-Pérez & Elías-Gutiérrez, 1997 (Ciro-Pérez & Elías-Gutiérrez 1997) is probably not related to a stygobiotic way of life but to life in a turbid environment.

A common characteristic, observed in Cladocera from all types of groundwater is their inability to swim (Dumont 1987, Dumont & Negrea 1996). This is not an exclusive character of stygobiotic cladocerans, given that all stygobiotic Cladocera belong to littoral-benthic genera (*Alona* s. l. and *Karualona*) and are poor swimmers, but a reduced swimming ability may represent a useful pre-adaptation to life in

groundwater. In fact, an ability to swim in a porous aquifer, where space is limited, has no advantage, and could be even counterproductive in caves, where the water level can rise instantly and swimming animals would be washed away. The loss of the ability to swim implies a benthic mode of feeding, and hence adaptations to this benthic feeding are expected.

The morphological adaptations of subterranean *Alona* to feeding are neither spectacular nor conspicuous when observed under low magnification, and are similar in species from both types of groundwater. They are most prominent in the food-manipulating limbs 1 and 2, and are best appreciated using SEM. In *Alona*, as in all members of the suborder Radopoda, the setulation of the long setae of the inner (IDL) and outer (ODL) distal lobe of the first pair of swimming legs (P1), and of the eight scrapers of the second pair (P2) can be of two types. Type I consists of narrowly spaced setules, all of similar size, with an intersetular distance of the order of 1 µm. Type II consists of longer and more spaced setules on P1, and at least some of the scrapers of P2 with coarse teeth instead of fine setules. The “modified” scraping setae may be any seta after the second one, where more than one calibre of teeth may occur on different scrapers (see examples in Dumont & Negrea 2002: Fig. 46). Functionally, the “modified” scrapers are in fact true scrapers, while the finely setulated ones function as combs or brushes, collecting particles of bacterial size (Fryer 1968). Brushes are also longer than true scrapers; the latter must exert force to remove particles from surfaces, and therefore require a robust stem. Differences in the number of setules on scrapers 5–8 of P2 in five *Alona* species are given in Table 2 and other relevant morphological characters are presented in Fig. 1. In all groundwater Cladocera known to date, IDL, ODL setae and scrapers are of type I: long, filiform and finely setulated. Scrapers 1–8 are relatively similar in shape and are much more slender and elongated than in epigeal taxa (Figs 1 and 2). In epigeal species, both types I and type II

Table 2. Number of setules on scrapers 5–8 of P2 and their type in adult females of five *Alona* species (epigeal, porous aquifer and karstic aquifer) (S = scraper).

Taxon	Mode of life	Type of scrapers	S-5	S-6	S-7	S-8
<i>Alona affinis</i>	epigeal	type II	30–33	28–30	25–28	20–21
<i>Alona guttata</i>	epigeal	type II	32–35	27–28	23–25	19–20
<i>Alona phreatica</i>	porous aquifer	type I	35–37	34–35	32–33	30–32
<i>Alona protzi</i>	porous aquifer	type I	41–43	42–43	41–42	36–38
<i>Alona hercegovinae</i>	karstic aquifer	type I	52–55	47–51	50–51	38–41

