Charles University in Prague, Faculty of Science, Department of Ecology
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Freshwater fishless pools: from the metacommunities to the systematics

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Ph.D. Thesis
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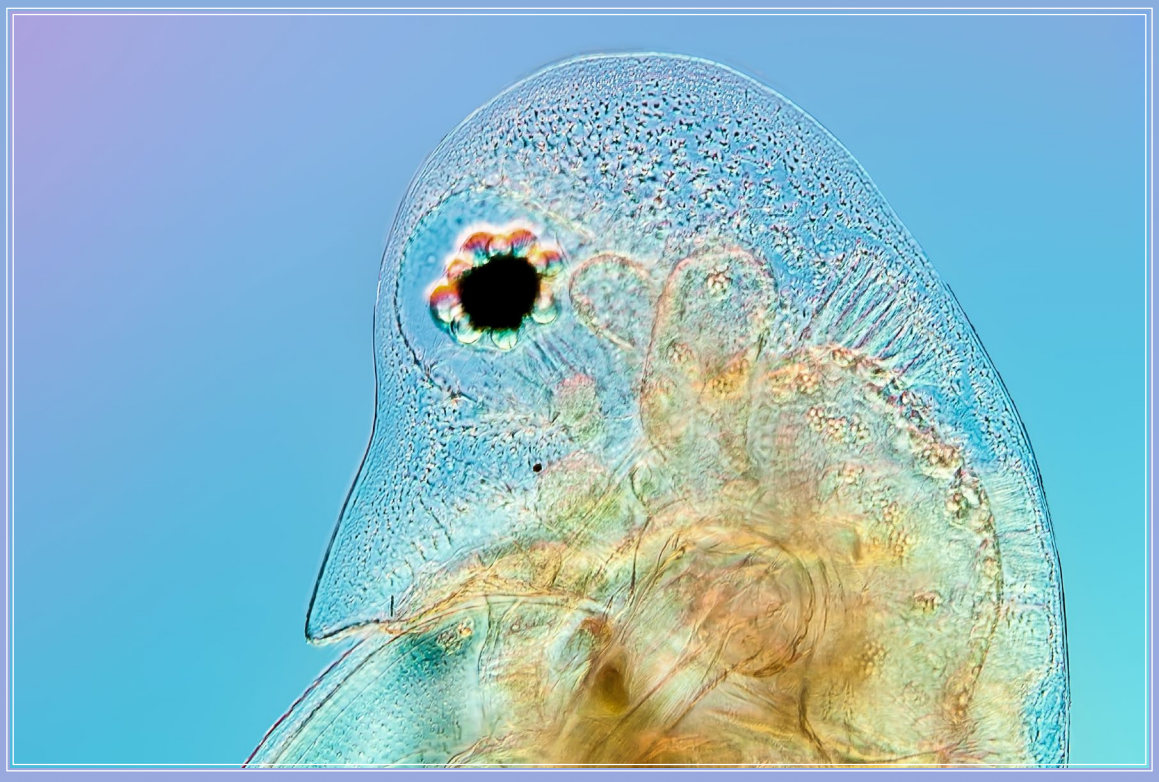

I declare that this thesis has not been submitted for the purpose of obtaining of the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts. All literature sources I used when writing this thesis have been properly cited.

In Prague, 5th April 2016

Petr Jan Juračka

Dedicated to prof. Vojtěch Jarošík (1958 - 2013),
founder of the Department of Ecology at the Faculty of Science,
Charles University in Prague.

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*Vojta gave me a very valuable advice with the statistical analyses,
especially at the beginning of this project.*

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Attached publications and manuscript

Part I. Metacommunity ecology of the invertebrates in newly created freshwater pools

1. **Juračka P.J.**, Declerck S.A.J., Vondrák D., Beran L., Černý M., Petrusek A. (2016): A naturally heterogeneous landscape can effectively slow down the dispersal of aquatic microcrustaceans. *Oecologia* **180(3)**: 785-796.
2. **Juračka P.J.**, Dobiáš J., Boukal D., Šorf M., Beran L., Černý M., Petrusek A. (*unpublished manuscript*): Space matters in species composition of passively as well as actively dispersing freshwater invertebrates in a heterogeneous landscape.

Part II. Systematics, ecology and morphology of *Daphnia*

3. **Juračka P.J.**, Kořínek V., Petrusek A. (2010): A new Central European species of the *Daphnia curvirostris* complex, *Daphnia hrbaceki* sp. nov. (Cladocera, Anomopoda, Daphniidae). *Zootaxa* **2718**, 1-22.
4. **Juračka P.J.**, Laforsch C., Petrusek A. (2011): Neckteeth formation in two species of the *Daphnia curvirostris* complex (Crustacea: Cladocera). *Journal of Limnology* **70**, 359-368.
5. **Juračka P.J.**, Sacherová V., Dobiášovská I., Bovšková D., Novosadová Z., Kořínek V., Petrusek A. (2016): Simplification of preparation techniques for scanning electron microscopy of Cladocera: preparing filtering limbs and ephippia for efficient studies of ultrastructure. *Crustaceana* **89**, 47-62.

ABSTRAKT (in Czech)

Sladkovodní tůně bez ryb mohou významně přispívat ke zvyšování regionálního druhového bohatství bezobratlých živočichů i cévnatých rostlin, neboť mnohdy i přes jejich spíše drobnou velikost obsahují značně složitá společenstva. Podstatná část této dizertační práce pramení právě z takových tůní, které byly pro účely ochrany přírody nově vytvořeny v rámci Chráněné krajinné oblasti Kokořínsko, známé svými hlubokými údolími a pískovcovými skalami. V kraji je rovněž velmi málo tekoucích vod či větších vodních ploch, následně je zde tedy i poměrně málo vodního ptactva, jež zásadně přispívá k šíření vodních organismů. V rámci této práce jsme se zaměřili na metaspolečenstva drobných korýšů vybraných 42 tůní z oblasti zhruba 220 km^2. Pomocí dekompozice variability druhového složení, analýzy druhového bohatství a kolonizačního pokusu přímo ve studované oblasti jsme zjistili, že prostorové uspořádání a množství okolních dalších vodních habitatů hrají hlavní roli v uspořádávání jednotlivých společenstev korýšů. To nás vedlo k závěru, že zdejší krajinná rozmanitost funguje jako omezení pro šíření korýšů mezi tůněmi. Následně jsme se rozhodli porovnat tyto výsledky s jinými bezobratlými živočichy s rozličnými schopnostmi šíření, od pasivně se šířících vířníků a vodních měkkýšů, po místně hojné skupiny vodního hmyzy (ploštice, brouky a vážky). U všech pasivních skupin a vodních brouků jsme vysvětlili výrazně více variability druhového složení pomocí prostorových charakteristik, než pomocí místních podmínek prostředí. Nicméně, velkou část variability druhového složení ploštic i vážek jsme vysvětlili skrze podmínky prostředí, které byly samy prostorově uspořádány. Na základě těchto výsledků usuzujeme, že strmé srázy zpomalují šíření nejen pasivně se šířících bezobratlých (které mezi tůněmi přenáší zejména vodní ptactvo a velcí savci), ale také pro aktivně létající hmyz, který za letu pravděpodobně kopíruje tvar terénu. Drobné a odlehlé tůně zvyšují druhovou rozmanitost také jako refugia pro vzácné druhy, které by jinak mohly být vytlačeny z větších či více dostupných lokalit konkurenčně úspěšnějšími druhy. Takovým případem je možná i perloočka *Daphnia hrbaceki* Juračka, Kořínek & Petrusek, 2010, kterou jsme přímo ze sledovaných tůní popsali jako nový druh pro vědu. Tyto hrotnatky byly snadno rozpoznatelné díky specifickému prohnutí karapaxu („hrbu“) a zachovanému týlnímu zoubku u dospělců. Takto charakteristické morfotypy však známe pouze z přírody a jen z velmi limitovaného množství vzorků. V laboratorních chovech *D. hrbaceki* tyto rysy ztrácí a je vzhledově takřka totožná s nejblíže příbuzným druhem *D. curvirostris*. Pokusili jsme se tedy navodit tvorbu hrbatého fenotypu v rámci experimentu s kairomony, látkami signalizujícími přítomnost predátora ve vodě. Přestože tento pokus nevyšel, podařilo se nám zdokumentovat značnou meziklonální variabilitu v tvorbě týlních zoubků u obou druhů v pokusu, jak *D. hrbaceki*, tak i *D. curvirostris*. Spolehlivé odlišení těchto dvou druhů vyžadovalo rozsáhlou morfologickou analýzu bohatého materiálu pomocí rastrovacího elektronového mikroskopu (SEM). V průběhu nejen této práce jsme tak postupně zjednodušili pracovní postup přípravy vzorků perlooček pro SEM, zejména jejich filtračních končetin a schránek trvalých vajíček, efipií. Námi optimalizovaný postup umožňuje velmi rychlou a hlavně spolehlivou přípravu těchto struktur, které nesou taxonomicky hodnotné znaky. Doufáme, že proto bude přínosný v dalších projektech (jak našich, tak zahraničních kolegů) zaměřených na funkční morfologii a systematiku perlooček.

ABSTRACT

Despite their small size, freshwater fishless pools often contain complex communities and substantially increase regional invertebrate and macrophyte biodiversity. The main core of this thesis originates from such habitats, which were newly created for the conservation purposes in the Protected Landscape Area Kokořínsko, Czech Republic. This landscape consists of deep valleys separated by steep sandstone ridges and is characteristic for very sparse stream network and low number of large water habitats, which consequents in generally low abundance of waterfowl. We studied microcrustacean metacommunities of 42 selected pools scattered over the area of approximately 220 km^2. Using variation partitioning of the species composition, analyses of the species richness and colonization experiment in the study area, we identified that spatial distribution of the habitats and number of neighbouring aquatic habitats play a major role in assembly of local communities. This led us to the conclusion that the landscape heterogeneity served as a partial barrier to dispersal of microcrustaceans. Subsequently, we compared this pattern of the microcrustacean metacommunity with other invertebrates of various dispersal modes, from passively dispersing rotifers and aquatic molluscs, to locally common and actively flying insects (true bugs, aquatic beetles and dragonflies). Substantially more variation in species composition variability was explained by the spatial structure than by local conditions in all passively dispersing groups and in aquatic beetles. However, shared fraction of spatial and local variables explained a major part of variation of species composition in dragonflies and true bugs. Therefore, we hypothesize that steep ridges serve as dispersal barriers not only for passive dispersers (whose vectors are waterfowl and large mammals), but also for actively flying insects, which probably follow the local topography in flight. Small and remote habitats may increase a regional diversity also as refugia of rare species, which could be outcompeted in larger or more connected habitats. This might be the case for *Daphnia hrbaceki* Juračka, Kořínek & Petrusek, 2010, a species which we described from our study area. It was very conspicuous by the humped shape of dorsal margin of the carapace, and by retaining neckteeth in adults. However, these specific morphotypes are known only from very limited number of field-collected samples. In laboratory cultures, *D. hrbaceki* loses its specific shape and resemble its closest relative, *D. curvirostris*. We attempted to induce the humped morphotypes experimentally by exposure to predator kairomones. This was not successful but we observed high interclonal variability in formation of neckteeth in both *D. hrbaceki* and *D. curvirostris*. When looking for stable morphological characteristics allowing reliable differentiation of these two species, a large number of specimens were analysed in detail by the Scanning Electron Microscopy (SEM). During this work (and in other projects), we simplified methods for preparation cladocerans, particularly their ephippia and trunk limbs, for SEM analyses. Our workflow allows safe and quick preparation of these body parts that may carry taxonomically valuable structures, and we hope it will be useful also in future projects on cladoceran functional morphology or systematics.

PREFACE

For me, the water means alpha and omega of basically everything. Without the water there would not be a life on our planet, as well as there would not be a topic of my thesis. I have been attracted by the freshwater since I was very young. The first real touch I remember was when I was three years old and fell to a fishpond from a small boat. As I was too young to swim, I was sitting on the bottom and waiting for the rescue, which fortunately came - my father found me and got me back to the air. The second touch came when I was five - being allowed to go alone further from our home. The most interesting thing all around was a small smelly stream Ředička, about three hundred meters from the house. Since then, I have been catching various invertebrates and keeping them in my bedroom till my high school. I loved to watch whirligig beetles on my table every evening, they were soooo nice! So nice I got stuck with the water until today! Although I switched for reptiles for some years, in 2001 I met Adam Petrusek at the summer camp for young biologists (Arachne). I was 16 and he was the first real hydrobiologist I had a chance to talk with. I realized that he is an interesting guy and decided to visit him during my first study year at the Faculty of Science, Charles University, in 2004. I remember that we talked about the possibility of cooperation and my only wish was to avoid any work with the *Daphnia*, which I considered to be boring. That is maybe why we described a new species of this incredible genus in 2010! I believe that this thesis educated me a lot. I hope I have learned to critically read scientific papers, to plan and perform a research, to calculate appropriate statistical analyses, and I certainly managed to publish my results. Although I have no clue how much time I will spend on the science communication and how much on the basic research in the coming years, I am grateful for all of that and I hope to use these skills every day.

Supervisor of this thesis, prof. Adam Petrusek,
sampling ephippia of a newly described cladoceran species
Daphnia hrbaceki Juračka, Kořínek, Petrusek, 2010 at its type locality.
(Nosálov, Czech Republic, 19 July 2010)

ACKNOWLEDGEMENTS

There is a long queue of incredible people, who made this thesis possible. For sure, I would not be able to see the queue end today, even if the sky is clear, because it is just too long. That is why I would like to thank here only those staying at the front position, and to thank all the others when meeting them in the real life.

First of all, I would like to thank myself for choosing a proper supervisor. Without Adam I would certainly never finish this thesis in such extent. Without him I would write even worse than I do right now, but at the same time I would think it is not too bad. He invested a substantial part of his life to giving me the feedback I really needed. Well, time to time one would like to hear more positive voice… but this would probably be wrong. Hence, I consider him to be the right choice and I am happy we spent more than ten years working together!

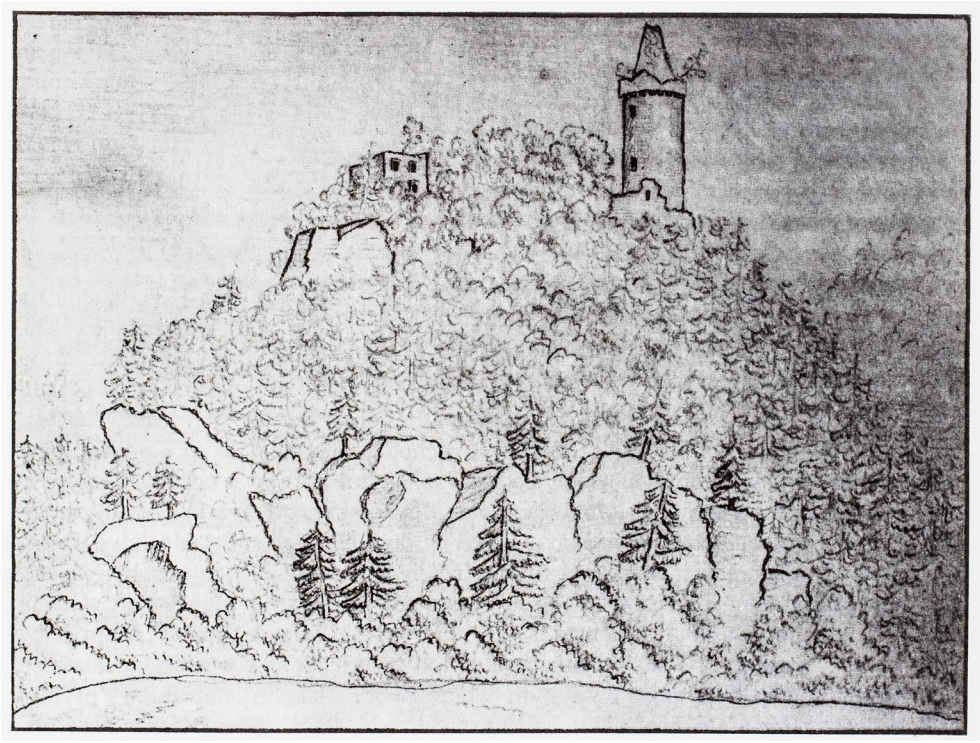
The second person in the queue I see is my wife Zuzka. During my work on this thesis she split herself twice, so now I thank also to our two replicates – Majda and Venda. I would like to thank them both for every hour they slept and made me able to write, and every hour they were awake and made me smile. Without all three of them I would not be satisfied in my life and had much less motivation to write. I would like to thank my parents as well, as they supported me since the childhood till today, without asking me what the things I do all the time are good for. They believed that all of it has some sense. Thanks to their hard work with me I did not lose my vision as predicted by the doctors, but conversely, I turned to the microscopes!

Most of the work presented here I did at the Department of Ecology. This is not the department only. It is the professional team as well as pretty fine gang of friends supporting each other with the great ideas and help. Without Jan Fott, Vladimír Kořínek or Veronika Sacherová I would never be able to determine the microcrustaceans in the right way. Without Lenka Krejčiříková and twice Jana Vokurková I would not know when the coffee is ready, when and where I have to sign something, or where I should be. I would like to thank also Radka Sychrová from the Department of Zoology, who introduced me into the world of ostracods and their identification. However, research described in the fourth chapter of my thesis was not performed in the Prague. I did our ecological experiments at Ludwig-Maximilians-Universität München under the supervision of Christian Laforsch. He taught me a lot not only about *Daphnia* and ecological experiments, but also about time scheduling and survival in the country the language of I don't speak.

One of the most important persons playing a major role in this thesis is also Steven Declerck, the referee of my MSc. thesis in 2009. Since then, we decided to work together, and he became my guide in the world of ecological statistics. Beside Steven, I would like to thank all of my co-authors!

Finally, I have to thank Czech Railways and Railway Infrastructure Administration. Passing one hundred kilometres to the job and back every day was never boring with their service, and thanks to common delays on the rail I was able to type this thesis a bit more than usually planned.

This thesis contains results of the research funded by the EuroCORES/EuroDIVERSITY projects BIOPOOL and STRESSFLEA (supported through the Czech Science Foundation Projects No. DIV/06/E007 and EEF/10/E022), the Grant Agency of the Charles University (132510), the Czech Science Foundation (P506/10/P167) and the German Research Foundation – DFG (LA 2159/6-1).



"Ouzká pěšinka vede mezi rozsedlinami skal a hustým křovím od jedné chatrče k druhé až dolů do oudolí k ouzkému sice, avšak dlouhému a hlubokému jezeru, na jehožto modravé hladině vodní lilie rozkládají své široké temnozelené listí, a bledé jejich květy plynou co stříbrné korunky nad temným zrcadlem vod. Jestliže, jak mnozí učenci jistí, v pradávných časích celá země česká jen jediné veliké jezero byla: tedy jest toto malý pozůstatek z oné nesmírné vodní hladiny, a ploché, ouzké břehy jeho jsou s obou stran veliké stupně písčité skaliny, která, kdo ví jak daleko, se stápí v prohlubeň vodní; neb dle ujišťování tamějších obyvatelů ještě žádný nenalezl dna ve vodě této."

Karel Hynek Mácha, Cikáni (1857; obr. 1832).

"A narrow path leads from one hut to another among the crevices of the rocks, through the dense undergrowth, down into the valley, to a narrow, but long, deep, lake, upon whose bluish surface water-lilies extend their broad, dark green leaves, and their pale blossoms drift like silver coronets above the dark mirror of the waters. If, as many scholars aver, the entire Czech land was simply a single great lake in the most ancient times, then this is a tiny remnant of those immense waters, and its narrow, flat banks on either side are great steps in the sandy rock that extends, who knows how far, into the watery depths; for, as far as the local inhabitants know, no one has ever plumbed the depths of these waters."

Karel Hynek Mácha, Gypsies (1857; image 1832), translation by Geoffrey Chew.

Karel Hynek Mácha was an important Czech romantic writer and poet, well-known for his poems and paintings of Kokořínsko, where most of this thesis originated.

INTRODUCTION

Metacommunity ecology of the freshwater pools

Some 150 years ago, when Karel Hynek Mácha got inspirations for his romantic books and poems during his pilgrim journeys through Bohemia, European landscape looked quite different than today. Urbanisation, agriculture, development of traffic infrastructure and many other human activities resulted in a substantial change of various landscape characteristics. Among many other habitats, swamps, fishless pools and ponds, and other small freshwater habitats are changing (Oertli *et al.* 2009), or even disappearing (e.g., Strayer 2006; McCauley, Jenkins & Quintana-Ascencio 2013). Loss of such habitats is, however, not a question of aesthetics or landscape planning only. Small and isolated aquatic habitats play an irreplaceable role in keeping high local biodiversity (Oertli *et al.* 2004; Biggs *et al.* 2005; Boix *et al.* 2012). There are multiple mechanisms how pools and other small freshwater habitats might do all of that. Despite their small size, it is possible to find various microhabitats within them, which can make possible a coexistence of multiple species with various ecological niches (March & Bass 1995). Generally, smaller habitats are less stable than the large ones, which might lead to lower alpha biodiversity observed at one moment (Juračka *et al.* 2016a). However, within a longer time scale, such habitat dynamics might allow more various species able to settle than in bigger and more stable localities (Scheffer *et al.* 1993). Finally, smaller habitats have also lower probability to be discovered by large animals as mammals or birds, which serve as dispersal vectors for various other small invertebrates. Therefore, these habitats may stay relatively isolated and serve as refugia for some rare species, which would be outcompeted by more common and stronger competitors in larger and more connected localities (Scheffer *et al.* 2006).

Fishless freshwater pools stay as an excellent and popular model for ecology, evolutionary and conservation biology studies for decades (Oertli *et al.* 2004; De Meester *et al.* 2005). The reasons for such type of habitats are numerous. Small pools are well and easily defined in the landscape. They might be very common in not fully urbanized landscapes and whole water column can be usually easily sampled. Moreover, it is possible to manipulate with their environmental characteristics, or build mesocosms inside. However, many of those benefits are lost in the presence of fish. When the fish are introduced, habitats can quickly transform to very different turbid state (Zedler 2003) and many ecological processes will play a minor role or disappear with preyed species at all (Fairchild, Faulds & Matta 2000; Nolby *et al.* 2015). This is why most of the ecological studies on the pools focus on the fishless habitats only (see De Meester *et al.* 2005).

Small habitats should not be studied separately as isolated islands of suitable habitats in „dry ocean". The pools are not as isolated as it might look like; there is usually a certain exchange of specimens due to dispersal. Altogether, organisms inhabiting patchy habitats can be understood not as unique communities, but rather as one large „metacommunity" (Leibold *et al.* 2004); or „metapopulation" when speaking about one species (Figuerola & Green 2002; Céréghino *et al.* 2008). Metacommunity and metapopulation ecology experienced a large boom in the last decades. Four fundamental „metacommunity paradigms", i.e., the most common scenarios how the metacommunities are affected by environmental filtering, dispersal and habitat stability, were postulated and studied for large variety of organisms worldwide (Fig. 1): 1) Species sorting, when the community composition is strongly affected by local environmental conditions, is a paradigm found to be the most common in the freshwater environment at all. Cottenie (2005) analysed 158 previously published data sets and tried to compare the species composition variability explained by

the spatial and environmental factors separately. The most common scenario, i.e., the species sorting, was identified in 44% of cases; 2) Mass effect is an opposite scenario, as it supposes that the communities are structured mostly by frequent and massive dispersal, while the local conditions play a minor role. The species are found at localities where they just get in, regardless of the habitat type or species requirements. 3) The third metacommunity paradigm, patch dynamics, might be of particular importance under the conditions of high instability of the studied habitats. In the case of the freshwater pools, this might be a case for localities with very short hydroperiod, which do not allow all the species to finish the life cycle. 4) The last scenario, the neutral model suggested by Hubbell (2001), is more or less theoretical. In essence, it assumes that species are not filtered by the environmental conditions and that they can simultaneously disperse very fast. Observed species composition in the field therefore responds more to the species „random walk", than to abiotic or biotic characteristics. This paradigm, although not realistic in most natural conditions, is a suitable null hypothesis to compare with predictions of other paradigms mentioned above.

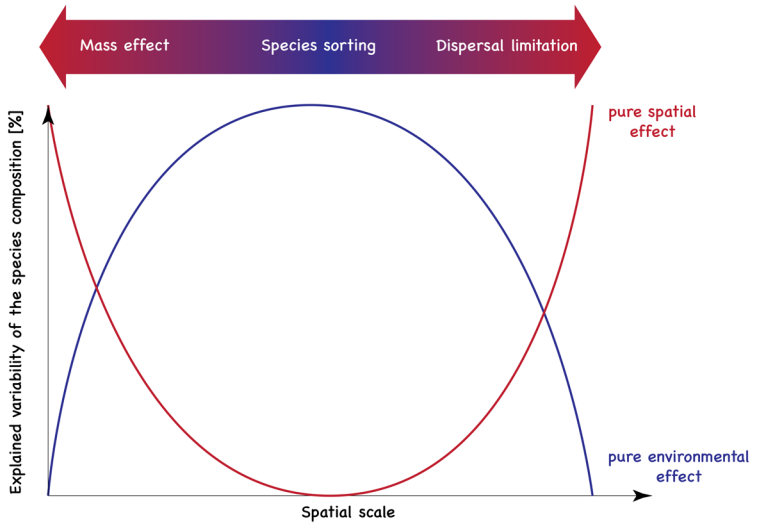


Fig. 1 Relationship between pure spatial and environmental effects under different dispersal rates. Adapted from Heino et al. (2015)

Numerous ecological studies were published with the focus on the pools and other small freshwater habitats last two decades. In the European context, probably the biggest projects in this field – and certainly most important for us – were BIOPOOL (2006-2009) under ESF Biodiversity programme, and MANSCAPE (2000-2005) followed by PONDSCAPE (2006-2011). All these projects covered very wide spectrum of the studies, from the field surveys (De Bie *et al.* 2012; e.g., Nédli *et al.* 2014) to large ecological experiments with mesocosms (e.g., Verreydt *et al.* 2012; Thielsch *et al.* 2015). Despite some delay, this thesis also originates from the BIOPOOL project. Elsewhere, freshwater pools serve as popular ecological model as well. Many important studies have been done for example in the United States (e.g., Jenkins & Buikema 1998; Shurin 2007) and Latin America (Declerck *et al.* 2011).

As mentioned above, a lot of the research on freshwater pools in the last decades has focused on the significance of the spatial structuring of the communities. Typically, with increasing spatial scale, spatial significance increases as well, as it is more difficult to disperse among more distant places (Declerck *et al.* 2011; Heino *et al.* 2015). However, within the freshwater habitats, environmental filtering is commonly observed to play a major role in structuring invertebrate communities (Cottenie 2005), and it might also interact with the spatial structure substantially (Cottenie 2005; Heino *et al.* 2015). However, simple comparisons of significance and explained variability of the species composition by local (i.e. environmental) and spatial variables is a bit out of the fashion today. Different and more complex questions are asked. How the community functions change at various spatial scales? And what are the real biological mechanisms behind the spatial metacommunity structuring? The first two chapters of this thesis follow this trend.

Invertebrate dispersal

„...A Ferda se ponořil, podplul jednu vodoměrku a tititi, zašimral ji na bříšku. „Íiiiiii!" Vypískla vodoměrka a uletěla..."

Obrázek a text (c) Ondřej Sekora, 1948

„... And Ferdy sank below the surface, passed under one water measurer and cootchie-cootchie-coo, tickled her belly. "Tee hee!", squeaked the water measurer and flied away…"

Image and text (c) Ondřej Sekora, 1948
Translation by Kateřina Matrasová, 2016

Considering all mentioned above, it is clear that effective dispersal is a key skill for various animals inhabiting freshwater pools and other small patchy habitats. Although there are numerous ways, how the invertebrates may disperse, there are two most important categories of dispersal: 1) passive (i.e., endo- & ectozoochory, via wind, water currents etc.); and 2) active (i.e., flying, walking or swimming). These aspects are reviewed in detail by Bilton et al. (2001), Stendera et al. (2012) and Incagnone et al. (2015). Although one could suggest that active flying or swimming should be the most effective way how to move from one habitat to another, the efficiency of passive dispersal attracts researchers for decades (McAtee 1917; Maguire 1959). Resting stages and eggs of the invertebrates (Fig. 2) are most commonly carried by the waterfowl (Charalambidou 2005; Green *et al.* 2013), as well as by various mammals as wild boar (Vanschoenwinkel *et al.* 2008c), Viable propagules can be carried not only on the vector surface but also in their gut (Charalambidou & Santamaría 2002; Waterkeyn *et al.* 2010a). Under specific conditions, amphibians might eventually carry invertebrate resting eggs as well, but such dispersal seems rather rare (Bohonak & Whiteman 1999; Vanschoenwinkel *et al.* 2008b). Under specific circumstances, invertebrate propagules can be spread with the wind (Brendonck & Riddoch 1999;

Vanschoenwinkel *et al.* 2008a), or floods (Fahd, Serrano & Toja 2000; Frisch & Threlkeld 2005). Recently, humans became also important vectors, including the researchers, fishermen or tourists (Waterkeyn *et al.* 2010b).

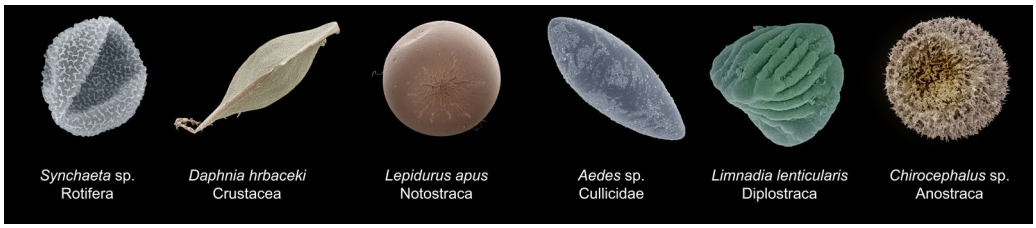


Fig. 2 Resting stages of invertebrates commonly serve as dispersal propagules among the freshwater pools. Coloured Scanning Electron Microscope (SEM) image, objects are not in scale.

Many species, especially small ones, as the protozoans or rotifers, were considered to have worldwide distribution for a long time, as populations without any noticeable morphological differences have been found on multiple continents (Finlay 2002). However, with increasing usage of the genetic tools it is more and more evident that that this might be actually an expectation rather than rule, as it is possible to reliably distinguish independent genetic lineages on the regional or continental scale (Adamowicz *et al.* 2009; Nédli *et al.* 2014; Vanormelingen *et al.* 2015). Within the freshwater pools, such cases might be found in diatoms (Van den Wyngaert *et al.* 2015), microcrustaceans (Hamrová *et al.* 2012; Adamczuk 2015), as well as rotifers (Xiang *et al.* 2011). This might lead to conclusions that the passive dispersal of such species is not as unlimited as previously assumed. However, evidence for dispersal limitations in freshwater invertebrates comes from the field observations rather rarely, most often from various extreme environments (Soininen *et al.* 2007), or from the studies based on large spatial scales (De Bie *et al.* 2012; Zhai *et al.* 2015).

Predation in freshwater pools

Both environmental (i.e., local) conditions and the spatial distribution of the habitats affect invertebrate metacommunities substantially. From the species point of view, however, inoculation to the suitable habitat positioned at the right place is not enough to found a new population. Despite their small size, freshwater pools often contain very complex communities (De Meester *et al.* 2005; Colburn, Weeks & Reed 2007) and various biological interactions play a major role in the final species composition. One of the key ecological relationships influencing local communities is the predation, having a substantial influence both on the composition and the species richness of the pools. Predators, however, may not manipulate with the presence and absence of its prey in the pools only. They can also induce substantial changes of life history as well as phenotypes of their prey. Various defences against the predation are commonly induced simply by the presence of the predator in water, as the prey can commonly "smell" it by identification of specific chemical agents (kairomones) associated with the specific predators. These chemicals are of variable chemical nature, and do not have to origin from the predator itself. For example, bacteria living on the fish surface apparently affect the way how cladocerans react to presence of the fish in the water (Beklioglu, Telli & Gozen 2006). In the last decades, a large attention has been paid on effects of kairomones on various prey species, cladocerans of the genus *Daphnia,* belonging among frequently chosen experimental models. Inducible antipredator defences in *Daphnia* might have various forms, including morphological (Mikulski, Lipowska & Pijanowska 2004; Petrusek *et al.* 2009), life history (Schwartz

1984; Vijverberg, Doksæter & van Donk 2006), as well as behavioural responses (Dodson 1988; Boersma, Spaak & De Meester 1998).

Among well-known morphological responses to the presence of an invertebrate predator are tiny structures on the dorsal part of *Daphnia* carapace called neckteeth (Tollrian 1993), being induced by phantom midges larvae (Diptera: *Chaoborus* sp.; Fig. 3). Despite their small size of several micrometres, they are well visible, especially in young specimen. An impact of such tiny structure against much larger predators has been discussed for a long time (Havel & Dodson 1984; Tollrian 1995). An acoustic microscopy study by Laforsch et al. (2004) revealed that neckteeth are just a proverbial "tip of the iceberg", while the most important defence against the predation stays in the carapace strengthening.

Fig. 3 Phantom midge larva (*Chaoborus* sp.) and its prey, *Daphnia*.

HISTORY OF THIS THESIS AND OUTLINE OF THE CHAPTERS

Kokořínsko Protected Landscape Area

In the Czech Republic, several projects focused on the restoration and creating new small freshwater pools. Among the large ones, project in the Ramsar Wetland Conservation Area Kokořínsko took our interest, as new numerous pools were restored or created there between the years 1996 and 2004, mostly for the purpose of enhancing biodiversity. These pools are distributed over a wide area of approximately 220 square kilometres and became soon inhabited by molluscs, amphibians and macrophytes, many of which are ranked as rare or vulnerable in Central Europe. We visited the area for the first time in May 2004 and immediately decided to pay attention to those pools, as we got interested how the pools get colonized. Following the tradition and focus of the hydrobiology group of the Department of Ecology, we focused first on microcrustaceans. Therefore, we collected samples from 42 selected localities scattered throughout the landscape, and tried to assess the most important factors influencing both species richness and species composition of the copepods and cladocerans, quite popular subjects in ecological studies of freshwater pools (e.g., Louette & De Meester 2005; Shurin, Cottenie & Hillebrand 2009; Frisch *et al.* 2012). However, copepods and cladocerans were not the only microcrustaceans sampled. We often observed also the

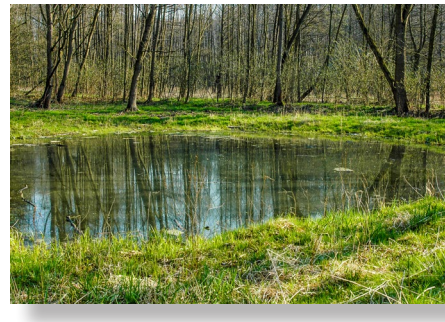
ostracods, which tend to be neglected by most of the researchers due to their relatively difficult identification (that usually requires dissection of the shell and individual limb analyses). As we presumed ostracods may potentially show different ecological characteristics than the copepods and cladocerans, I decided to analyse them as well. This was a big challenge for me, as that work needed very high precision and steady hand.

This research is summarized in the **chapter 1** of this thesis (Juračka *et al.* 2016a). The most important result is the finding how the heterogeneous landscape affects both species richness and composition of the microcrustaceans by substantially slowing down their dispersal. We identified this dispersal limitation through three independent lines of evidence: 1) number of potential source localities (i.e., habitats in the vicinity, specifically in the 3 km radius around each study site) stayed among the most important variables correlating with the microcrustacean species richness, as well as the species composition of the pools; 2) the "valley distance" (distance measured not as the bird flies but along the canyon axes) served as the strongest predictor of the species composition; and 3) we observed very slow colonization rates within the field experiment with newly excavated pools directly in the study area.

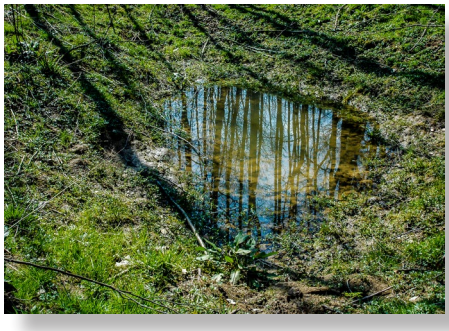
While we studied three different groups of the microcrustaceans within the first chapter, all these groups are of similar size, and all are passive disperses. However, importance of the body size and dispersion modes has been recently highlighted (Beisner & Peres-Neto 2009; De Bie *et al.* 2012), as even subtle differences in the dispersal ability might lead to substantial differences in the spatial structuring within one taxonomic group (Akdemir *et al.* 2016). Number of studies focusing on various invertebrate groups and their spatial structuring stays very limited until today. This is one of the reasons we subsequently processed data not only on microcrustaceans, but also on other common groups of invertebrates inhabiting studied pools. We compared both passive and active dispersers within the unpublished **chapter 2**, and found that not only passive dispersers, but also actively flying insects are distributed according to the landscape structure (Juračka et al., unpublished). Similar spatial structure of the passive and active dispersers might have a biological reason. The study area consist of very steep ridges and deep valleys, which likely influence the movement patterns of key vectors of passively dispersing invertebrates. We hypothesize that same factors that affect large mammals and waterfowl, which both avoid crossing too steep ridges, may also affect actively flying invertebrates.

Fig. 4 Larva (left) and adult (right) of the Great Diving Beetle (*Dytiscus marginalis*).

Pool 8 with high macrophyte cover in the autumn
(14th November 2008)

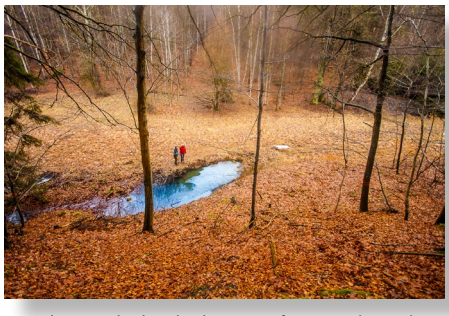


Pool 41 with low macrophyte cover in the spring
(3rd April 2007)

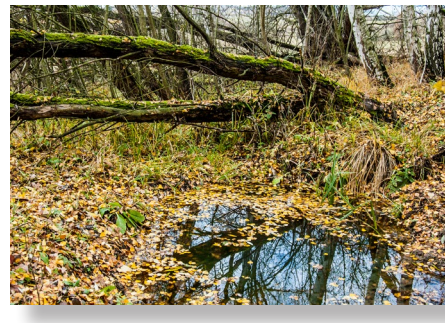


Pool 37, the smallest of the studied habitats
(3rd April 2007)

Pool 40, the largest of the studied habitats
(26th March 2008)



Pool 12 on the border between forest and meadow
(3rd March 2009)



Pool 34 shaded with the forest
(7th November 2007)

Fig. 5: Variability among freshwater pools studied within the first two chapters of this thesis was large. Examples are shown in this figure.