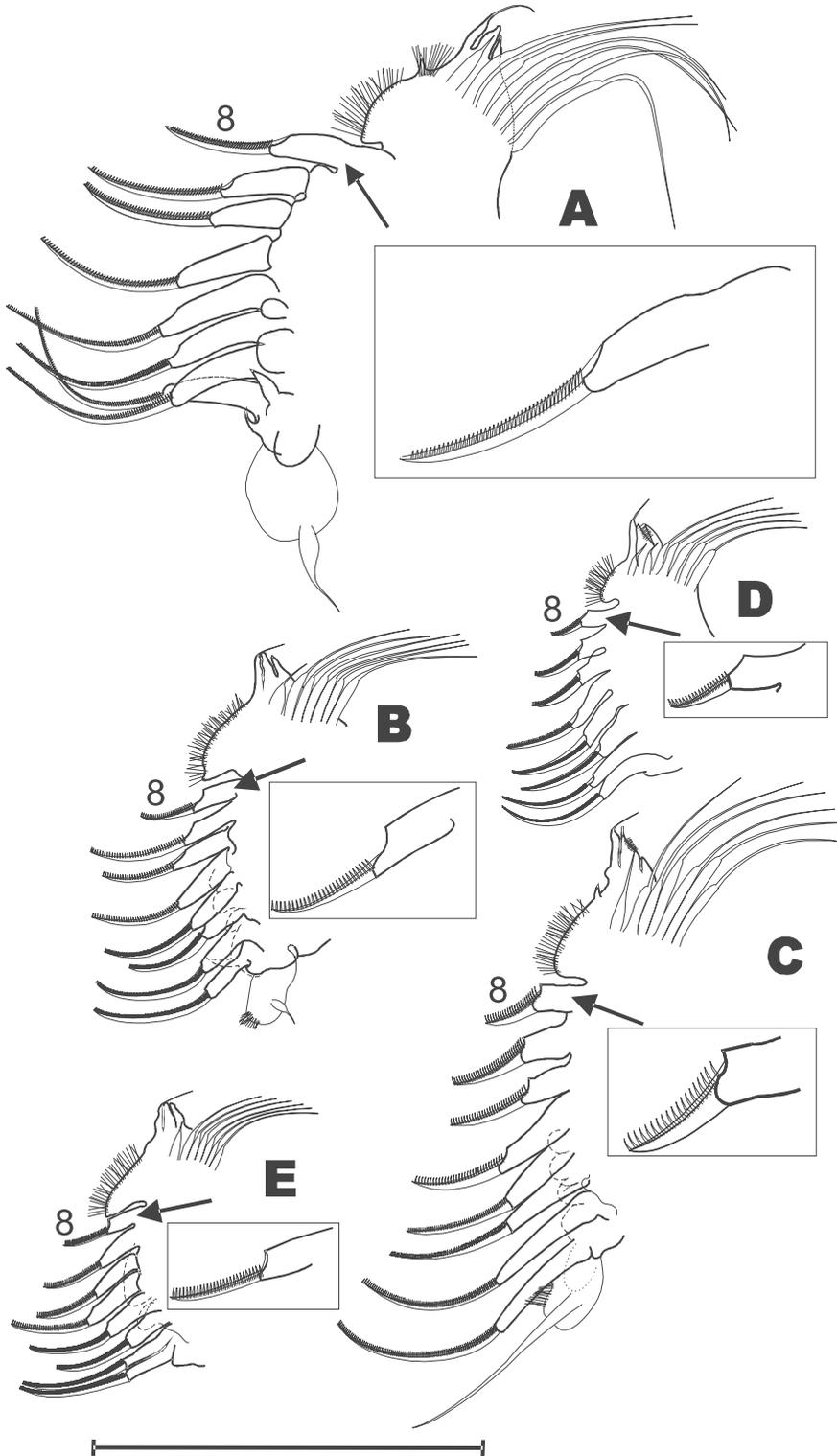


Fig. 1. The second trunk limb (P2) in five species of genus *Alona* (s. l.) related to their habitat. **A** – *Alona hercegovinae* Brancelj, 1990 (karstic aquifer); **B** – *Alona protzi* Hartwig, 1900 (porous aquifer); **C** – *Alona affinis* (Leydig, 1860) (epigean); **D** – *Alona guttata* G. O. Sars, 1862 (epigean); **E** – *Alona phreatica* Dumont, 1983 (porous aquifer). Enlarged figure of scraper 8 (in rectangle; see also black arrow and number) for each species is added. Scale bar: 100 μ m (not for scraper No. 8).

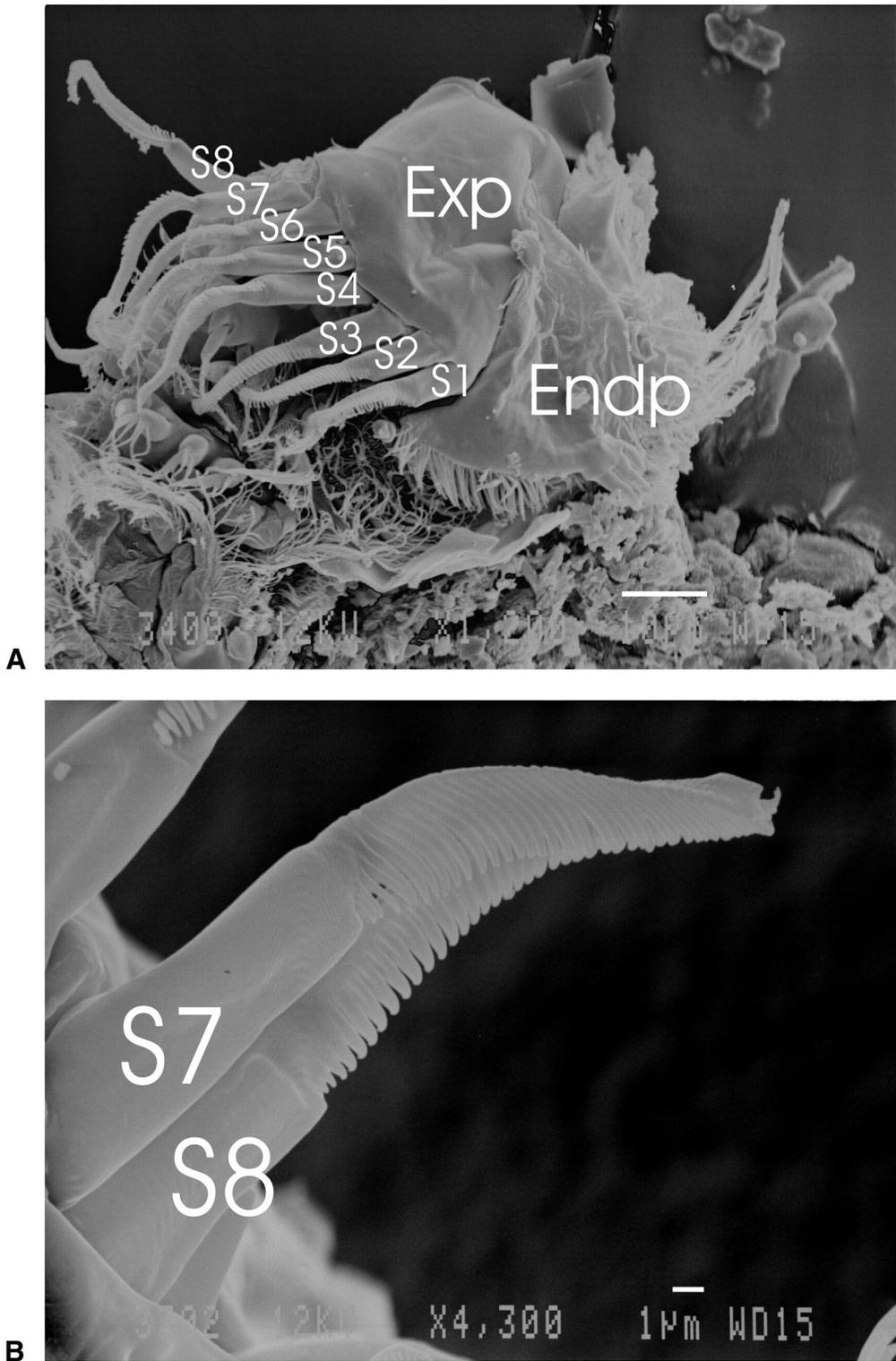


Fig. 2. SEM pictures. **A:** P2 of *Alona phreatica* Dumont, 1983 (scale bar: 100 µm); **B:** details of scrapers 7 & 8 on P2 of *Alona protzi* Hartwig, 1900 (scale bar: 1 µm). (S = scraper; Exp = exopodite, Endp = endopodite).

may occur. *Alona rectangulara* is an example of epigean species with type I setulation, while *Alona verrucosa* is an example of type II (for illustrations, see

Alonso 1996, Dumont & Negrea 2002 and Fig. 1 in this paper). Species that occur in epigean as well as in hypogeal environments, like *Alona guttata* and *A.*

elegans show type I setulation as well. Species with type I scrapers are thus potential colonizers of the porous aquifer. The length and armature of the eight scrapers on the second trunk limb in Cladocera from porous and karstic groundwater reflect the kind of food consumed. In porous groundwater there is still plenty of coarse and hard particulate organic material. It seems that the Radopoda which occur here do not collect such large particles, but prefer smaller food, partly decomposed by bacteria and fungi. However, in karstic environment, with predominantly fine suspended organic material and clay, rich in bacteria, long scrapers, equipped with fine lamellas, are necessary for survival.

In Calanoida, groundwater life caused a decrease in size, anophthalmia, and depigmentation (Galassi 2001), beside morphological adaptations related to feeding. Most conspicuous is the elongation of the maxillipeds, second in length only to the antennulae. In epigeic taxa, the maxillipeds are shorter than the swimming legs (see Dussart & Defaye 2001). The right antennula of epigeic males has expanded 13th–18th segments; in subterranean taxa these are of the same width as the other segments or are only slightly enlarged (in *Spelaodiaptomus* and *Stygodiaptomus*). An exception is *Argyrodiaptomus cavernicolax*, with antennulae similar to epigeic species. The setal armature of the antennula in stygobiotic taxa is as in epigeic taxa but with a tendency towards a reduction of the number of aesthetascs and a relative elongation of the setae. Another characteristic of stygobiotic taxa is a reduction of the number of segments of the swimming legs (for details see Brancelj 2005) as well as an elongation of the remaining segments.

Biological and ecological adaptations

Some of the ecological adaptations that support life of stygobiotic Cladocera and Calanoida in the subterranean realm are shared with other groundwater organisms, whereas other are specific to the diaptomids or radopods involved.

A reduction of the number of eggs per brood is common in invertebrates in low-temperature environments constrained by food (Moldovan 2005). A larger size of the offspring compensates for their low number (Calow 1998). In the subterranean environment, large and well-developed newborns are competitively superior and can reproduce early. Most epigeic Cladocera, including benthic species, have more than two eggs per brood. These develop to miniature versions of their mothers within the mother's brood

pouch. Such direct development of apomictic (i. e. non-fertilised) eggs is characteristic of all Cladocera. Little is known about egg-size and number of neonates in stygobiotic Cladocera, but our preserved material of *A. phreatica*, *A. hercegovinae* and *A. protzi* revealed that about 80% of females carried one egg and about 20% two eggs. Also, published figures indicate that the size of eggs and neonates is larger than in their epigeic relatives (see Petkovski & Flößner 1972, Dumont 1987, Brancelj 1990). This suggests that, like in copepods and other stygobiotic animals, K-selection (Rouch 1968, Odum 1971, Moldovan 2005) and/or A-selection (Greenslade 1983) may occur, an aspect of adaptation of stygobiotic Cladocera that will need attention in the future.

Usually, but not always, ephippium production is linked with a (usually brief) episode of amphimixis (i. e. sexual reproduction) (Dumont & Negrea 2002). But, so far, males have only been recorded in the stygobiotic *A. phreatica*, *A. protzi* and in the stygophilic *A. elegans*. Absence of ephippia is potentially a sign of a stable environment and of absence of sexual recombination. Ephippia and males have been found only in the hyporheic zone (i. e. up to 1 m deep), where variations (daily/seasonal) in temperature, food, and water level are more frequent than in karstic aquifers, where water level and current velocity may change sharply but with a low frequency. Ephippia have been found in stygobionts twice, in *K. alsafadii* and *A. phreatica*. Cladocera from karstic aquifers, living in an environment with annual temperature fluctuations in the range of 1 °C, might behave like the plankton of large tropical lakes, where “a total absence of sexual recombination over many years has been suspected” (Dumont & Negrea 2002). Clonal populations of parthenogenetic females perpetuate these populations. Periods without males (whose absence reduces competition for food) combined with the long life-span characteristic of karstic groundwater organisms (Rouch 1968), may last for decades or even centuries. The price to pay for such a strategy is, of course, the build-up of a genetic load and, ultimately, slow evolution (Chiambeng & Dumont 2005). The relatively frequent incidence of males (and consequently ephippia) and the presence of populations in locations with porous characteristics indicate that *A. protzi*, *A. phreatica* and *K. alsafadii* are relatively younger inhabitants of the subterranean realm than the possibly asexual “karstic *Alona*” from caves.