Daphnia hrbaceki and scanning electron microscopy

When analysing samples for the research summarized in the first chapter, I commonly observed predatory phantom midge larvae (*Chaoborus* sp.) and juvenile daphnids with the characteristic antipredator structures, the neckteeth. The neckteeth usually disappear before *Daphnia* reaches its maturity. This was not the case of adult parthenogenetic females we observed in one sample from Kokořínsko, whose neckteeth together with a very specific shape of the dorsal part of carapace resembled North American species *Daphnia minnehaha* (Fig. 6). The specimen was so weird that I was unsure about its identification, and we decided to characterize it by molecular barcoding, i.e., sequencing mitochondrial DNA markers that may allow its identification by comparison with other *Daphnia* species. Surprisingly, we found out that this is apparently a new, undescribed species. As the genus *Daphnia* belongs among the most studied model organisms at all, finding a new species so close to Prague was surprising.

However, while the "humped" specimens from the first samples were easy to differentiate from other daphnids, this was not the case for specimens cultivated in the lab or in other field samples taken later. The humped carapace as well as the neckteeth disappeared. Finding stable morphological differences allowing reliable identification of the new species took us five years. However, we finally described *Daphnia hrbaceki* within the **chapter 3**, using both genetic and morphological characteristics (Juračka, Kořínek & Petrusek 2010). The species is dedicated to a prominent Czech hydrobiologist, Jaroslav Hrbáček, who worked with *Daphnia* for most of his life. Furthermore, his name means in the Czech language "a small hunchback", which perfectly fits to the humped specimens of this *Daphnia*.

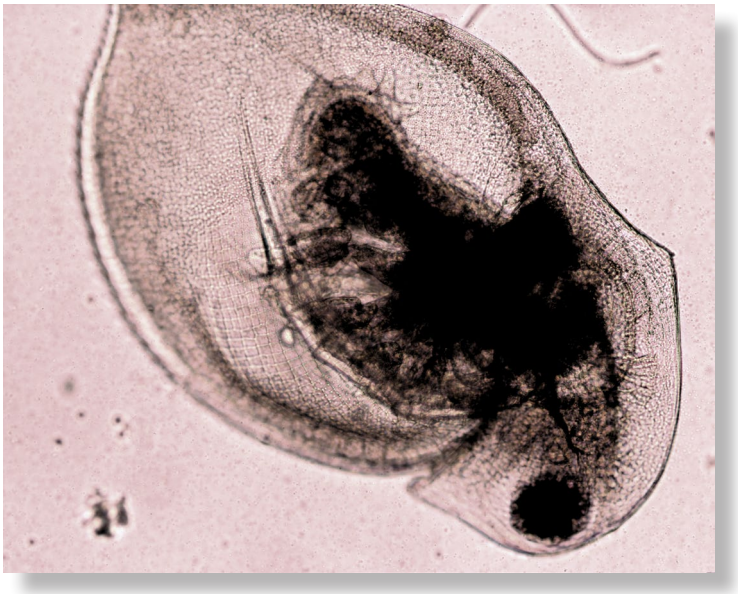


Fig. 6 The first observed specimen of *Daphnia hrbaceki* (November 2005) with the specific hump-shaped dorsal carapace and the neckteeth.

Since the first samples, we presumed the unusual body shape of *Daphnia hrbaceki* was an inducible defence against the invertebrate predators, locally abundant phantom midge larvae (*Chaoborus*). This assumption was further strengthened when next generations kept in the laboratory conditions lost this shape, and became similar to the common species *D. curvirostris*. Hence, we decided to study that mechanism experimentally and tried to induce the hunchback morphotype in laboratory clones, and compare the responses of *Daphnia hrbaceki* with *D. curvirostris.* The experiment was performed at Ludwig-Maximilians-Universität München, under the supervision of Christian Laforsch. Although we did not succeed in inducing the hunchback shape, we observed interestingly high interclonal variability in the life history response to a predator presence. Results of that experiments are summarized within the **chapter 4** (Juračka, Laforsch & Petrusek 2011). Additionally to the phantom midge larvae (*Chaoborus crystallinus*) presented in the manuscript, we tested three clones of both *D. hrbaceki* and *D. curvirostris* also for the backswimmers (*Notonecta* sp.) and stickleback fish, but none of these predators induced the humped shape.

Fig. 7 Experimental setup in Munich. *Chaoborus*, *Notonecta* and stickleback kairomones were added to separate vessels.

Probably the best morphological feature distinguishing newly described *D. hrbaceki* from its closest relative, *D. curvirostris*, is the ultrastructure of ephippia (**chapter 3**). Such patterns of the ephippium ultrastructure are very simple to observe under the Scanning Electron Microscope (SEM). Moreover, ephippia can be collected very easily from the sediments of most habitats inhabited by *Daphnia*. As they are resistant to harsh environmental conditions and stay in the sediments well preserved for ages, they allow researchers to map history of *Daphnia* centuries ago (e.g., Frey 1987, Mergeay et al. 2004, Kotov and Wappler 2015), or even deeper in the past (Kotov & Wappler 2015; Kirillova *et al.* 2016). Once collected in the field, ephippia can be stored for years in the lab prior to the analyses. These features make ephippia a suitable target for studies aiming of compare large numbers of the habitats and their history. Therefore, we focused on simplification of the ephippia preparation for the SEM and described a new simplified methods enabling cleaning ephippial ultrastructure from unwanted biofilm with consequent quick preparation, in the **chapter 5** (Juračka *et al.* 2016b).

However, most of the tiny taxonomically important morphological features in Cladocera have not been described from ephippia but from the trunk limbs. I have spent a lot of time processing these fragile structures when working on the description of the new species, as well as later when collaborating with prof. Kořínek on other projects focusing on cladoceran systematics. This is why we tried to apply both dehydration and cleaning methods also on the dissected limbs. Because the limbs are small, any manipulation with them is extremely risky, as it is very easy to damage their structure, or even to lose them. Therefore, we also provide in this chapter an optimized workflow for the preparation and manipulation with the cladoceran thoracopods that should provide repeatable and reliable results.

FOR WHOM THIS WORK IS RELEVANT?

So, what is this study good for, and who might be interested in? Direct applications of the basic biological research are rare and usually follow the research with a substantial delay, but I hope there are some visible footprints already. From the public point of view, the strongest result of the thesis probably comes from finding a new species, *Daphnia hrbaceki*, in the Protected Landscape Area. New species of various taxa are commonly described worldwide almost every day but usually the finding stays only in the scientific literature and becomes of interest to a very narrow group of experts. In our case, however, the new species already starts to serve as a flag species, i.e., an argument for nature conservation and creating new freshwater habitats in the area. This species became also a subject of our public outreach, as we published several popularizing articles in Czech journals focusing on popular science (*Živa*, *Vesmír*) or travelling (*Koktejl*). We showed the species in public exhibitions, and I gave numerous popularization talks where the species was mentioned. This way, we could attract the public to aquatic biodiversity research and conservation. I hope that we have also shown that small isolated freshwater habitats might serve as potential refugia for rare species, as we hypothesize that the habitat isolation might play a crucial role for *Daphnia hrbaceki*, which has never been observed anywhere else, with the exception for similar sites in Slovakia 50 years ago.

In contrast to wide public outreach boosted by *Daphnia hrbaceki*, results of other chapters of the thesis remain relevant primarily for the scientific community. Our metacommunity studies demonstrating how important the landscape structure may be for both passive dispersers and for various actively flying invertebrates add another piece to our understanding of metacommunity structure and dynamics but we hope at least some of the results will be of interest for a wider audience than experts on the specific studied group. That is not the case of the last chapter, which targets specifically cladocerologists. We hope, however, that SEM methods described in it may make life of some colleagues easier, and open a gate for some wider (paleo)ecological studies, especially of the large lentic habitats, as the lakes, fishponds or permanent swamps worldwide.

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CHAPTER 1

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COMMUNITY ECOLOGY - ORIGINAL RESEARCH

A naturally heterogeneous landscape can effectively slow down the dispersal of aquatic microcrustaceans

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Abstract Several studies have suggested that aquatic microcrustaceans are relatively efficient dispersers in a variety of landscapes, whereas others have indicated dispersal limitation at large spatial scales or under specific circumstances. Based on a survey of a set of recently created ponds in an area of approximately 18 × 25 km, we found multiple indications of dispersal limitation affecting the community assembly of microcrustacean communities. Spatial patterns in the community composition were better explained by the geomorphological structure of the landscape than by mere geographic distances. This suggests that ridges separating the network of valleys act as dispersal barriers, and as such may channel the dispersal routes of the studied taxa and, likely, also of their animal vectors. Dispersal limitation was further supported by a strong positive relationship between species richness and the abundance of neighboring water bodies, suggesting that isolation affects colonization rates. Finally, the apparent dispersal limitation of microcrustaceans is further corroborated by the observation of low colonization rates in newly dug experimental ponds in the study area.

Keywords Zooplankton dispersal · Dispersal limitation · Metacommunity ecology · Microcrustaceans · Dispersal barriers

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Introduction

Dispersal limitation and its impact on the community assembly of isolated natural communities remains an intensively studied topic since the original formulation of the Island Biogeography Theory (MacArthur and Wilson 1967). Organisms with limited dispersal capabilities in particular should be studied in a regional rather than local context (see Hanski 1998; Leibold and Norberg 2004). Inhabitants of inland aquatic habitats, which are assumed to be physically more separated than terrestrial environments, have been reported to have larger dispersal capacities than terrestrial taxa of the same taxonomic groups (Kappes et al. 2014). Within the terrestrial realm, spatial structuring at very local scales is particularly obvious for soil organisms (e.g., Jiménez et al. 2014), which are usually more dispersal limited than their above-ground relatives (Lindo and Winchester 2009). However, the majority of terrestrial passive dispersers, including plants (e.g., Auffret and Plue 2014; Soons and Ozinga 2005; Nathan and Muller-Landau 2000) and various invertebrates (see review in Bell et al. 2005), can be quite effectively transported by wind or animal vectors. Such taxa tend to be more dispersal limited when they are habitat specialists (e.g., Ellis 2012; Löbel

et al. 2006; Brunet et al. 2011) or at biogeographic rather than regional scales (e.g., Gonçalves-Souza et al. 2014).

Passive dispersal is one of the key adaptations of life in isolated freshwater habitats (see Maguire 1963), and has been studied in a considerable number of aquatic invertebrates (see Beisner et al. 2006; Bohonak and Jenkins 2003) and plants (e.g., Alahuhta et al. 2014; Viana et al. 2014). Microcrustaceans, especially cladocerans and copepods, are frequently studied model groups both in the field and in outdoor mesocosm experiments. Most studies on microcrustaceans have focused on relatively small spatial extents (at the scale of kilometres or less), and have found microcrustaceans to be efficient dispersers (e.g., Cohen and Shurin 2003; Michels et al. 2001). In contrast, at very broad spatial scales (hundreds to thousands of kilometres), a limited number of studies have suggested some level of dispersal limitation (e.g., De Bie et al. 2012; Zhai et al. 2015). Under some specific circumstances, evidence of dispersal limitation at smaller spatial scales has also been reported from both experiments (Jenkins 1995) and field-based observational studies (Soininen et al. 2007). At intermediate scales (tens of kilometres), Declerck et al. (2011b) found growing spatial structuring with increasing spatial scale. However, field studies assessing potential dispersal limitation among microcrustacean communities at such intermediate scales are rare.

In our study, therefore, we evaluated to what extent dispersal of microcrustaceans to newly created aquatic habitats can be affected by landscape structures that may act as potential barriers for passive dispersal. Over an area of ca 18 × 25 km, we studied 42 newly created fishless and spatially clustered pools (Fig. 1) in a landscape with four important characteristics: (1) a lack of large water bodies, (2) low connectivity among aquatic habitats, (3) a relatively low density of waterfowl (Šťastný et al. 2006), known to be one of the most important vectors of microcrustacean dispersal (Figuerola and Green 2002), and (4) the presence of steep canyons that can restrict the movement of dispersal vectors, including both waterfowl and terrestrial mammals. We focused on the species richness and composition of three major taxonomic groups of microcrustaceans (cladocerans, copepods, and ostracods).

In this naturally fragmented landscape, we hypothesized that the local species richness and composition of microcrustaceans should be structured more by the landscape structure (i.e., by natural spatial clustering and connectivity of the pools determined by the topography) than by pure geographical distance. Alternatively, if dispersal was unlimited, we expected to observe only weak spatial patterns (if any), which could not be explained by the environment. Consequently, we also analyzed multiple statistical models that took into account the effects of potentially confounding environmental variables (which may be also spatially structured) and the species composition of invertebrate predators in the studied pools. Under the assumption of dispersal limitation, we also expected microcrustacean species richness in young pools to be related to the number of other aquatic habitats in the neighborhood of the studied localities, which likely serve as the source of immigrant species (Louette and De Meester 2005). To evaluate this migration, we also performed a field experiment with new pools dug directly in the study area. Three independent aspects make this study unique in the context of other spatially-oriented analyses of microcrustacean metacommunities: (1) the intermediate spatial scale, which is underrepresented in other studies, (2) the taxonomic coverage, and (3) the heterogeneous landscape structure.

Materials and methods

Study site and localities

The 42 studied pools, selected according to the their position, size and age, are located within the Kokořínsko Protected Landscape Area (ca 18 × 25 km, 50°23′–50°38′N, 14°24′–14°42′E), Czech Republic (Fig. 1). The local landscape is mostly forested and consists mainly of deep and narrow rocky valleys (with depths often reaching about 100 m), the alluvial plains of two larger streams, as well as open meadows and fields located at higher elevations. Moreover, this area is characterized by a sparse stream network (due to the sandstone subsoil; see inset in Fig. 1) and a scarcity of large aquatic habitats. Subsequently, there is also a relatively low abundance of waterfowl (Šťastný et al. 2006), key long-distance vectors of aquatic invertebrates and plants (Figuerola and Green 2002). The pools were created between 1997 and 2004 for conservation purposes, to provide suitable habitats for vulnerable amphibian, invertebrate and macrophyte taxa. Most of them (34 pools, i.e., 81 %) were new, the remaining ones (8 pools, 19 %) were renewed at places where a pool or a wetland had been located in recent decades but more recently had no open-water habitat remaining.

The pool surface areas spanned several orders of magnitudes (see Supplementary Table S1), with maximum values per pool in the studied years (2005 and 2006) varying from 0.5 to 2400 m^2 (median 150 m^2). Maximum pool depths varied from 0.2 to 2 m (median 0.85 m). Seven pools are connected to very small streamlets, while the remainder are not connected to any running water, even during occasional spring floods. Two of the pools occasionally dry out if the groundwater level is too low in summer; all of them freeze over in winter. All pools were intentionally kept fishless for the whole study period to promote the diversity of other animal and plant taxa. All also contained at least some macrophyte stands or littoral vegetation. More details on the basic environmental parameters of each pool are given in Supplementary Table S1.

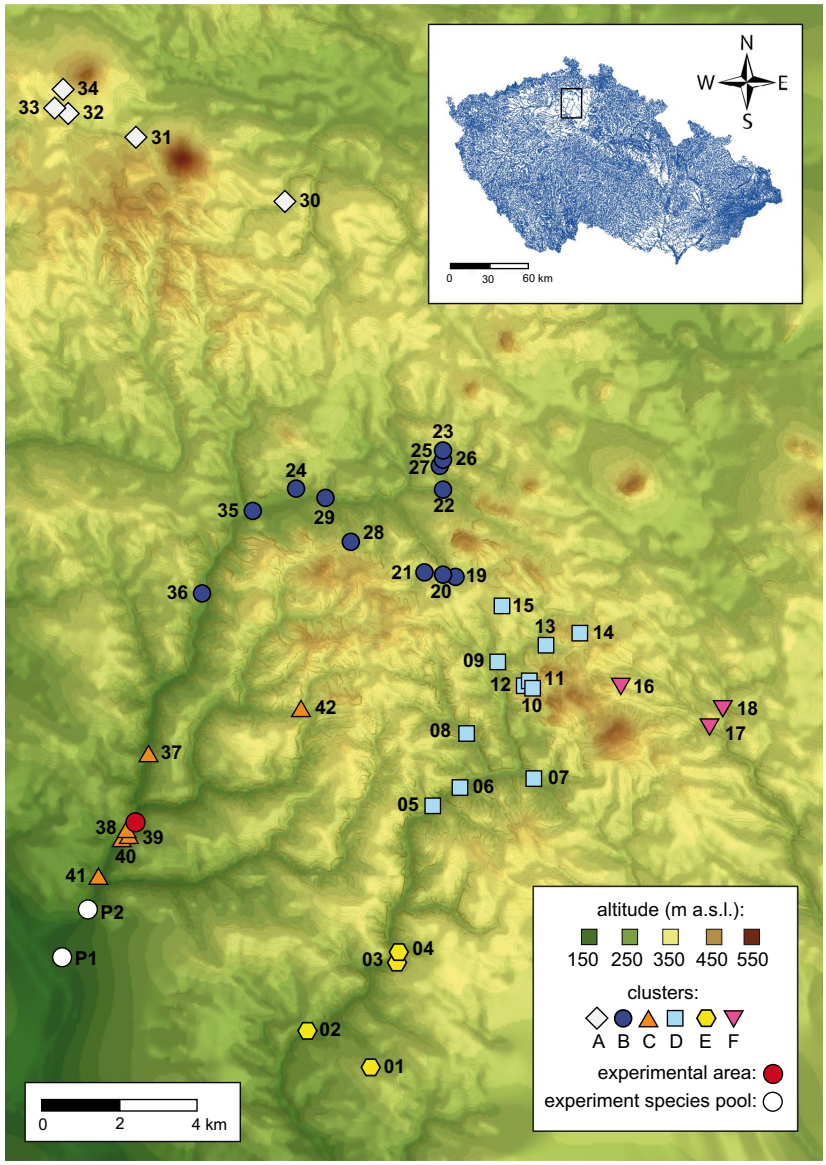

Fig. 1 Position of the studied localities ($n = 42$) within the heterogeneous landscape, and their affiliation to spatial clusters ($n = 6$) defined by pool geographical position in relation to deep canyons representing potential barriers for dispersal vectors, and by connectivity by streams. *Inset* the position of the studied area within the river network of the Czech Republic

Field sampling and sample processing

The pools were sampled in two consecutive years (2005–2006), three times per year: in spring (late April to early May), summer (July) and autumn (October to early November). Two pools were completely dry in summer 2006, and three samples were accidentally lost during the fieldwork; we thus processed a total of 252 samples. We used plankton nets with mesh sizes of 40–200 μm to collect samples of microcrustaceans (cladocerans, copepods, and ostracods). A 200-μm throwing net was used to collect samples from open water, while a pole-attached 100-μm net was swept

through the littoral vegetation (the finer mesh size ensuring that even the smallest chydorid cladocerans living in the vegetation were collected). These samples were preserved in 90 % ethanol. Furthermore, we collected a formalin-preserved sample at every site (primarily for an independent analysis of rotifer species composition but also to validate the presence/absence of crustacean species). When the macrophyte cover of the pond area exceeded 25 % but open water was also present, both 100- and 200-μm net samples were taken and inspected separately; results were then pooled for the respective pond during the data analyses. The plankton nets were carefully washed between sampling of different pools.

During each sampling, we measured water temperature, dissolved oxygen concentration, conductivity and pH with a multiparameter water quality probe (YSI 556 MPS; YSI, Yellow Springs, USA) in the middle of the water column. However, only conductivity was used in further statistical analyses, as the other parameters substantially vary on a diurnal basis. Coverage of macrophytes was rated categorically, as low (up to 25 % of the pool bottom), medium (25–75 %) and high (over 75 %). Pool depth and approximate surface area were also noted on each sampling date.

Chlorophyll a, nitrogen and phosphorus levels were not measured in 2005 and 2006, but we had the opportunity to measure these variables during a later sampling campaign in spring 2008, when all other measured characteristics and the overall appearance of the pools were very similar to preceding years. In these samples, total nitrogen and total phosphorus were analyzed from water filtered through plankton net of 40 μm mesh size to remove large seston. Nitrogen was then analyzed by high temperature combustion using a Formac Total Organic Carbon/Total Nitrogen analyzer (Skalar Analytical, Breda, Netherlands). Total phosphorus was measured colorimetrically after perchloric acid digestion according to Kopáček and Hejzlar (1993). Chlorophyll a level was estimated in vivo with an AquaFluor Fluorometer (Turner Designs, Sunnyvale, USA) and regarded as an indicator of the overall trophic state and food availability for herbivorous microcrustaceans.

Microcrustacean species were identified under a light microscope and stereomicroscope; copepods and ostracods were first treated with lactic acid to improve the observation of detailed morphological traits. Whenever possible, animals were identified to the species level using Amoros (1984), Flößner (2000), Šrámek-Hušek (1953), Meisch (2000), Einsle (1996) and several unpublished keys on local fauna. In a few samples, too small and therefore undeterminable copepod larvae were observed; these were not included in further analyses. Whenever possible, at least 300 individuals from sub-samples of known volume were identified from each sample, otherwise all specimens were identified and counted. In open water samples, *Chaoborus* larvae were frequently found together with microcrustaceans. As these predators are known to influence their communities substantially (e.g., Jäger et al. 2011; Luecke and Litt 1987; Yan et al. 1991), the relative abundance of *Chaoborus* within the sample was used in the analyses as a semi-quantitative variable [modified from the Braun-Blanquet scale; (Braun-Blanquet et al. 1932) and consisting of seven categories: <1, 1–5, 5–10, 10–20, 20–40, 40–80, ≥80 %].

To evaluate potential predation pressure on microcrustacean communities, we simultaneously took samples of other macroinvertebrates living in the pools using a sweep net. These were always collected by the same person, applying a standardized sampling effort (15 min sampling time per site). According to the local conservation policy, the sampling of invertebrates had to be performed in order to maximize species richness but not to collect macroinvertebrates quantitatively; thus neither the abundance nor biomass of individual species could be quantified. Therefore, data on potential predatory taxa present in the sampled pools, i.e., true bugs, dragonfly larvae, and aquatic beetles, were available as presence/absence data only (Supplementary Table S2). All these taxa were identified by experts on the respective groups (see Acknowledgements).

Data analysis

The main aim of our analyses was to evaluate the extent to which colonization of newly constructed ponds by microcrustacean zooplankton may be constrained by dispersal limitation. This was achieved through multiple analyses of microcrustacean community composition data, as well as through the analysis of species richness patterns.

We created three sets of a priori spatial predictors based on pool locations and topography to evaluate potential dispersal limitation. Categorical dummy variables identified clusters of pools (Fig. 1), within which we hypothesized among-pond dispersal is more frequent than among different clusters. In addition, for all pairs of the pools we computed two geographic distance matrices: a Euclidean distance matrix from geographical coordinates, and a distance matrix that will further be referred to as the "valley distance" matrix. The latter matrix comprised the shortest distances measured between each pair of pools following the course of the main canyons and valleys. We hypothesized that this "valley distance" would reflect connectivity among the localities by animal vectors (particularly terrestrial ones, which we assumed play a major role in the area). These expectations were made a priori, without any information on existing patterns of community differentiation. From both distance matrices, we calculated principal coordinates (PCoA) and used the most important orthogonal axes (vectors) with positive eigenvalues according to Borcard et al. (2011). Given that the probability for a pond

to be reached by species may also depend on the availability of source populations in the neighborhood, we also quantified the number of all lentic aquatic habitats present in a radius of 3 km around each pool (according to Louette et al. 2008). We applied partial redundancy analysis (RDA) and the variation partitioning procedure of Peres-Neto et al. (2006) to assess the unique explanatory power of each type of spatial predictor variable groups and the strength of their collinearities. Finally, we created a spatial RDA model composed of the variables with significant contributions.

To assess how much the community structure reflects the spatial structure and how much is confounded by variables related to other important ecological processes, we performed a second variation partitioning to challenge the spatial model with two categories of other explanatory variables: (1) a set of environmental variables (the 'environmental' matrix), and (2) variables representing the invertebrate predator communities (the 'predator' matrix).

The environmental matrix included key characteristics of the aquatic environment potentially affecting zooplankton communities, i.e. phytoplankton chlorophyll a concentration, concentration of total nitrogen and phosphorus, conductivity, a binary variable representing the historical presence/absence of a water body at the site, the age of the pool, macrophyte cover, surface area, and maximum depth. This matrix also included information on the characteristics of the immediate neighborhood, i.e., the presence or absence of a connection to a stream, position of the pool at the bottom of a canyon or in a permanently shadowed area. The predator matrix contained variables reflecting various aspects of invertebrate predation pressure and consisted of predator species richness, semi-quantitative density of *Chaoborus* larvae in open water samples, and five variables representing the major axes of variation in the species composition of invertebrate predators (i.e. the five most important PCoA axes built from a Sørensen dissimilarity matrix that was calculated from predator presence–absence data).

Presence–absence data indicate the spatial distribution patterns of species. When based on abundance data, the interpretation of patterns of community differentiation may be confounded by the relative ecological success of species at sites, as well as possible sampling bias when using different mesh sizes. For this reason, we expected presence–absence data to be more straightforward for the interpretation of metacommunity patterns that had been caused by dispersal limitation (Declerck et al. 2011b). All RDA and variation partitioning analyses in this study were therefore based on microcrustacean presence–absence data. To avoid the problem of too many zeros in the presence–absence data (the so-called double-zero problem), we applied the procedure of distance-based redundancy analysis (Borcard et al. 2011; Legendre and Anderson 1999). We first compiled species lists for each site based on all observations of our 2-year study period. We then used this presence–absence matrix to calculate a Jaccard distance matrix, and subsequently extracted principal coordinates which were then used as species variables in the redundancy and variation partitioning analyses. To prevent negative eigenvalues, we applied a Lingoez correction in these principal coordinate analyses. All continuous environmental explanatory variables were log-transformed prior to analyses. Prior to variation partitioning, we applied the AIC stepwise forward selection procedure according to Crawley (2007) on the environmental and predator variables, using the R function "step". Variation partitioning was computed using the functions "vegdist" and "varpart" of the R library vegan (Oksanen et al. 2011). Principal coordinate analysis was done using the function "pcoa" of the package ape (Paradis et al. 2004).

To identify and evaluate the most important variables affecting crustacean species richness, we constructed a regression tree relating local species richness with the entire set of explanatory and spatial variables. Regression trees are appropriate for exploring complex data including multiple and unknown interactions (e.g., Allen and Dodson 2011; Davidson et al. 2012). To construct the tree, we applied binary recursive partitioning using the R library tree (Ripley 2011); afterwards, we reduced the model complexity by using the function "prune.tree" based on a cost–complexity measure according to Crawley (2007).

Colonization experiment

We complemented our survey with a colonisation experiment based on the expectation that colonization of newly created habitats represents a lower boundary of dispersal rates. We built a set of 20 experimental pools in an area of 120 × 160 m in a meadow within the study area (Fig. 1). The pools had a circular shape of 5 m in diameter, with an average depth of ca 0.5 m and a surface area of approximately 10 m^2. The bottom of the pools was covered by plastic foil to reduce variations in water level fluctuation among pools. Pools were filled with water from a nearby brook that had been double filtered through a plankton net (40 µm mesh). For the sake of other research objectives, half of the experimental pools were inoculated with 500 adult females of *Daphnia curvirostris* per pool; these units were not taken into account for the present study. We were able to collect data from only 8 of the remaining pools because 2 dried out soon after the start of the experiment due to damage to the foil.

Sampling of the pools started 4 weeks after pool construction (July 2007). A 6-l water sample from the whole water column was collected at multiple places in each pool using a tube sampler, and filtered through a plankton net

with 40 µm mesh size. Samples were then preserved with formalin. To avoid contamination, the equipment was carefully washed between sampling of different pools. For each pool, crustacean species composition (presence/absence) was analysed from six samples collected in the first year of the existence of the pool (collected in 3-week intervals between August and December 2007), and five samples from the second year (collected in ca 2-month intervals between March and December 2008).

In addition, we sampled all water bodies within a radius of 3 km of the colonisation experiment, assuming that these water bodies were the most likely candidate sources of microcrustaceans colonizing the experimental pools (except for *D. curvirostris*, which could colonize from adjacent inoculated pools). These water bodies consisted of five pools from cluster F (Fig. 1, no. 37–41; distance: 0.5–2 km), one small pond in a castle park (P1; distance: ca 3 km) and a set of 5 interconnected shallow fishponds (P2; 0.2–2 ha; distance: 2–3 km). We are not aware of any other relevant freshwater habitats located closer than 6.2 km to the experimental area. We assessed the local species pool from these water bodies by sampling them once during the spring and once during the summer during the experiment, except for pools 37–41 that were regularly sampled in the framework of the survey described above.

Results

Microcrustaceans found in the pools

We identified 54 microcrustacean taxa (Supplementary Table S3): 30 species of cladocerans, 15 cyclopoids, 1 calanoid, and 8 ostracods. The most common species were the cyclopoids *Eucyclops* gr. *serrulatus* (40 pools) and *Megacyclops viridis* (26 pools), the cladocerans *Chydorus sphaericus* (40 pools) and *Simocephalus vetulus* (30 pools), and the ostracods *Cypridopsis vidua* (30 pools) and *Notodromas monacha* (28 pools). One of the cladocerans found during this study, *Daphnia hrbaceki*, was recently described as a new species (Juračka et al. 2010), and the most common cyclopoid, *E.* gr. *serrulatus*, was shown to actually be a diverse species complex (Hamrová et al. 2012).

The average number of species per sample reached 4.4, whereas the maximum species count in a single sample was 13. However, the cumulative species richness per pool over the whole study period ranged from 7 to 21 species (mean 11.7; see Supplementary Table S1). Pools created up to 2 years before the onset of the study ($n = 17$) already hosted relatively rich microcrustacean communities (7–19 spp., median 12), and the species richness of this category of young pools was not significantly lower or higher than that of older pools ($n = 25$, 8–21 spp., median 11).

Variation partitioning of community composition

Valley distances were superior to Euclidean distances in explaining variation in the microcrustacean community composition (adj. R^2 of conditional effect: 7 %, $p < 0.005$ for the former vs. 0.7 %, $p = 0.24$ for the latter; results not shown). Valley distances (Fig. 2a; adj. R^2 of the conditional effect: 6.7 %; $p < 0.005$), and a priori defined spatial clusters (Fig. 2a; adj. R^2 of the conditional effect: 4.0 %; $p = 0.035$) each explained a significant portion of the compositional variation independently. The conditional effect of neighboring source habitats was lower and insignificant (Fig. 2a; adj. R^2 of the conditional effect: 1.0 %; $p = 0.095$). However, the number of neighboring habitats was significantly collinear with both previously mentioned spatial matrices (Fig. 2a; adj. R^2 of the marginal effect: 2.6 %; $p < 0.005$). Subsequently, we merged all three predictor variable categories into one matrix (further referred

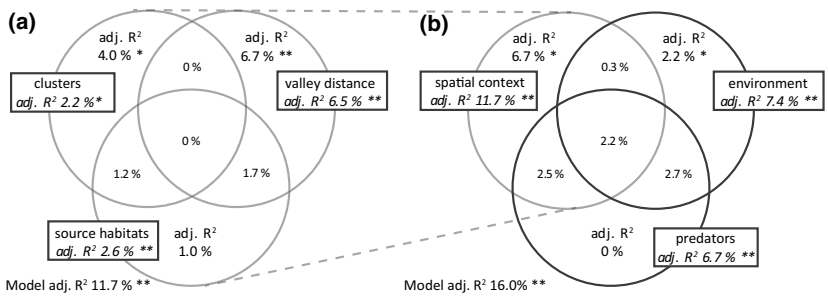

Fig. 2 Venn diagram with variation partitioning results of microcrustacean species composition explained by: **a** three spatial variable categories (i.e., affiliation of pools to a priori defined clusters, the number of other lentic habitat within a radius of 3 km, and principal coordinates of the 'valley' distance dissimilarity matrix); **b** three variable categories representing spatial context, pool characteristics, and invertebrate predator richness. **values significant at the 0.005 level; *0.05 level (tested with partial RDA at 200 permutations)

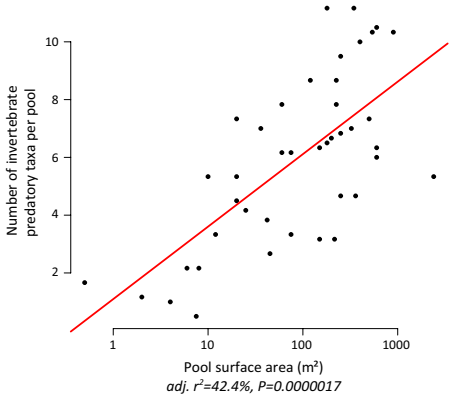

Fig. 3 Relationship between the invertebrate predator species richness and pool surface area

to as the 'spatial context') and partitioned the crustacean community variation among this matrix and the most parsimonious predictor matrices for pool environment (consisting of the variables pool surface area and hydrological history) and invertebrate predator communities (i.e., number of predator taxa). Each of the three matrices (spatial, environmental, predator) explained the crustacean species composition significantly when tested on their own (marginal effects: $p \leq 0.005$; Fig. 2b). Spatial context explained a total of 11.7 % ($p < 0.005$) of the community variation, of which 6.7 % ($p < 0.005$) was unique and of which 5 % was collinear with the other predictor variables. Environmental variables explained a total of 7.4 % ($p < 0.005$) of the community variation, of which 2.2 % ($p < 0.05$) was collinear. Predator species richness explained 6.7 % ($p < 0.005$) of the microcrustacean species composition, but all of this variability was found to be collinear with the spatial and environmental variables. Indeed, a strong positive association was found between pool surface area and predator species richness (Fig. 3). The whole model explained 16 % of the microcrustacean community variation and was highly significant ($p < 0.005$).

Species richness

The best regression tree (Fig. 4) explained 64 % of the variation in local species richness. The strongest predictor of this tree, explaining 36 % of species richness variation, was the number of lentic aquatic habitats within a 3 km radius around the target pools. The 20 pools surrounded by less than 8 potential source habitats hosted significantly fewer microcrustacean species than the remaining 22 pools (median values of 10 and 13.5 species, respectively). The latter group was further split into a group of 10 smaller, relatively species-poor pools (surface area: <200 m^2; median of species richness: 12 species), and 12 larger pools with higher species richness (median: 14 species). Macrophyte cover was identified as the most important variable affecting the species richness in pools with low numbers of nearby source habitats. Pools with a macrophyte cover lower than 25 % of the pool surface area had lower species richness (median: 9 species) than pools with a higher macrophyte cover (median: 11 species). Pool surface area, the number of nearby source habitats, and macrophyte cover all showed positive associations with species richness (see Fig. 4).

Colonization experiment

During the two surveyed seasons, the ensemble of experimental pools was colonized by a total of six microcrustacean taxa (Supplementary Table S4), two found very early after the start of the experiment and four additional ones in the course of the second year (Fig. 5). The number of taxa observed at any point in time was usually very low (Fig. 5). *D. curvirostris*, which most probably originated from the nearby inoculated pools, was already found in four pools during the first sampling, then colonizing one more pool 1 month later, and an additional one in the second year. Other early colonizers were the cladoceran *Bosmina longirostris* (found in two pools on the first sampling date) and juvenile copepods (in four pools; these, however, could not be identified to the species level). Copepod adults, identified as *Mesocyclops leuckartii* and *Eucyclops* gr. *serrulatus*, were found in the second year of the study (each in two pools). Additional taxa observed during the second year included one cladoceran (*Scapholeberis mucronata*, in two pools), and an ostracod (*Notodromas monacha*, eventually found in seven pools). All these species were also found in nearby water bodies during the field survey of local species pool (Supplementary Table S4).

Discussion

In a landscape characterised by scarce freshwater habitats located within valleys demarcated by steep slopes, we aimed to evaluate the extent to which the colonisation process of microcrustacean communities in newly created pools may be hampered by dispersal limitation. Three independent lines of evidence indeed suggest some level of dispersal limitation. First, a priori-defined clusters of the pools, based on their location in canyons or deep valleys, significantly predicted microcrustacean species composition. Also, valley distances explained community composition substantially better than Euclidean distances between pools. Second, pools with more aquatic habitats in their vicinity contained more species than more isolated

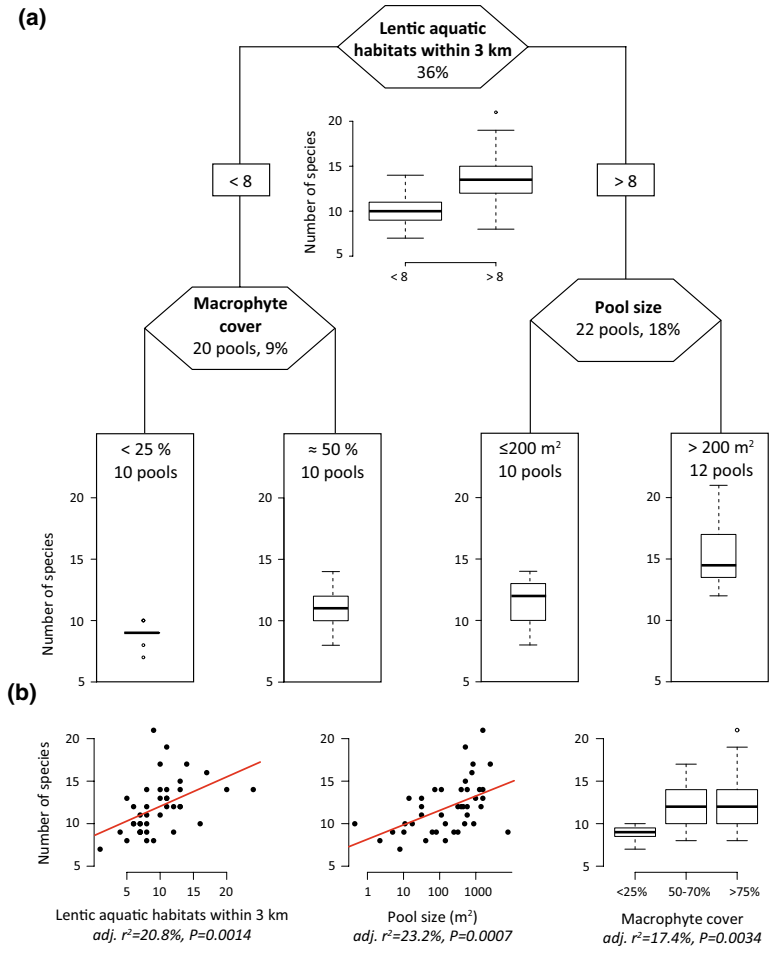

Fig. 4 **a** Regression tree identifying the most important variables influencing the microcrustacean species richness within each of the studied pools. This tree explains 63.7 % of the variation in species richness. Boxplots under each tree node compare species richness between groups of the pools defined by these nodes. **b** Relationship between species richness and the most important variables selected in the regression tree

ones, suggesting that microcrustacean communities in the pools are substantially influenced by dispersal from nearby sources. And, third, in an outdoor colonization experiment, only a very limited subset of the local microcrustacean species pool (6 out of 31 spp.) was able to successfully colonize newly created ponds within a time span of 1.5 years.

The degree to which a metacommunity of organisms is affected by dispersal limitation depends on the age of the habitat, distances between habitat patches, and the presence and spatial configuration of dispersal barriers, in addition to other important factors such as overall landscape connectivity and the dispersal capabilities of the organisms under consideration (Leibold et al. 2004). Our study is unique in that it addresses spatial community patterns of relatively young microcrustacean communities at an intermediate geographic scale (tens of kilometres). In contrast, most studies on young communities have so far been largely experimental, based on the monitoring of community trajectories in mesocosms or newly dug ponds within short time frames and at very local scales (e.g., Cohen and Shurin 2003; Jenkins 1995). Survey-based studies of cladoceran metacommunities at larger scales have mainly been limited to an analysis of the spatial structure of older, established metacommunities (e.g., Declerck et al. 2011b; Viana et al. 2014). Although experimental studies have often documented the rapid colonization of newly created pond habitats at least by a regionally occurring subset of crustacean zooplankton species (e.g., Cohen and Shurin 2003; Louette et al. 2008; but see Jenkins and Buikema 1998), several survey-based studies have also revealed indications of some degree of dispersal limitation in naturally occurring established zooplankton metacommunities. For example, in studies of habitats spatially arranged in a hierarchic manner, Ng et al. (2009) and Declerck et al. (2011b) reported

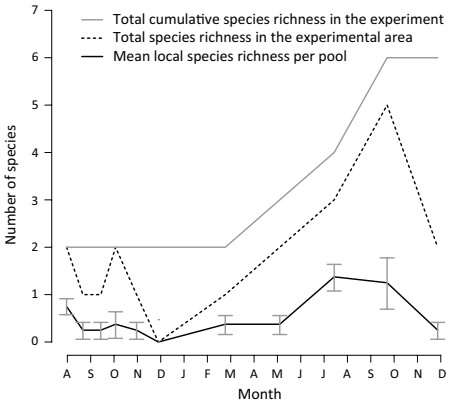

Fig. 5 Microcrustacean species richness in experimental pools ($n = 20$) in a meadow within the study area, since their creation in August 2007 until December 2008. *Whiskers above the line* showing species richness per pool indicate standard errors of the mean. The *dashed line* represents the number of species actually observed in all experiment pools for the specific month, while the *gray line* shows a cumulative curve of the same number

an increased signature of dispersal limitation with increasing spatial extent. Our study therefore fills a gap by studying the signatures of dispersal limitation in recent pond habitats at larger than local spatial scales.

Studies comparing spatial patterns among organism groups have indicated weaker spatial patterning in zooplankton taxa when compared to larger-bodied passively dispersing organism groups such as macroinvertebrates and fish, but stronger patterning than in microorganisms (Beisner et al. 2006; De Bie et al. 2012; Shurin et al. 2009). For recently established pond habitats, our results suggest that the zooplankton assembly can be substantially affected by dispersal limitation, to such an extent that the signature of this dispersal limitation is still noticeable several years after pond creation. It should be noted, however, that our study may only be representative for pools and ponds in landscapes that are characterized by a low abundance of freshwater habitats and the presence of important dispersal barriers. Furthermore, at longer time scales, the impact of dispersal limitation might be weaker, and community composition will likely better reflect environmental conditions (the so-called "quorum effect"; see Jenkins and Buikema 1998), possibly mediated by priority effects (Allen et al. 2011).

Abiotic vectors, such as wind, rain or water currents, have occasionally been shown to play a role in the overland dispersal of crustacean zooplankton organisms at small spatial scales (e.g., Cacéres and Soluk 2002; Cohen and Shurin 2003; Sciullo and Kolasa 2012). Given the relatively large spatial scale of our study area and the low hydrological connectivity among pool habitats, animal vectors most likely played an important role in crustacean dispersal. The most important candidate vectors in the area are large mammals (e.g., Vanschoenwinkel et al. 2008b; Waterkeyn et al. 2010) and waterfowl (e.g., Figuerola and Green 2002); other animal groups reported to disperse freshwater crustaceans, such as amphibians (Vanschoenwinkel et al. 2008a) and large aquatic insects (Schlichting and Sides 1969; Van de Meutter et al. 2008), are probably less relevant. Large mammals, particularly roe deer and wild boar, are locally abundant (Beran et al. 1999), and their footprints were often observed at the edge of studied pools during the sampling. Despite relatively low waterfowl densities in the region when compared to the rest of the Czech Republic, we also frequently observed mallard ducks (*Anas platyrhynchos*) at the studied localities, known to disperse dormant stages of aquatic invertebrates (Green et al. 2002; Proctor 1964) and even living ostracods (Green et al. 2013). Thus, waterfowl may contribute to pool colonization, and the signs of dispersal limitation may rather represent the limited mobility of vectors among pools from different clusters than their overall scarcity.

The amount of community variation that was explained by local environmental variables was low. Such low explanatory power of environmental variables may occur when important latent environmental variation is missed by the survey, and when spatial processes influence the species composition more than species sorting (Padial et al. 2014). Furthermore, priority effects (Frisch and Green 2007) and habitat monopolization (De Meester et al. 2002; Louette et al. 2007) by first colonizers may also contribute to a poor match between environment and species composition (Jenkins and Buikema 1998; Schulz et al. 2012). Pool surface area and hydrological history of the habitat were the only abiotic environmental variables that we found to be significantly associated with microcrustacean community composition. The variation explained by these factors was, however, almost entirely collinear with the taxonomic richness of predatory invertebrates and spatial context (Figs. 3, 4). Indeed, ponds with a large surface area contain a more diverse invertebrate fauna than smaller ponds (Angeler and Alvarez-Cobelas 2005; Anusa et al. 2012; March and Bass 1995).

Splitting the explained variability of the species composition into spatial and environmental contexts via variation partitioning based on eigenvector-based spatial filters (including PCoA used by us) has been recently disputed, as this approach may lead to inaccurate estimations of explained variability when inappropriately used (e.g., Diniz-Filho et al. 2012; Gilbert and Bennett 2010; Smith and Lundholm 2010). After reviewing numerous variation partitioning studies, Soininen (2014) strongly suggested considering not only spatial and environmental matrices

but also biotic interactions, which may mask a species sorting mechanism (i.e., the effect of the "environment fraction" on the species composition). In our study, we indeed analyzed data on predator presence that may have influenced the observed communities substantially (e.g., Shurin 2001; Verreydt et al. 2012). Furthermore, our conclusions that microcrustacean dispersal is limited in the studied heterogeneous landscape are based not only on variation partitioning of the species composition but also on the analysis of species richness.

During our entire study, we detected a total of 54 taxa for the whole region under consideration, which is in agreement with the only previous study on microcrustaceans in the area (Omesová 2006), which reported 24 cladocerans and 16 copepods from 30 comparable habitats. Regression tree analysis revealed that microcrustacean species richness in ponds was best related to the number of other aquatic habitats in the immediate surroundings. This pattern most likely reflects the decreasing likelihood of colonization of a pond with an increasing degree of isolation. The pool area was the second most important factor influencing microcrustacean species richness, with larger pools being more species-rich than smaller pools. Populations in larger pools are less prone to extinction than populations in smaller pools (Frisch et al. 2006) and have a higher probability of receiving dispersing propagules than small patches (e.g., MacArthur and Wilson 1967). Larger pools may also harbor higher microhabitat diversity and therefore provide higher niche diversity, allowing the coexistence of higher numbers of species (March and Bass 1995). The positive association between macrophyte cover and species richness indeed suggests an important influence of microhabitat diversity for crustacean diversity in these ponds (e.g., Cornell and Lawton 1992; Declerck et al. 2007, 2011a; Shiel et al. 1998).

Despite the absence of active transport, documented colonization rates of microcrustaceans colonizing new habitats are usually relatively high, at least at small to intermediate spatial scales (e.g., Frisch et al. 2012; Louette and De Meester 2005). Our study demonstrates that, in sufficiently complex landscapes with a low density of and connectivity among waterbodies, microcrustacean communities may be substantially affected by dispersal limitation, at least in the early stages of their existence. The application of distance measures that take into account the landscape complexity, such as the "valley distance" used in our case, can help elucidate scale-dependent biodiversity patterns.

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Author contribution statement LB and AP originally formulated the idea. PJJ and LB conducted the fieldwork. DV and MČ conducted the field experiment. PJJ and DV analyzed the samples. PJJ, SAJD and AP proceeded the statistical analyses. PJJ, AP and SAJD wrote the manuscript.

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A naturally heterogeneous landscape can effectively slow down the dispersal of aquatic microcrustaceans

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Electronic Supplementary Material (ESM)

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Table 1

Pool	Cluster	Name	Lattitude (N)	Longitude (E)	Elevation [m a.s.l.]	Surface area [m^2]	Maximum depth [cm]	Conductivity [$\mu S.cm^{-1}$] mean (range)	Origin	Created	Macrophyte cover	Neighbourhood	Other habitats in 3 km range	Total P [$\mu g.l^{-1}$]	Total N [$mg.l^{-1}$]	Chlorophyll a [$mg.l^{-1}$]	Species richness			
																	Cladocera	Copepoda	Ostracoda	all groups
1	E	Střemy	50° 23' 12"	14° 34' 30"	285	42	30	385 (169-605)	R	2004	low	C,S	24	107	3.1	38	8	3	2	13
2	E	Štampach	50° 23' 43"	14° 33' 12"	204	600	130	379 (297-530)	N	2003	low		20	15	0.6	19	6	5	2	13
3	E	Harasov I	50° 24' 50"	14° 34' 48"	221	345	150	233 (163-286)	N	2001	medium		14	80	0.8	16	6	8	3	17
4	E	Černínov	50° 24' 56"	14° 34' 48"	221	225	100	488 (406-648)	N	2001	medium		13	73	1.3	37	7	4	3	14
5	D	Hlučov	50° 27' 13"	14° 35' 10"	235	225	120	143 (80-224)	N	2003	high		11	41	0.7	21	7	8	2	17
6	D	Boudecký mlýn	50° 27' 32"	14° 35' 35"	238	250	150	353 (318-405)	N	2000	low		8	166	0.7	26	4	4	3	11
7	D	Ráj	50° 27' 48"	14° 37' 19"	250	250	100	126 (80-157)	N	2003	medium		8	127	1.2	27	6	4	2	12
8	D	Planý důl	50° 28' 19"	14° 35' 37"	250	20	100	121 (80-208)	N	2000	medium	C	5	51	2.0	44	4	6	4	14
9	D	Rozínkova tůň	50° 29' 26"	14° 36' 8"	291	360	150	382 (285-450)	N	1997	medium	S	6	51	0.8	24	5	4	1	10
10	D	Černý důl I	50° 29' 11"	14° 36' 60"	332	0.5	40	764 (594-987)	N	1997	high	C	7	58	2.0	23	4	3	3	10
11	D	Černý důl II	50° 29' 12"	14° 36' 59"	324	150	70	890 (858-957)	R	2001	low		7	17	0.7	11	4	4	2	10
12	D	Černý důl III	50° 29' 9"	14° 36' 58"	357	45	100	773 (664-866)	N	2001	low	C,S	7	136	0.6	33	2	3	4	9
13	D	Houska	50° 29' 47"	14° 37' 12"	308	180	150	423 (301-564)	N	2001	medium	R	11	41	4.1	43	6	5	3	14
14	D	Kbely	50° 30' 1"	14° 37' 41"	402	600	80	404 (330-455)	R	2000	high		9	161	1.3	23	9	10	2	21
15	D	Blatce	50° 30' 16"	14° 36' 4"	368	216	50	396 (225-482)	R	2003	medium		6	150	1.2	97	2	5	2	9
16	F	Český příkop I	50° 29' 19"	14° 39' 3"	343	250	120	90 (62-124)	R	1998	high	C	13	254	1.9	144	4	8	3	15
17	F	Český příkop II	50° 28' 53"	14° 41' 11"	289	10	200	50 (30-74)	R	2004	low	C,S	11	525	1.7	44	5	6	3	14
18	F	Bezdědice	50° 29' 11"	14° 41' 24"	287	60	100	748 (498-1180)	N	1999	medium	C,S	8	110	0.8	40	5	5	4	14
19	B	Beškov	50° 30' 36"	14° 34' 48"	279	7.5	50	462 (50-555)	N	1996	low	C,S	7	51	5.1	8	2	4	2	8
20	B	Kluk I	50° 30' 39"	14° 34' 36"	283	2	20	449 (399-630)	N	1997	medium	C,S	5	37	0.8	6	3	3	2	8
21	B	Kluk II	50° 30' 38"	14° 34' 15"	286	4	30	504 (80-638)	N	2003	low	C,S	4	38	6.1	6	5	1	3	9
22	B	Nedamov I	50° 31' 54"	14° 34' 22"	261	600	50	328 (287-408)	N	1999	medium	R	10	20	1.6	23	4	6	3	13
23	B	Nedamov II	50° 32' 21"	14° 34' 14"	271	540	120	473 (330-571)	N	2000	high		11	30	0.7	33	6	3	3	12
24	B	Deštná	50° 31' 37"	14° 31' 6"	249	150	80	423 (263-539)	N	2004	low		13	10	0.5	9	6	4	2	12
25	B	Nedamov III	50° 32' 15"	14° 34' 13"	269	20	90	413 (375-444)	N	2003	low	R	12	9	0.7	26	5	5	2	12
26	B	Nedamov IV	50° 32' 11"	14° 34' 12"	267	400	100	386 (357-450)	N	2003	low	R	11	12	0.4	7	6	4	2	12
27	B	Nedamov V	50° 32' 10"	14° 34' 13"	265	900	50	394 (331-425)	N	2003	low	R	10	29	0.5	9	8	4	3	15
28	B	Vrabcov I	50° 30' 59"	14° 32' 20"	275	75	50	402 (351-444)	R	2002	low	C,S	8	14	0.6	57	2	3	3	8
29	B	Vrabcov II	50° 31' 32"	14° 31' 37"	238	120	120	250 (219-278)	N	2000	medium		12	73	1.1	42	2	6	0	8
30	A	Loubí	50° 35' 46"	14° 29' 48"	293	12	50	534 (427-699)	N	1999	high	C,S	16	12	1.1	56	6	2	2	10
31	A	Hvězda	50° 36' 24"	14° 26' 10"	316	180	40	419 (364-484)	N	2003	high	R	13	38	0.7	21	7	3	2	12
32	A	Blíževedly I	50° 36' 34"	14° 24' 31"	333	20	80	915 (705-1070)	N	2004	medium	R	10	111	1.3	13	5	4	2	11
33	A	Blíževedly II*	50° 36' 38"	14° 24' 15"	333	25	80	860 (841-1272)	N	2004	medium	S	9	80	5.1	33	4	2	2	8
34	A	Ronov	50° 36' 55"	14° 24' 24"	350	8	40	1007 (822-1426)	N	2004	low	S	8	14	4.1	10	4	3	3	10
35	B	Zakšín	50° 31' 12"	14° 29' 54"	235	500	130	540 (244-772)	N	1998	high	S	10	105	1.3	89	4	4	5	13
36	B	Medonosy	50° 30' 6"	14° 29' 7"	230	36	40	351 (260-507)	N	1996	low		8	327	5.0	102	3	4	1	8
37	C	Sv. Kryštof*	50° 27' 23"	14° 28' 28"	208	6	30	432 (452-600)	N	2003	low	C,S	1	29	12.3	31	3	2	2	7
38	C	Tupadly	50° 26' 11"	14° 28' 12"	185	75	120	189 (109-285)	N	2002	low		7	19	0.6	26	2	5	3	10
39	C	Tupadly	50° 26' 5"	14° 28' 12"	184	60	50	855 (100-1589)	N	2001	medium		7	17	0.6	32	4	6	2	12
40	C	Tupadly	50° 26' 1"	14° 28' 5"	191	2400	150	386 (183-762)	N	2004	low		7	16	0.5	7	6	2	1	9
41	C	Želízy	50° 25' 28"	14° 27' 43"	176	324	80	590 (468-791)	N	2000	medium	S	17	21	2.1	20	6	5	6	17
42	C	Vidim	50° 28' 22"	14° 31' 48"	286	200	150	270 (220-305)	R	1999	medium		6	210	1.3	18	4	5	3	12

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Table 2

Group / species	Localities	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
			Presence in pools																																									
Coleoptera																																												
Acilius sulcatus	14	16	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	2	1
Agabus biguttatus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Agabus bipustulatus	14	17	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	2	2	1	0	1	0	0	1	0	1	0
Agabus congener	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Agabus didymus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Agabus guttatus	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Agabus melanarius	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Agabus nebulosus	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Agabus paludosus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Agabus sturmi	10	14	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	0	0	2	0	0	0	0	0
Agabus undulatus	4	7	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Agabus sp.	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Colymbetes fuscus	8	8	0	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Dytiscus marginalis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Gyrinus marinus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gyrinus substriatus	11	14	1	0	0	1	0	0	0	1	1	0	2	1	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0
Hydaticus seminiger	2	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Hydaticus transversalis	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroglyphus geminus	3	3	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Hydrochara caraboides	3	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus angustatus	6	7	0	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Hydroporus erythrocephalus	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Hydroporus incognitus	11	15	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	4	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	2	1	0	0	0	0	0	0	1
Hydroporus palustris	16	35	0	1	2	0	0	2	0	3	0	0	0	1	0	0	0	4	3	4	0	0	0	0	0	0	0	0	1	1	2	0	0	2	0	0	1	0	0	0	1	0	3	4
Hydroporus planus	12	14	0	0	1	0	0	0	0	1	0	0	0	0	0	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	1	1	0	0	0	1	0	1	1	0	1
Hydroporus pubescens	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroglyphus pussilus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus striola	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus umbrosus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hygrotus impressopunctatus	6	7	0	0	2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Hygrotus inaequalis	15	21	0	1	4	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	2	1	0	1	0	2	1	1	0	0	1	0	0	0	0	0	2
Hyphydrus ovatus	20	40	0	2	3	3	0	1	1	0	1	0	0	0	4	0	0	1	0	3	0	0	0	0	1	0	1	2	3	0	1	0	0	0	0	0	4	1	0	1	2	0	1	4
Ilybius ater	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ilybius fuliginosus	17	22	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	2	0	0	0	2	1	1	2	1	0	1	2	0	1	0	0	0	1	2	0	0	1	0	1	0
Noterus clavicornis	8	14	0	0	0	1	0	0	0	0	0	0	0	0	1	4	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	1	0	0	3	0	0	0	0	0	0	1
Noterus crassicornis	5	6	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Platambus maculatus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus exsoletus	2	2	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus frontalis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus suturalis	9	11	0	1	2	1	1	1	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Scarodytes halensis	5	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	3	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Suphrodytes dorsalis	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Hemiptera																																												
Callicorixa praeusta	4	4	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
Corixa dentipes	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corixa punctata	23	43	0	1	3	0	2	3	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	3	3	1	3	2	1	1	0	1	1	0	0	1	3	0	5	1	2	2	0
Cymatia coleoptrata	5	13	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Gerris argentatus	6	6	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0
Gerris gibbifer	5	5	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
Gerris lacustris	38	83	3	1	2	2	4	5	1	3	3	1	4	1	2	1	2	4	2	2	0	1	0	4	2	2	3	3	0	1	4	1	1	1	3	2	1	1	2	2	3	0	2	1
Gerris lateralis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gerris odontogaster	10	12	0	1	1	0	0	2	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Gerris sp.	2	2	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gerris thoracicus	5	5	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
Hesperocorixa linnaei	9	13	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	2	0	2	0
Hesperocorixa sahlbergi	19	24	0	1	1	2	2	1	0	0	0	0	0	0	1	1	1	0	2	0	0	0	0	0	3	0	1	1	0	1	0	0	1	0	0	0	1	1	1	1	1	0	0	0
Ilyocoris cimicoidea	12	41	0	2	2	0	1	5	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	4	0	0	2	4	0	5	0	0	0	0	0	5	0	0	0	5	0	0	0
Nepa cinerea	10	16	0	0	0	0	0	0	1	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	2	0	0	2	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	3
Notonecta glauca	32	92	3	2	4	4	5	3	3	2	2	0	1	1	6	2	0	1	1	0	0	0	0	5	4	4	3	5	3	0	3	0	2	2	5	0	3	2	0	3	2	3	2	1
Notonecta lutea	4	6	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Notonecta maculata	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2	0	0	0	0	1	0	1	0	2	0	0
Notonecta sp.	17	20	0	1	2	1	1	1	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	2	1	1	0	1	0	2	0	0	0	0	0	0	0	0	0	1	0
Notonecta viridis viridis	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plea minutissima	10	20	0	0	1	2	0	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3	0	0	2	3	0	1	0	0	0	0	0	2	0	0	1	0	0	0	0
Sigara concinna	2	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara distincta	6	7	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Sigara falleni	13	22	0	2	1	0	0	0	0	1	1	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	1	2	0	2	0	2	0	0	0	0	0	0	1	0	3	0	1
Sigara fossarum	4	4	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara lateralis	13	14	0	1	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	2	0	0
Sigara nigrolineata	9	14	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	2	1	0	0	3	1	0	0	2	0	0
Sigara semistriata	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara sp.	7	8	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Sigara striata	7	8	0	0	1	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
Velia caprai	3	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Odonata																																												
Aeshna cyanea	33	103	2	0	0	1	0	0	3	6	4	6	6	5	2	1	3	4	3	6	0	0	0	5	2	1	5	2	2	5	2	5	2	2	0	2	1	4	2	1	0	1	2	5
Aeshna grandis	5	9	0	0	2	1	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Aeshna mixta	6	11	0	3	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Anax imperator	5	8	0	3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Anax parthenope	1	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coenagrion puella	28	71	1	0	3	4	1	4	3	0	4	0	0	0	4	3	1	1	0	1	0	0	0	2	3	2	1	3	5	0	3	1	2	1	0	0	4	2	0	1	4	0	4	3
Coenagrion pulchellum	18	29	0	1	1	4	1	3	2	0	2	0	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	0	2	0	2	0	1	0	0	0	1	1	0	1	1	0	2	0
Coenagrion sp.	28	80	1	3	2	6	4	5	4	0	2	0	0	1	4	2	0	1	0	1	0	0	0	3	4	3	3	4	3	0	4	0	1	0	0	0	3	3	0	3	5	1	3	1
Cordulia aenea	6	6	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythromma viridulum	5	5	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Ischnura elegans	13	17	0	4	1	0	1	0	1	0	1	0	0	0	2	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1
Ischnura pumilio	2	5	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes dryas	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes sponsa	6	8	0	2	0	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes viridis	7	10	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	2	1
Libellula depressa	15	34	3	4	1	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	3	0	2	0	0	5	0	4	1	1	0	2
Platycnemis pennipes	1	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pyrrhosoma nymphula	30	76	1	1	1	4	2	3	2	4	4	0	5	3	2	0	1	0	3	2	0	0	0	6	2	2	5	2	2	3	1	6	2	2	2	0	1	0	0	1	0	0	0	1
Somatochlora metallica	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympecma fusca	7	8	0	1	2	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympetrum depressiusculum	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympetrum sanguineum	5	7	0	0	2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0
Sympetrum striolatum	9	13	0	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	1	2	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
Sympetrum vulgatum	11	12	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	2	1	0	0	0	0	0	0	0	0	0	0
Diptera																																												
Chaoborus spp.	39	163	3	6	5	6	5	5	3	6	1	2	2	2	6	3	4	6	6	5	1	0	0	0	5	5	5	5	6	6	3	4	3	1	4	4	5	5	2	6	5	5	4	3

Table 3

Group / species	Localities	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
			Presence in pools																																									
Cladocera																																												
Acroperus harpae	**2**	**2**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona elegans	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona guttata	**3**	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Alona quadrangularis	**3**	**3**	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona rectangula	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alonella exigua	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alonella nana	**2**	**3**	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Biapertura intermedia	**3**	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bosmina longirostris	**7**	**10**	2	0	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia affinis	**1**	**1**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia megops	**4**	**4**	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia reticulata	**6**	**9**	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	1	0	0	2	0	0	1
Daphnia curvirostris	**4**	**6**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	0	0
Daphnia hrbaceki	**2**	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daphnia longispina	**10**	**22**	0	0	0	2	5	4	4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	2	1	1
Daphnia obtusa	**17**	**41**	3	1	0	0	0	0	0	2	0	1	2	5	1	0	0	0	2	5	0	0	0	1	0	1	6	1	0	0	0	0	0	0	1	3	1	5	0	0	0	0	0	0
Daphnia parvula	**1**	**1**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daphnia pulex	**19**	**44**	3	0	0	1	0	0	0	4	0	1	4	0	0	4	3	3	0	0	2	1	0	0	0	2	0	0	1	1	0	1	2	0	0	0	0	0	3	0	0	2	1	5
Diaphanosoma sp.	**1**	**1**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Graptoleberis testudinaria	**1**	**1**	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chydorus sphaericus	**40**	**161**	2	4	3	3	5	3	6	3	3	6	5	6	4	4	5	5	4	2	1	0	0	5	4	3	5	6	5	6	5	6	5	6	5	1	2	5	1	5	3	3	4	2
Pleuroxus aduncus	**10**	**15**	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	0	2	3	0	0	0	0	0	0	0	1	1	0
Pleuroxus trigonellus	**2**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pleuroxus truncatus	**11**	**21**	1	4	1	0	3	0	4	0	2	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Polyphemus pediculus	**6**	**9**	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scapholeberis mucronata	**4**	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
Scapholeberis rammeri	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Sida crystalina	**2**	**3**	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Simocephalus exspinosus	**14**	**19**	1	0	0	0	0	2	1	1	0	0	0	0	0	1	0	0	1	2	0	0	0	0	1	1	0	1	0	0	0	2	0	2	0	2	0	0	0	0	0	0	1	0
Simocephalus vetulus	**30**	**101**	1	4	5	4	5	5	5	0	2	0	0	0	5	1	0	0	0	1	0	2	1	4	4	3	4	4	5	0	3	1	4	4	3	1	4	0	0	5	4	2	5	0
Cyclopoida																																												
Acanthocyclops einslei	**10**	**12**	1	0	0	0	1	0	0	0	0	1	2	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Acanthocyclops vernalis	**16**	**22**	0	1	3	0	2	0	0	1	0	2	0	0	0	1	1	1	1	0	0	0	0	0	1	1	1	2	0	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0
Cyclops strenuus	**7**	**10**	0	0	2	0	0	0	0	1	0	0	3	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Diacyclops bicuspidatus	**10**	**20**	0	0	0	1	0	1	0	0	0	0	0	0	0	2	2	0	0	1	1	0	0	0	0	0	0	0	0	4	1	5	0	0	0	0	0	0	0	0	0	0	2	0
Diacyclops bisetosus	**1**	**1**	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diacyclops crassicaudis	**2**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Ectocyclops phaleratus	**7**	**7**	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Eucyclops macruroides	**2**	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Eucyclops gr. *serrulatus*	**40**	**132**	1	2	4	3	2	0	2	6	1	3	2	5	4	1	1	3	2	3	6	5	5	6	4	1	4	4	4	3	4	6	5	3	3	5	5	3	0	1	4	1	4	1
Macrocyclops albidus	**14**	**24**	0	1	1	1	1	0	0	0	1	0	0	0	3	2	0	0	0	0	1	0	0	2	6	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	2	0	0	0
Macrocyclops fuscus	**15**	**34**	0	1	4	1	3	3	1	2	0	0	0	0	2	1	0	0	0	2	0	0	0	4	0	3	0	1	1	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0
Megacyclops gigas	**19**	**34**	0	1	0	0	0	1	0	2	4	0	0	0	0	1	2	1	3	1	0	2	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	2	3	2	1	1	2	3
Megacyclops viridis	**26**	**60**	2	0	2	0	3	4	4	2	0	0	1	2	1	0	0	5	4	3	2	0	0	1	0	2	0	0	0	3	0	0	3	5	2	1	2	1	1	1	1	0	0	2
Mesocyclops leuckartii	**10**	**14**	0	0	1	0	2	0	0	0	2	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	1	2	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0
Thermocyclops crassus	**4**	**4**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
Calanoida																																												
Eudiaptomus vulgaris	**7**	**10**	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1
Ostracoda																																												
Candona candida	**9**	**12**	0	0	0	1	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2	0	2	1	0	0	0	0	0	1	0
Candona neglecta	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyclocypris ovum	**5**	**8**	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	1	0
Cypria ophtalmica	**17**	**49**	0	0	0	0	0	1	0	0	0	6	1	4	0	0	0	4	4	3	2	4	2	0	0	0	0	0	0	3	0	6	0	0	1	0	2	0	2	0	0	0	2	2
Cypria cf. *subsalsa*	**6**	**7**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Cypridopsis vidua	**30**	**90**	3	5	1	3	6	4	5	2	0	0	0	0	5	5	4	2	3	4	0	0	0	2	4	2	3	5	4	1	0	0	2	3	1	1	1	3	2	2	0	0	2	0
Ilyocypris gibba	**5**	**7**	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Notodromas monacha	**28**	**56**	0	2	1	3	4	1	3	0	1	2	0	2	3	2	1	0	0	2	0	0	0	1	1	2	3	2	4	2	0	1	1	0	0	0	1	0	0	4	2	2	2	1
Sum of species			**14**	**14**	**17**	**15**	**19**	**11**	**12**	**13**	**10**	**10**	**9**	**9**	**14**	**21**	**10**	**14**	**13**	**14**	**9**	**8**	**9**	**13**	**12**	**12**	**12**	**13**	**17**	**8**	**9**	**10**	**12**	**11**	**8**	**10**	**14**	**9**	**7**	**10**	**11**	**9**	**16**	**12**

Table 4

	Experimental pools								Regional species pool						
	A2	**A4**	**B1**	**B4**	**C3**	**D2**	**D4**	**E3**	**37**	**38**	**39**	**40**	**41**	**F1**	**F2**
Cladocera															
Bosmina longirostris	N	N	1	N	N	N	1	N	N	N	N	N	N	N	Y
Daphnia curvirostris	1	N	N	1	1	1	2	13	Y	N	N	N	N	N	N
Scapholeberis mucronata	N	N	N	N	N	15	N	13	N	Y	Y	Y	Y	N	N
Acroperus harpae	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	N
Ceriodaphnia megops	N	N	N	N	N	N	N	N	N	Y	Y	N	N	N	N
Ceriodaphnia reticulata	N	N	N	N	N	N	N	N	N	N	Y	N	Y	N	N
Chydorus sphaericus	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	N
Daphnia longispina	N	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	N
Daphnia pulex	N	N	N	N	N	N	N	N	Y	Y	N	Y	Y	N	N
Eurycercus lamellatus	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	N
Pleuroxus aduncus	N	N	N	N	N	N	N	N	N	Y	N	Y	Y	N	N
Pleuroxus denticulatus	N	N	N	N	N	N	N	N	N	Y	N	Y	N	N	N
Pleuroxus truncatus	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N
Simocephalus exspinosus	N	N	N	N	N	N	N	N	N	Y	N	N	Y	N	N
Simocephaus vetulus	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	N
Copepoda															
Eucyclops serrulatus	17	N	N	N	N	N	N	15	N	Y	Y	Y	Y	Y	N
Mesocyclops leuckartii	N	N	N	15	N	N	15	N	N	Y	N	N	N	N	N
copepoda larvae	1	N	1	15	1	2	1	4	Y	Y	Y	Y	Y	N	N
Acanthocyclops vernalis	N	N	N	N	N	N	N	N	N	N	N	N	Y	N	N
Cyclops strenuus	N	N	N	N	N	N	N	N	Y	Y	Y	N	Y	N	Y
Diacyclops bicuspidatus	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	N
Macrocyclops albidus	N	N	N	N	N	N	N	N	N	Y	Y	Y	N	N	N
Macrocyclops distinctus	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	N
Macrocyclops fuscus	N	N	N	N	N	N	N	N	N	Y	N	N	N	Y	N
Megacyclops gigas	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	N	N
Megacyclops viridis	N	N	N	N	N	N	N	N	Y	Y	Y	N	N	N	N
Ostracoda															
Notodromas monacha	13	13	N	10	13	13	N	13	0	Y	0	Y	Y	N	N
Candona candida	N	N	N	N	N	N	N	N	0	0	0	0	Y	N	N
Cyclocypris ovum	N	N	N	N	N	N	N	N	0	0	0	0	Y	N	N
Cyclocypris vidua	N	N	N	N	N	N	N	N	Y	Y	0	Y	Y	N	N
Cypria ophtalmica	N	N	N	N	N	N	N	N	0	0	0	0	Y	Y	N

CHAPTER 2

Juračka P.J., Dobiáš J., Boukal D., Šorf M., Beran L., Černý M., Petrusek A.:
Space matters in species composition of passively as well as actively dispersing freshwater invertebrates in a heterogeneous landscape.
(unpublished manuscript)

Space matters in species composition of passively as well as actively dispersing freshwater invertebrates in a heterogeneous landscape

(manuscript draft)

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SUMMARY

1. Assessing the effect of spatial distribution of patchy freshwater habitats on the structure of aquatic metacommunities remains an actual topic. Dispersal abilities of different taxonomic groups have potential to strongly influence the resulting patterns. Most of the recent studies, however, still focus on one or group or a narrow selection of taxa, and those comparing more groups with various dispersal capabilities are very limited.
2. We studied spatial patterns in species richness and composition of three passively dispersing and three actively flying invertebrate groups (rotifers, microcrustaceans and molluscs vs. true bugs, aquatic beetles and dragonflies) within a system of 42 newly created fishless pools in a highly heterogeneous Central European landscape consisting of deep valleys and steep ridges. We hypothesized that all groups are affected by dispersal barriers in such landscape but to different extent depending on their dispersal mode.
3. The habitat size, measured as the pool surface area or pool depth, was the most important characteristics influencing the species richness for every studied group, following the classical island biogeography pattern. Valley distances, defined as the shortest distance among each pair of the studied habitats that avoids crossing the steep ridges, explained substantially more variation in species composition of all groups than pure geographic distances.
4. In all passively dispersed taxa and in the aquatic beetles, spatial variables (the valley distance matrix, a priori assigned pool clusters according to position in the landscape, and the number of all other aquatic habitats in the neighbourhood as an estimate of potential inoculation sources), explained substantially more variability of the species composition than basic local environmental characteristics. However, in the dragonflies and true bugs, a significant part of the species composition was explained by the shared fraction of spatial and local variables.
5. We conclude that the landscape spatial structure affected dispersal and metacommunity assembly of both actively and passively dispersing invertebrates. This is likely because flying insects follow the same geomorphological structures as the key animal vectors of passive dispersers.

Keywords: metacommunity ecology, freshwater pools, dispersal limitation, invertebrates, dispersal barriers

INTRODUCTION

Small aquatic habitats scattered throughout the landscape represent an active connection between terrestrial and aquatic environments. Therefore, they are inhabited by both purely aquatic animals as well as those associated with water during various stages of their life cycle. This might lead to relatively higher biodiversity than in exclusively aquatic or terrestrial habitats (Zedler 2003). However, there are more independent mechanisms by which freshwater pools, swamps and ponds play an irreplaceable role in supporting high local species biodiversity. 1) Being an ecotone, even very small habitats might offer high microhabitat or niche diversity (March & Bass 1995), as well as shelter against predation (Compte *et al.* 2016). 2) Small habitats are usually more dynamic environments than large ones; hence, they may offer in different periods of time suitable conditions for different species, and therefore the overall species richness might be higher than in bigger and more stable localities (Oertli, Joye & Castella 2002; Williams *et al.* 2004). 3) Very small pools can have lower frequency of colonization by passively dispersing species. At the landscape spatial scale, presence of habitats with different degree of isolation can lead to higher regional species diversity than under the conditions of only well connected ones (Scheffer *et al.* 2006).

As the environmental conditions may substantially and quickly change in small habitats, all groups of animals living there have to cope with such instability, including water level fluctuation or even complete drying (e.g., Frisch, Moreno-Ostos & Green 2006; e.g., Stendera *et al.* 2012). Therefore, abilities to disperse to other habitats or to produce resistant dormant stadia are key factors determining the success in colonization of new habitats and survival in rapidly changing environments (Shurin *et al.* 2000; Doi, Chang & Nakano 2010). Dispersal of freshwater invertebrates may be both active (e.g., flying or swimming) and passive, mediated by other vectors including animals (birds, mammals, amphibians and invertebrates) or wind; see reviews by Bilton (2001) and Incagnone et al. (2015). Considering their dispersal ability, populations and communities of aquatic organisms living in these "islands" of suitable environment should not be considered isolated but rather as large metapopulations and metacommunities. These can overcome dynamic changes of individual local habitats easier due to continuous dispersal among the patches in time (Cottenie & De Meester 2004; Leibold *et al.* 2004). Analyses of metacommunity structure at different spatial scales has been in focus of numerous recent studies (e.g., Declerck *et al.* 2011; Da Silva & Hernández 2015; Heino *et al.* 2015). These revealed that structuring of the communities can be strongly influenced by the dispersal mode of the studied taxa (e.g., De Bie *et al.* 2012; Santos *et al.* 2015; Akdemir *et al.* 2016), as well as the character of the landscape and connectivity of individual patches (Michels *et al.* 2001; Van De Meutter, Stoks & De Meester 2006).

In highly heterogeneous Central European landscape with newly created or restored pools scattered among deep valleys, we observed strong spatial structuring in passively dispersed microcrustaceans (Juračka *et al.* 2016). Although the microcrustacean taxa studied by us (cladocerans, copepods, and ostracods) may differ to some extent in their dispersal abilities, the focal group of our study was taxonomically relatively homogeneous. Comparable studies focusing simultaneously on multiple freshwater invertebrate groups of different dispersal abilities still remain scarce (as highlited in two most recent ones, i.e., De Bie et al. 2012; Curry & Baird 2015). Therefore, we decided to compare the patterns observed in microcrustaceans with a wide spectrum of taxa inhabiting the same small freshwater habitats with widely differing dispersal abilities, from aquatic molluscs to several orders of actively flying insects. Despite the fact that propagules of passive

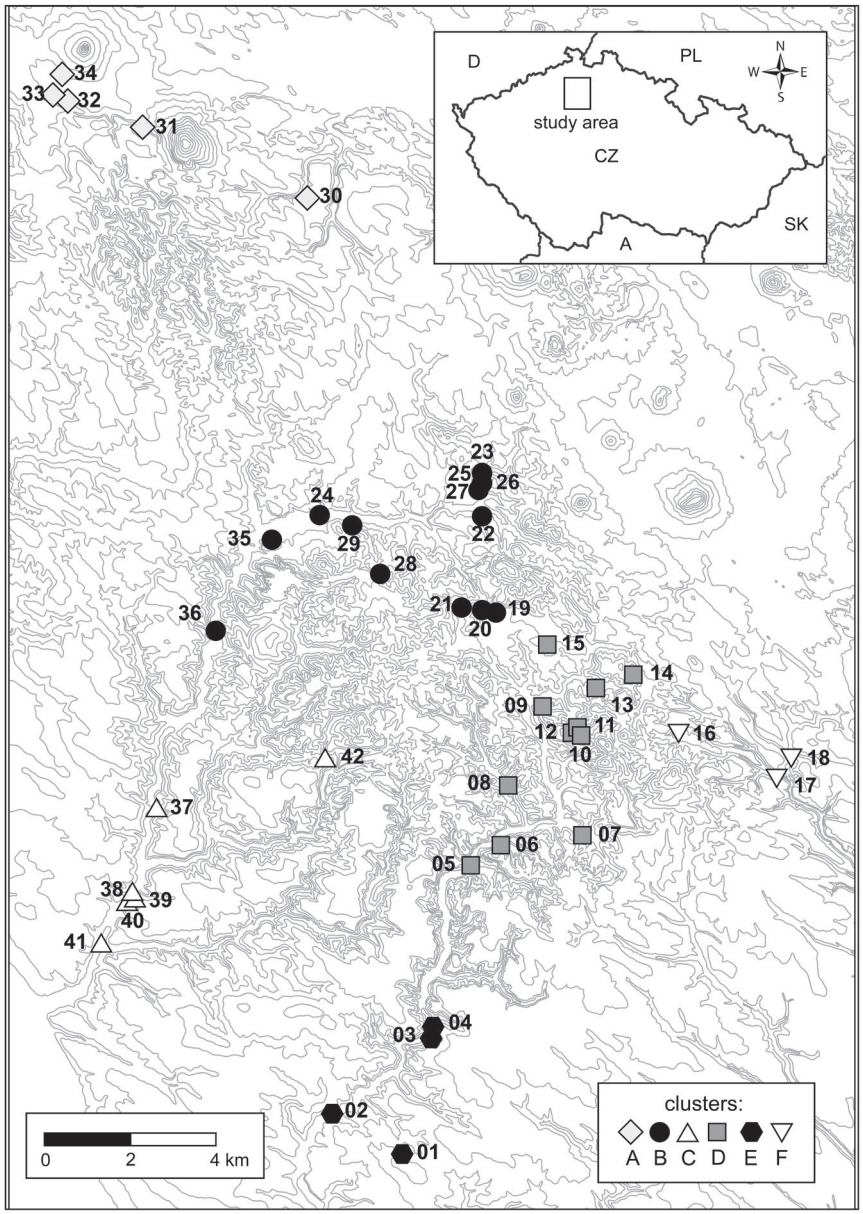

Fig. 1 Position of studied pools within the canyons, and their affiliation to a priori defined spatial clusters. Altitudinal gradients are shown by contour lines with interval of 20 m. Characteristics of the pools are listed in the Supplementary Table S1.

dispersers may be quickly transported for large distance by active vectors (e.g., Havel & Shurin 2004; Frisch, Green & Figuerola 2007), we assumed actively flying insects to be less spatially structured than the passive dispersers (crustaceans, rotifers or molluscs). Nevertheless, we hypothesized that in a complex landscape, species composition of both passively and actively dispersing groups will reflect the local topography more than geographic distances alone, as steep ridges and canyons may substantially affect movement patterns of actively dispersing aquatic invertebrates as well as vertebrate vectors carrying the propagules of passive dispersers (Juračka *et al.* 2016).