Calanoida from karstic aquifers are the only filter-feeders in groundwater that have maintained a plank-

tonic way of life. But such planktonic animals inevitably have limited capabilities to resist currents. Thus the question arises how they maintain viable populations in cave lakes subjected to flash-floods after torrential rains on the surface. Furthermore, these planktoners feed on suspended particles, which occur only in turbulent conditions, whereas cave water is usually crystal-clear. Turbulence in karstic aquifers requires longitudinal currents (as opposed to vertical fluxes in surface water bodies). It follows that stygobiotic Calanoida, with a habitus still adapted to swimming in the water-column, must have strategies not to be washed out from their caves. Stagnant water can occur for weeks and even months on end; flash floods are punctuated, short-time events. Water just turbulent enough to keep food particles suspended occurs only in between these periods. Calanoida must therefore be able to collect particles from the water column and from the bottom as well. In a laboratory population of *Troglo diaptomus* we observed both types of feeding. In a glass jar, containing a mixture of water and sediment, specimens of *T. sketi* maintained their position in the same “gliding” way as epigean taxa. After the suspended material had settled, they began diving to the bottom, remaining there for some time with vibrating maxillipeds before returning to the water column, their digestive tract filled with clay and mineral particles (Brancelj 2005).

Anticipating strong currents is of vital importance to cave Calanoida. Thus, they avoid channels with permanent currents, their main habitat being channels and cracks protected from currents; in such a microhabitat, small-bodied taxa are at an advantage (Bowman 1986). Further, as the mineralization of organic material in subterranean environments increases with residence time and depth in the karst, filter-feeders should avoid deep saturated zone. In accordance, Calanoida occur predominantly in the saturated zone, where the water table oscillates.

Epigean calanoid females carry 10–60 eggs per clutch; in *Troglo diaptomus*, one to three eggs per clutch are found, but each egg is at least as big as the genital segment (Fig. 3 in Petkovski 1978), 2–3 times the diameter in epigean species. An important final adaptation is the shortened development of some intermediate stages, recorded also in cave-dwelling beetles (Moldovan 2005). Little information on this is available in Calanoida, but we observed that in *T. sketi*, the embryo develops to a stage resembling a small copepodid rather than a nauplius. In contrast to Cladocera, all stygobiotic Calanoida are bisexual with an adult sex ratio close to 1:1.

Notes on geographic distribution

The geographic distribution of stygobiotic cladocerans and calanoids provides insights to understand the dynamics of the invasion of the subterranean realm by these organisms. At present, stygobiotic Cladocera appear largely restricted to Europe (Fig. 3). Various species have been found in freshwater-wells and caves in India, China and Africa (Dumont, pers. obs., Ranga Reddy, pers. obs.), but invariably a possibility of surface-water mixing was present, and/or the species were common lacustrine taxa, except for *A. elegans* which was abundant in guelta Zemmour, a semi-permanent lakelet (drying out on average every 5–10 years) in Rio de Oro, Western Sahara. This “guelta” intersects with the groundwater, like many other localities (mainly man-made wells) in Algeria and Tunisia where *A. elegans* was collected (Dumont 1979). In central Spain and Italy (Sardinia and Trieste Karst), *A. elegans* has been reported from temporary waters (Margaritora 1985, Alonso 1996) that may connect to the groundwater table. In South Australia, cladocerans were collected by one of us (HJD) in December 1982, in river gravel samples (*Alona* cf. *gut-tata*, *Neothrix* sp.), but these specimens are no longer available for study. There are no data on cladocerans from North and South American groundwater. In Europe, in contrast, subterranean Cladocera were abundant in collections performed in 2002 and 2003, during an EU project (PASCALIS project 2004) on the groundwater fauna of the alluvial plain of rivers in Belgium (the Walloon Karst) and France (basins of Rhône and Loire; vicinity of Lyon). Subterranean Cladocera in Europe were previously known from few locations in the groundwater of a small river (Couze Pavin) in the French Massif Central (Dumont 1983) and basin of the river Ter (in Gerona Province, Spain) (Sabater 1987, Alonso 1996). Nowadays, *A. phreatica* is on record for all the above-mentioned countries, in more than 30 locations. Strict groundwater populations of *A. protzi* were also found at one location in Belgium and 12 localities near Lyon (France). *A. bessei* was collected only once, in the basin of the river Rhône. *A. elegans* was recorded in the porous aquifer in Belgium (one location and one specimen in the Walloon karst), France (two locations in the Roussillon area, with 30 specimens, and 8 locations in the Jura region, with about 300 specimens) and in Slovenia (one location and one specimen in the psammolittoral zone of an Alpine lake). All collecting sites are gravel beds, at depths >50 cm. All remaining stygobiotic Cladocera listed in Table 1 are

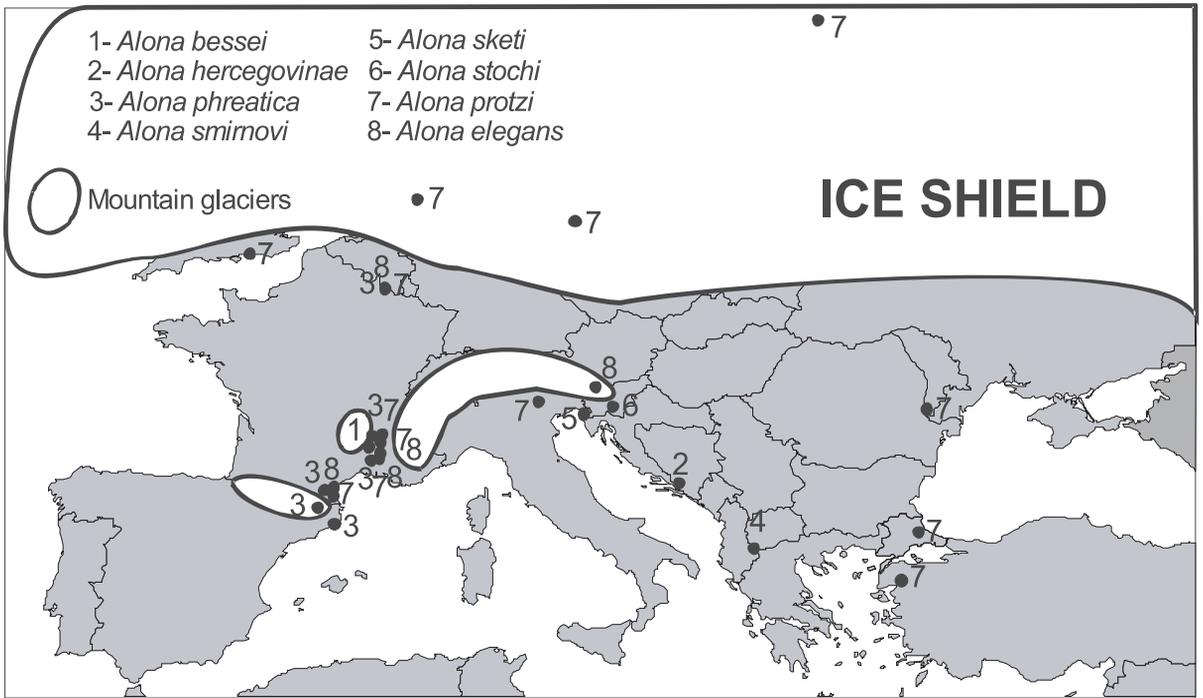


Fig. 3. Distribution of known localities of stygobiotic Cladocera (incl. stygophilic *A. elegans*) in Europe, and maximum extent of ice during the Pleistocene.

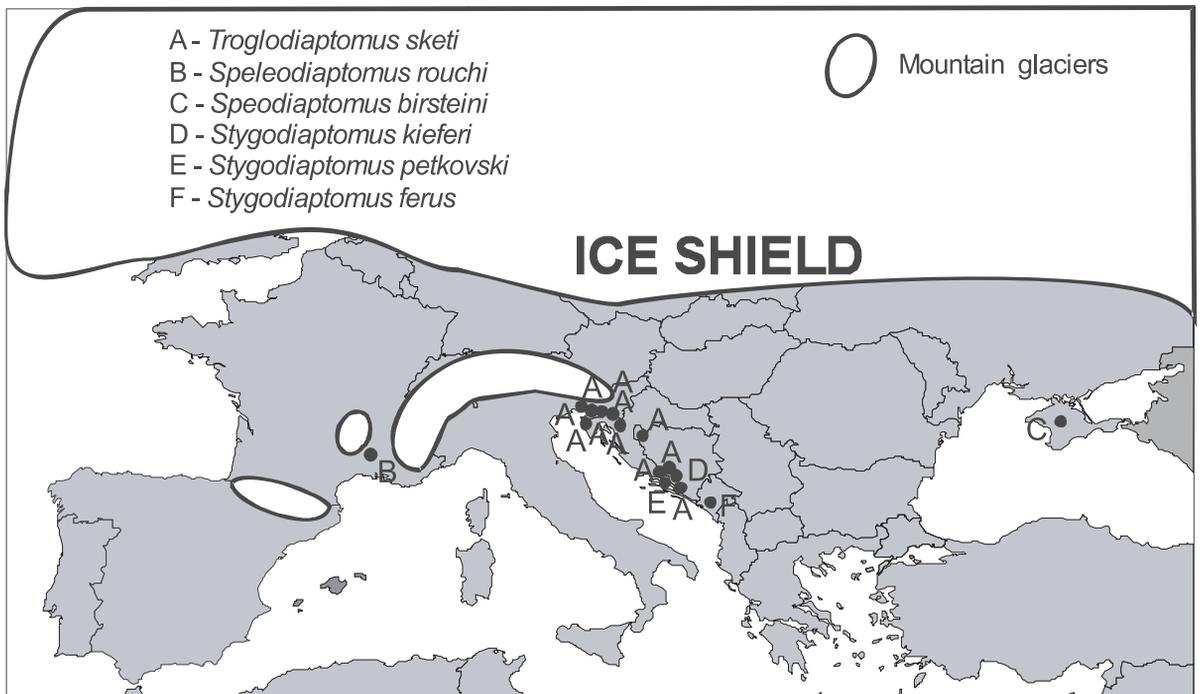


Fig. 4. Distribution of known localities of stygobiotic Calanoida in Europe, and maximum extent of ice during the Pleistocene.

restricted to the Dinaric area and to Macedonia (Lake Ohrid). Most of them are known from a single locality (cave or spring), except *A. hercegovinae*, which

occurs in three caves few kilometres apart in Hercegovina (north of Popovo Polje). The distribution of stygobiotic Cladocera in porous aquifers in Europe,

as currently known, ranges between north-eastern Spain (river Ter, Gerona Province) (latitude 42° N), the upper Rhône and Loire (45° N – 46° N), and southern Belgium (the Walloon Karst – 50° N). Few specimens of *Alona protzi* were collected in seven locations north and east of this core range (Fig. 3). The currently known distribution of populations of *A. elegans* in porous aquifers in central Europe is the same as *A. protzi*, with an additional location in Slovenia. Another narrow area where stygobiotic Cladocera were collected in karstic aquifers (caves and springs) runs in northwest – southeast direction along the Dinarids and east to Lake Ohrid (latitude 45.5° N–41° N).