METHODS:

Study area and localities

We studied 42 fishless pools within the Kokořínsko Protected Landscape Area, Czech Republic (N 50° 23'-50° 38', E 14° 24'-14° 42'; see Fig. 1 and Supplementary Table S1). The pools were either newly created or restored between 1996 and 2004 for conservation purposes, as they can host rare species of the macrophytes, molluscs and amphibians present in the area. Studied habitats are spread among forested deep valleys of highly heterogeneous landscape, formed mostly with the sandstone rocks. This region of approximately 18×25 km is known for sparse stream network (consisting of just two brooks and few smaller springs), due to which there is low abundance of waterfowl when compared with the rest of the Czech Republic (Šťastný & Bejček 2006).

Sampling and species determination

Sampling and measurements of local variables followed the methods already used in the area and recently published by Juračka et al. (2016). Samples of six locally common invertebrate groups (monogonont rotifers, molluscs, true bugs, aquatic beetles, dragonflies and microcrustaceans, i.e., cladocerans, copepods and ostracods) were taken three times per year (spring, summer, autumn) in two consequent years (2005, 2006). Rotifers and microcrustaceans were sampled with plankton nets of mesh sizes of 40 and 100 μm, respectively. Generally, pooled sample resulted from sampling in the open water and within the macrophytes and shore vegetation if presented. Aquatic insect larvae and molluscs were sampled by sweeping a sieve (mesh size ca 2 mm) among the macrophyte vegetation, open water and bottom detritus. Samples were preserved with 4% formaldehyde (rotifers), or 90% alcohol (all others) directly in the field. All taxa were determined to the lowest species level possible (Table S2). The exceptions were damselfly larvae of the genus *Coenagrion* (which are included in the dataset as *Coenagrion* sp.), and some juvenile copepods, whose determination to species level is not reliable (these were noted but not included in the dataset).

Measuring local characteristics

The same dataset for local characteristics of the pools was used as in Juračka et al. (2016). During the sampling, we used a multi-parameter water quality probe (YSI 556 MPS, YSI Inc., Yellow Springs, USA) to measure multiple parameters, but only conductivity that does not show short-term fluctuations was used in subsequent analyses. On each sampling day, we also distinguished among three macrophyte coverage categories: low (up to 25% of the pool area), medium (25%–75%) and high (over 75%). Simultaneously, we estimated the pool depth and area. Due to technical reasons, it was not possible to measure chlorophyll a, nitrogen and phosphorus levels in the years of study (2005 and 2006), so we included in the analyses data from the subsequent sampling campaign (see Juračka et al. 2016).

Implementing the spatial scale

We assessed a spatial scale by three independent methods following Juračka et al. (2016): 1) We constructed two independent spatial matrices: a Euclidean, representing distance calculated directly from the geographical coordinates, and the valley distance matrix, measured as the shortest distance between all pairs of the pools when avoiding crossing steep ridges. 2) We identified six spatial clusters of the pools accordingly to the location within the deep canyons, where we assumed a

dispersion to be more frequent within the clusters, than among them. Finally, 3) we counted the number of neighbouring habitats in 3 km radius around each pool, which might serve as a potential source of incoming species. The same radius was used for microcrustaceans in Juračka et al. (2016) as well as in Louette and DeMeester (2008) but apparently is relevant also for actively dispersing insects; for example, dispersal across 2-km distances seems common in damselflies (as revealed in population genetic analyses of population structure crossed by (Lorenzo-Carballa *et al.* 2015).

Statistical analyses

All continuous variables were log-transformed prior to statistical analyses. Species observed only once during the study period were not included into the analyses of species composition, but are retained in the species list (Supplementary Table S2) and were used in the species richness analyses. To find the most important predictors of the observed species richness, we used regression trees with binary recursive partitioning and appropriate tree pruning according to Crawley (2007).

To analyse patterns of species composition, we compiled a presence-absence species list for every locality and the whole study period. We therefore calculated a Jaccard distance matrix from this data and extracted principal coordinates using a function pcoa() of R library PCNM (Dray, Legendre & Peres-Neto 2006), which were used instead of raw species data in the subsequent analyses. When extracting the coordinates, we applied Lingoez correction to avoid a problem with negative eigenvalues (Legendre & Legendre 1998). Consequently, we applied AIC stepwise forward selection (Crawley 2007) to identify the most important environmental variables influencing the species composition of each focal taxonomic group. As the identified factors overlapped among the groups, four environmental factors affecting the overall species composition were used as an environmental matrix in the analyses. This matrix consisted of: 1) water surface area; 2) water depth; 3) previous presence-absence of any other aquatic habitat on the place of currently studied habitat; and 4) chlorophyll a concentration. Comparison of the explained variability by this environmental matrix, and key environmental variables selected for each taxonomic group, is shown in the Supplementary Table S3. To contrast the variability in species composition explained by local (environmental) and spatial processes, we performed variation partitioning using the function varpart () of R library vegan (Oksanen *et al.* 2015).

Assessing potential bias caused by different species richness among studied groups

We observed large differences in observed species richness among the focal taxonomic groups (from 18 to 92 per group). Therefore, we were aware of that explained variability among the groups could be biased by substantially different number of dimensions. For the direct comparison, we therefore performed 200 variation partitioning analyses of randomly selected species lists containing 18 species (number of species in the least species-rich taxon, molluscs) for all studied groups except molluscs.

RESULTS

Species richness

During two consecutive study seasons (2005-2006), we identified altogether 258 taxa within six most common groups of aquatic invertebrates in the 42 studied pools: 92 monogonont rotifers, 54 microcrustaceans, 40 aquatic beetles, 31 true bugs, 23 dragonflies and 18 molluscs (Table S2). Recorded invertebrate species richness pooled across both seasons varied from 15 to 93 species per pool (Table S2). The most important variable affecting positively the species richness, identified for every studied group by regression trees (Fig. S4), was the habitat size, measured as either water surface area or as a pool depth (Table 1). For true bugs (Fig S4A), pool surface area explained 55.2% of the species richness variation, as it is possible to divide the pools into three size categories, with substantially increasing number of species with the habitat size. We observed very similar trend for dragonflies (Fig. S4B) and rotifers (Fig. S4E), where the surface area explained 62.3% and 50.8% of the species richness variation, respectively; low number of species were found in pools with surface area below ca 8 m^2. Number of other aquatic habitats in the nearest neighbourhood affected positively, as the strongest predictor, the species richness of actively flying aquatic beetles (27.2%; Fig. S4C), as well as passively dispersed crustaceans (Fig. S4F; 36.4%), and explained after a pool depth a substantial fraction of the species richness of molluscs (18.8%). Other measured variables affected the species richness only marginally. We did not observe any consistent differences in the explained species richness between actively and passively dispersing taxa.

	passive dispersers			*active dispersers*		
SPECIES RICHNESS	**Mollusca**	**Crustacea**	**Rotifera**	**Coleoptera**	**Hemiptera**	**Odonata**
number of neighbouring localities	18.8	36.4	-	27.2	-	-
pool surface area	-	18.6	50.8	-	55.2	62.3
pool depth	20.5	-	-	10.1	-	-
chlorophyll a concentration	-	-	1.4	-	-	10.9
previous hydrological history	11	-	-	-	-	-
conductivity	-	-	-	9.9	-	11
macrophyte cover	-	8.7	-	-	-	-
pool age	-	8.7	-	-	10.7	-
total	**50.3**	**63.7**	**52.2**	**47.2**	**65.9**	**84.2**

Table 1 Species richness variation (in %) explained by the environmental variables chosen by the regression trees (shown in Supplementary Figure S4) for each taxonomic group. Only variables selected in at least one of the regression trees are included in the table.

Species composition

A valley distance was much stronger predictor of the species composition than pure geographic coordinates in all studied groups (Table 2A). Therefore, we used valley distance instead of Euclidean distance or geographic coordinates as a proxy of the habitat spatial distribution. The spatial matrix used in the data analyses included also the number of other aquatic habitats in the neighbourhood and habitat affiliation to the a priori defined spatial clusters (shown in Fig. 1). Consequently, we found the spatial structure to be a very significant predictor of the species composition of all studied invertebrate groups. Spatial matrix explained substantially higher fraction of the species composition than the evaluated local variables in all passively dispersing taxa, and in actively flying beetles (Fig. 2, Table 2B). In true bugs and dragonflies, pure effect of the spatial pattern on the species composition

seemed less important than the local parameters, but shared fraction (i.e., explained by both spatial and local variables) was a substantial part of the overall variability in both groups (Fig. 2, Table 2).

SPECIES COMPOSITION

A)		*passive dispersers*			*active dispersers*	
Valley vs. Euclidean distance	**Mollusca**	**Crustacea**	**Rotifera**	**Coleoptera**	**Hemiptera**	**Odonata**
valley distance	2.6	7.1	9.9	6.9	5.9	7.3
shared	5	0	1.3	0	0	1.6
Euclidean distance	1.3	0.9	0.2	1.8	0.6	0

B)	**Mollusca**	**Crustacea**	**Rotifera**	**Coleoptera**	**Hemiptera**	**Odonata**
Spatial variables						
valley distance	3,4	6,7	6.9	6,8	5,7	6.8
No. neighbouring localities	1,6	1,1	0	2,2	3,4	4.3
cluster affiliation	2,8	4	3.4	6,2	3,1	2.7
total (including shared variability)	**11,1**	**11,7**	**14.1**	**14,3**	**10,7**	**14.7**

C)	**Mollusca**		**Crustacea**		**Rotifera**		**Coleoptera**		**Hemiptera**		**Odonata**	
Spatial vs. Local variables	18 species		54 species	18 species	92 species	18 species	38 species	18 species	31 species	18 species	24 species	18 species
local variables	2,6		4,7	3.8 ± 2.0	0,7	1.3 ± 1.5	4,1	3.9 ± 2.2	5,5	4.6 ± 1.8	5,5	4.3 ± 0.8
shared	0,8		2,9	2.4 ± 1.5	3,3	2.6 ± 1.8	3,8	3.2 ± 1.7	7,9	7.1 ± 1.8	9,2	8.3 ± 1.2
spatial variables	10,4		8,6	6.4 ± 3.5	10,8	7.7 ± 3.5	10,5	8.2 ± 4.0	2,8	1.9 ± 1.5	4,6	4.7 ± 1.1
total (including shared variability)	**13,7**		**16,3**	12.5 ± 3.9	**14,8**	11.1 ± 4.0	**18,4**	15.2 ± 4.6	**16,2**	13.3 ± 2.9	**19,4**	17.3 ± 2.0

Table 2 Decomposition of variation (in %) explained by various matrices of spatial and environmental variables. A) Variation in the species composition (in %) of every studied taxonomical group partitioned among a valley distance matrix, a Euclidean distance matrix computed directly from the geographic coordinates, and shared contribution of both. As a Euclidean distance was much poorer predictor of the species composition, only the valley distance was used in the subsequent analyses. B) Variation explained by different spatial variables: valley distance matrix, the number of neighbouring aquatic habitats, and affiliation to a priori defined clusters. C) Variation explained by the three spatial variables as in B (a spatial matrix) and the local environmental parameters (selected with stepwise AIC selection; Supplementary Table S3). For all the groups except molluscs, variation partitioning was repeated 200 times for randomized selection of 18 species, i.e., the number of observed mollusc species. Means and standard deviations of the permuted dataset are shown.

DISCUSSION

Being well defined habitats commonly present in the landscape, freshwater pools are a good model system for ecological, evolutionary and conservation biology studies (Oertli *et al.* 2004; De Meester *et al.* 2005). Our study stands out among others focusing on aquatic invertebrate dispersal by a wide taxonomical coverage, and the setting in a unique heterogeneous landscape where steep ridges may serve as dispersal barriers. We explained higher amounts of the species composition variability by the spatial than environmental patterns not only in all studied groups of passive dispersers (i.e., rotifers, molluscs and crustaceans), but also in the aquatic beetles. Therefore, we conclude that high landscape heterogeneity substantially affected the species composition of these groups, as we have previously shown for microcrustaceans (Juračka et al. 2016). In such context, it might be less surprising that the matrix of valley distances served as the strongest spatial predictor of species composition in all studied taxa, both active and passive dispersers. In passive dispersers, we consider this effect to be a reflection of the movement patterns of animals vectors, i.e., of waterfowl and large mammals (wild boar or roe deer). Even for taxa with active mode of dispersion, i.e., aquatic beetles, true bugs and the dragonflies, we suppose that the steep valley ridges become dispersal barriers Although these insect groups are capable of flying high, they prefer to follow major topographic structures (Russell *et al.* 1998). Furthermore, active dispersers in particular are likely

affected also by different wind patterns in and out of the valleys that reflect the local landscape structure (Bertin *et al.* 2015).

However, spatial processes affecting species compositions are substantially more complex. The structure of local communities may reflect not only the connectivity among the habitats (Cottenie & De Meester 2003; Doi *et al.* 2010), landscape structure (Michels *et al.* 2001; Juračka *et al.* 2016), and dispersal capability of the studied organisms (Shurin, Cottenie & Hillebrand 2009; Incagnone *et al.* 2015; Akdemir *et al.* 2016) but also spatially structured environmental characteristics (Koenig 1999; Peres-Neto & Legendre 2010) or even dispersal-mediated biological interactions (Verreydt *et al.* 2012). Identification of the mechanisms leading to the strong spatial structuring might be therefore not straightforward, especially in the field studies (Ng, Carr & Cottenie 2009).

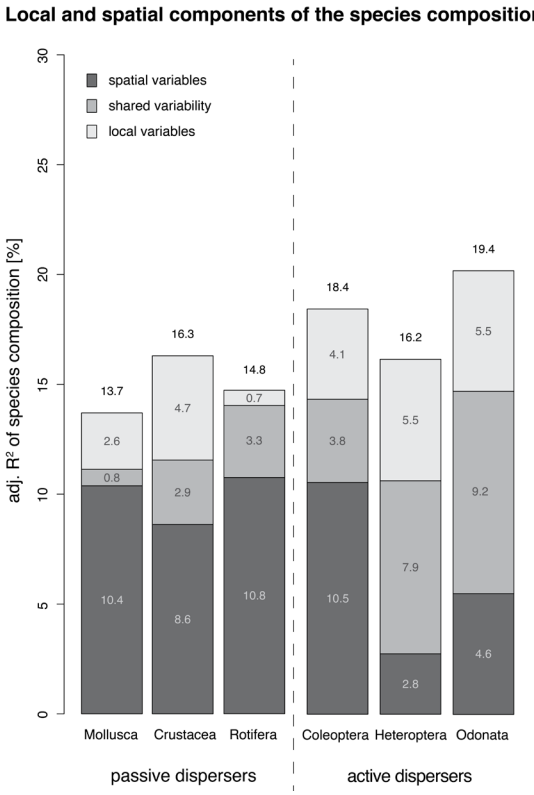

Fig. 2 Species composition partitioned to contribution of local (environmental) and spatial variables (expressed as percentages of explained variation). For more details, see Table 2C.

Despite the fact that local conditions appeared as less important predictors of the species composition than the spatial context, they still significantly influenced the local communities, especially observed species richness. We found the habitat size, measured as the pool surface area as well as a pool depth, to be the most important variable affecting the species richness of all studied invertebrates. Higher species richness in larger habitats is a general pattern resulting from increased dispersal (MacArthur & Wilson 1967). Larger habitats may also contain more diverse microhabitats leading to a coexistence of more complex communities (March & Bass 1995; Tolonen *et al.* 2003; Compte *et al.* 2016). They also tend to be less sensitive to seasonal fluctuations (Frisch *et al.* 2006), which might be particularly important for permanent macrofauna. Indeed, we observed higher number of mollusc species in deep pools, and a strong effect of habitat size was observed for dragonflies, which stay in the larval stages even for years. Increasing habitat size promoted higher dragonfly diversity in 80 ponds studied in Switzerland by Oertli et al. (2002). However, within that study the aquatic beetles and molluscs did show such trend, which contrasts to our results. We consider this difference to be a consequence of generally smaller size of studied habitats in our system.

We suppose that strong spatial structuring of aquatic beetle communities in our study responds to their biology. Although they can fly, aquatic beetles might be considered more or less resident species in permanent freshwater habitats, as their flight period tends to be restricted to breeding dispersal, overwintering, or in response to habitat deterioration including drying (Fernando &

Galbraith 1973; e.g., Boda & Csabai 2013). Many species of aquatic beetles are also often observed to be closely associated with the macrophytes (Boukal *et al.* 2007; Gioria *et al.* 2010) but we observed only a weak and non-significant positive effect of macrophyte coverage on beetle species richness. Most species observed in our study are ubiquists that can be found in a wide range of habitats, even if their populations would be most abundant in vegetated water bodies.

As we originally hypothesized, we suppose that the strong spatial structuring of passively dispersed microcrustaceans and molluscs results from their limited dispersal capacity in comparison with actively flying insects. However, it has to be noted that in other systems, both aquatic molluscs (Van Leeuwen *et al.* 2013), and microcrustaceans (Louette & De Meester 2005; Frisch *et al.* 2007) can be surprisingly efficient disperses, and their populations might be structured more by the niche-assembly mechanisms than by the dispersal (Cottenie & De Meester 2004; Hoverman *et al.* 2011). We observed strong effect of the spatial structure also in the rotifers, which have been identified to be very effective dispersers (e.g., Frisch *et al.* 2012). Rotifers are usually among the first organisms colonizing newly created habitats (Jenkins & Buikema 1998; Cáceres & Soluk 2002), due to dormant eggs and generally high abundances (Frisch *et al.* 2012). The most plausible cause of their high dispersal capacity might be small body size (De Bie *et al.* 2012) that presumably facilitates their long-range wing dispersal (Jenkins & Underwood 1998; Frisch *et al.* 2012). In case of such heterogeneous landscape as in our case, however, their most important dispersal vector might be waterfowl (Frisch *et al.* 2007) that mediates also crustaceans, aquatic snails (Van Leeuwen *et al.* 2013), and at least occasionally even insects (Charalambidou & Santamaría 2002; Figuerola, Green & Michot 2005).

We originally expected to observe more species in older habitats, as we supposed that more time could offer more occasions for colonization. Indeed, more species of the aquatic beetles have been observed in older habitats (Fairchild, Faulds & Matta 2000). However, we have not observed any significant effect of the pool age on either species composition or richness. Partly, this might have been caused by strong priority effects (Allen, VanDyke & Caceres 2011; Pu & Jiang 2015) that limit dispersal success of later arrivals. Furthermore, most of the studied pools were relatively small, or with poor environmental characteristics (excessive shading, steep shores, fluctuating water levels) to constitute optimal habitats for rich invertebrate assemblages that might show such patterns. Eight of the studied pools were restored on the places where a wetland or a pool had been present in the past. We supposed that patterns of colonization of such pools might differ, for example due to the potential presence of sediment egg banks. However, previous hydrological history of the pools influenced only marginally species richness of the molluscs (Supplementary Figure S4D) that do not form long-lived dormant propagules, and species composition of the crustaceans (Supplementary Table S3).

Conclusions

Variation partitioning of the species composition revealed strong spatial structuring of the metacommunity composition of both passively dispersed and actively flying invertebrates in highly heterogeneous landscape consisting of deep valleys and steep ridges. Furthermore, pools that may be more connected by dispersal (i.e., with more other aquatic habitats in their neighbourhood) contained more species of aquatic beetles, molluscs and microcrustaceans. We suppose that similar trends in the metacommunity assembly of passive and active dispersers reflect the fact that actively flying insects respect the landscape topography, similarly to the passive disperser vectors, which

move within the valleys more than across the ridges. Decomposition of the species composition variation into local and spatial matrices as a proxy of dispersal ability has been critically discussed (e.g., Gilbert & Bennett 2010; Smith & Lundholm 2010), as such approach may lead in some cases to overestimation of importance of spatial patterns in case of insufficient environmental data. However, populations of various actively dispersing taxa living in small freshwater habitats have been consistently observed to be less spatially structured than passive disperses both in lotic (Curry & Baird 2015; Kärnä *et al.* 2015) as well as lentic ecosystems (Rundle *et al.* 2002; Rádková *et al.* 2014), which is in agreement with the presumed impact of dispersal mode. These differences suggest that resulting patterns, in previous as well as in our study, are not just statistical artefacts but are related to underlying ecological phenomena.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1 The list of all study sites with their environmental characteristics. Origin "R"(renewed) indicates pools created on places with some previous hydrological history. Origin "N" indicates new pools at sites without no previous wetland habitat.

Supplementary Table S2 Invertebrates identified in the study. Numbers represent the number of samples (max. 6) in which a species was observed. Aquatic beetles were determined by David Boukal and Jan Klečka, crustaceans by Petr Jan Juračka, true bugs by Petr Kment and Tomáš Soldán, rotifers by Michal Šorf, dragonflies by Jakub Dobiáš and Martin Černý, aquatic molluscs by Luboš Beran.

Supplementary Table S3 Environmental variables selected with AIC forward selection procedure according to their contribution to the species composition. Altogether, only four variables were chosen across all taxonomic groups. Subsequently, two redundancy analyses were calculated for each group - with only the variable(s) selected by the procedure above, and with all four variables.

Supplementary Figure S4 The most important variables affecting the species richness identified by regression trees for each of studied taxa. Explained variation of species richness (in %) is shown for the whole trees as well as for each node.

SUPPLEMENTARY TABLE S1

Pool	Cluster	Name	Lattitude (N)	Longitude (E)	Elevation [m a.s.l.]	Surface area [m^2]	Maximum depth [cm]	Conductivity [$\mu S.cm^{-1}$] mean (range)	Origin	Created	Macrophyte cover	Other habitats in 3 km range	Total P [$\mu g.l^{-1}$]	Total N [$mg.l^{-1}$]	Chlorophyll a [$mg.l^{-1}$]
1	E	Střemy	50° 23' 12"	14° 34' 30"	285	42	30	385 (169-605)	R	2004	low	24	107	3.1	38
2	E	Štampach	50° 23' 43"	14° 33' 12"	204	600	130	379 (297-530)	N	2003	low	20	15	0.6	19
3	E	Harasov I	50° 24' 50"	14° 34' 48"	221	345	150	233 (163-286)	N	2001	medium	14	80	0.8	16
4	E	Černínov	50° 24' 56"	14° 34' 48"	221	225	100	488 (406-648)	N	2001	medium	13	73	1.3	37
5	D	Hlučov	50° 27' 13"	14° 35' 10"	235	225	120	143 (80-224)	N	2003	high	11	41	0.7	21
6	D	Boudecký mlýn	50° 27' 32"	14° 35' 35"	238	250	150	353 (318-405)	N	2000	low	8	166	0.7	26
7	D	Ráj	50° 27' 48"	14° 37' 19"	250	250	100	126 (80-157)	N	2003	medium	8	127	1.2	27
8	D	Planý důl	50° 28' 19"	14° 35' 37"	250	20	100	121 (80-208)	N	2000	medium	5	51	2.0	44
9	D	Rozínkova tůň	50° 29' 26"	14° 36' 8"	291	360	150	382 (285-450)	N	1997	medium	6	51	0.8	24
10	D	Černý důl I	50° 29' 11"	14° 36' 60"	332	0,5	40	764 (594-987)	N	1997	high	7	58	2.0	23
11	D	Černý důl II	50° 29' 12"	14° 36' 59"	324	150	70	890 (858-957)	R	2001	low	7	17	0.7	11
12	D	Černý důl III	50° 29' 9"	14° 36' 58"	357	45	100	773 (664-866)	N	2001	low	7	136	0.6	33
13	D	Houska	50° 29' 47"	14° 37' 12"	308	180	150	423 (301-564)	N	2001	medium	11	41	4.1	43
14	D	Kbely	50° 30' 1"	14° 37' 41"	402	600	80	404 (330-455)	R	2000	high	9	161	1.3	23
15	D	Blatce	50° 30' 16"	14° 36' 4"	368	216	50	396 (225-482)	R	2003	medium	6	150	1.2	97
16	F	Český příkop I	50° 29' 19"	14° 39' 3"	343	250	120	90 (62-124)	R	1998	high	13	254	1.9	144
17	F	Český příkop II	50° 28' 53"	14° 41' 11"	289	10	200	50 (30-74)	R	2004	low	11	525	1.7	44
18	F	Bezdědice	50° 29' 11"	14° 41' 24"	287	60	100	748 (498-1180)	N	1999	medium	8	110	0.8	40
19	B	Beškov	50° 30' 36"	14° 34' 48"	279	7,5	50	462 (50-555)	N	1996	low	7	51	5.1	8
20	B	Kluk I	50° 30' 39"	14° 34' 36"	283	2	20	449 (399-630)	N	1997	medium	5	37	0.8	6
21	B	Kluk II	50° 30' 38"	14° 34' 15"	286	4	30	504 (80-638)	N	2003	low	4	38	6.1	6
22	B	Nedamov I	50° 31' 54"	14° 34' 22"	261	600	50	328 (287-408)	N	1999	medium	10	20	1.6	23
23	B	Nedamov II	50° 32' 21"	14° 34' 14"	271	540	120	473 (330-571)	N	2000	high	11	30	0.7	33
24	B	Deštná	50° 31' 37"	14° 31' 6"	249	150	80	423 (263-539)	N	2004	low	13	10	0.5	9
25	B	Nedamov III	50° 32' 15"	14° 34' 13"	269	20	90	413 (375-444)	N	2003	low	12	9	0.7	26
26	B	Nedamov IV	50° 32' 11"	14° 34' 12"	267	400	100	386 (357-450)	N	2003	low	11	12	0.4	7
27	B	Nedamov V	50° 32' 10"	14° 34' 13"	265	900	50	394 (331-425)	N	2003	low	10	29	0.5	9
28	B	Vrabcov I	50° 30' 59"	14° 32' 20"	275	75	50	402 (351-444)	R	2002	low	8	14	0.6	57
29	B	Vrabcov II	50° 31' 32"	14° 31' 37"	238	120	120	250 (219-278)	N	2000	medium	12	73	1.1	42
30	A	Loubí	50° 35' 46"	14° 29' 48"	293	12	50	534 (427-699)	N	1999	high	16	12	1.1	56
31	A	Hvězda	50° 36' 24"	14° 26' 10"	316	180	40	419 (364-484)	N	2003	high	13	38	0.7	21
32	A	Blíževedly I	50° 36' 34"	14° 24' 31"	333	20	80	915 (705-1070)	N	2004	medium	10	111	1.3	13
33	A	Blíževedly II	50° 36' 38"	14° 24' 15"	333	25	80	860 (841-1272)	N	2004	medium	9	80	5.1	33
34	A	Ronov	50° 36' 55"	14° 24' 24"	350	8	40	1007 (822-1426)	N	2004	low	8	14	4.1	10
35	B	Zakšín	50° 31' 12"	14° 29' 54"	235	500	130	540 (244-772)	N	1998	high	10	105	1.3	89
36	B	Medonosy	50° 30' 6"	14° 29' 7"	230	36	40	351 (260-507)	N	1996	low	8	327	5.0	102
37	C	Sv. Kryštof	50° 27' 23"	14° 28' 28"	208	6	30	432 (452-600)	N	2003	low	1	29	12.3	31
38	C	Tupadly	50° 26' 11"	14° 28' 12"	185	75	120	189 (109-285)	N	2002	low	7	19	0.6	26
39	C	Tupadly	50° 26' 5"	14° 28' 12"	184	60	50	855 (100-1589)	N	2001	medium	7	17	0.6	32
40	C	Tupadly	50° 26' 1"	14° 28' 5"	191	2400	150	386 (183-762)	N	2004	low	7	16	0.5	7
41	C	Želízy	50° 25' 28"	14° 27' 43"	176	324	80	590 (468-791)	N	2000	medium	17	21	2.1	20
42	C	Vidim	50° 28' 22"	14° 31' 48"	286	200	150	270 (220-305)	R	1999	medium	6	210	1.3	18

SUPPLEMENTARY TABLE S2

Group / species	Localities	Samples	Presence in pools																																									
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Coleoptera																																												
Acilius sulcatus	14	16	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	2	1
Agabus biguttatus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Agabus bipustulatus	14	17	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	1	2	2	1	0	1	0	0	1	0	1	0
Agabus congener	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Agabus didymus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Agabus guttatus	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Agabus melanarius	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Agabus nebulosus	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Agabus paludosus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Agabus sturmii	10	14	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	3	0	0	2	1	0	0	1	0	0	1	0	0	0	0	2	0	0	0	0	0
Agabus undulatus	4	7	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Agabus sp.	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Colymbetes fuscus	8	8	0	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Dytiscus marginalis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Gyrinus marinus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gyrinus substriatus	11	14	1	0	0	1	0	0	0	1	1	0	2	1	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0
Hydaticus seminiger	2	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Hydaticus transversalis	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroglyphus geminus	4	4	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Hydrochara caraboides	3	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus angustatus	6	7	0	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Hydroporus erythrocephalus	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Hydroporus incognitus	11	15	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	4	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	2	1	0	0	0	0	0	0	1
Hydroporus palustris	16	35	0	1	2	0	0	2	0	3	0	0	0	1	0	0	0	4	3	4	0	0	0	0	0	0	0	0	1	1	2	0	0	2	0	0	1	0	0	0	1	0	3	4
Hydroporus planus	12	14	0	0	1	0	0	0	0	1	0	0	0	0	0	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	1	1	0	0	0	1	0	1	1	0	1
Hydroporus pubescens	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus striola	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydroporus umbrosus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hygrotus impressopunctatus	6	7	0	0	2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Hygrotus inaequalis	15	21	0	1	4	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	2	1	0	1	0	2	1	1	0	0	1	0	0	0	0	0	2
Hyphydrus ovatus	20	40	0	2	3	3	0	1	1	0	1	0	0	0	4	0	0	1	0	3	0	0	0	0	1	0	1	2	3	0	1	0	0	0	0	0	4	1	0	1	2	0	1	4
Ilybius ater	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ilybius fuliginosus	17	22	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	2	0	0	0	2	1	1	2	1	0	1	2	0	1	0	0	0	1	2	0	0	1	0	1	0
Noterus clavicornis	8	14	0	0	0	1	0	0	0	0	0	0	0	0	1	4	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	1	0	0	3	0	0	0	0	0	0	1
Noterus crassicornis	5	6	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Platambus maculatus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus exsoletus	2	2	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus frontalis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhantus suturalis	9	11	0	1	2	1	1	1	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
Scarodytes halensis	5	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	3	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Suphrodytes dorsalis	3	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Crustacea [Cladocera]																																												
Acroperus harpae	**2**	**2**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona elegans	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona guttata	**3**	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Alona quadrangularis	**3**	**3**	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alona rectangula	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alonella exigua	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alonella nana	**2**	**3**	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Biapertura intermedia	**3**	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bosmina longirostris	**7**	**10**	2	0	0	0	1	0	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia affinis	**1**	**1**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia megops	**4**	**4**	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceriodaphnia reticulata	**6**	**9**	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	1	0	0	2	0	0	1
Daphnia curvirostris	**4**	**6**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	0	0
Daphnia hrbaceki	**2**	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daphnia longispina	**10**	**22**	0	0	0	2	5	4	4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	2	1	1
Daphnia obtusa	**17**	**41**	3	1	0	0	0	0	0	2	0	1	2	5	1	0	0	0	2	5	0	0	0	1	0	1	6	1	0	0	0	0	0	0	1	3	1	5	0	0	0	0	0	0
Daphnia parvula	**1**	**1**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daphnia pulex	**19**	**44**	3	0	0	1	0	0	0	4	0	1	4	0	0	4	3	3	0	0	2	1	0	0	0	2	0	0	1	1	0	1	2	0	0	0	0	0	3	0	0	2	1	5
Diaphanosoma sp.	**1**	**1**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Graptoleberis testudinaria	**1**	**1**	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chydorus sphaericus	**40**	**161**	2	4	3	3	5	3	6	3	3	6	5	6	4	4	5	5	4	2	1	0	0	5	4	3	5	6	5	6	5	6	5	6	5	1	2	5	1	5	3	3	4	2
Pleuroxus aduncus	**10**	**15**	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	0	2	3	0	0	0	0	0	0	0	1	1	0
Pleuroxus trigonellus	**2**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pleuroxus truncatus	**11**	**21**	1	4	1	0	3	0	4	0	2	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Polyphemus pediculus	**6**	**9**	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scapholeberis mucronata	**4**	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0
Scapholeberis rammeri	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Sida crystalina	**2**	**3**	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Simocephalus exspinosus	**14**	**19**	1	0	0	0	0	2	1	1	0	0	0	0	0	1	0	0	1	2	0	0	0	0	1	1	0	1	0	0	0	2	0	2	0	2	0	0	0	0	0	0	1	0
Simocephalus vetulus	**30**	**101**	1	4	5	4	5	5	5	0	2	0	0	0	5	1	0	0	0	1	0	2	1	4	4	3	4	4	5	0	3	1	4	4	3	1	4	0	0	5	4	2	5	0
Crustacea [Cyclopoida]																																												
Acanthocyclops einslei	**10**	**12**	1	0	0	0	1	0	0	0	0	1	2	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Acanthocyclops vernalis	**16**	**22**	0	1	3	0	2	0	0	1	0	2	0	0	0	1	1	1	1	0	0	0	0	0	1	1	1	2	0	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0
Cyclops strenuus	**7**	**10**	0	0	2	0	0	0	0	1	0	0	3	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Diacyclops bicuspidatus	**10**	**20**	0	0	0	1	0	1	0	0	0	0	0	0	0	2	2	0	0	1	1	0	0	0	0	0	0	0	0	4	1	5	0	0	0	0	0	0	0	0	0	0	2	0
Diacyclops bisetosus	**1**	**1**	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diacyclops crassicaudis	**2**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Ectocyclops phaleratus	**7**	**7**	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Eucyclops macruroides	**2**	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Eucyclops gr. *serrulatus*	**40**	**132**	1	2	4	3	2	0	2	6	1	3	2	5	4	1	1	3	2	3	6	5	5	6	4	1	4	4	4	3	4	6	5	3	3	5	5	3	0	1	4	1	4	1
Macrocyclops albidus	**14**	**24**	0	1	1	1	1	0	0	0	1	0	0	0	3	2	0	0	0	0	1	0	0	2	6	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	2	0	0	0
Macrocyclops fuscus	**15**	**34**	0	1	4	1	3	3	1	2	0	0	0	0	2	1	0	0	0	2	0	0	0	4	0	3	0	1	1	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0
Megacyclops gigas	**19**	**34**	0	1	0	0	0	1	0	2	4	0	0	0	0	1	2	1	3	1	0	2	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	2	3	2	1	1	2	3
Megacyclops viridis	**26**	**60**	2	0	2	0	3	4	4	2	0	0	1	2	1	0	0	5	4	3	2	0	0	1	0	2	0	0	0	3	0	0	3	5	2	1	2	1	1	1	1	0	0	2
Mesocyclops leuckartii	**10**	**14**	0	0	1	0	2	0	0	0	2	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	1	2	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0
Thermocyclops crassus	**4**	**4**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
Crustacea [Calanoida]																																												
Eudiaptomus vulgaris	**7**	**10**	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1
Crustacea [Ostracoda]																																												
Candona candida	**9**	**12**	0	0	0	1	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2	0	2	1	0	0	0	0	0	1	0
Candona neglecta	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyclocypris ovum	**5**	**8**	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	1	0
Cypria ophtalmica	**17**	**49**	0	0	0	0	0	1	0	0	0	6	1	4	0	0	0	4	4	3	2	4	2	0	0	0	0	0	0	3	0	6	0	0	1	0	2	0	2	0	0	0	2	2
Cypria cf. *subsalsa*	**6**	**7**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Cypridopsis vidua	**30**	**90**	3	5	1	3	6	4	5	2	0	0	0	0	5	5	4	2	3	4	0	0	0	2	4	2	3	5	4	1	0	0	2	3	1	1	1	3	2	2	0	0	2	0
Ilyocypris gibba	**5**	**7**	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Notodromas monacha	**28**	**56**	0	2	1	3	4	1	3	0	1	2	0	2	3	2	1	0	0	2	0	0	0	1	1	2	3	2	4	2	0	1	1	0	0	0	1	0	0	4	2	2	2	1
Mollusca																																												
Acroloxus lacustris	**1**	**1**	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anisus leucostoma	**2**	**6**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anisus vortex	**2**	**10**	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bithynia tentaculata	**1**	**6**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0
Ferrissia fragilis	**2**	**7**	0	2	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Galba truncatula	**12**	**26**	0	4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3	0	0	1	0	1	0	2	3	3	2	0	0	0	1	2	0	3	0
Gyraulus albus	**8**	**28**	0	1	4	5	1	0	2	0	3	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
Gyraulus crista	**9**	**25**	0	3	6	6	1	2	0	0	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0
Hippeutis complanatus	**6**	**26**	0	0	6	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	2	4
Lymnaea stagnalis	**3**	**14**	0	0	5	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Musculium lacustre	**3**	**7**	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Pisidium casertanum	**16**	**38**	0	0	1	0	0	0	0	0	0	3	0	2	2	0	0	1	0	0	5	6	5	1	0	0	0	0	1	2	0	1	3	1	0	2	2	0	0	0	0	0	0	0
Pisidium milium	**2**	**2**	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pisidium obtusale	**1**	**5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