In contrast to Cladocera, Calanoida extend from Mexico in the west to South China in the east. *M. cookeri* has been found in caves along the Sierra de El Abra in Mexico (latitude 23° N), *A. cavernicolax* in Kwantung Province (South China) and *H. dumonti* in North Vietnam, Asia (latitude 20° N – 22° N). The remaining stygobiotic Calanoida are restricted to southern and south-eastern Europe: from southern France (Department Gard) over the Dinaric region to the Crimean Peninsula, in a narrow area between 42.5° N and 45° N (Fig. 4). All locations with stygobiotic Calanoida are in karstic aquifers.

The ratio between sampling effort and sampling success can be used to evaluate the validity of these geographically restricted (Cladocera) or less restricted (Calanoida) ranges. So far, there have been few systematic sampling campaigns, but in the PAS-CALIS project, where about 300 alluvial gravel beds in six regions (one each in Spain, Belgium, Italy and Slovenia, and two in France) were sampled using a standard methodology. About 10 % of these were positive for Cladocera from porous aquifers, but they were strongly geographically localized (one region in Belgium and two in France). For stygobiotic Calanoida the ratio was even smaller: only one location in Slovenia tested positive. In Western Australia, two regions have been systematically sampled (Murchison and Pilbara) (Eberhard & Halse 2004). Several tens of locations have been surveyed but so far without positive results for stygobiotic Calanoida or Cladocera (Humpreys et al. 2004).

Discussion

Stygobiotic Cladocera and Calanoida represent two contrasting ecological groups. Subterranean Cladocera are *Alona*-like, of littoral-benthic origin, small to

extremely small (size range 0.2–0.5 mm), with laterally flattened body. Members of epigeal as well as stygobiotic taxa live in close contact with the substratum from which they brush small food particles. Many epigeal taxa move among stones, pebbles and coarse sand, but most of them penetrate deeper layers only occasionally. According to their size, mode of locomotion and feeding, several groups (viz. Aloninae and Chydorinae among the Chydoridae, Macrothricidae, Neothricidae, and Ilyocryptidae), are potential colonizers of the subterranean environment. Most of these, except few Macrothricidae and Neothricidae, have a globular body shape, and they failed to colonize the subterranean environment. As far as known today, only the laterally compressed Aloninae successfully invaded the porous as well as karstic aquifers, albeit with only a few taxa. Inhabitants of karstic aquifers exhibit stronger stygobiotic adaptations and are considered older lineages than those of porous aquifers. But if the spherical shape is what prevented the Chydorinae and other families from colonizing alluvium, there is no reason why they should not have become established in karst, where space is not restricted. The reason for their absence in this environment is a major unanswered question.

Calanoida differ from Cladocera in several respects. All Calanoida are planktonic, and stygobiotic members of the group maintain that same way of life, making them unique among stygobionts. Stygobiotic Calanoida are so far known only from karstic aquifers (caves), while Cladocera are known from porous and karstic aquifers. Stygobiotic Calanoida are scattered over the globe, while Cladocera appear mainly restricted to Europe. Calanoida retain a sex ratio 1:1 in groundwater while in Cladocera males are rare in porous aquifers and (completely?) absent in karstic aquifers. Differences are especially contrasting as both groups are relatively close in their taxonomic position. Nevertheless, they share some common characteristics (see chapter on adaptations).

The habitats occupied by stygobiotic Cladocera and Calanoida

Aloninae living in the subterranean environment fall into two groups. The first group (group I), present in Central and Western Europe, Asia Minor, North Africa, the Arabian Peninsula, and probably elsewhere, inhabits gravel beds or alluvial plains (Dumont & Negrea 1996). The second group (group II), seems restricted to south-eastern Europe, living in karstic systems.

The PASCALIS project showed that representatives of group I live in gravel beds between 0.5 and 1 m depth (hyporheic and top-most phreatic zone). Here, the physical and chemical characteristics of the water slightly differ from those of surface water. Most values (pH, conductivity, O₂ saturation, temperature) are within 20 % of the values measured at the surface (data from the PASCALIS project 2004). Differences depend on the porosity of gravel beds and consequently on the intensity of water exchange between the surface and deeper layers of the stream beds (Boulton et al. 1998). Some rivers harbouring groundwater Cladocera are small, often representing the headwater sections of rivers (altitude about 1000 m); with average annual discharge around 1 m³ s⁻¹. In contrast, some collecting sites are located in lowlands (River Ter; alt. 100 m; Dumont & Negrea 1996) where physical and chemical conditions of the hyporheic zone differ from the headwater zone. There is no information on water quality from the Arabian location (for *K. alsafadii*), except that it was a fast-running, clear-water stream, with a well-developed cyprinid fish fauna.

Group II (4 species) is restricted to saturated/unsaturated karst. *A. smirnovi* from Lake Ohrid was found around a spring, and specimens had likely been washed out of it. With a modal body length of c. 0.3 mm, this is one of the smallest representatives of *Alona*, suggesting it lives in the porous aquifer (see below). If this is the case, group II would be reduced to three related, strictly karst-dwelling species: *A. hercegovinae*, *A. stochi* and *A. sketi* (Brancelj 1990, 1992, 1997). These species live in small pools in unsaturated karst zone, which are reached by the water table during floods. After each lowering of the water table, specimens are trapped in these pools, together with stygobiotic Copepoda, Isopoda, Amphipoda, and *Proteus*. In two caves in Slovenia (Osapska Jama and Kompoljska Jama), *Alona sketi* and *A. stochi* were only collected after floods when the unsaturated zone was filled with groundwater. This is a strong indication of the phreatic origin of these Cladocera. In addition, they were never collected from dripping water in the epikarst (vadose) zone (Brancelj, pers. obs.).

In caves where stygobiotic Cladocera were collected (*Alona hercegovinae*: Ljelješnica, Obod = Obodska Pečina, Sušica; *A. sketi*: Osapska Jama) there was little organic debris in the pools or syphons (Brancelj 1990, 1992). After heavy snow melts or rain all these caves function as temporary springs. The walls of the galleries are usually polished by water expelled from the caves. In caves Ljelješnica,

Obodska Pečina, Osapska Jama and Kompoljska Jama, specimens of stygobiotic *Alona* (i.e. *A. hercegovinae*, *A. sketi* and *A. stochi*) were always accompanied by the stygobiotic calanoid *T. sketi*.

Stygobiotic Calanoida are swimmers, restricted to karstic aquifer with stagnant water pools, syphons, lakes or pools inside caves, subterranean rivers, or springs. The habitat most commonly occupied by stygobiotic Calanoida is the top-most saturated and unsaturated zone in karst. This is related to their feeding behaviour. *T. sketi* was found for the first time in a layer of freshwater in an abyss (a vertical cave) near Rovinj (Istria, Croatia). The abyss is about 10 m deep, several tens of meters from the sea shore. During heavy rain, the water level rises about 3–4 m. The upper layer is used as drinking water; below, salinity increases to 16–28 ‰ at the bottom. During sampling, water temperature was 13 °C (Petkovski 1978). All other locations with *T. sketi* contain only freshwater, as is the case for all other stygobiotic Calanoida elsewhere in the world. Little additional information on water quality exists for the caves inhabited by other stygobiotic Calanoida. Elías-Gutiérrez & Suárez-Morales (1998) reported temperatures in the caves in Mexico as high as 26–27 °C, an oxygen concentration as low as 1.2 mg l⁻¹, a pH range 6.88–8.00 and conductivity 700 µS. Experience from Slovenia, Herzegovina and Vietnam suggests that Calanoida inhabit subterranean waters with suspended organic material, or clay deposits rich in organic material, or bacterial films. Suspended organic material is indicator of groundwater connection with sinking rivers.

Origin of stygobiotic Cladocera and Calanoida

The relatives of stygobiotic Cladocera and Calanoida live in surface fresh water. All confirmed stygobiotic Cladocera are small-sized members of a single subfamily of the suborder Radopoda – the Aloninae. All taxa of stygobiotic Calanoida are small-sized members of sub-families Diaptominae, Speodiaptominae, and Microdiaptominae (Dussart & Defaye 2001) (for details see Brancelj 2005). Stygobiotic Cladocera and Calanoida may have “entered” the subterranean environment on several occasions and in different ways but probably following similar ecological pressures. Their “occupation” of the subterranean environment can be explained following the model of Stoch (2004), by direct multiple colonization followed by speciation. Obviously, karst-dwellers cannot be older than the karst and caves themselves, and therefore,

these taxa must have evolved after the Alpine Orogenesis, in the late Eocene or Miocene. Beside the physical constraint (the very existence of karstic aquifer), other pressures may have helped colonization. Glaciation during the Pleistocene certainly provided a pressure to colonize the subterranean environment in areas that were either ice-covered or adjacent to such areas. Indeed, all locations with Cladocera in porous aquifer in Europe are either outside or close to the former boundary of Pleistocene glaciers (Fig. 3). However, the taxonomic relations between and among different taxa within Calanoida and Cladocera also supports the hypothesis of an older, pre-Pleistocene colonization of subterranean realm. Indeed, some karst-dwelling calanoids have evolved to subfamily status, and two groups of groundwater cladocerans deserve to be removed from *Alona* s. s. as separate new Aloninae genera. Since cladocerans speciate more slowly than calanoids (Dumont & Negrea 2002) the subterranean representatives of both groups may be of a similar, Cenozoic age. This hypothesis is supported by affinity of the exclusively karstic group to the *Alona*-like taxa (*A. diaphana*) with subtropical-tropical range. As noted earlier, cave formation also dates back to mid-Cenozoic times.

All these explanations suggest that the groundwater fauna of Europe began evolving from surface-dwelling taxa under the warm climate that preceded the Miocene cooling. By the end of the Pliocene, this cooling may have eliminated many surface dwellers, while well-sheltered subterranean forms survived. Indirect evidence for this view is the fact that Radopoda are not the only group to show this phenomenon (see earlier, Bathynellacea) and the few species that survived the cooling of the epigeal habitats are found in persistent ancient lakes, like Lake Baikal. Rather than being mutually exclusive, the Pleistocene and the Miocene-origin hypotheses may represent a continuous process that persists to date, since colonization of groundwater may have been favoured by different pressures at different times (cooling, drying, predation...). Species of Cladocera from porous aquifers have only few morphological adaptations characteristic of strict stygobionts (e. g. absence of eyes and ocelli), and may still occur in ground- as well as in epigeal water (like *A. protzi* or *A. elegans*), which is the reason why we rated these species as younger than the karst-group.