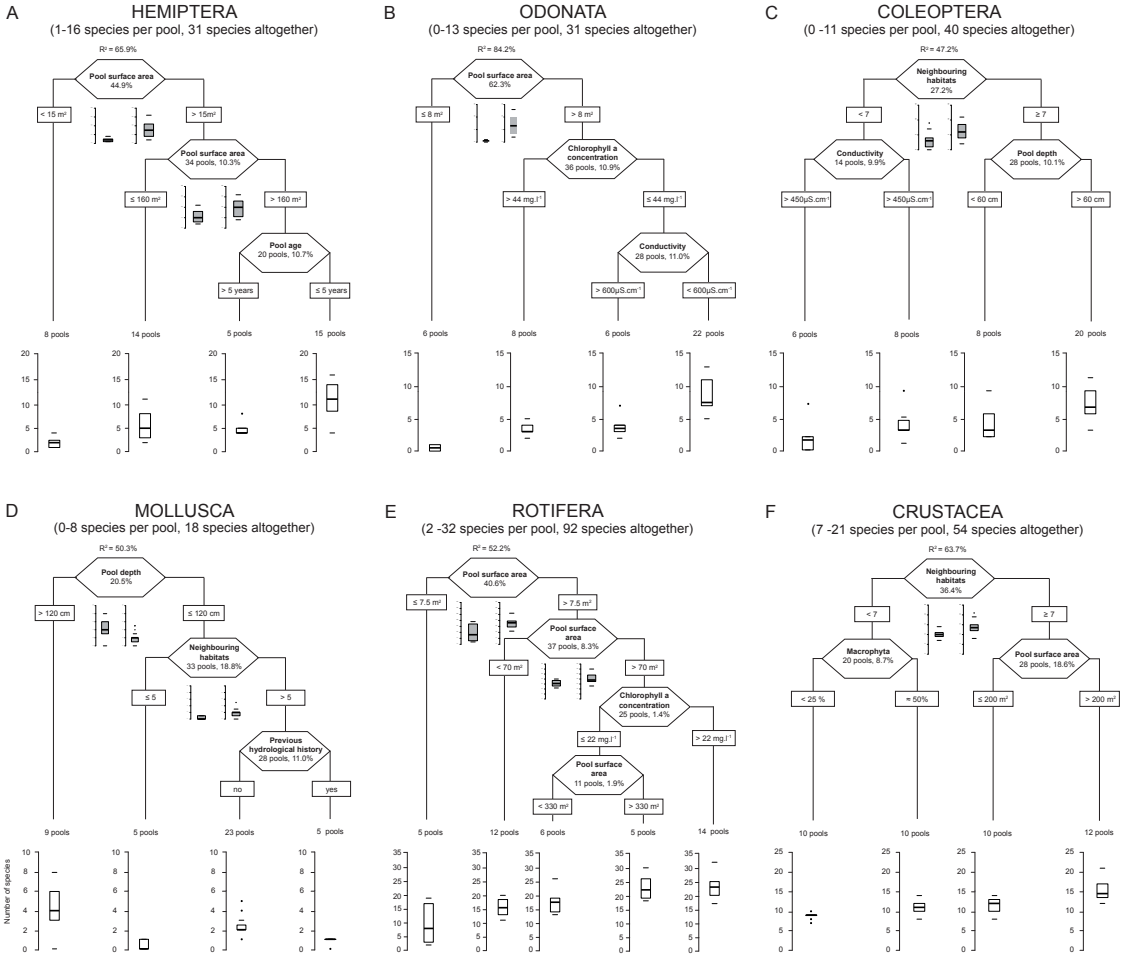
Pisidium personatum	**2**	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Radix auricularia	**8**	**27**	0	0	1	4	0	0	0	0	3	0	0	0	6	3	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	6	0	0	0
Radix labiata	**20**	**75**	0	1	0	0	0	4	2	0	1	4	1	5	0	0	0	0	5	2	6	0	0	4	4	2	5	5	6	0	0	6	5	0	0	0	6	1	0	0	0	0	0	0
Sphaerium corneum	**1**	**1**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hemiptera																																													
Callicorixa praeusta	4	4	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
Corixa dentipes	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corixa punctata	23	43	0	1	3	0	2	3	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	3	3	1	3	2	1	1	0	1	1	0	0	1	3	0	5	1	2	2	0
Cymatia coleoptrata	5	13	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Gerris argentatus	6	6	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0
Gerris gibbifer	5	5	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
Gerris lacustris	38	83	3	1	2	2	4	5	1	3	3	1	4	1	2	1	2	4	2	2	0	1	0	4	2	2	3	3	0	1	4	1	1	1	3	2	1	1	2	2	3	0	2	1
Gerris lateralis	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gerris odontogaster	10	12	0	1	1	0	0	2	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Gerris sp.	2	2	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gerris thoracicus	5	5	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1
Hesperocorixa linnaei	9	13	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	2	0	2	0
Hesperocorixa sahlbergi	19	24	0	1	1	2	2	1	0	0	0	0	0	0	1	1	1	0	2	0	0	0	0	0	3	0	1	1	0	1	0	0	1	0	0	0	1	1	1	1	1	0	0	0
Ilyocoris cimicoides	12	41	0	2	2	0	1	5	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	4	0	0	2	4	0	5	0	0	0	0	0	5	0	0	0	5	0	0	0
Nepa cinerea	10	16	0	0	0	0	0	0	1	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	2	0	0	2	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	3
Notonecta glauca	32	92	3	2	4	4	5	3	3	2	2	0	1	1	6	2	0	1	1	0	0	0	0	5	4	4	3	5	3	0	3	0	2	2	5	0	3	2	0	3	2	3	2	1
Notonecta lutea	4	6	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Notonecta maculata	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2	0	0	0	0	1	0	1	0	2	0	0
Notonecta sp.	17	20	0	1	2	1	1	1	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	2	1	1	0	1	0	2	0	0	0	0	0	0	0	0	0	1	0
Notonecta viridis viridis	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plea minutissima	10	20	0	0	1	2	0	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3	0	0	2	3	0	1	0	0	0	0	0	2	0	0	1	0	0	0	0
Sigara concinna	2	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara distincta	6	7	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Sigara falleni	13	22	0	2	1	0	0	0	0	1	1	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	1	2	0	2	0	2	0	0	0	0	0	0	1	0	3	0	1
Sigara fossarum	4	4	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara lateralis	13	14	0	1	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	2	0	0
Sigara nigrolineata	9	14	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	2	1	0	0	3	1	0	0	2	0	0
Sigara semistriata	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigara sp.	7	8	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Sigara striata	7	8	0	0	1	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
Velia caprai	3	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Odonata																																													
Aeshna cyanea	33	103	2	0	0	1	0	0	3	6	4	6	6	5	2	1	3	4	3	6	0	0	0	5	2	1	5	2	2	5	2	5	2	2	0	2	1	4	2	1	0	1	2	5
Aeshna grandis	5	9	0	0	2	1	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Aeshna mixta	6	11	0	3	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Anax imperator	5	8	0	3	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Anax parthenope	1	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coenagrion cf. puella	28	71	1	0	3	4	1	4	3	0	4	0	0	0	4	3	1	1	0	1	0	0	0	2	3	2	1	3	5	0	3	1	2	1	0	0	4	2	0	1	4	0	4	3
Coenagrion cf. pulchellum	18	29	0	1	1	4	1	3	2	0	2	0	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	0	2	0	2	0	1	0	0	0	1	1	0	1	1	0	2	0
Coenagrion sp.	28	80	1	3	2	6	4	5	4	0	2	0	0	1	4	2	0	1	0	1	0	0	0	3	4	3	3	4	3	0	4	0	1	0	0	0	3	3	0	3	5	1	3	1
Cordulia aenea	6	6	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythromma viridulum	5	5	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Ischnura elegans	13	17	0	4	1	0	1	0	1	0	1	0	0	0	2	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1
Ischnura pumilio	2	5	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes dryas	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes sponsa	6	8	0	2	0	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lestes viridis	7	10	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	1
Libellula depressa	15	34	3	4	1	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	3	0	2	0	0	5	0	4	1	1	0	2
Platycnemis pennipes	1	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pyrrhosoma nymphula	30	76	1	1	1	4	2	3	2	4	4	0	5	3	2	0	1	0	3	2	0	0	0	6	2	2	5	2	2	3	1	6	2	2	2	0	1	0	0	1	0	0	0	1
Somatochlora metallica	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympecma fusca	7	8	0	1	2	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympetrum depressiusculum	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sympetrum sanguineum	5	7	0	0	2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0
Sympetrum striolatum	9	13	0	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	1	2	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
Sympetrum vulgatum	11	12	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	2	1	0	0	0	0	0	0	0	0	0	0
Rotifera																																													
Collotheca sp.	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Asplanchna priodonta	3	6	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asplanchna girodi	2	2	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asplanchna sieboldi	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anuraeopsis fissa	29	69	0	3	6	5	2	4	0	3	2	0	0	0	3	1	3	0	0	2	0	0	0	2	3	2	2	3	3	3	3	0	1	1	0	0	1	2	3	1	1	2	1	1
Brachionus angularis	11	14	0	0	1	0	1	0	0	0	0	0	1	1	0	4	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0
Brachionus bidentata	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brachionus calyciflorus	5	6	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brachionus diversicornis	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brachionus quadridentatus	4	6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	3	0	0	0	0	0	0
Brachionus rubens	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Brachionus urceolaris	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Notholca acuminata	2	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Notholca squamula	10	12	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2	1	1	0	0	0	0	0	1	0	0	0	1	0	2	1	0	0	0	0	1	0	0	0
Kellicottia longispina	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Keratella cochlearis	22	37	2	0	4	2	4	2	0	1	4	1	2	0	1	3	0	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0
Keratella quadrata	24	53	3	0	3	0	5	1	1	0	1	1	5	1	1	3	0	0	0	0	0	0	1	1	0	1	1	3	4	0	0	0	0	1	0	0	3	2	3	5	1	2	0	0
Keratella testudo	33	89	2	0	3	3	0	3	0	6	3	2	4	4	4	1	2	2	6	3	0	0	0	4	1	3	4	3	2	2	2	1	2	1	0	0	2	2	2	2	2	4	0	2
Keratella ticinensis	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Keratella valga	7	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	1	0	1	1	0	0	0	1	0
Keratella sp.	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Platyias quadricornis	2	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Colurella adriatica	3	3	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colurella hindenburgi	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colurella uncinata	29	40	1	1	0	1	2	2	1	0	0	1	1	2	0	1	1	0	1	1	0	0	1	3	1	1	1	1	0	2	2	1	1	2	3	0	0	1	0	1	1	0	2	0
Lepadella acuminata	5	8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	2	0	0	0
Lepadella oblonga	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Lepadella ovalis	14	17	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	1	0	0	1	2	1	0	1	1	1	1	1	0	0	1	0
Lepadella patella	29	67	4	2	4	0	1	1	0	2	0	3	1	3	3	3	3	4	3	0	0	0	0	2	1	2	0	0	0	1	2	2	0	0	2	3	4	1	3	0	1	2	2	2
Lepadella rhomboides	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Squatinella mutica	7	8	0	2	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Squatinella rostrum	14	21	0	1	1	1	3	3	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	0	0	0	0	2	2	0	1	0	0	0	0	0
Epiphanes senta	12	15	0	0	0	0	0	0	0	1	0	3	0	2	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0	0
Euchlanis deflexa	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Euchlanis dilatata	11	14	0	0	1	2	0	3	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0
Euchlanis lyra	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Euchlanis triquetra	5	5	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Ascomorpha ecaudis	5	6	0	0	1	0	0	0	0	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gastropus hyptopus	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lecane bulla	5	6	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0
Lecane closterocerca	28	48	2	1	3	0	3	2	2	0	1	0	3	0	0	0	0	2	1	1	0	0	1	2	2	0	0	1	2	1	2	1	3	0	1	2	1	0	3	1	1	2	1	0
Lecane elsa	5	8	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
Lecane flexilis	8	11	0	0	1	0	2	0	2	1	1	2	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lecane ludwigii	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lecane luna	11	20	0	1	1	0	1	0	2	0	0	0	0	0	0	0	2	5	0	0	0	0	0	2	1	0	0	1	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Lecane lunaris	24	45	1	3	1	2	2	1	2	3	0	0	1	2	3	1	2	0	0	0	0	0	0	3	2	2	1	0	2	2	3	0	1	2	0	0	0	0	0	2	1	0	0	0
Lecane stichaea	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lecane sp.	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Lophocharis oxysternon	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lophocharis salpina	10	17	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	3	1	4	0	0	0	0	1	1	0	0	0	1	2	0
Mytilina bisulcata	7	8	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	1	1	0	1
Mytilina mucronata	22	38	1	0	1	0	0	0	1	2	0	0	0	0	1	0	0	1	0	1	0	0	0	2	1	1	0	0	2	2	3	5	2	1	1	2	3	0	0	0	0	2	2	1
Mytilina ventralis	13	17	0	0	3	0	0	1	0	1	1	0	0	0	1	1	2	0	0	0	0	0	0	0	2	0	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0
Cephalodella sp.	25	42	3	0	2	1	0	3	2	0	0	2	0	3	0	1	0	1	2	0	0	0	0	1	1	1	0	0	1	4	1	0	2	3	2	1	1	0	1	1	1	0	0	1
Cephalodella catellina	2	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

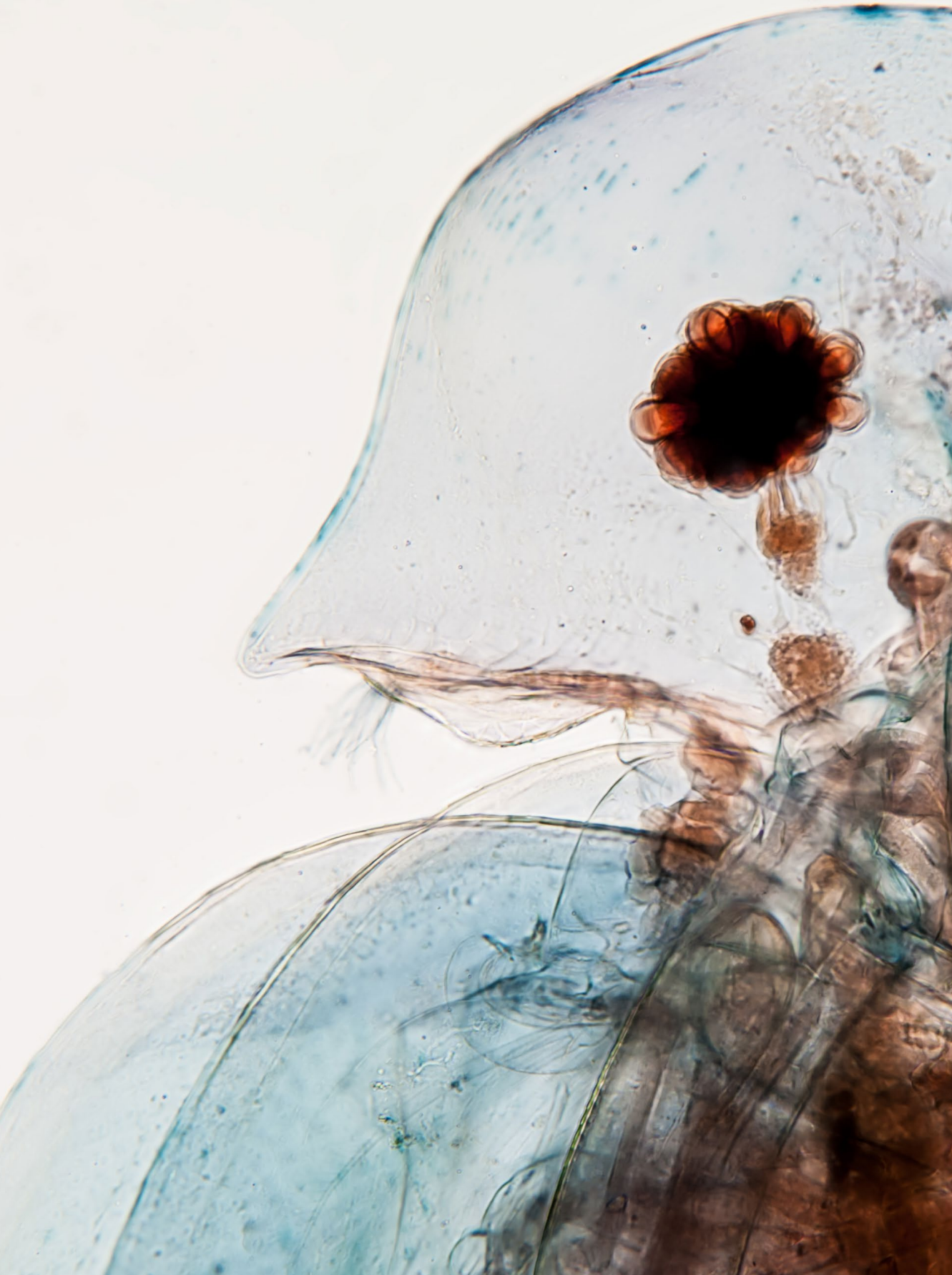
Cephalodella gibba	8	8	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	1	0	0	0	1	0
Cephalodella ventripes	4	5	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Monommata sp.	7	8	0	0	0	2	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Notommata sp.	1	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scaridium longicaudum	2	3	0	0	0	0	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Polyarthra dolichoptera	34	115	1	5	6	5	5	5	2	4	5	1	4	3	3	4	2	0	3	4	0	0	0	2	2	6	5	5	3	5	2	0	0	1	0	2	3	1	3	4	3	4	0	2
Polyarthra major	8	9	0	0	0	2	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Polyarthra vulgaris	10	13	0	0	0	0	2	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	1	0	1	1	2	1	0	0
Synchaeta tremula	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Synchaeta oblonga	26	64	1	2	5	5	1	1	4	5	5	4	6	1	1	3	0	0	0	0	0	0	2	1	0	0	1	2	0	1	0	0	0	0	2	1	2	0	0	2	2	2	2	0
Synchaeta pectinata	19	51	1	0	0	0	4	1	0	0	0	0	0	0	1	0	0	0	4	1	0	0	0	2	3	5	5	4	4	2	3	0	0	0	0	0	0	1	0	5	2	2	0	1
Synchaeta sp.	5	5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Trichocerca bicristata	4	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0
Trichocerca bidens	14	18	0	1	0	0	1	3	2	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	2	0
Trichocerca brachyura	6	9	0	0	1	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca cf. cylindrica	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca elongata	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Trichocerca gracilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca iernis	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Trichocerca inermis	2	3	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca parvula	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Trichocerca porcellus	4	5	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Trichocerca pussila	4	6	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Trichocerca rattus	21	34	0	0	0	1	2	2	1	1	3	3	0	0	1	1	1	1	3	1	0	0	0	0	1	0	0	0	2	0	2	0	2	1	0	2	0	0	0	1	0	0	2	0
Trichocerca rousseleti	3	3	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca weberi	2	3	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichocerca sp.	7	10	0	0	3	1	1	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichotria pocillum	6	8	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichotria tetractis	4	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Conochilus (Conochiloides) dossuarius	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Filinia longiseta	15	25	0	0	1	1	0	0	0	0	1	1	3	3	1	0	4	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	2	1	0	0	0	1	0	2
Filinia terminalis	3	5	3	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
not determined Flosculariacea	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexarthra mira	3	3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testudinella mucronata	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testudinella patina	34	64	2	1	2	1	1	1	1	2	0	3	4	3	1	1	3	1	0	0	0	0	1	1	1	0	1	1	2	2	2	1	3	4	3	3	0	0	1	2	2	3	2	2

SUPPLEMENTARY TABLE S3

AIC selection	*passive dispersers*						*active dispersers*					
local variables selected with the model	**Mollusca**		**Crustacea**		**Rotifera**		**Coleoptera**		**Hemiptera**		**Odonata**	
pool surface area	-		x		x		-		x		x	
pool depth	x		-		-		x		-		-	
chlorophyll a concentration	-		-		-		-		x		x	
previous hydrological history	-		x		-		-		-		x	
RDA	**R^2**	**AIC**	**R^2**	**AIC**	**R^2**	**AIC**	**R^2**	**AIC**	**R^2**	**AIC**	**R^2**	**AIC**
model with selected local variables	3.2	-32.7	7.5	-52.4	3.6	-53.6	5.4	-42	12.7	-51.9	18.3	-55.4
model with four local variables	5.3	-30.1	7.7	-50.7	4.4	-51.3	7.8	-40.4	13.4	-50.4	18.4	-54.6

SUPPLEMENTARY FIGURE S4





CHAPTER 3

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A new Central European species of the *Daphnia curvirostris* complex, *Daphnia hrbaceki* sp. nov. (Cladocera, Anomopoda, Daphniidae)

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Abstract

Although systematics of the cladoceran genus *Daphnia* (Cladocera: Daphniidae) has been intensively investigated for decades using both morphological and genetic approaches, new lineages are being discovered on all continents, including in well-studied regions. Among Holarctic daphnids, *Daphnia curvirostris* Eylmann, 1887 held an interesting position, sharing some morphological characters of both the *D. pulex* and *D. longispina* groups. Recently, additional species of the *D. curvirostris* complex have been discovered in the Eastern Palaearctic. Here, we describe a new species in this complex from Central Europe, *D. hrbaceki* **sp. nov**. It was discovered in small, newly created fishless pools in the Czech Republic, and an additional sample of apparently the same taxon was collected in 1951 in Slovakia. *D. hrbaceki* is the closest yet known relative of *D. curvirostris*, but remains genetically divergent from all members of the complex (based on the sequences of three mitochondrial genes: 12S, COI, and ND2). In general, adult females of this species are morphologically very similar to *D. curvirostris*. Unlike the latter species, *D. hrbaceki* may develop a specific hump-shaped dorsal outline of the carapace, presumably an inducible defence against invertebrate predators. Juveniles of the new species occasionally form neckteeth, which may also be retained in adult individuals. The species also shows substantial variation in the size of spines in the middle pecten of the postabdominal claw, similarly as in the Japanese member of the species complex, *D. tanakai* Ishida, Kotov & Taylor, 2006. This variable character of spine size in the postabdominal middle pecten (a transition from the *pulex* to the *longispina* group character), as well as a bent and heavily setulated terminal seta on the male 2nd endopodite (considered as the *pulex* group character), are typical for the new species. *D. hrbaceki* also differs from *D. curvirostris* as well as other members of the complex in the ephippial surface ultrastructure. Our study demonstrates the utility of such ultrastructural characters in *Daphnia* taxonomical studies.

Key words: taxonomy, new species, inducible defences, ephippia ultrastructure

Introduction

Water fleas of the genus *Daphnia* (Anomopoda: Daphniidae) are an important group in the zooplankton of inland water bodies, particularly in temperate zones. Their position in pelagic food webs, linking primary producers in phytoplankton and planktivorous consumers, especially fish, makes daphnids some of the keystone taxa in lake ecosystems. In addition, several *Daphnia* species have become model organisms in a number of research fields, including evolutionary biology or applied sciences such as ecotoxicology (Peters & de Bernardi 1987; Benzie 2005). In comparison with other cladoceran taxa, the genus *Daphnia* can be considered extremely well-known (Forró *et al*. 2008), and is among the most intensively studied aquatic invertebrates. However, there are still substantial gaps in knowledge of the diversity and systematics of this ecologically important model taxon. As in other cladoceran groups, undescribed lineages are being discovered in all biogeographic regions (see, e.g., Adamowicz *et al*. 2009), and many apparently widespread taxa turn out to be cryptic species complexes if studied in detail (Forró *et al*. 2008).

Until recently, *Daphnia curvirostris* Eylmann, 1887 belonged to a group of rather unusual *Daphnia* species which turned out to belong to the same genetic lineage in different biogeographic zones, despite its very broad distribution including the Palaearctic, Africa and North America (Benzie 2005). However, two new closely related species from the *D. curvirostris* complex were recently described from the eastern Palearctic: *Daphnia tanakai*

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Ishida, Kotov & Taylor, 2006 from Japan and *Daphnia sinevi* Kotov, Ishida & Taylor, 2006 from East Russia. Additional genetic evidence (Kotov *et al*. 2006) indicates that the diversity within this species complex in the eastern Palaearctic is even higher; apparently, this region may have been a diversification centre of the complex.

The *D. curvirostris* complex has several interesting morphological features. Despite belonging phylogenetically to the *D. longispina* group (Adamowicz *et al*. 2009) that mostly consists of pelagic taxa from larger water bodies, members of the *D. curvirostris* complex usually inhabit smaller water bodies, and share some ecological as well as morphological characteristics with the *D. pulex* group. Among the *D. longispina* group, the *D. curvirostris* complex is unique in having an enlarged middle pecten of spines on the postabdominal claw (i.e., a pecten of the *pulex* type, which has been used as the main differentiating character between the *longispina* and *pulex* groups; see, e.g., Glagolev 1995). Interestingly, it has been shown for *D. tanakai* that this feature, believed to be very stable in higher taxonomic groups, can be variable within a single species, and even within a single population (Ishida *et al*. 2006). However, *D. tanakai* remains so far the only taxon within the *D. curvirostris* species complex for which such variation has been documented. Another morphological character that has received recent attention was the ability to form neckteeth, an antipredator morphological structure, in *D. sinevi*, another newly described Far East taxon of the complex (Kotov *et al*. 2006). By documenting for the first time that such feature also exists in *curvirostris*-like taxa (Kotov *et al*. 2006), this discovery provided additional evidence that neckteeth apparently originated several times independently in *Daphnia* (Colbourne *et al*. 1997).

In Europe, from which *D. curvirostris* was originally described (Eylmann 1887), this taxon seemed to be very homogeneous. However, the *Daphnia* fauna of the Western Palaearctic regions is far from fully explored, as documented by recent discoveries of a number of cryptic lineages within the genus in this biogeographic region (Petrusek 2003; Petrusek *et al*. 2008; Adamowicz *et al*. 2009; Petrusek *et al*. 2009). In this paper, we describe a new species from the *D. curvirostris* complex, *Daphnia hrbaceki* **sp. nov**., collected from small pools in Central Europe (Czech Republic and Slovakia). A single sample of *Daphnia* of unusual morphology was collected in Slovakia in 1951 (from a pool at the village Rimavská Baňa); however, no additional material of this taxon was available until recently, when similar individuals were found in a newly recreated small fishless pool in the Czech protected landscape area Kokořínsko. The finding of this apparently rare species, which shares several characteristics with the above-mentioned Eastern Palaearctic taxa, demonstrates that pond and pool habitats may harbour substantial cryptic diversity even in seemingly well-explored regions.

Material and methods

Sampling. Zooplankton samples were collected by plankton nets (mesh sizes 100–200 μm). Localities in the protected landscape area Kokořínsko (Central Bohemia, Czech Republic) were visited three times per year (spring, summer, autumn) in five consecutive years from 2005 to 2009. All samples were preserved either with 96% ethanol or by the addition of formalin to a resulting formaldehyde concentration of approx. 4%. The sample from Rimavská Baňa (southern Slovakia) was collected in 1951 during a student field course and preserved with formalin.

Morphological analyses. We used samples of several related or morphologically superficially similar taxa for comparison with the putative new species (Table 1): *Daphnia curvirostris*, *D. tanakai*, *D. sinevi*, *Daphnia longispina* (O. F. Müller, 1776), *Daphnia minnehaha* Herrick, 1884, *Daphnia pulex* Leydig, 1860, and *Daphnia* sp. (morphotype FLO9), a North American taxon labeled in several publications as *D. arenata* which nevertheless must be considered a *nomen nudum* (see below for a discussion of its nomenclature).

Material used for mounting in permanent slides was transferred to ethanol and stained with lignin pink and chlorazol black E dyes for 24 hours. After staining, specimens were dehydrated with 2-2-dimethoxypropane for 10–15 minutes, then transferred into xylene and mounted in Canada balsam (Kořínek 1999). To see details of the exoskeleton, some specimens were heated for 30 minutes in 10% potassium hydroxide or lactic acid, and washed in distilled water before mounting.

For morphological analyses, we used optical as well as scanning electron microscopy (SEM). Photographs were taken by a Nikon DXM1200F digital camera attached to a Nikon Eclipse E400 optical microscope. Every object under the microscope was photographed several times with different depths of focus. Resulting pictures were consequently merged into one completely sharp picture (Extended Depth of Field).

TABLE 1. Material examined in this study. Abbreviations of country names: BG—Bulgaria, CA—Canada, CH—Switzerland, CZ—Czech Republic, DE—Germany, IL—Israel, JP—Japan, PL—Poland, RU—Russia, SK—Slovakia, UG—Uganda, US—United States of America. Abbreviations of personal names: AGK—A. G. Kirdyasheva, AP—A. Petrusek, AYS—A. Y. Sinev, DV—D. Vondrák, EK—E. Kočárek, MČ—M. Černý, FK—F. Kubíček, HK—H. Kling, HL—H. Loffler, JH—J. Hrbáček, KO—K. Okamoto, OA—O. Albertová, PDNH—P. D. N. Hebert, PJJ—P. J. Juračka, VK—V. Kořínek. Samples used for genetic analyses are marked with asterisks. Precision of geographic coordinates depends on availability of data or size of the locality.

Locality	Geographical coordinates	Sampling date	Collected by:
***Daphnia hrbaceki* sp. nov.**			
CZ: Kokořínsko, pool #17 in Český příkop (type locality)*	N 50°28'54" E 14°41'10'	12 July 2006	PJJ
CZ: Kokořínsko, pool #18 in Žďárský důl	N 50°29'11" E 14°41'24'	10 November 2006	PJJ
SK: Rimavská Baňa, shallow pool	N 48° E 19°	27 April 1951	OA
Daphnia curvirostris			
CZ: Libický luh near Velký Osek, fluvial pools	N 50°06' E 15°10'	April 2007	VK
CZ: Přerov, fluvial pools (including Karasí pool)	N 50°10' E 14°48'	13 samples between 1964 and 2007	VK
CZ: Kokořínsko, Tupadly, experimental pools	N 50°26'16" E 14°28'20"	23 October 2007	DV
CZ: Kadov, fishpond Paseka	N 49°25'25" E 13°47'50"	22 August 1991	VK
CZ: Tchořovice, fishpond Radov	N 49°25'28" E 13°49'13"	22 June 1985	VK
CZ: Slatina, large temporary marsh on a meadow	N 49°23'48" E 13°44'55"	28 April 2008	VK
CZ: Kateřina, nature reserve Soos, pool	N 50°09' E 12°24'	19 April 1959	JH
CZ: Lednice, pools in the Dyje River alluvial plain	N 48°48' E 16°50'	15 samples between 1948 and 2007	VK + other collectors
CZ: Havraníky, shallow pool	N 48°48'54" E 16°00'16"	7 June 2001	VK
CZ: Mutěnice, forest pool	N 48°54' E 17°04'	23 April 1969	FK
CZ: Kunovice, forest fluvial pool	N 49°02' E 17°30'	31 March 2007	MČ
CZ: Moravičany, temporary fluvial pools	N 49°45'21" E 16°58'40"	2 April 2007	MČ
SK: Vinné, Vinianské Lake	N 48°49'06" E 21°59'12"	22 May 1964	JH
PL: Wipsowo, small pool in a peat bog east of village	N 53°54' E 20°49'	21 August 1958	JH
BG: Chelopechene, shallow puddle at fish farm	N 42°44' E 23°27'	14 October 1987	VK
IL: Netanya, temporary pool Dora	N 32°17'25" E 34°50'45"	20 January 2004	AP
UG: Ruwenzori Range, Bujuku Lake	N 0°22'36" E 29°53'35"	September 1967	HL

continued next page

TABLE 1. (continued)

Locality	Geographical coordinates	Sampling date	Collected by:
RU: Borok, temporary puddles	N 58°03' E 38°13'	11 June 2004	AGK
Daphnia minnehaha			
CA: Ontario, Experimental lake area: Lake #81 (Patalas,1971)	N 49°38'49" W 94°04'27"	24 September 1971	HK
CA: vicinity of lake #81, small pool	as above	29 August 1971	VK
***Daphnia* morphotype FLO9**			
US: Oregon, Florence, coastal pond #9	N 44°, W 124°	16 May 1989 15 April 1993	PDNH MČ
US: Oregon, Florence, Sutton Lake	N 44°03'40" W 124°05'21"	16 April 1993	MČ
Daphnia tanakai			
JP: Honshu, Tateyama Mountains, Lake Mikuriga-ike	N 36°34'54" E 137°35'49"	25 September 1978	KO
Daphnia sinevi			
RU: Nakhodka, pond in Avangard	N 42°48' E 132°53'	25 September 2004	AYS
Daphnia longispina			
CZ: Mirovice, abandoned clay pit	N 49°31'01" E 14°03'23"	21 September 1986	VK
DE: Ismaning, Ismaninger Fischteiche, large fishpond	N 48°13'00" E 11°46'08"	22 September 2004	AP
CH: Valais, shallow pond above Great St. Bernard pass	N 45°52'16" E 07°10'12"	6 September 2005	AP
Daphnia pulex			
CZ: Chlístovice, pond	N 49°53'06" E 15°13'30"	1 October 1995	VK
Daphnia obtusa			
CZ: Kokořínsko, Medonosy, small shallow pool *	N 50°30'06" E 14°29'07"	9 March 2010	PJJ

Specimens preserved in 96% ethanol or formalin solution were used for SEM analyses. To clean the surface of foreign particles, specimens were treated with hot 10% potassium hydroxide for 5 to 10 minutes. Remnants of alkali were washed out in distilled water. Specimens were then dehydrated in a graded acetone series and then dried either by critical point drying (using the dryer BAL-TEC CPD 030) or with organic volatile matter hexamethyldisalazane (Laforsch & Tollrian 2000). Dehydrated specimens or body parts were gold-coated for 5 minutes in argon plasma at 10^{-1} millibar vacuum in the BAL-TEC Sputter Coater SCD 050. Gold-coated objects were observed in the JEOL JSM-6380 LV scanning electron microscope at 15 kV. Background surrounding the object was replaced in the micrographs by solid black.

Genetic analyses. To characterise the morphologically unusual *Daphnia* population from the Czech Republic, we amplified three mitochondrial genes commonly used in *Daphnia* diversity studies. Genes for the small ribosomal subunit (12S rRNA) and for the cytochrome c oxidase subunit I (COI) have been traditionally used in studies on *Daphnia* phylogeny (e.g., Schwenk *et al*. 2000; Colbourne *et al*. 2006; Petrusek *et al*. 2009), and are available for the vast majority of *Daphnia* species so far genetically analysed (see Adamowicz *et al*. 2009). Sequences of these genes deposited in the public database (GenBank accession numbers HM625747 for 12S and HM625748 for COI) are therefore useful for any future studies analysing new or rare species in a wider context. The third chosen marker, the rapidly evolving gene for NADH dehydrogenase subunit 2 (ND2), has recently been used to characterise Eastern Palaearctic members of the *D. curvirostris* complex and their phylogenetic relationships (Ishida *et al*. 2006; Kotov *et al*. 2006), and it remains the only mitochondrial marker available for

those taxa. We therefore used it to reconstruct the phylogenetic position of the Czech taxon within the *D. curvirostris* complex, as well as in the wider phylogenetic context. In particular, we included in the phylogenetic analysis the specimen representing *Daphnia obtusa* Kurz, 1874, a species common in the studied area and co-occurring with the studied taxon at its type locality.

Nucleic acid isolation, amplification and sequencing followed previously published protocols. DNA was extracted from single *Daphnia* individuals preserved in ethanol by proteinase K digestion (Schwenk *et al*. 1998). Fragments of 12S rDNA and COI genes were amplified using standard protocols as in Schwenk *et al*. (2000). For ND2, we followed the protocol provided in Ishida *et al*. (2006), using the primer combination MetF2 and TrpR. PCR products were purified and sequenced on ABI 3730XL capillary sequencers by a third party (Macrogen, Seoul, Korea). Resulting sequences (deposited in GenBank under accession numbers HM625747-HM625750) were aligned with sequences of other relevant *Daphnia* species (retrieved from GenBank) using the ClustalW algorithm (Thompson *et al*. 1994) in MEGA version 4 (Tamura *et al*. 2007). The alignments were checked by eye and corrected according to the translated amino-acid alignment, and sequence divergences (Kimura 2-parameter model) were calculated by the same software.

Phylogenetic relationships among species within the *Daphnia curvirostris* complex, including selected taxa from other species complexes of the *D. longispina* group and three members of the *D. pulex* group as an outgroup, were subsequently assessed using a part of the ND2 gene, which was available for all relevant taxa (alignment length 932 bp). We used jModeltest (Posada 2008) to select the best model of nucleotide substitution, and assessed the phylogeny using the Bayesian inference (BI) in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003), and Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses in PAUP* 4.0b10 (Swofford 2002). In BI, two parallel runs of four Monte Carlo Markov chains were run for 3 million generations, trees were sampled every 100 generations, and the first 20% of sampled trees were discarded as a burn-in phase. In PAUP, heuristic searches were conducted with tree bisection-reconnection branch swapping and 10 random sequence taxon additions; branch support was evaluated by nonparametric bootstrapping with 100 (ML) and 1000 (MP) pseudoreplicates.

Abbreviations. CL—Chlístovice, Czech Republic; ELA—Ontario, Canada; FL—Florence, Oregon, USA; H—Havraníky, Czech Republic; I—Ismaning, Germany; K—Kokořínsko (type locality), Czech Republic; KP—Karasí pool, Czech Republic; LL—Libický luh, Czech Republic; LM—Lake Micuriga, Japan; RB—Rimavská Baňa, Slovakia; GSB—Great St. Bernard pass, Switzerland.

Results

Taxonomy

Order Anomopoda Sars, 1865

Family Daphniidae Straus, 1820

Genus *Daphnia* Müller, 1785

Daphnia hrbaceki sp. nov.

(Figs 1–8)

Etymology. The new species is dedicated to the eminent Czech hydrobiologist Jaroslav Hrbáček (1921–2010), who initiated complex ecological studies of *Daphnia* populations in the former Czechoslovakia. The name in the Czech language also reflects the hunched body shape of some individuals.

Type locality. A small fishless, recently (2004) excavated pool in the valley Český příkop (protected landscape area Kokořínsko, Czech Republic); N 50°28'54", E 14°41'10", alt. 289 m above sea level. The pool is 7 m long and 3 m wide with maximal depth ca. 2 m, situated in a deep, shaded valley with a cold microclimate. The type series was collected on 5 November 2007 by P. J. Juračka.

Holotype. Adult parthenogenetic female (total body length 1.7 mm) mounted in Canada balsam and stained with a mixture of lignin pink and chlorazol black E; Natural History Museum, London (NHM 2010.39).

Allotype. Adult male (body length without shell spine 1.0 mm) mounted and stained as above (NHM 2010.40).

Paratypes. Males and females (45 specimens), preserved in 96% ethanol and a small amount of glycerol (NHM 2010.53-62). Additional specimens from the type series are deposited in the collection of the National Museum, Prague (P6E3005).

Ephippial female (total body length 1.5 mm) stained and mounted as above (NHM 2010.41).

Dissected parthenogenetic female treated with hot 10% potassium hydroxide and mounted as above (NHM 2010.42).

Females and males (13 specimens) stained and mounted as above (NHM 2010.43-52).

Diagnosis. *Parthenogenetic female* with median keel on head shield, some populations with induced necktooth on its posterior margin. Similar neckteeth may be present in juveniles and males. Antennule completely reduced, median mound strongly vaulted with reticulated apex. Ocellus pigmented. Shallow cervical depression. Shell spine short or absent. Gnathobase of second thoracic limb extended distally into angular projection. Postabdominal claw with second (middle) pecten of spinules or teeth of variable size and shape: either spinules slightly longer than those in proximal pecten, or large teeth longer than width of claw.

Ephippium saddle-shaped, dorsal ridge smooth (without spinules), only reticulated; posterior carapace margin included into ephippium. Ephippial surface ultrastructure with many minute pits surrounded by fine lamellae.

Male with medium-sized rostrum hardly covering antennular socket. Antennule short, two to three times longer than wide. One of the three terminal setae on 2nd endopodite bent and heavily setulated. Pre-anal margin of postabdomen weakly depressed, anal margin convex.

Size. Total body length (without shell spine): parthenogenetic female 1.0–1.7 mm; ephippial female 1.2–2.0 mm; male 0.9–1.2 mm.

Description. Parthenogenetic female. *Head*: high, strongly vaulted apical part with median keel increasing in width dorsally. Keel extremely developed in some individuals; forming hump-shaped structure (Figs. 1C, E; 2B, F). Neckteeth rarely present in adult females (Figs. 1E; 2B, E). Dorsal margin with shallow cervical depression (Fig. 1B). Frontal contour of head concave above rostrum. Rostrum not prominent, its tip bent ventrally in some specimens. Tip of rostrum obtusely rounded and split into two lobes by suture or line between head shield and ventral side of head in lateral aspect (Fig. 4C, E). Mid-antennular mound well developed, markedly reticulated on apex. Optic vesicle contiguous with frontal part of head. Ocellus pigmented. Fornix rounded at base of second antenna.

Antennule: not protruding, its body reduced, seen as lateral areole on median mound with 9 sensory setae; single lateral seta anterior to areole (Fig. 4C, E).

Antenna: Setal formula of natatory setae: 0-0-1-3/1-1-3. Presumably sensorial setae and spinules: two setae on concertina-like basal joint, one apical spine-like on its outer side, one seta on inner side between both branches, one apical spinule on dorsal margin of second segment 4-segmented branch. Dark rings at base of distal part of swimming setae may be present in some individuals or populations. Surface of all segments covered with transversal groups of small teeth.

First maxilla: carrying three robust, curved and heavily setulated setae and one short stump-like distal seta.

Carapace: approximately sub-ovoid, length of posterior spine variable, forming up to 15% of body length (without shell spine) or completely reduced. Spinules on ventral margin cover 1/3 to 2/3 of its length, spinulation on dorsal margin developed only in posterior 1/4 of margin or only near posterior spine. Spinulation of dorsal margin completely missing in some individuals. Fringe of sub-marginal setae absent.

Thoracic limbs: agree with the re-description of *Daphnia curvirostris* in Ishida *et al*. (2006) with the exception of 2nd limb gnathobase, which extends in front of longest clearing seta into noticeable rectangular corner or small lobe (Fig. 5E, F).

Postabdomen: elongated, tapering distally, pre-anal face even, covered with scattered groups of fine spinules, anal margin slightly convex, fringed with up to 15 strong teeth that increase in length distally. Distal portion of postabdominal setae slightly shorter than proximal one. Abdominal processes gradually diminishing distally, first twice as long as second, third reduced to 1/3 up to 1/2 of second one in specimens preserved in formalin. Terminal claw long, with three groups (pectens) of teeth and spinules. Proximal one of 13–19 minute spinules, middle pecten variable in size: either 8–9 large teeth markedly longer than width of claw or 11–13 spinules that only slightly exceed in length those of other two pectens. Distal row of about 60 fine spinules, not reaching tip of claw. Differences in size and length of claw spinules were observed among samples collected in different times of season, and between individuals from the wild and those cultured in laboratory (Fig. 3A–D).

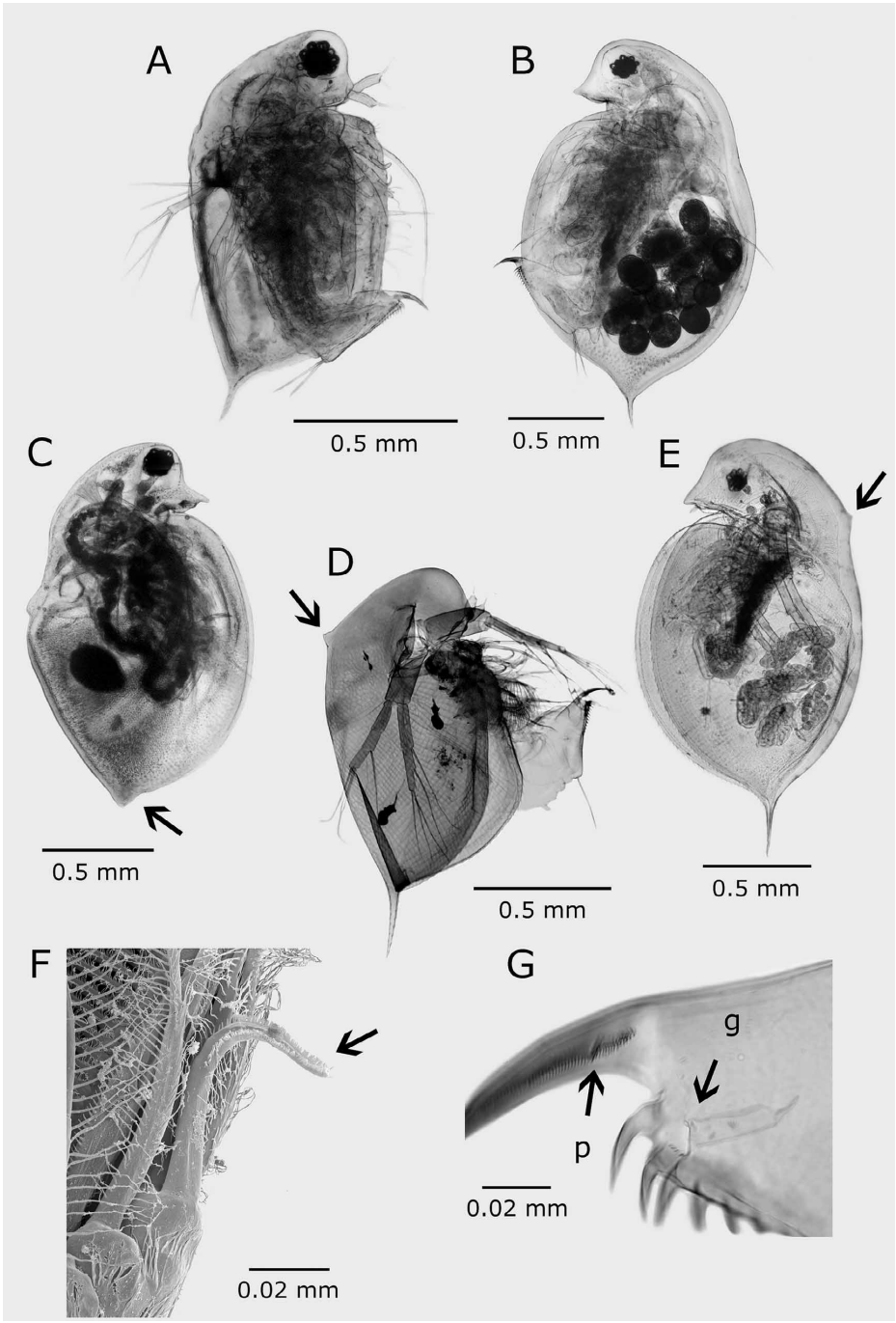

FIGURE 1. *Daphnia hrbaceki*. A. Adult male (K). B. Adult parthenogenetic female (K). C. Adult ephippial female (K). D. Adult male (RB) with necktooth indicated by arrow. E. Adult parthenogenetic female (RB) with morphology presumably induced by invertebrate predators; arrow indicates a hump-shaped dorsal outline of the carapace. F. Adult male (RB), hook-like apical seta (2nd limb) indicated by arrow. G. Adult male (RB), postabdomen (contrast increased at gonopore area); arrows indicate gonopore (g) and middle pecten on postabdominal claw (p).

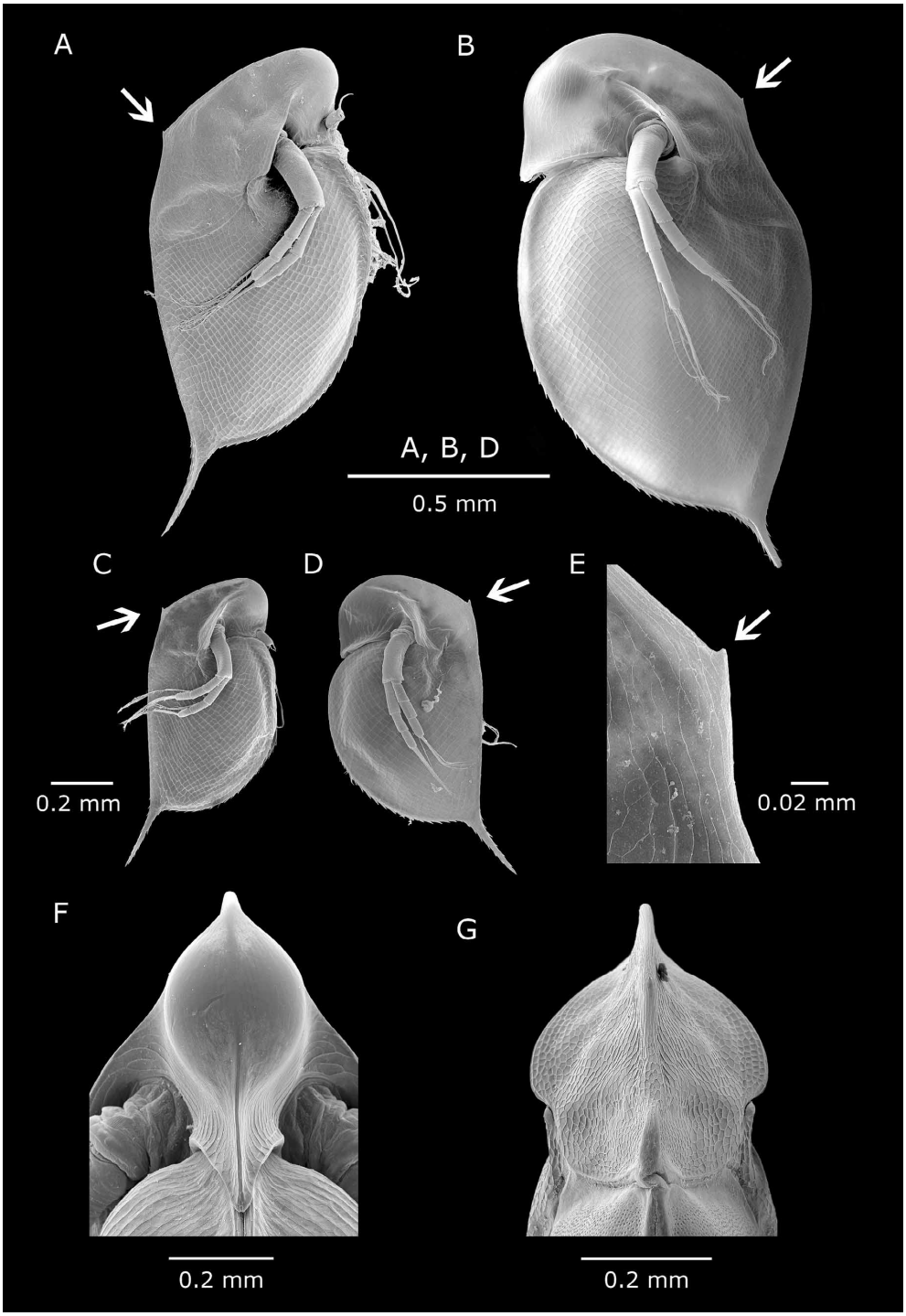

FIGURE 2. *Daphnia hrbaceki*. Arrows indicate neckteeth. A. Adult male (RB); B. Adult parthenogenetic female (K); C. Juvenile male (RB); D. Juvenile female (RB); E. Subadult female (RB), detail of necktooth; F. Head of adult parthenogenetic female (K) in antero-ventral aspect. G. Head of adult ephippial female (K) in dorsal aspect.

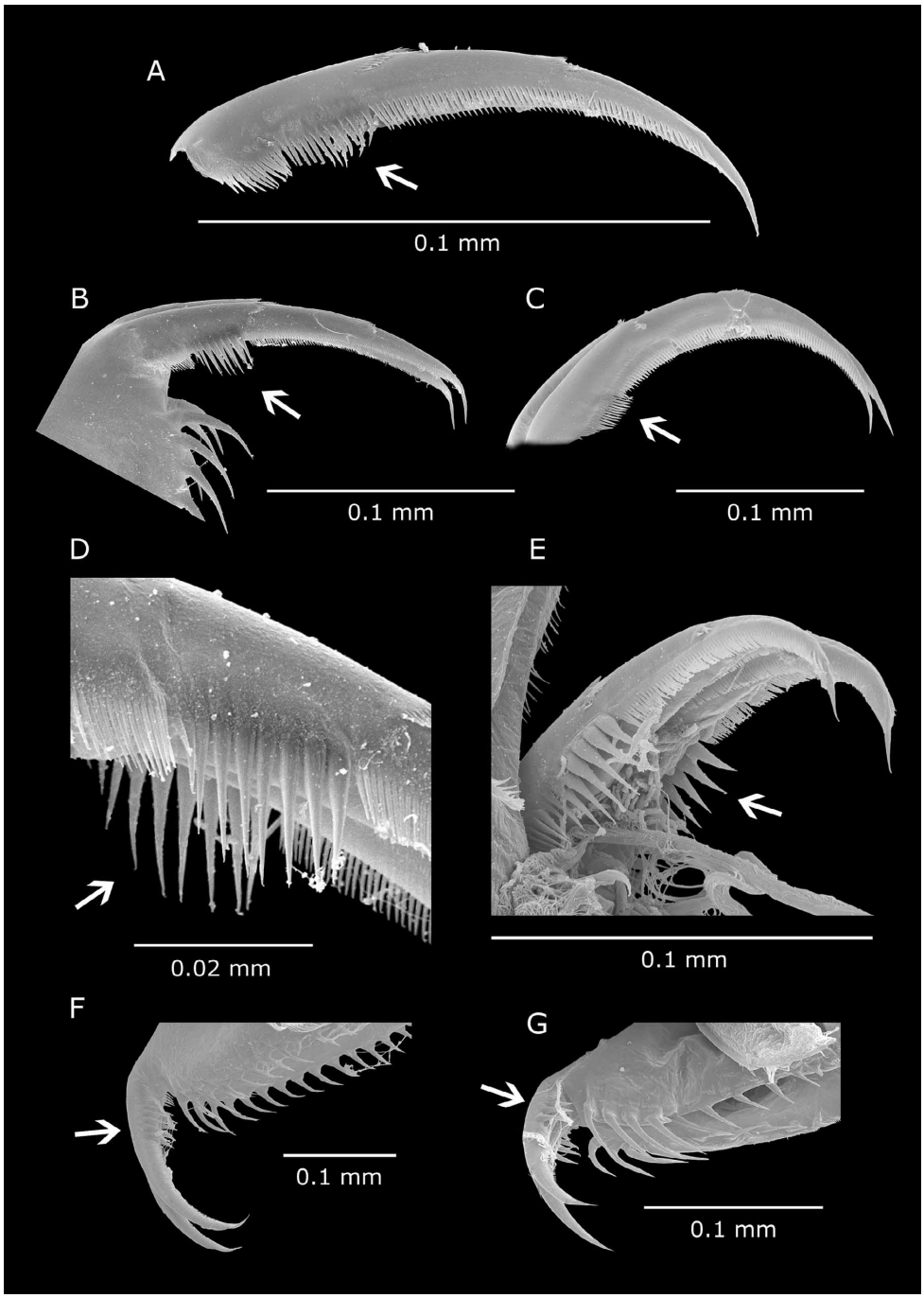

FIGURE 3. Comparison of postabdominal claws. Arrows indicate second (middle) pecten of spinules or teeth of postabdominal claw. A. *Daphnia hrbaceki*, adult female (K). B. *D. hrbaceki*, adult female from laboratory culture (K). C. *D. hrbaceki*, adult female (RB). D. *D. hrbaceki*, adult female from laboratory culture (K); detail of middle pecten. E. *D. hrbaceki*, adult male from laboratory culture (K). F. *Daphnia* sp. (morphotype FLO9), adult female (FL). G. *D. minnehaha*, adult female (ELA).

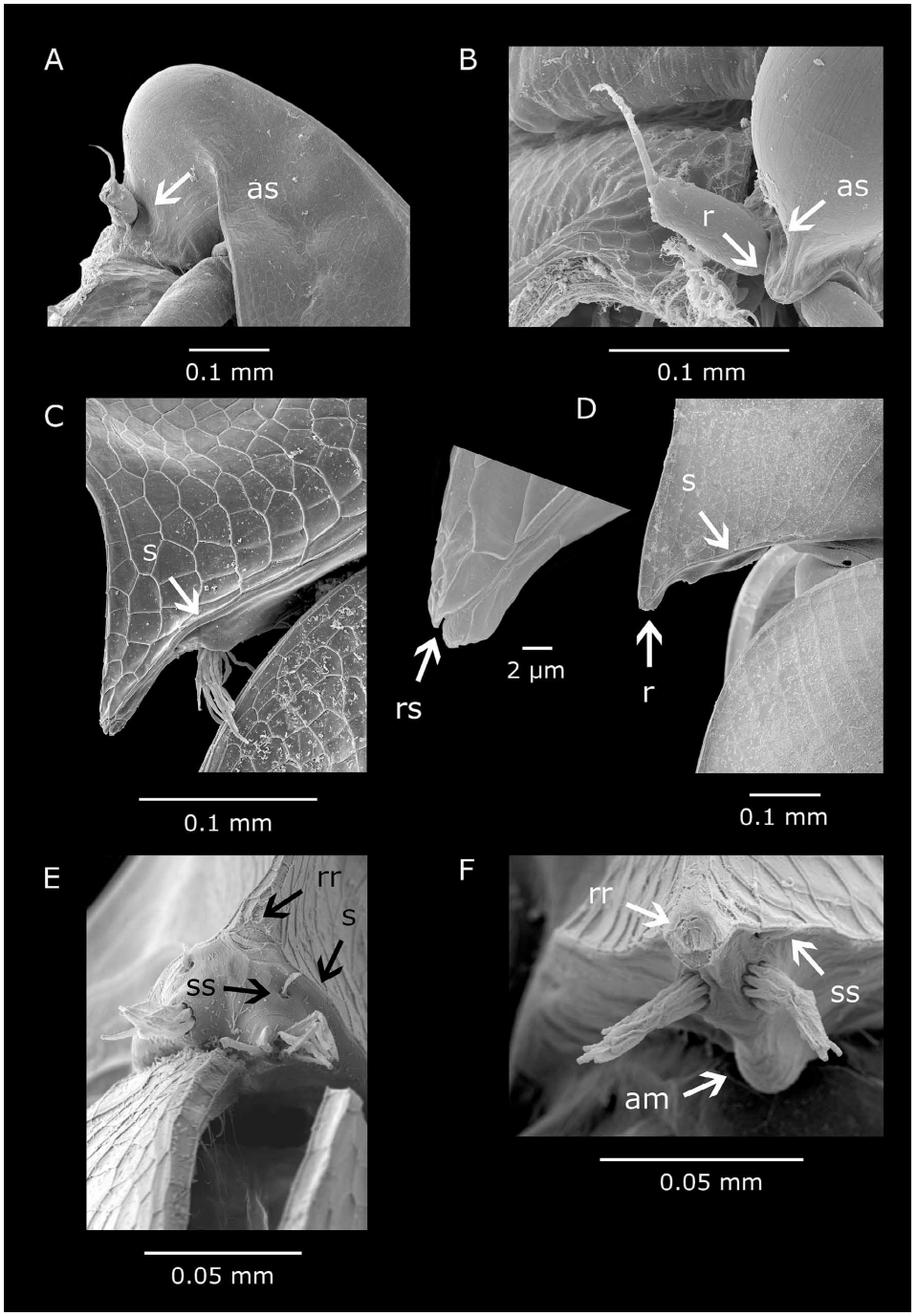

FIGURE 4. *Daphnia hrbaceki* and *Daphnia curvirostris*. Arrows indicate male antennular socket (as), suture between head shield and ventral side of head (s), single lateral seta anterior to areole (ss), rostrum (r), reticulation on the tip of rostrum (rr), apparent split of the rostrum (rs) and antennular mound (am). A, B. *D. hrbaceki,* head of adult male (RB) C. *D. hrbaceki*, adult female (K); rostrum and antennule, lateral aspect. D. *D. curvirostris*, adult female (H); rostrum and antennule, lateral aspect; detail of the rostrum tip in lateral aspect shown on the left. E. *D. hrbaceki*, adult female (K); rostrum, postero-frontal aspect. F. *D. curvirostris*, adult female (KP); rostrum, frontal aspect.

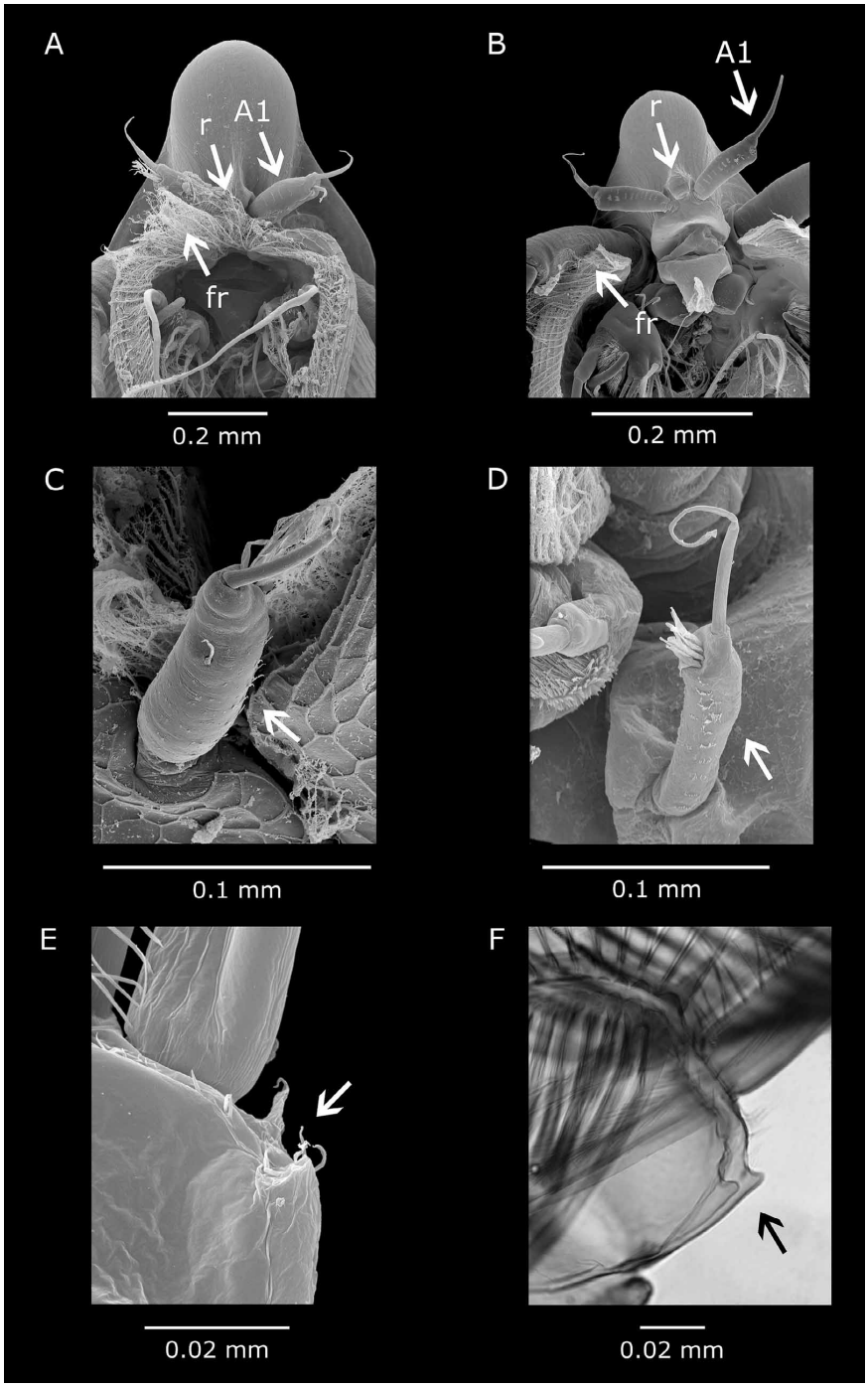

FIGURE 5. *Daphnia hrbaceki* and *Daphnia curvirostris*. A. *D. hrbaceki*, adult male (RB); antennules (A1) and rostrum (r), arrow (fr) indicates valves fringed with row of long, sub-marginal feathered setae. B. *D. curvirostris,* adult male, arrows as in Fig. 5 A (LL). C. *D. hrbaceki*, adult male (K); antennule (indicated by arrow). D. *D. curvirostris,* adult male (KP); antennule (indicated by arrow). E, F. *D. hrbaceki*, adult females (RB); 2nd thoracic limb, gnathobase, arrows indicate gnathobase extending distally into angular projection.

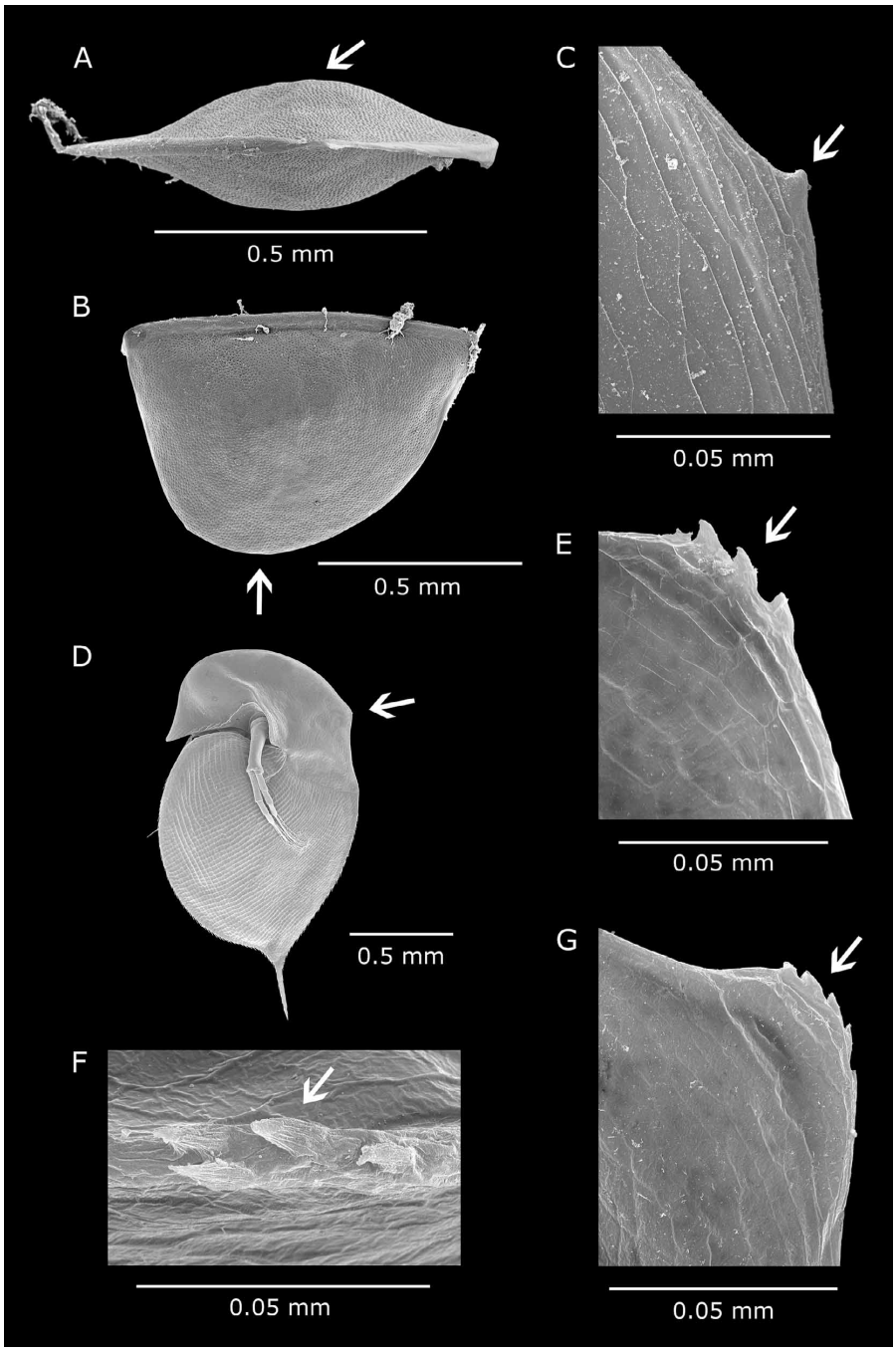

FIGURE 6. Ephippium and neckteeth. A. *Daphnia hrbaceki*, dorsal aspect of free ephippium (K); arrow indicates evenly shaped convex outline lacking any concavity between the two egg chambers. B. *D. hrbaceki*, lateral aspect of free ephippium (K); arrow indicates position of maximal width of the ephippium. C. *D. hrbaceki*, adult female (RB); detail of necktooth (indicated by arrow). D. *D. minnehaha*, adult female (ELA) with neckteeth (indicated by arrow). E. *D. minnehaha*, adult female (ELA); detail of neckteeth (indicated by arrow). F. *D. minnehaha*, juvenile female (ELA); dorsal aspect, detail of neckteeth (indicated by arrow). G. *Daphnia* sp. (morphotype FLO9) (FL); detail of neckteeth (indicated by arrow).

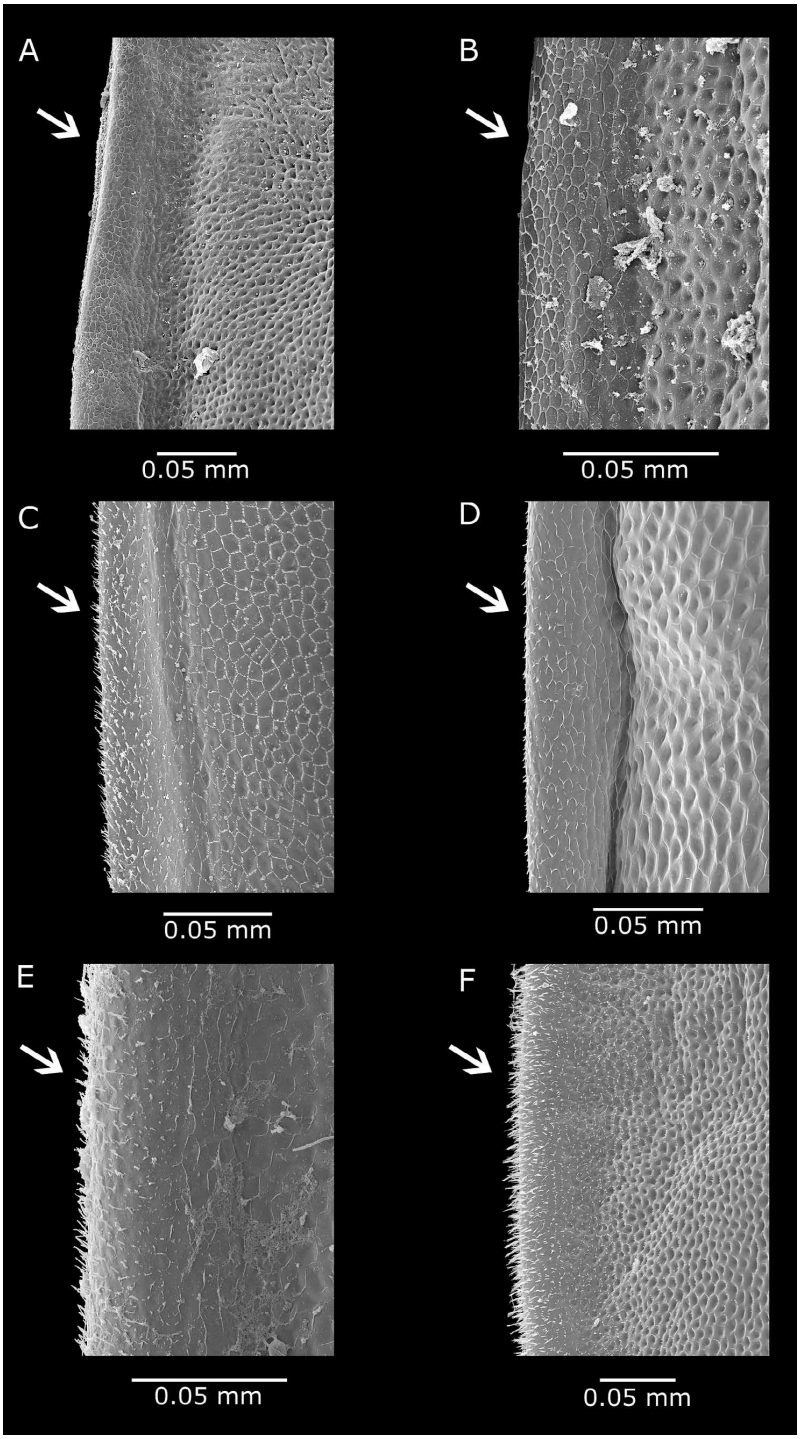

FIGURE 7. Comparison of ultrastructures of ephippial dorsal ridges with various development of spinulation or reticulation (indicated by arrows). A, B. *Daphnia hrbaceki* (K). C. *D. curvirostris* (H). D. *D. tanakai* (LM). E. *Daphnia* sp. (morphotype FLO9) (FL). F. *D. minnehaha* (ELA).

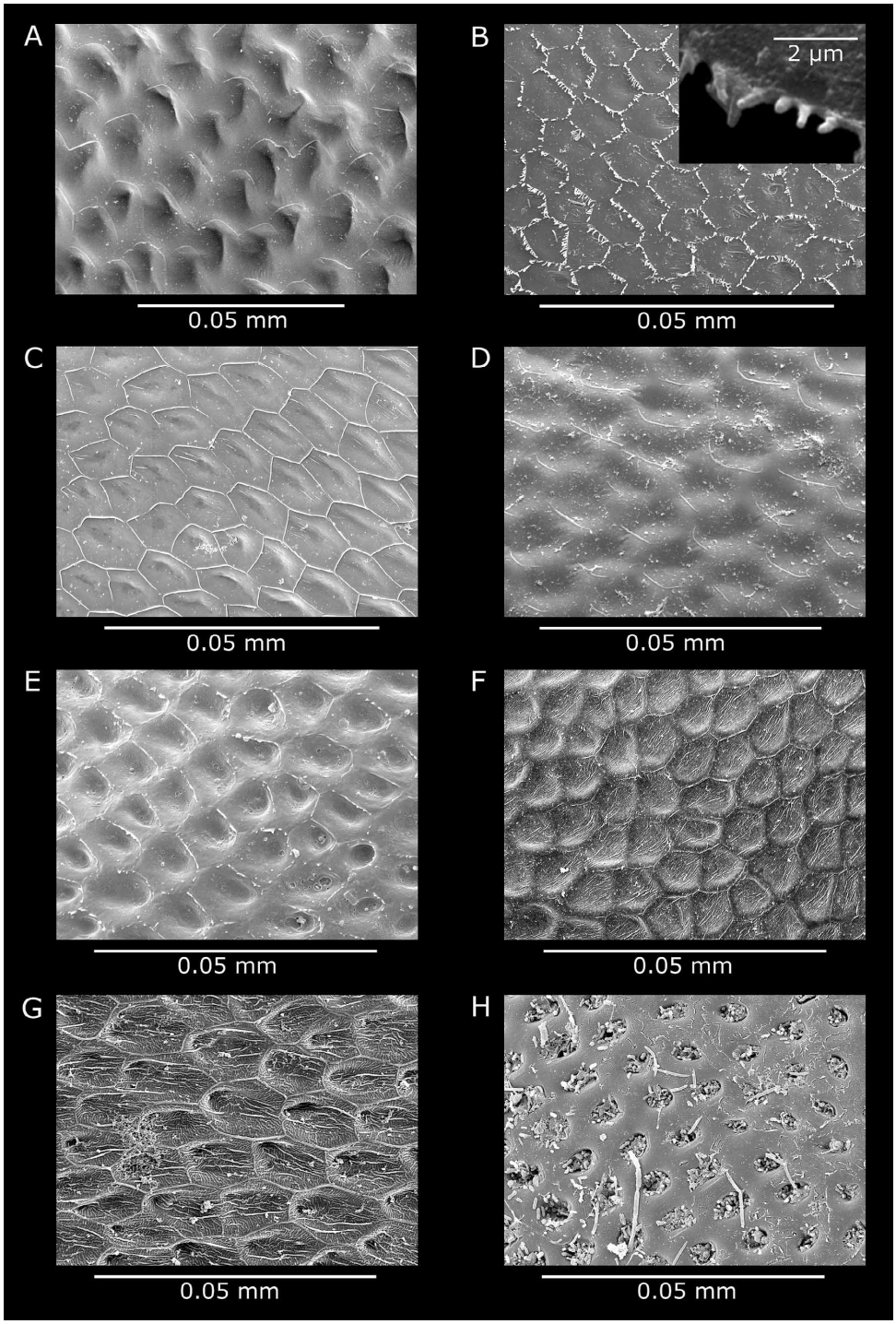

FIGURE 8. Comparison of ephippial surface ultrastructures. A. *Daphnia hrbaceki* (K). B. *D. curvirostris* (LL); detail shown in inset. C. *D. tanakai* (LM). D. *Daphnia* sp. (morphotype FLO9) (FL). E. *D. minnehaha* (ELA). F. *D. pulex* (CL). G. *D. longispina* from high-altitude temporary pool (GSB). H. *D. longispina* from lowland fishpond (I).

Ephippial female. Dorso-posterior part of head shield swollen, forming bulge over dorsal suture between carapace and head shield (Figs. 1C, 2G). Ephippial surface covered with sclerotized pneumatic cells reaching up to postero-dorsal angle of shell without any gap. Two resting eggs perpendicular to dorsal margin, egg chambers well separated from each other. Free post-molting ephippium (Fig. 6A, B) asymmetrically saddle-shaped, with maximal width between centre and proximal third of its length. Dorsal ridge without any spinescence, only reticulated (Fig. 7A, B). Postero-dorsal corner includes part of vaulted posterior margin and remnants of short shell spine, which is lost in older, freely floating ephippia. Surface ultrastructure with many minute pits surrounded by fine lamellae (Fig. 8A).

Male. *Head*: rounded in frontal part around optic vesicle, apical contour only feebly convex, gradually descending dorsally to level of attachment of posterior antennal muscle or to necktooth (if present) (Figs. 1A, 2A, 4A). Compound eye large, filling half of frontal portion of head shield, ocellus pigmented. Obtuse rostrum short, covering only antennular socket. Antennular (ventral) part of head extends ventrally forming posterior wall of antennular sockets (Fig. 4A, B).