West-Mediterranean *A. elegans* may have spent the glacial period in porous groundwater. Like in *K. alsafadii*, its mechanism of colonization may have been an adaptation to the risk of drought, due to the

evaporation of surface water under a regime of high temperatures, and to the seasonality of rainfall. Because some water is likely to remain as porous groundwater in depressions filled with sand above an impermeable layer, animals might have moved there to survive. If this hypothesis is correct, not yet discovered species should be expected to exist in the semi-arid zones of any continent. As stated earlier, there are indications that such faunas exist in Australia, and perhaps in Africa. One of us (HJD) explored the waters in the Sahara and Sahel in the 1970s, and found *Ceriodaphnia quadrangula* in damp sand at the bottom of a recently evaporated desert lakelets in the Ahaggar Mountains (Guelta Imeleoulaouene, May 1980), together with dragonfly larvae and desert toads. Since *Ceriodaphnia* is a large, globular species, not adapted to life underground, this strategy may have offered only temporary survival.

The ancestors of karst-dwelling Cladocera and Calanoida probably entered the caves as benthic dwellers and planktonic swimmers through sinkholes around the Miocene, when humid, subtropical climate extended across Europe. The glacial period severed their connection with their epigeal relatives; and they had probably become stygobionts before the Pleistocene cooling, and continued evolving afterwards. Cladocera and Calanoida from karst aquifers had enough time to evolve all the attributes of typical stygobionts. In calanoids, the highest degree of troglomorphic adaptations, like blindness, oligomerisation, and loss of geniculation of the male right antenna is found in *T. sketi*, *S. birsteini*, *M. cookeri* and *H. dumonti* (sub-families Speodiaptominae and Microdiaptominae) which we consider the oldest subterranean among Calanoida. These species are scattered over the globe (Mexico, Europe, and Vietnam). *T. sketi* and *M. cookeri* might have a common ancestor (Elías-Gutiérrez & Suárez-Morales 1998) whereas *H. dumonti* belongs to a group that invaded the subterranean environment independently. The European genera *Spelaeodiaptomus*, *Stygodiaptomus* and the Chinese *Argyrodaptomus* represent a younger group (no oligomerisation of legs; only slight modification of A1 in *Stygodiaptomus* and *Spelaeodiaptomus*) that probably became trapped in karst aquifers (i. e. caves) around the onset of the glaciations. Why the geniculation of the A1, a character believed to relate to mating, should have regressed with time is uncertain. Perhaps in the simple pelagic community of caves, the cost of maintaining a modified antennal structure was superfluous because in such a community there is no risk for calanoids to misidentify a mating part-

ner. It follows, those old as well as young lineages of groundwater calanoids should be exclusive to caves, as long as there is enough space for them to swim. They should be distinguishable age-wise by their degree of morphological specialization. Any cave in the world in contact with phreatic waters, regardless of local climate, is therefore a candidate for housing more karst-dwelling calanoids; in theory, the same is true for karst-dwelling cladocerans. One of the main enigmas of karst cladocerans at this time is why not more of them have been found outside Europe, even if Europe has a much longer tradition in speleobiological studies than the rest of the World.

Stygobiotic Cladocera and Calanoida are rare and their distribution is patchy: many collecting campaigns fail to produce a single specimen. Moreover, they are represented by a limited number of taxa. Both groups combined (13 taxa) represent about 0.01% of all known stygobionts in Europe (1600 taxa in Western Europe, and probably as many in SE and E Europe) (Botosaneanu 1986; PASCALIS project 2004). But even in Europe much remains to be discovered, and the PASCALIS project substantially increased the number of locations with stygobiotic Cladocera and Calanoida; appropriate sampling techniques revealed much richer faunas than expected. In fact, knowledge on geographical distribution of stygobiotic Cladocera in Europe increased dramatically when the Bou-Rouch pump became the standard collecting tool in alluvium instead of the Karaman-Chappuis method (Pospisil 1992). For example, before 2000, only about 10–15 specimens of *A. protzi* were known (Flößner 2000), but as soon as sampling by Bou-Rouch method was conducted on a wide scale in Europe, the species was found regularly in the interstitial zone at > 50 cm (PASCALIS project 2004). In karstic aquifers, Calanoida may also be underestimated by the type of plankton net. The most common nets have a mesh size > 150 µm, and do not retain Calanoida. Lakes and pools in caves should be in future sampled with nets of mesh size of 100 µm or smaller to collect more small-bodied representatives of stygobionts there.

Acknowledgements

Part of this study was supported by EU project PASCALIS (Protocols for the Assessment and Conservation of Aquatic Life in the Subsurface – EVK2-CT-2001-002121). The authors thank Dr. Kazimir Drašlar for taking SEM pictures the scrapers and Drs Kay van Damme for his comments on the scrapers of *Alona*. We also thank our colleagues Karanovic, Stoch and Ranga Reddy for sharing information on porous or karstic

waters, where stygobiotic Calanoida and Cladocera were collected and to Jim Green for linguistic improvements of the manuscript. Two anonymous reviewers provided numerous useful comments.

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Submitted: 20 January 2006; accepted: 15 June 2006.