Antennule: in adult males directed towards compound eye, its segment short, two to three times longer than wide, reaching hardly to pigmented part of compound eye. Flagellum inserted on conical butt elevated over shallow socket for sensory papillae. Dorsal seta inserted distally at about four fifths of antennular length (Figs. 4B, 5C).

Antenna: surface sculpture of all segments weaker than in female.

Carapace: ventral aspect: wide anterior gap between valves fringed with row of long, sub-marginal feathered setae. Setae most densely spaced along anterior fold of valves, gradually shortened to mid carapace margin (Fig. 5A). No gap or sub-marginal setae at distal part of ventral margin, only small marginal spines and groups of sub-marginal setules present. Dorsal margin feebly convex.

Thoracic limbs conform with the description of *Daphnia curvirostris* male in Ishida *et al*. (2006). Hook-like seta of 2nd limb is shown in Fig. 1F.

Postabdomen: all abdominal processes reduced, proximal one very small, others mostly missing. Pre-anal part with shallow depression, anal margin convex, fringed with up to 12 lateral spines (Fig. 1D). Gonopores open ventrally of last three largest marginal spines (Fig. 1G). Distal part of postabdominal setae slightly shorter than their proximal part. Middle pecten on terminal claw with either 6–7 spines or 10–12 spinules (Fig. 3E).

Differential diagnosis. The new species has to be differentiated from several other taxa present in the region of its occurrence: *Daphnia curvirostris*, members of the *Daphnia pulex* group, and *Daphnia longispina* (O. F. Müller, 1776), as well as related taxa in Asia and two taxa showing some similarities in North America. The main differential characters are listed in Tab. 2. Among locally occurring species, females in the *D. pulex* group are clearly distinguished by well developed antennules protruding from the antennular mound which contrast with the reduced, non-protruding antennule of *Daphnia hrbaceki*. *Daphnia longispina* has a flat, reduced inter-antennular mound, but parthenogenetic females in some of the populations are difficult to distinguish from those of *D. hrbaceki* that do not have enlarged middle pecten of the postabdominal claw. The *Daphnia longispina* ephippium is also widest in the anterior third of its length, its dorsal ridge covered with spinules and a shell spine always part of the free ephippium compared with the smooth dorsal ridge of the *Daphnia hrbaceki* ephippium whose greatest width is about mid-length. The apical stiff seta on the male second endopodite of *Daphnia longispina* is S-shaped, armed with a row of robust teeth or thorns; that of *D. hrbaceki* is hook-like (Fig. 1F), its distal part fringed on both margins with dense rows of spinules. The *Daphnia curvirostris* ephippium is asymmetrically saddle-shaped and widest at the proximal third of its length, with the dorsal ridge covered densely with minute spinules (Fig. 8B). Ephippial surface covered with small pits framed with rows of small, blunt spinules (Fig. 8B). *D. curvirostris* males have a longer basal segment of the antennule reaching nearly to the anterior margin of the pigmented part of the compound eye (Fig. 5D), whereas this reaches only the posterior contour of the eye in *D. hrbaceki* (Fig. 5C).

Two other related species have been described from eastern Asia (Japan and the Russian Far East): *Daphnia tanakai* and *Daphnia sinevi*. The ephippium of *Daphnia tanakai* does not include the carapace posterior margin; the postero-dorsal corner of the ephippium is obtusely rounded. The ephippial dorsal ridge is covered with sparsely distributed fine spinules (Fig. 7D). A wide gap is present between ephippial surfaces (covered with large sclerotized cells) and the posterior margin of carapace, separated by ecdysial suture. Ephippial surfaces are covered with shallow dimples and a pattern of hexagonal fine lamellae (Fig. 8C). Males have a reduced rostrum and a long antennule. *Daphnia sinevi* has a robust inter-antennular mound with slightly protruding tips of antennules. The

TABLE 2. Main differential morphological characters among *Daphnia hrbaceki*, *Daphnia curvirostris*, *Daphnia tanakai*, *Daphnia sinevi*, *Daphnia longispina* (populations from small, fishless habitats), *Daphnia minnehaha* and *Daphnia* sp. (morphotype FLO9, denoted as *D. arenata* in some studies).

Character	*D. hrbaceki* **sp. nov.**	*D. curvirostris*	*D. tanakai*	*D. sinevi*	*D. longispina*	*D. pulex*	*D. minnehaha*	morphotype FLO9
Female								
head								
documented ability to produce neckteeth (Fig. 6)	yes	yes (rarely)	no	yes	yes	yes	yes	yes
antennule body (Fig. 4)	reduced	reduced	reduced	reduced	reduced	prominent	slightly prominent	slightly prominent
carapace								
sub-marginal row of long setae	absent	absent	absent	absent	absent	absent	absent	present
thoracic limbs								
2nd gnathobase-posterior margin (Fig. 5)	extended, keel-like	rounded	rounded	rounded	rounded	rounded	rounded	rounded
postabdomen - terminal claw								
middle pecten (Fig. 3)	large teeth or fine spinules	large teeth	large teeth of fine spinules	large teeth	fine setules	large teeth	large teeth	large teeth
ephippium								
postero-dorsal portion of carapace incorporated in ephippium	yes	yes	not	yes	yes	yes	yes	yes
dorsal spinescence (Fig. 7)	absent	present	present	present	present	present	present	present
surface ultrastructure (Fig. 8)	minute pits surrounded by fine lamellae	minute pits surrounded by rows of blunt spinules	shallow dimples with remnants of longitudinal striation	not studied	variable	dimples surrounded with well developed lamellae	shallow pits surrounded by toothed lamellae	shallow dimples with remnants of surrounding lamellae
Male								
2nd limb, terminal seta on last endite	bent	bent	bent, but variable	strongly bent	not bent	bent	bent	robust. slightly bent
distal portion postabdomen	setulated	setulated	setulated	setulated	with strong teeth	setulated	setulated	setulated
dorsal margin, anal region	even	even	slightly convex	slightly convex	even	shallow depression	deep depression	deep depression
1st postabdominal processes	reduced	reduced	reduced	reduced	reduced	long, reaching over insertion of postabdominal setae	short, reaching up to insertion of postabdominal setae	short, reaching up to insertion of postabdominal setae

ephippium is saddle-shaped, widest at the posterior third of its length, its dorsal ridge with fine spinules. The postero-dorsal corner of the ephippium is horn-shaped, not rounded. Male has long, slender antennule.

The body shape of some individuals of *D. hrbaceki* may superficially resemble *Daphnia minnehaha* (Fig. 6E) and *Daphnia* sp. (morphotype FLO9, denoted as *D. arenata* in some studies) occurring on the North American continent. Both species have antennular tips partly protruding from the base of the head shield, and individuals of the FLO9 morphotype carry a row of sub-marginal plumose setae similar to those in the *D. obtusa* complex or in the subgenus *Ctenodaphnia* (this characteristic of the American taxon was omitted in Hebert 1995); such a row of plumose setae is not observed in *Daphnia hrbaceki* and *D. curvirostris*. Dorsal ridges of ephippia of both American species are covered with spinules (Fig. 7E, F) in contrast to the smooth reticulated dorsal ridge of *D. hrbaceki*. Males of American species have a deep depression in the pre-anal part of the postabdomen contrasting with the even or slightly convex anal region in *D. hrbaceki*.

Other material examined. *Daphnia hrbaceki*: Czech Republic, Kokořínsko, small pool (N 50°29'11", E 14°41'24"), 13 July 2006, P. J. Juračka legit. *Daphnia* cf. *hrbaceki*: Slovakia, Rimavská Baňa, (48.5° N, 19.9° E) fluvial pool, 27 April 1951, O. Albertová legit. The first author sampled most pools in the vicinity of Rimavská Baňa village recently (three times in 2006–7) but without success. In the original sample from the mid 20th century, no other *Daphnia* species was present.

Distribution. So far, *Daphnia hrbaceki* has only been found in two isolated pools in Central Bohemia and at another locality in south-eastern Slovakia (for the Slovak sample, no DNA data is available). Apart from the type locality, the species was found in a similar pool created in 1999, located about 500 m away. Cladoceran fauna of the region where *D. hrbaceki* was discovered had been studied for at least one century. The species is thus certainly very rare and it is difficult to judge the area of its distribution. However, other populations may have escaped detection (being confused with *D. curvirostris* or other species) if individuals did not exhibit the characteristic hump-shaped body profile.

Ecology. The species was sampled in the summer zooplankton and survived up to the beginning of winter. It was outcompeted in spring by co-occurring *Daphnia obtusa*. Both species coexisted in summer. Summer water conditions: conductivity fluctuated within the range 39–768 $\mu S.cm^{-1}$; pH 5.7–7.8; temperature up to 17.4 °C; dissolved oxygen 1.8–10.4 $mg.l^{-1}$. The species was successfully cultivated in the laboratory on a diet of green algae (mostly *Scenedesmus*).

Genetic analyses. All analysed mitochondrial genes of the analysed Czech *Daphnia* clearly showed a considerable divergence from all other so-far genetically characterised species in the genus: the genetically most similar species, *Daphnia curvirostris*, diverged by 13% at 12S, 23% at COI, and 41% at ND2 (all Kimura 2-parameter distances); other analyzed species, including all other known members of the *D. curvirostris* complex, diverged substantially more (over 46.8% at ND2; Fig. 9). The divergence of the syntopically occurring *D. obtusa* (belonging to the *D. pulex* group) from *D. hrbaceki* exceeded 63% at ND2. No variation in sequences of any of the three mitochondrial genes was observed in several analysed individuals of *D. hrbaceki*.

The GTR+I+G model of nucleotide substitution consistently performed best among the different approaches to model selection, based on the 932 bp long alignment of ND2 sequences. All applied methods of phylogenetic reconstructions supported the sister relationship between the new species and *D. curvirostris* despite their relatively high divergence. The support for monophyly of the *D. curvirostris* complex was weaker but the whole complex was unambiguously assigned as a sister taxon of the *D. longispina* complex (Fig. 9).

Taxonomic and nomenclatural comments. *Daphnia hrbaceki* could be characterized both morphologically and genetically. Its morphological peculiarities have been known for more than fifty years, but difficult to evaluate as there was only a single sample from Slovakia available. The recent discovery of populations in Central Bohemia allowed DNA analyses and a comparison of both morphology and genetics with recently described East Asian taxa. The morphological diagnosis of the species and its membership within the *D. curvirostris* complex were thus substantiated.

Comparison with other taxa described over century ago from Japan (*Daphnia whitmani* Ishikawa, 1895 and *Daphnia morsei* Ishikawa, 1895) is difficult as the original drawings are inadequate and the descriptions do not mention some important characters. For instance, the ephippium of *D. whitmani* is traced as not reaching to the posterior margin of carapace in Fig. 4 in Ishikawa (1895), but clearly incorporating it in Fig. 4b in the same work. In general, *D. whitmani* seems to be similar to the recently described *Daphnia sinevi*. The male of *D. morsei* has a remarkably deep depression of the pre-anal or anal part of the postabdomen. A genetically clearly divergent

Daphnia population found recently in Japan may have belonged to this taxon (Kotov *et al*. 2006). Recent genetic analysis (Kotov & Taylor 2010) nevertheless suggested that the above-mentioned taxa described by Ishikawa likely belong to the *D. pulex* group and are therefore unrelated to the *D. curvirostris* complex.

Both American species mentioned in the differential diagnosis are in great need of re-description. Hebert (1995) documented some of their morphology on his CD-ROM on North American *Daphnia* fauna. While *Daphnia minnehaha* was described by Herrick (1884) according to the rules applied in the time of publication and the use of this name is not in doubt, the description of *Daphnia arenata* is lacking some of the attributes required by the International Code of Zoological Nomenclature. No types were designated, the description contained neither a short diagnosis nor differential diagnosis, and the text of the description itself was substituted by a set of microphotographs illustrating selected morphological characters. Coastal pond #9 at Florence (Oregon) was designated the type locality. The name *Daphnia arenata* has already been used in other regular publications (e.g., Colbourne *et al*. 1997; Benzie 2005; Mergeay *et al*. 2008). This situation clearly suggests that the name has to be considered a *nomen nudum*. The problem with the nomenclature of several North American taxa first named in Hebert (1995) is discussed in details in Benzie (2005). Therefore we prefer to label our comparative material as *Daphnia* sp. (morphotype FLO9).

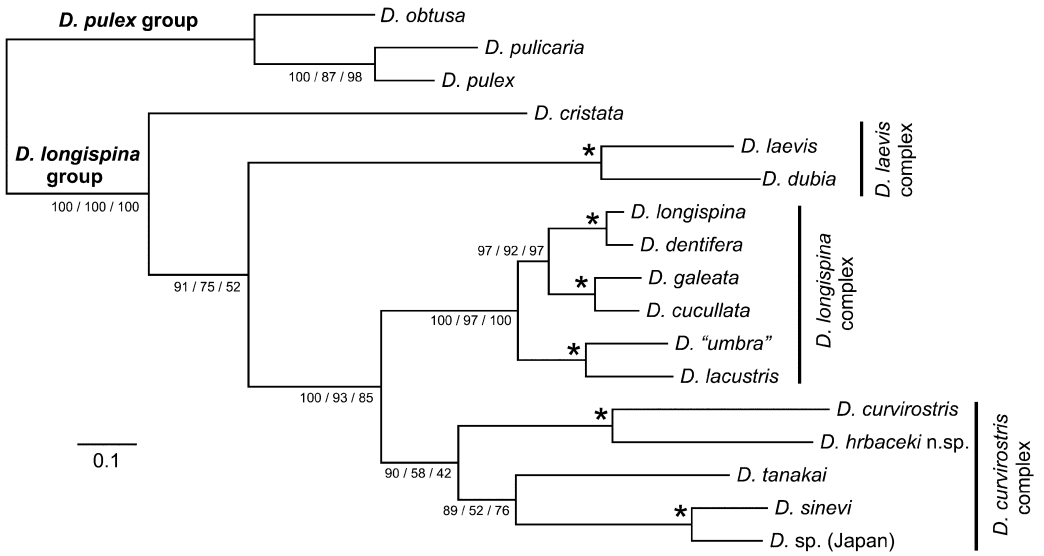

FIGURE 9. Relationship among species of the *Daphnia curvirostris* complex and its position relative to other species complexes (represented by selected taxa) of the *D. longispina* group (nomenclature of the *D. longispina* complex follows Petrusek *et al*. 2008). Three members of the *D. pulex* group, including *D. obtusa* coexisting with *D. hrbaceki*, were used as outgroups. The tree was constructed by the Bayesian inference of phylogeny from a partial sequence of the mitochondrial ND2 gene. Node support is provided for Bayesian inference, Maximum Likelihood and Maximum Parsimony analyses, asterisks indicate sister species with support at least 99% in all three analyses. Vertical bars delineate species complexes, scale indicates 10% divergence.

Discussion

Daphnia hrbaceki is the closest relative of *D. curvirostris* identified to date, although the level of genetic divergence between these two species is substantial. The new species shares several characteristics with other recently described species of the *D. curvirostris* complex. It is the second *Daphnia* species of the *curvirostris* complex after *D. tanakai* that shows substantial variation in the size of the middle postabdominal pecten even within the same population. This confirms that this character may not be as stable as previously thought, and populations differing solely in such a feature should be carefully compared by other means (see also Ishida *et al*. 2006 and Kotov *et al*. 2006 for discussion).

Our study has some implications for the use of certain morphological characters in *Daphnia* taxonomy. In particular, it demonstrates the usefulness of structures on the ephippial surface; the ephippial ultrastructure is a

character reliably differentiating *D. hrbaceki* from morphologically similar *D. curvirostris*. On the other hand, we could not support the use of the differential character for the species in the *D. curvirostris* complex introduced in Kotov *et al*. (2006): the lateral bilobate aspect of the rostral part of the head. The detailed analysis of our SEM pictures (Fig. 4C–F) shows that ventral (antennular) part of the head is separated by a more or less noticeable suture present in all *Daphnia* species. The more or less swollen or vaulted tip of the antennular plate is variably expressed in living individuals and may be influenced with formalin or ethanol preservatives.

Known populations of *D. hrbaceki* are characterised by the presence of antipredator morphological structures. Juveniles commonly formed neckteeth (Fig. 2C, D), previously documented within the *D. curvirostris* complex only in the recently described *D. sinevi* (Kotov *et al*. 2006). However, neckteeth seem to be occasionally observed in *D. curvirostris* as well. Careful inspection of the comparative material originating from Czech pools with *Chaoborus* larvae revealed that a small necktooth in the first and occasionally in the second juvenile instars is commonly present but missing in older instars and adults. Interestingly, it might be retained also in adult males, as seen in some specimens collected in Central Bohemia (D. Vondrák, unpubl. data). The presence of this morphological feature in the *D. curvirostris* complex therefore deserves further attention. Small fishless pools, the habitat of the above-mentioned species as well as of *D. hrbaceki*, are often characterised by strong invertebrate predation (Arnott & Vanni 1993). Larvae of *Chaoborus* phantom-midges, which are commonly observed in the type locality of *D. hrbaceki*, are among the most important predatory invertebrates in such habitats (e.g., Kvam & Kleiven 1995; Young & Riessen 2005). Neckteeth, formed especially in juvenile individuals of various *Daphnia* species (Colbourne *et al*. 1997; Kotov *et al*. 2006), have long been known to efficiently increase resistance to this predator (Havel & Dodson 1984; Repka *et al*. 1995). Additionally, Laforsch *et al*. (2004) recently showed that the defensive mechanism accompanying neckteeth formation is much more complex, and involves substantial strengthening of the whole carapace.

D. hrbaceki is able to retain the neckteeth after achieving maturity (Figs. 1D, E; 2A, B, E), a feature rarely observed in other *Daphnia* species. Such *D. hrbaceki* adults usually exhibit morphotypes with a hump-shaped dorsal body outline, a prominent feature that first suggested that the studied population is unique. Among other congeneric species, the North American *D. minnehaha* (which also tends to form hump-shaped morphs in the presence of predators) may retain neckteeth after maturity, usually in conditions of low food concentration and high *Chaoborus* predation pressure. With a better food supply, adults of this species may tend to lose neckteeth (Riessen & Young 2005). *Daphnia hrbaceki* seems to show a similar reaction to food conditions and predator density, as suggested by changes of the prevailing morphotypes in the type locality over time. A year after the habitat was created, under high transparency (Secchi depth over 1 m) and apparently low food densities, hump-shaped adults with neckteeth were frequent in the population (around 80% of all adult individuals). Two to three years later, the nutrient content of the pool seems to have increased: transparencies dropped to 20 cm, chlorophyll-a concentration reached 50 $\mu g.l^{-1}$ in summer, and the pool surface started to be overgrown by macrophytes. Correspondingly, hump-shaped *Daphnia* forms were very rare in the population, and adults with neckteeth were not observed in three consecutive seasons (2007–9). As adults of *D. hrbaceki* not showing antipredator defence structures are hardly distinguishable from *D. curvirostris*, it is not surprising that this species would have escaped attention even if it was common in the Central European landscape.

Apparent morphological similarity is the most common reason why cryptic species are overlooked in nature (Pfenninger & Schwenk 2007). It is therefore possible that *D. hrbaceki* lives also in other regions but has not been recorded in the recent decades. However, genetic analyses of different European populations of *D. curvirostris* suggest that cryptic species within this complex are rare. Černý and Hebert (1999) screened 17 Czech and Slovak populations using allozyme analysis. All analysed populations belonged apparently to a single species despite substantial intraspecific variation. Similar results were obtained by Michels *et al*. (2003) from an analysis of ten Belgian populations; in that case, allozyme analysis was verified by sequencing of a mitochondrial gene. Screening of COI variation of selected *D. curvirostris* individuals from various habitats across the Western Palaearctic, from Spain to Israel, also did not reveal any cryptic lineage (A. Petrusek, unpublished data).

D. hrbaceki therefore seems to be relatively rare species in Europe. Possibly, its centre of distribution is not in Central Europe from which we describe it but elsewhere, and it was introduced to the region from some distant source. Several non-indigenous cladoceran species, including *Daphnia*, have widely dispersed across continents thanks to human activities (e.g., Havel & Medley 2006; Mergeay *et al*. 2006), and at least one *Daphnia* species, *D. ambigua* Scourfield, 1947, was actually described from its invaded range. First recognized as a distinct species in

Europe, it is a North American invader spreading only in the recent decades (Dumont 1974; Žofková *et al*. 2002). An unusual genetic lineage with *D. similis*-like morphology but genetically clearly divergent, discovered in a temporary pool in Munich, Germany (Petrusek 2003; Adamowicz *et al*. 2009), might also be a case of a long-range introduction within the Palaearctic region.

The failure to recognize *D. hrbaceki* earlier, despite its potential to form conspicuous morphotypes, may also have an ecological explanation. This species seems to be a relatively weak competitor, at least in comparison with *D. obtusa* inhabiting similar habitats in the landscape surrounding the type locality. The latter species coexists with *D. hrbaceki* in both its presently known Czech localities, and outcompetes it in the spring and early summer. The type locality, artificially re-created at a site which used to be a wetland with a tiny ephemeral pool, offered an opportunity for colonization by a species that might not be successful in later stages of succession. It is not unlikely that *D. hrbaceki* will be completely replaced by *D. obtusa* in the future. A similar case was documented in Belgium, where a population of the *Daphnia atkinsoni* complex, previously not recorded in that country, colonized a newly created pool. Originally reaching high densities, it was largely replaced by *D. magna* which appeared in the pool later (Louette & De Meester 2004). It is possible that *D. hrbaceki* is favoured in young habitats in the beginning of the zooplankton assemblage process, especially in the studied region where zooplankton is apparently not dispersal-limited (P. Juračka, unpublished data). However, we cannot rule out that this species used to live at the site in the past, and the present population was founded from the resting egg bank.

Daphnia hrbaceki was discovered in newly created pools, which were dug in the Kokořínsko landscape protected area for conservation purposes of rare species of aquatic macrophytes, molluscs and amphibians. Conservation of those well-known vulnerable flagship taxa may have large impact on other organisms as well (Walpole & Leader-Williams 2002). Newly created pools have an important role as refuges from predators found in permanent waters, particularly fish (Wellborn *et al*. 1996), as biocorridors and habitats for a wide range of aquatic taxa (Santamaría 2002), and may offer opportunities for species that are usually outcompeted by other dominant species later during succession (Zedler 2003). Our discoveries of a new *Daphnia* species in Central European pools and other cryptic lineages of the genus found in such habitats in the Western Palaearctic (e.g., Adamowicz *et al*. 2009; Petrusek *et al*. 2009) stress the importance of small and temporary waters for preserving aquatic biodiversity.

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CHAPTER 4

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Neckteeth formation in two species of the *Daphnia curvirostris* complex (Crustacea: Cladocera)

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ABSTRACT

Cladocerans of the genus Daphnia *show different morphological adaptations against invertebrate predation. Among those, the formation of neckteeth has attracted substantial attention. Morphotypes exhibiting neckteeth better resist predation from larvae of phantom midges* Chaoborus *(Diptera). These morphological structures are known from several species of the* Daphnia longispina *and* D. pulex *complexes; recently they have also been reported in the* D. curvirostris *complex, within which they are well documented from the Far East species* D. sinevi *and from Central European* D. hrbaceki*. Much scarcer are indications of the formation of these structures in the widespread species* D. curvirostris*. Careful inspection of samples from pools with* Chaoborus *larvae nevertheless revealed that a small necktooth in the first few instars of* D. curvirostris *is not uncommon, but probably has been mostly overlooked in the past. Occasionally, even adult* D. curvirostris *males may carry this feature. We provide documentation, particularly by scanning electron micrographs, of neckteeth in field-collected* D. curvirostris*, and in juvenile individuals of its sister species* D. hrbaceki*. In addition, we tested the response of three clones each of* D. curvirostris *and* D. hrbaceki *to* Chaoborus *kairomones in laboratory experiments. Two clones of the former species and all three of the latter responded to this predator cue with neckteeth formation. First-instar juveniles of* D. hrbaceki *also occasionally carried neckteeth in control treatments without* Chaoborus *kairomones, but second and third instars did not. We also observed strong interclonal variation in neonate length in the presence of kairomones in this species. We provide a summary table listing all* Daphnia *species presently known to exhibit neckteeth, and propose that the ability to form these structures may be more widespread among common* Daphnia *species than previously assumed.*

Key words: Daphnia hrbaceki*,* Chaoborus*, interclonal variability, inducible defences, neckteeth, predation*

1. INTRODUCTION

Inducible morphological defences are among the most interesting antipredator adaptations, as they are often very conspicuous traits. They have been documented in most groups of organisms, ranging from bacteria to vertebrates (Tollrian & Harvell 1999). In aquatic environments, prey responses are often initiated by detecting predator kairomones, i.e., infochemicals associated with a particular predator (Dicke & Sabelis 1988). In cladocerans of the genus *Daphnia*, phenotypic plasticity in antipredator defensive traits has been frequently studied. Various *Daphnia* species show striking protective morphological structures, such as helmets of various shapes in *D. cucullata* Sars, 1862 (Tollrian 1990) or *D. longicephala* Hebert, 1977 (Grant & Bayly 1981), sharp spines in *D. lumholtzi* Sars, 1885 (Sorensen & Sterner 1992; Tollrian 1994; Dzialowski *et al*. 2003), or spiny head lobes called the "crown of thorns" in the *D. atkinsoni* complex (Laforsch *et al*. 2009; Petrusek *et al*. 2009). Other morphological antipredator defences are much less obvious. In the presence of predatory phantom-midge (*Chaoborus*) larvae, a number of *Daphnia* species form neckteeth (Tab. 1), characteristic small spines on the dorsal part of their carapace. Although it had been shown that neckteeth efficiently increase the resistance of *Daphnia* to *Chaoborus* predation (Havel & Dodson 1984; Repka *et al*. 1995), the mechanism of this protective effect remained unclear. Laforsch *et al*. (2004) nevertheless showed that the phenotypic changes accompanying neckteeth formation are much more complex, and involve not only superficially visible structures but also substantial strengthening of the carapace.

Neckteeth can be formed by various species of the subgenus *Daphnia* (*sensu* Johnson, 1952; i.e., including both *D. longispina* and *D. pulex* groups), especially in juvenile individuals (Colbourne *et al*. 1997; Kotov *et al*. 2006). The presence of neckteeth also recently received attention in the *D. curvirostris* complex, shown to contain several lineages in the Palaearctic region (Ishida *et al*. 2006; Kotov *et al*. 2006; Juračka *et al*. 2010). Species of this complex often live in small fishless pools where invertebrate predation is usually strong (Arnott & Vanni 1993) and *Chaoborus* larvae are common (e.g., Kvam & Kleiven 1995; Sell 2006). Despite this, an observation of neckteeth in a member of the *D. curvirostris* complex was pointed out in the literature only recently, for *D. sinevi* Kotov, Ishida & Taylor, 2006, a species newly described from the Russian Far East (Kotov *et al*. 2006). This discovery provided additional

Tab. 1. List of *Daphnia* lineages known to produce neckteeth, arranged according to their phylogenetic relationships. Species complexes are labelled according to Adamowicz *et al.* (2009), their phylogenies are provided in Adamowicz *et al.* (2009) and Juračka *et al.* (2010). Nomenclature of *D. longispina* follows Petrusek *et al.* (2008). Nomenclature of the *D. pulex* complex is not resolved (see, e.g., Mergeay *et al*. 2008); the lineage indicated as FLO9 was named *D. arenata* in Hebert (1995) and several subsequent publications but has never been formally described; different lineages are labelled *D. pulicaria* and *D. pulex* in the Old and the New World.

Species	Neckteeth type	Habitat	Distribution	References
***D. pulex* complex**				
Daphnia sp. FLO9	several teeth in a row	coastal ponds	Western Nearctic	Hebert (1995); Benzie (2005); Juračka *et al.* (2010)
D. pulex Leydig	rosette	pools, ponds, lakes	Palaearctic and Ethiopian	Tollrian (1993); Sell (2000); Laforsch *et al*. (2004)
D. "pulex" (American lineage)	rosette	ponds, lakes	Nearctic, Panarctic	Havel (1985); Parejko & Dodson (1991); Riessen & Trevett-Smith (2009)
D. pulicaria Forbes	N/A	ponds, lakes	Neartic, alpine lakes in Europe	Luecke & Litt (1987)
D. "pulicaria" (European lineage)	single tooth	ponds, lakes	Palaearctic	V. Kořínek, pers. observation
***D. catawba* complex**				
D. catawba Coker	single tooth	ponds, lakes	Eastern Nearctic	Haney *et al*. (2010)
D. minnnehaha Herrick	single to multiple teeth in a row or rosette	ponds	North Eastern Nearctic	Colbourne *et al*. (1997); Benzie (2005); Riessen & Trevett-Smith (2009); Juračka *et al*. (2010)
***D. obtusa* complex**				
D. obtusa Kurz	single to multiple teeth in a row	puddles, pools	Western Palaearctic	P. J. Juračka, pers. observation
***D. longispina* complex**				
D. dentifera Forbes	rosette	ponds, lakes	Nearctic	Benzie (2005)
D. longispina O.F. Müller (including *hyalina* and *rosea* forms)	single to multiple teeth in a row or rosette	pools, lakes	Palaearctic and Ethiopian	Negrea (1983); Boronat & Miracle (1997); Benzie (2005)
***D. curvirostris* complex**				
D. curvirostris Eylmann	single to multiple teeth	ponds, pools, various temporary water bodies	Palaearctic, Ethiopian, Nearctic	Glagolev (1986); Kirdyasheva (2010); Hudec (2010, this study)
D. hrbaceki Juračka, Kořínek & Petrusek	single tooth	pools	Western Palaearctic	Juračka *et al*. (2010)
D. sinevi Kotov, Ishida & Taylor	single tooth	ponds	Eastern Palaearctic	Kotov *et al.* (2006)

support for the conclusions of Colbourne *et al*. (1997) that neckteeth in *Daphnia* originated several times independently.

Another species of the *D. curvirostris* complex recently described from Central Europe, *Daphnia hrbaceki* Juračka, Kořínek & Petrusek, 2010, exhibits this protective structure as well (Juračka *et al*. 2010). Juračka *et al*. (2010) observed neckteeth in both male and female juveniles and even in adults in the *D. hrbaceki* type locality, which was inhabited by *Chaoborus* larvae. In some natural populations, adults carrying neckteeth had a conspicuous hump-shaped dorsal body outline, presumably a phenotype accompanying the formation of inducible antipredator structures under certain environmental conditions. Similar forms are known from the North American species *D. minnehaha* Herrick, 1884 (Hebert 1995). *D. hrbaceki* escaped recognition and formal description for a long time, although its hump-shaped phenotypes strikingly differ from other European *Daphnia* species. Apparently, this is due to the fact that such morphs occur only under specific environmental conditions: while observing the population at the species' type locality for several years, we noted that hump-shaped phenotypes slowly disappeared, despite the continuing presence of *Chaoborus* larvae (Juračka *et al*. 2010).

Even the most widespread member of the species complex, *Daphnia curvirostris* Eylmann, 1887, which has been known for more than a century, may apparently form neckteeth. However, this has been largely overlooked. When comparing *D. hrbaceki* to *D. curvirostris* to elucidate species-specific traits, we observed a single necktooth in juveniles and even adult males in some Central European populations of the latter species; we therefore searched for evidence for this feature in the available literature. To our knowledge, the only drawings of *D. curvirostris* with one necktooth have been given by Glagolev (1986) and Kirdyasheva (2010) from a Russian population. Additionally, Matile (1890) provided documentation of neckteeth formation in adult *Daphnia* specimens of a taxon described by him as *D. dentata*, which might belong to the *D. curvirostris* complex, from the vicinity of Moscow. Apart from formation of a single neckteeth in *D. curvirostris*, Hudec

Tab. 2. Material analyzed morphologically in this study. Abbreviations of collector names: AGK: A.G. Kirdyasheva, DV: D. Vondrák, PJJ: P.J. Juračka, VK: V. Kořínek, VKr: V. Kraslová. All localities except Borok (Russia) are in the Czech Republic. *Chaoborus* was identified to species level if material was available; otherwise its presence is noted. NA indicates a pre-sorted sample where presence of this predator could not be evaluated.

Locality	Coordinates	Locality type	Date	*Chaoborus*	Collector
Daphnia curvirostris					
Tupadly	N 50°26'16" E 14°28'20"	experimental pools	23 October 2007, 6 October 2008	*C. crystallinus* *C. obscuripes*	DV
Vrbno, near Smyslov pond	N 49°25'07" E 13°48'10"	temporary pool	4 June 2010	present	VK
Tchořovice, near Radov pond	N 49°25'17" E 13°49'22"	forest pool	May 2010	present	VK
Tvrdonice	N 48°44'54" E 17°01'25"	temporary pool	15 April 2008	*C. pallidus*	VKr
Borok (Russia)	N 58°03' E 38°13'	temporary puddles	11 June 2004	present	AGK
Daphnia hrbaceki					
Nosálov (type locality)	N 50°28'54" E 14°41'10"	pool	7 samples between May 2005 and November 2007	*C. crystallinus*	PJJ
Nosálov	N 50°29'11" E 14°41'24"	pool	10 November 2006	*C. crystallinus* *C. flavicans*	PJJ
Drásov	N 49°41'37" E 14°06'19"	temporary pool	3 July 1995	NA	VK
Daphnia obtusa					
Nosálov	N 50°29'11" E 14°41'24"	pool	4 July 2005, 13 October 2005	*C. crystallinus*	PJJ
D. "pulicaria" (European lineage)					
Pole	N 49°25'23" E 13°48'03"	pool	30 July 2010	present	VK

(2010) documented a juvenile female ascribed to this species with multiple neckteeth from a Slovakian population, and Kirdyasheva (2010) reported that some juveniles from one of the Russian populations also carried three or more neckteeth. Several independent observations therefore confirm that *D. curvirostris* is able to form neckteeth; unfortunately, the above-cited works are mostly difficult to access.

The present study has two aims: 1) to provide light and scanning electron microscopy documentation of neckteeth in *D. curvirostris*, and compare them with those of its sister species *D. hrbaceki*; 2) to experimentally test whether neckteeth formation in both of these European members of the *D. curvirostris* complex can be induced by *Chaoborus* kairomones under laboratory conditions. Neckteeth induction has been successfully demonstrated in laboratory experiments with other *Daphnia* species (e.g., Havel & Dodson 1987; Tollrian 1995; Sell 2000; Riessen & Trevett-Smith 2009); we therefore hypothesized that both species would be responsive to *Chaoborus* cues.

2. METHODS

2.1. Material examined

The studied populations of *Daphnia*, particularly *D. curvirostris* and *D. hrbaceki*, used for neckteeth documentation and for laboratory experiments, are listed in table 2. If present in the samples, *Chaoborus* was identified to species level according to Rozkošný *et al*. (1980).

For the first laboratory experiment, each species was represented by three different clones, distinguishable from each other by alleles at seven microsatellite loci (described in Brede *et al*. 2006): Dp281NB, DaB17/17, SwiD14, Dgm105, Dgm112, SwiD4, and SwiD18 (A. Thielsch, unpublished data). Two of those clones per species, together with a clone of *Daphnia pulex* Leydig, 1860 known to be well responsive to predator cues, were used in the second experiment. The *D. pulex* clone was included as a control for neckteeth formation; it was provided by Ralph Tollrian and has been cultured in the laboratory for several years.

2.2. Documentation of neckteeth from field samples

To document neckteeth from natural populations, we used both light and scanning electron microscopy (SEM). Photographs were taken by a Nikon D300 digital camera attached to an Olympus BX51TF optical microscope. A selected specimen was photographed 10 times with different depths of focus, and the resulting image was merged to gain extended depth of field with Helicon Focus 5.1.2. and Adobe Photoshop CS3 software.

Specimens used for SEM were dehydrated in a graded acetone series and then dried with organic volatile matter hexamethyldisalazane (Laforsch & Tollrian

2000). Dehydrated specimens were gold-coated in a BAL-TEC Sputter Coater SCD 050 for 5-7 minutes in argon plasma at 10^{-1} millibar vacuum. Then, they were imaged with a JEOL JSM-6380 LV scanning electron microscope.

2.3. Experimental design

We used three clones each of *D. curvirostris* and *D. hrbaceki*, sampled in late August 2006, to test their response to *Chaoborus* kairomones. *D. curvirostris* clones originated from shallow temporary pools near Přerov nad Labem (N 50°10', E 14°49'), *D. hrbaceki* from its type locality near Nosálov (see Tab. 2). The animals were reared in the laboratory under constant conditions (20 ºC ± 0.5, 16 hours of light per day) in artificial medium (according to Jeschke & Tollrian 2000); local groundwater from Planegg-Martinsried was used instead of tap water. Daphnids were fed daily with *Scenedesmus obliquus* (1.5 mg carbon L^{-1}).

For the first experiment, we randomly selected six juvenile females of each clone and placed them into separate beakers (volume 1.5 L). Into each of these beakers, we put a small plastic cage with the bottom made from a 200 µm mesh, allowing the flow of infochemicals but not physical contact with the predator. In three beakers, the cage contained five specimens of the 4th larval instar of *Chaoborus crystallinus*. The other three beakers containing daphnids of each clone served as control treatments without the predator presence. *Chaoborus* were fed with *Daphnia* neonates of the same clone as in the respective beaker to maximize the expression of morphological defences, as predators consuming conspecific prey are known to increase the formation of inducible defences (Stabell *et al*. 2003; Laforsch *et al.* 2006). To ensure sufficient mixing of predator kairomones and prey alarm substances with the culture medium, each cage with *Chaoborus* larvae was raised almost out of the medium and lowered back down twice a day. The medium in each beaker was changed with every reproductive event.

Daphnia individuals with which the experiment started (the "mother generation") were exposed to the predator cues to take maternal effects into account (Agrawal *et al.* 1999). We then used individuals from the third clutches of these females to evaluate the response of the next-generation juveniles to predator kairomones (neonates of the first and second clutch were removed and used as feed for *Chaoborus*). The third clutch neonates were counted, individually photographed to measure body and spine length (see below), and checked for the presence of neckteeth. Immediately afterwards, we randomly selected five individuals from the clutch (or less in cases of smaller clutches) and transferred them to separate 0.1 L beakers (the smaller flask volume was used due to space limitations) to follow the life history and morphological changes of each daphnid individually. The media were changed twice a day in each beaker. In *Chaoborus* treatments, the beakers contained culture medium with predator-conditioned water prepared as described for the mother generation (see above). The control medium contained only algal food but no predator or prey infochemicals. We took a second measurement of morphometric parameters of each individual *Daphnia* at the age of first reproduction, and evaluated the number of offspring in their first clutch.

To compare neckteeth formation among first three juvenile instars, we performed a second experiment using two clones of each species tested in the first experiment (*D. hrbaceki* clones 2 and 3, and *D. curvirostris* clones 1 and 3). In addition, we also exposed a clone of *D. pulex* to *Chaoborus* kairomones to test for the efficiency of the predator cue, as this species is known to exhibit distinct neckteeth in response to *Chaoborus* (e.g., Tollrian 1995). The experimental design was similar to our first experiment, with the exception that we did not transfer the juveniles of the third brood of preconditioned mothers separately into small beakers but kept them in the original vessel to constantly expose the animals to predator cues. In addition, we used ten *Chaoborus* larvae per litre to increase the concentration of predator cues. We randomly selected 20 individuals (if available) in three consecutive days to collect animals of the first three instars. We checked for presence or absence of neckteeth in these instars under a Leica M10 stereomicroscope.

2.4. Measurements and statistical analyses

Photographs of each measured individual from the first experiment were taken by an Olympus ALTRA20 digital camera mounted on a Leica M10 stereomicroscope. Subsequently, we measured two morphometric parameters in the software Olympus cell^P: body length (defined as the length between the upper edge of the compound eye to the base of the tail spine) and tail spine length (a straight line between the base of the tail spine and its top). Occasional juvenile individuals that were substantially larger than the others were removed from the dataset, as we suspected them of already being in the second instar. We also measured the body length of randomly selected neckteeth-carrying individuals from one population of each species (*D. curvirostris*: Tvrdonice, 15 April 2008; *D. hrbaceki*: type locality near Nosálov, 17 August 2006), to evaluate their size distributions and thus check whether neckteeth are present in different instars.

We used Pearson's Chi-square test to compare ratios of induced (i.e., with neckteeth) and uninduced specimens within each species in both experiments. Since we used 3 tests in the second experiment, we applied consequent manual Hochberg's p-value adjustment (Benjamini & Hochberg 1995) for multiple testing. The morphometric parameters were compared between individuals in *Chaoborus* and control treat-

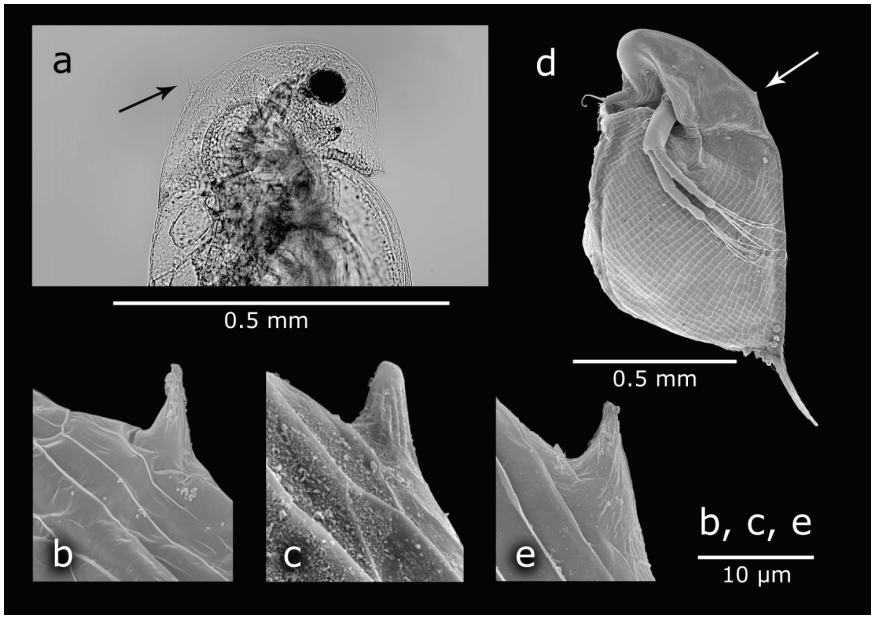

Fig. 1. Neckteeth in Czech populations of the *Daphnia curvirostris* complex. *D. curvirostris* from Tupadly: head (**A**) and necktooth detail (**B**) of juvenile females; an adult male in lateral view (**D**); detail of an adult male necktooth (**E**). *D. hrbaceki*: necktooth of a juvenile female from Nosálov (**C**). More figures of *D. hrbaceki* with neckteeth are available in Juračka *et al*. (2010). Arrows indicate neckteeth.

ments by a series of non-parametric Wilcoxon's signed-rank test with consequent manual Hochberg's *p*-value adjustment. As the sizes of different specimens within one clutch cannot be considered independent replicates, we averaged them for each clutch, and used a single value for the whole clutch. Wilcoxon's signed-rank test was also used for comparing the size of clutches from controls and *Chaoborus* treatments.

3. RESULTS

In the samples of *Daphnia curvirostris* originating from Czech and Russian pools with *Chaoborus* larvae, most juveniles carried a small (5-10 µm) necktooth (Fig. 1a, b). A necktooth of approximately the same size was also occasionally retained in adult males (Fig. 1d, e), as seen in field samples from Tupadly, Czech Republic (but also documented from Borok, Russia; Kirdyasheva 2010). Neckteeth of juvenile *D. hrbaceki* (Fig. 1c) were of a similar morphology as those of *D. curvirostris*. In both species, neckteeth were carried by a wide size range of juveniles, clearly indicating that the structure is present in several juvenile instars: the size of measured neckteeth-carrying individuals ranged between 0.61 mm 1.26 mm in *D. curvirostris* from Tvrdonice, and between 0.52 and 1.04 mm in *D. hrbaceki* from its type locality.

Juveniles of both *D. curvirostris* and *D. hrbaceki* also formed neckteeth during the laboratory experiments (Tab. 3); these individuals did not differ phenotypically from those in the natural populations. In the first experiment, *D. hrbaceki* had a much stronger tendency to form these structures: in all three tested clones, all first-instar juveniles carried a necktooth in the treatment with *Chaoborus* kairomones. Interestingly, some first-instar juveniles with neckteeth were also found in the control treatments; their proportion was nevertheless significantly lower than in the *Chaoborus* treatments. A small proportion of individuals from one of the three tested *D. curvirostris* clones also formed neckteeth; however, there was no significant difference between controls and *Chaoborus* treatments. In the second experiment, however, almost all specimens of all three instars of both species produced neckteeth in the presence of *Chaoborus*, while those not exposed to predator cues only formed these structures in the first instar in *D. hrbaceki* (Tab. 3). No specimen of *D. hrbaceki* with a hump-shaped carapace (as found in the wild) was observed in the laboratory experiments.

Differences in daphnid morphometric and life history traits measured in the first experiment were not consistent between the *Chaoborus* and control treatments, either between the two tested species or among clones within species. We did not observe any clear trends or significant differences in size at first reproduction, clutch size, or relative spine length. The neonate size, however, showed interesting patterns (Fig. 2). *D. hrbaceki* clones 1 and 3 formed significantly larger neonates in the presence of *Chaoborus* than in controls

Tab. 3. Ratios of induced (with neckteeth) and uninduced neonates of *Daphnia hrbaceki* and *D. curvirostris* in the laboratory induction experiments. *D. pulex* served as a control for the efficiency of the predator cue in the second experiment. Significances of differences between *Chaoborus* and control treatments were tested by the Pearson's Chi-square test (adjusted *p*-values are given for the second experiment).

	Instar	Clone	% with neckteeth (total N)		Chi-square tests	
			Control	*Chaoborus*	χ^2	*p*-value
Experiment I						
Daphnia hrbaceki	1	1	100% (10)	100% (19)	12.2	<0.001
		2	50% (8)	100% (14)		
		3	71% (17)	100% (8)		
Daphnia curvirostris	1	1	0% (5)	15% (27)	2.46	0.12
		2	0% (10)	0% (3)		
		3	0% (16)	0% (23)		
Experiment II						
Daphnia hrbaceki	1	2	67% (6)	100% (1)	1.66	0.6
		3	100% (18)	100% (15)		
	2	2	0% (14)	100% (5)	63.43	<0.001
		3	0% (20)	94% (32)		
	3	2	NA	100% (1)	23.03	<0.001
		3	0% (20)	67% (30)		
Daphnia curvirostris	1	1	0% (20)	100% (20)	70	<0.001
		3	0% (20)	100% (10)		
	2	1	0% (20)	100% (20)	80	<0.001
		3	0% (10)	100% (20)		
	3	1	0% (20)	70% (20)	36.52	<0.001
		3	0% (20)	NA		
Daphnia pulex	1	1	0% (17)	100% (30)	47	<0.001
	2		0% (20)	100% (50)	70	<0.001
	3		0% (20)	100% (20)	40	<0.001

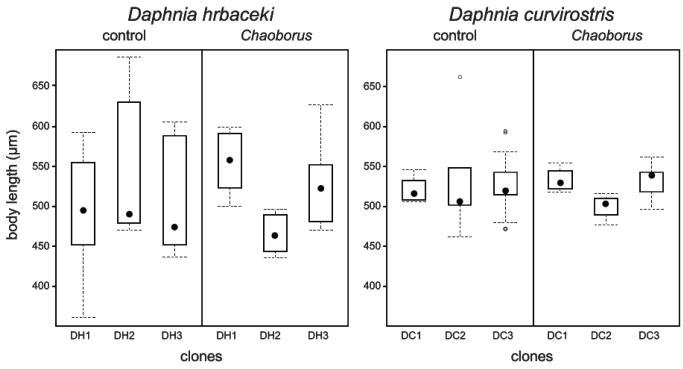

Fig. 2. Body length of *Daphnia hrbaceki* and *Daphnia curvirostris* third-clutch neonates in absence and presence of *Chaoborus* kairomones in the laboratory induction experiment. Median (dark circle), interquartile ranges (box) and non-outlier ranges (whiskers) are shown by the box-and-whisker plot; outliers are indicated by empty circles. *D. hrbaceki* clones 1 and 3 were significantly larger in the kairomone treatment than in the control, clone 2 was significantly smaller. In *D. curvirostris*, differences in neonate lengths from kairomone and control treatments were not significant.

(Wilcoxon's signed-rank tests with Hochberg's *p*-value adjustment; adjusted $p = 0.036$, W = 0 and 0.027, W = 0, respectively); on the contrary, clone 2 neonates were significantly smaller under the same conditions (adjusted $p = 0.027$, W = 58). *D. curvirostris* clones did not exhibit any difference in neonate size between treatments (adjusted $p > 0.376$, $W \leq 7$ in all three comparisons). We also did not observe any trade-off between neonate size within the clutch and clutch size in either of the tested species.

4. DISCUSSION

Our study confirms that both studied European species of the *Daphnia curvirostris* complex are able to form neckteeth in the field as well as under laboratory conditions, and in several juvenile instars. However, we observed neckteeth formation in the *D. hrbaceki* first instar not only in the presence of *Chaoborus* kairomones but also in the treatments without predator cues. Similar observations are known from some lineages of

the *D. pulex* complex, including European *D. pulex* Leydig, 1860 (Tollrian 1993) as well as the North American *D. "pulex"* (H. Riessen, personal communication); additionally, Kirdyasheva (2010) reported neckteeth in juvenile instars of *D. curvirostris* from a population where *Chaoborus* had not been observed. Spontaneous neckteeth development in neonates of these species may be explained by the fact that they occur in fishless habitats where invertebrate predation is often very strong. Hence, neckteeth development even in the absence or low concentration of *Chaoborus* cues may be a good start-up defence, as predation pressure may change rapidly.

Based on previous experimental work on other *Daphnia* species (Havel 1985; Tollrian 1993), we expected that our studied taxa should produce a higher ratio of neonates with neckteeth in *Chaoborus* treatments than in controls. The results were consistent with this hypothesis in both experiments (Tab. 3). In the first experiment, the trend was significant only for *D. hrbaceki*, in which all neonate individuals of all three tested clones carried neckteeth in the *Chaoborus* treatment. In the second experiment, the kairomone effect was much stronger, and differences between control and predator treatments were highly significant in almost all species and instars (with the exception of the *D. hrbaceki* first instar). The difference between the experiments may be explained by doubled kairomone concentration in the second one. This corresponds to results of previous studies reporting the influence of kairomone dose on the formation of protective traits (e.g., Tollrian 1993). In the first experiment that focused on neonates only, *D. curvirostris* formed neckteeth much less frequently than *D. hrbaceki* (only 15% of juveniles of a single clone in the *Chaoborus* treatment). This is in accordance with the infrequent field observations of *D. curvirostris* populations with neckteeth, and suggests that *D. hrbaceki* is more likely to respond with morphological defences under low kairomone concentrations.

In our experiments, individuals of both species showing neckteeth exclusively formed a single necktooth. We did not observe any rosette-like neckteeth formed by more dorsal spines, as documented in *D. curvirostris* by Hudec (2010) and Kirdyasheva (2010). Their field observations nevertheless suggest that the taxon is one of those *Daphnia* species that are plastic in their level of neckteeth expression (see Tab. 1).

In both experiments, we did not observe any hump-shaped morphs. The failure to produce inducible defences as strong as those seen in the wild is common in laboratory experiments (Dodson 1988; Tollrian 1994; Laforsch & Tollrian 2004; Tanner & Branstrator 2006). In our case, this may be due to various reasons. It could be due to an incomplete or insufficiently intense inducing stimulus. Tanner & Branstrator (2006) found a three-generation delay in *D. mendotae* Birge, 1918 producing a round helmet in reaction to the predatory cladoceran *Leptodora*; possibly, a dorsal hump in *D. hrbaceki* may only be formed in an experiment spanning several generations. Riessen and Young (2005) suppose that similar hump-shaped phenotypes in North American *D. minnehaha* are induced by the predator only under low-food conditions. This synergistic interaction would correspond to the field observations of *D. hrbaceki* from its type locality, a newly excavated pool. Hump-shaped morphs were common there during the first years of habitat existence, but disappeared two to three years later when the trophic status of the habitat substantially increased (Juračka *et al*. 2010). In addition, it has been shown that small scale turbulence evoked by the movement of predators can act synergistically with chemical cues to induce maximal trait responses (Tollrian & Laforsch 2006). Hence, synergistic effects of kairomones and environmental conditions are well known within the *Daphnia* genus (e.g., Weber 2001; Weetman & Atkinson 2002; Tollrian & Laforsch 2006), and may also explain the absence of hump-shaped morphs in our experiments.

Daphnia are known to react to the presence of predators not only through morphological changes, but also by adaptive shifts in their life history (e.g., Schwartz 1984; Weber & Declerck 1997; Boersma *et al*. 1998). Among the most common changes are alterations in the number and size of offspring through maternal effects, depending on the specific predators (Tollrian 1995; Agrawal *et al*. 1999). In the presence of predators preferring larger prey (particularly fish), some species tend to produce smaller neonates (Reede 1997; De Meester & Weider 1999; Spaak *et al*. 2000; Mikulski 2001). On the other hand, the same prey species may follow the opposite strategy in the presence of predators which are gape-limited, including *Chaoborus* (Pastorok 1981). In this case, females exposed to predator kairomones usually tend to produce large neonates (Riessen & Sprules 1990; Lüning 1992; Spitze 1992; Tollrian 1995; but for exception, see Spitze, 1992).

As both studied species occur in small fishless pools with frequent strong predation pressure by *Chaoborus* (Mura & Brecciaroli 2003; Louette & De Meester 2005; Juračka *et al*. 2010), a tendency to increase neonate size in the kairomone treatments could have been expected. However, although all three clones of *D. hrbaceki* reacted to *Chaoborus* cues with a significant change in neonate size, the direction of this change varied among the clones. Two clones produced significantly larger neonates, while the third one produced smaller ones (Fig. 2). Strong interclonal variability in the reaction to predator kairomones is well known from previous laboratory experiments in *Daphnia* (Parejko & Dodson 1991; Weber & Declerck 1997; Boersma *et al*. 1998), including opposite reactions within one species (Spitze 1992; Boersma *et al*. 1998; Pauwels *et al*. 2005).

Based on field observations, we suppose that neckteeth are induced relatively often as a defence against

Chaoborus predation, probably also by other species of the subgenus *Daphnia* in which this feature is not known. It is generally assumed that neckteeth in *Daphnia* originated multiple times independently, and this hypothesis has been suggested by several authors. Colbourne *et al*. (1997) and Kotov *et al*. (2006) came to this conclusion because neckteeth had been documented only sporadically in distinct *Daphnia* species complexes. The potential independent origin of neckteeth in *D. longispina* and *D. pulex* groups (treated as distinct subgenera) was also discussed by Beaton and Hebert (1997) in their study of the cellular basis of *Daphnia* head morphology. Representatives of the two groups differed in the number of polyploid cells in the muscle attachment region, which might be responsible for neckteeth formation. Colbourne *et al*. (1997) also claimed, in support of the multiple-origin hypothesis, that some *Daphnia* species living mostly in small turbid habitats without *Chaoborus*, e.g., North American members of the *D. obtusa* complex, do not produce neckteeth even in experiments with *Chaoborus* kairomones. However, Beaton & Hebert (1997) proposed a potential for neckteeth formation in three species of that complex, although they lacked evidence of this ability from field samples or laboratory collections. This is in agreement with field observations from Europe: *D. obtusa* Kurz, 1874 (*sensu stricto*) does produce neckteeth in pools with high *Chaoborus* abundances (P.J. Juračka, personal observation).

The growing evidence that neckteeth are more common than previously assumed among various daphnids from both the *pulex* and *longispina* groups may also give some support to an alternative scenario of evolution of neckteeth defences. Ontogenetic mechanisms allowing neckteeth formation could be a plesiomorphic character, expressed only in taxa where selection by predators strongly favoured them. This is further supported by the fact that some species apparently exhibit different forms of neckteeth (ranging from single to multiple arranged in a row or a rosette-like fashion). Further research into the genomic basis of neckteeth formation may reveal whether the different forms of neckteeth are homologous in unrelated *Daphnia* species or not.

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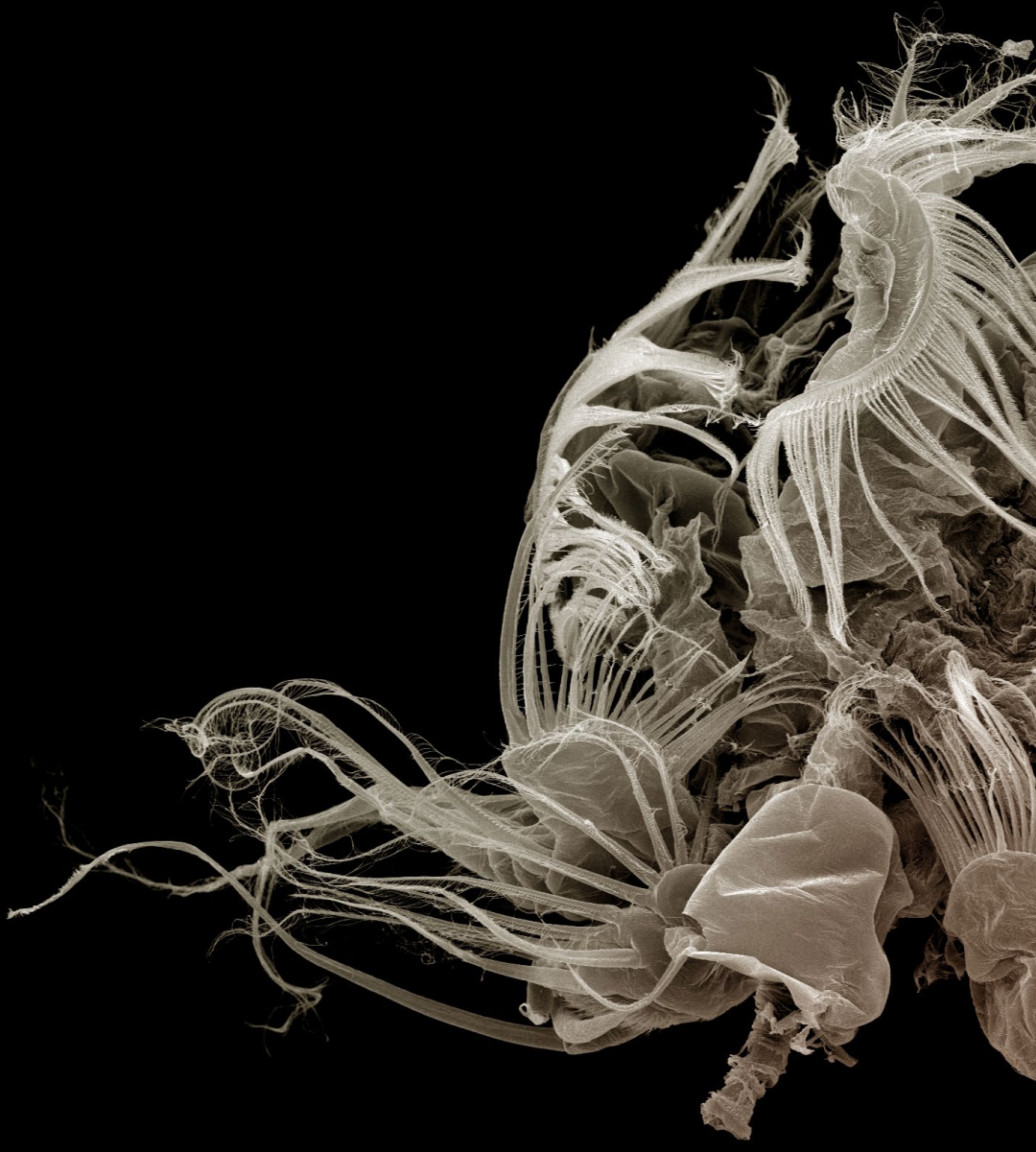

CHAPTER 5

Juračka P.J., Sacherová V., Dobiášovská I., Bovšková D.,
Novosadová Z., Kořínek V., Petrusek A. (2016):
Simplification of preparation techniques for scanning electron microscopy of Cladocera:
preparing filtering limbs and ephippia for efficient studies of ultrastructure.
Crustaceana **89**, 47-62**.**

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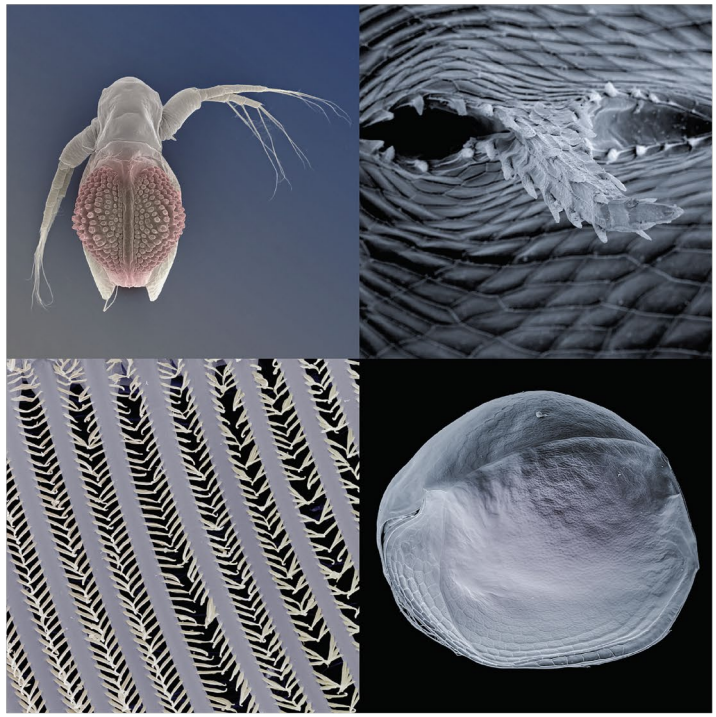

CRUSTACEANA
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Cover of the journal *Crustaceana* illustrates an article presented as **chapter 5** of this thesis.
Cover caption:
Four scanning electron microscope images of Cladocera. Specimens were prepared according to the methods suggested in: Juračka et al. (2016; Crustaceana, 89(1): 47-62). Top left, dorsal view of ephippial female of *Moina weismanni* Ishikawa, 1896; top right, caudal view (detail) of the spina of *Daphnia hrbaceki* Juračka, Kořínek & Petrusek, 2010, with the ventral side of the specimen to the left; bottom left, detail of the filtration limbs of the same species; bottom right, lateral view of the shell (carapace) of *Chydorus sphaericus* (O. F. Müller, 1776).

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CRUSTACEANA

SIMPLIFICATION OF PREPARATION TECHNIQUES FOR SCANNING ELECTRON MICROSCOPY OF CLADOCERA: PREPARING FILTERING LIMBS AND EPHIPPIA FOR EFFICIENT STUDIES OF ULTRASTRUCTURE

BY

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ABSTRACT

Scanning electron microscopy (SEM) is widely used in studies on crustacean systematics and functional morphology. The surface ultrastructure of cladoceran ephippia may carry valuable information for taxonomy, and its analysis may be also helpful in palaeoecological studies focusing on ephippia preserved in old sediments. SEM is also commonly used in analyses of cladoceran filtering limbs, which not only serve for filtering of food particles, but are also taxonomically important structures. In this study, we describe an efficient method for preparing both ephippia and limbs for SEM analyses. The workflow minimizes physical manipulation, which may reduce the risk of damage or loss of material, and allows a relatively large amount of material to be studied. We also evaluated the effects of two strong chemical agents used to remove unwanted biofilm from both ephippia and limb surfaces. This approach may further facilitate SEM analyses in systematic, ecological and palaeoecological surveys of Cladocera.

Key words. — Cladocera, ephippia, trunk limbs, SEM, ultrastructure, taxonomy, paleoecology, methods

RÉSUMÉ

La microcopie électronique à balayage (MEB) est largement utilisée dans les études sur la systématique et la morphologie fonctionnelle des crustacés. L'ultrastructure de la surface des éphippies de cladocères peut apporter des informations précieuses pour la taxonomie, et son analyse peut aussi être utile lors dans les études paléoécologiques des éphippies conservées dans les sédiments anciens. La MEB est aussi couramment utilisée dans l'analyse des appendices filtreurs des cladocères, qui ne servent pas seulement à la filtration des particules alimentaires, mais sont

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aussi des structures taxonomiquement importantes. Dans cette étude, nous décrivons une méthode efficace de préparation à la fois des éphippies et des appendices pour les analyses en MEB. Le processus utilisé minimise la manipulation physique, ce qui peut réduire le risque de dommage ou de perte de matériel, et permet l'étude d'une relative grande quantité de matériel. Nous avons aussi évalué les effets de deux puissants agents chimiques utilisés pour retirer le biofilm indésirable à la surface à la fois des éphippies et des appendices. Cette approche pourra ultérieurement faciliter les analyses en MEB pour l'étude systématique, écologique et paléoécologique des Cladocera.

Mots clés. — Cladocera, éphippie, appendices du tronc, MEB, ultrastructure, taxonomie, paléoécologie, méthodes

INTRODUCTION

Over the past few decades, scanning electron microscopy (SEM) has become more accessible and easier to use, even for relatively inexperienced researchers. Because of this, SEM has also become widely used in studies on functional morphology, as well as the systematics of minute planktonic species, including cladocerans, which are key organisms for recent, as well as paleoecological freshwater studies. A number of previously neglected evolutionary lineages of these crustaceans has been discovered recently, particularly by genetic tools (see Adamowicz & Purvis, 2005). However, morphological traits to distinguish them from already known taxa are often lacking. In addition to the traditional approach used for taxon identification, i.e., evaluation of characteristics that can be easily distinguished under a light microscope, very fine structures that are only apparent when using SEM might provide valuable insights (e.g., Østergaard & Bresciani, 2000; Juračka et al., 2010). SEM has also been very useful in studies on the functional morphology of cladoceran filtering limbs, especially in the genus *Daphnia* (e.g., Hartmann & Kunkel, 1991; Macháček, 1998). These structures are also of taxonomical importance and have been used for species descriptions or identification (e.g., Kotov, 2000; Van Damme et al., 2007).

Filtering limbs are just one example of a microscopic morphological structure of significance in ecological or systematic research. Ultrastructures on the surface of ephippia have been suggested by several authors to carry valuable information as well (Glagolev, 1983; Kokkinn & Williams, 1987; Lu, 2001), even for more general, evolutionary-focused studies (see review by Brendonck & De Meester, 2003). Ephippia can be found in the sediments of almost all inland waters with an aquatic phase long enough to allow cladocerans to colonize and finish their life cycle. Subfossil ephippia that can be often identified to species level lay in the sediments for hundreds (Mergeay et al., 2004) or thousands (Frey, 1987) of years. However, old ephippia, regardless whether from sediments or water surface, can be covered with various particles, bacterial mass, or other biofilm that prevents in-depth study of their surface ultrastructure. Nevertheless, even much older ephippia may retain surface characteristics in the fossil record; Kotov & Taylor (2011)

reported on exceptionally preserved *Daphnia* ephippia of Mesozoic age, which could be identified to the subgeneric level, and even their surface ultrastructure could be studied.

To obtain accurate scanning electron micrographs of recent and not fully-dehydrated samples of subfossil material extracted from wet sediments, two important material preparation steps need to be carefully performed: specimen surface cleaning and dehydration. Although appropriate material preparation is crucial for high-quality imaging in both light and electron microscopy, these methods have been evolving relatively slowly, especially in comparison with the microscopy itself. Most of the methods recently used were developed from the 1960s to the 1980s (e.g., Anderson, 1951; Kozloff & Galigher, 1971; Felgenhauer, 1987; Inoué & Osatake, 1988). The efficiency and suitability of various SEM preparation methods differ substantially, depending on the characteristics of the material and taxa studied. Therefore, the methods should be optimized for various objects to obtain the best results.

A BRIEF OVERVIEW OF SEM PREPARATION METHODS USED FOR CLADOCERA IN THE PAST

For Cladocera, the preparation methods for SEM need to be adjusted to their soft tissues and delicate structures, such as various setae on their trunk limbs or relatively soft and convex shells, which tend to collapse while dehydrating (Laforsch & Tollrian, 2000). Preparation of cladocerans for SEM usually requires material fixation and dehydration, and often includes also cleaning the surface of the cladoceran body from unwanted biofilm and particles.

Material fixation

Very good results have been obtained by fixing the tissues with glutaraldehyde in phosphatase buffer for freshwater crustaceans (Felgenhauer, 1987; Inoué & Osatake, 1988) and sodium cacodylate for marine crustaceans (Felgenhauer, 1987). Post-fixing with osmium tetroxide (OsO_4) greatly improves the quality of specimens by stabilizing lipids and increasing the contrast and stability under the electron beam, thus reducing charging (e.g., Kozloff & Galigher, 1971; Felgenhauer, 1987; Inoué & Osatake, 1988). Although most of the cladoceran material sampled in the past was preserved in just 4% formaldehyde, it is still well suited for SEM. However, in recent years zooplankton samples are often preserved in ethanol (with concentrations frequently exceeding 90%), avoiding potential toxic effects of formaldehyde and allowing subsequent DNA analyses. This preservation method, however, tends to cause crumpling of soft structures due to rapid dehydration and the denaturation of proteins.

Cleaning the surface of the cladoceran body

SEM analyses of the surface of any biological object are often complicated by the presence of debris or epibionts. Ephippia in particular are frequently covered with such unwanted organic material, as they are often sampled in sediments or at the water surface where they may float for a long time. Similarly, filtering limbs of Cladocera are often covered with various organic matters, such as food particles or other filtered material. Removing this material may be therefore a crucial step in preparing such material for any type of microscopy, although exceptionally clean samples may sometimes be available, particularly from oligotrophic habitats (Hartmann & Kunkel, 1991) or laboratory cultures.

Detailed methods for cleaning crustacean body surfaces of debris, epibionts, mucus, and bacteria or fungi have been listed by Felgenhauer (1987). These include using anionic surfactants and sonication for removal of debris, shaking in glycerol to remove mucus, and treating the living animals before preservation with antibiotics to remove bacteria. Simpler methods can be used to clear filtering structures of food particles in experimental studies; keeping live animals in sterile or artificial media for a short period of time prior to morphological analysis is often sufficient (Mangalo, 1987; Hartmann & Kunkel, 1991). Lactic acid or potassium hydroxide have also been used for cleaning Cladocera before examination by standard light microscopy; this is usually done to remove the soft tissues but to retain chitinous structures (Harrison & Anderson, 1975).

Material dehydration

After preservation, choosing a suitable dehydration method is the second crucial step to minimize the deformation of specimens, most frequently caused by surface-tension forces. Therefore, leaving material out in the air to dry out naturally is only suitable for hard shells or structures with very low liquid content (including cladoceran ephippia). However, this approach was successfully used in one study on filtering structures in *Daphnia*, with dissected limbs directly left to dry in a desiccator for three days; the author claimed this provided very good results as the filtering combs kept their natural appearance (Brendelberger, 1985). Similarly, Saha et al. (2011) washed the relatively hard-bodied cladocerans of the genus *Bosmina* with just distilled water, and let them air-dry. Although the results were very good in that case, without substantial carapace shrinkage, this method usually results in completely shrivelled specimens in other Cladocera. Suitable drying methods thus should overcome or reduce surface-tension forces, and preserve the original look of the material.

For soft or hydrated organisms, usually physical and chemical dehydration methods are used. The critical-point drying (CPD) method is the most popular

among physical methods (Laforsch & Tollrian, 2000). It was introduced by Anderson (1951), who showed that surface tension forces in submersed specimens can be eliminated by heating the liquid above its critical point where it changes into a gas. At that critical temperature the gas pressure and liquid phases are in equilibrium and there is no phase boundary between them. The CPD method was apparently first applied on microcrustaceans by Crittenden (1981), and since then it has also been the most frequently used dehydration method used in the preparation of Cladocera (e.g., Ganf & Shiel, 1985; Mangalo, 1987; Hartmann & Kunkel, 1991).

Chemical dehydration methods are usually based on sublimation of the solid phase of organic substances from the specimen under various conditions such as in a vacuum or at an appropriate temperature. Specimens are usually dehydrated by a series of increasing concentrations of ethanol or acetone, subsequently replaced by a chosen organic compound that is then allowed to sublimate from the sample. For dehydration of marine Cladocera, Nival & Ravera (1979) used paradichlorbenzene with a melting point of 53.5°C, and reported excellent results. Inoué & Osatake (1988) used *tert*-butyl alcohol, and this was then successfully used for studies of trunk limbs (Sacherová, 1998) and shell structures (Kořínek et al., 1997) of Cladocera. It has an even lower melting point (26°C), and the fine structures of dried material show almost no artefacts. Evaporation of organic substances is another widely used approach for chemical dehydration. A method that had been previously suggested to be a cheap and effective method for some animal samples (Bray et al., 1993) was adapted for use in daphnids by Laforsch & Tollrian (2000). Instead of a solid phase, they recommended liquid bis(trimethylsilyl)amine, also known as hexamethyldisilazane (HMDS), evaporating slowly at room temperature. This approach has been used frequently in *Daphnia* studies since then (e.g., Petrusek et al., 2009; Juračka et al., 2010).

Aims of the study

In this study, we describe the adaptation of some currently used SEM preparation techniques specifically for Cladocera. We had two main aims. First, we tested for potential undesirable influences of two strong chemical cleaning agents commonly used in light microscopy, potassium hydroxide and lactic acid, on the ultrastructures of ephippia and filtering limbs. Second, we aimed to facilitate work with individual thoracopods under full visual control, also allowing correction of their orientation, with minimal physical manipulation of the limbs as this introduces a substantial risk of limb damage or even loss.

MATERIAL AND METHODS

Material studied

Free ephippia and ephippial females of five common European *Daphnia* species (*D. pulex* Leydig, 1860, *D. longispina* (O. F. Müller, 1776), *D. curvirostris* Eylmann, 1887, *D. magna* Straus, 1820 and *D. obtusa* Kurz, 1875) were collected from seven small habitats in the Czech Republic (see table I). Ephippia were sampled from the water surface with a plankton net of mesh size 0.1 mm, and were kept in water until sample processing in the laboratory. Simultaneously sampled ephippial females were fixed with 80% alcohol. Filtering limbs were dissected from ethanol-preserved specimens of five *Daphnia* species varying in size, *D. ambigua* Scourfield, 1947, *D. atkinsoni* Baird, 1859, *D. magna*, *D. pulicaria* Forbes, 1893 and *D. tibetana* (Sars, 1903), and kept in ethanol (table I).

Preparation of ephippia and filtering limbs

To compare different preparation techniques, ephippial shells were separated into two halves with a sharp needle. One half was analysed without any treatment, while the second one was washed for 5 min in either 10% potassium hydroxide or 90% lactic acid at 80°C and subsequently washed in distilled water prior to observation. Both ephippial halves were then left to dry out in a desiccator for several days at room temperature. To enable microscopy of thoracopods, specimens preserved in ethanol were transferred to either a hot 10% potassium hydroxide bath or 90% lactic acid bath for 10 min. They were then passed through at least three baths of de-ionized water to wash out the remnants of the acid or the hydroxide. Afterwards, water was replaced with 70% ethanol with the addition of two specific stains — a mixture of chlorazol black E and lignin pink (Kořínek, 1999). Staining of otherwise transparent small limbs allows easier handling and reduces losses during subsequent procedures. Stained specimens were then transferred to 100% ethanol.

Preparation of the specimens for limb dehydration was done in three different ways, according to the animal size and character of the material: (1) In the case of very small species, e.g., *D. ambigua*, or old or rare material, whole adult females were dehydrated without any dissection prior to dehydration. (2) Most frequently, cladoceran specimens in alcohol were dissected with two sharpened tungsten-wire dissecting needles under a stereomicroscope. One needle was used to keep the specimen in a fixed position on a microscopic slide, the other to open the upper valve of the carapace and detach the inner part of the body, including all limbs and the postabdomen, at the location of mandibles. (3) Optionally, the filtering apparatus removed from the animal by the above-mentioned procedure

TABLE I
Material used in this study

Species	Locality	Geographical coordinates	Locality type	Sampling date	Preservation	Collector(s)
Ephippia						
Daphnia australis	Mason Bay, Australia	33°49′18″S 120°23′41″E	Hyposaline swamp	30 August 2003	Ethanol	MZ
Daphnia curvirostris	Experimental pool D1, Tupadly, Czech Republic	50°26′16″N 14°28′20″E	Fishless pool	30 November 2012	None	PJJ, DB, ID, ZN
Daphnia dolichocephala	Road Darling Malmesbury, W. Cape, South Africa	33°26′02″S 18°41′13″E	Artificial dam	6 September 2005	Ethanol	JM
Daphnia longispina	Vrbno, Czech Republic	49°24′37″N 13°48′16″E	Garden artificial pool	29 November 2012	None	PJJ, VK
Daphnia longispina	Farář fishpond, Bítovany, Czech Republic	49°53′27″N 15°51′56″E	Large fishpond	29 June 2011	Ethanol	PJJ
Daphnia middendorffiana	GR6, Greenland	67°01′05″N 52°27′03″W	Freshwater fishless pool	29 September 2012	Ethanol	PJJ
Daphnia obtusa	Medonosy, Czech Republic	50°30′06″N 14°29′07″E	Fishless pool	30 November 2012	None	PJJ, DB, ID, ZN
Daphnia magna	Býčkovice, Czech Republic	50°33′40″N 14°12′39″E	Small fire reservoir	30 November 2012	None	PJJ, DB, ID, ZN
	Prostřední fishpond, Lednice, Czech Republic	48°46′55″N 16°48′11″E	Large fishpond	26 July 2011	Formaldehyde	AP

TABLE I
(Continued)

Species	Locality	Geographical coordinates	Locality type	Sampling date	Preservation	Collector(s)
Daphnia pulex	Tchořovice, W. of Smyslov pond, Czech Republic	49°25′08″N 13°48′7″E	Pool	29 November 2012	None	PJJ,VK
	Přerov nad Labem, Czech Republic	50°10′14″N 14°48′60″E	Fluvial pool	30 November 2012	None	PJJ, DB, ID, ZN
Daphnia pulicaria	Las Vegas, Nevada, U.S.A.	36°N 115°W	Pond	1 January 1989	Ethanol	PDNH
Filtration limbs						
Daphnia ambigua	Sunningdale, Buckinghamshire, U.K.	51°22′20″N 0°36′58″W	Pond	28 May 2011	Ethanol	MB
Daphnia magna	Prostřední fishpond, Lednice, Czech Republic	48°46′55″N 16°48′11″E	Small pond at shore of large fishpond	19 June 1956	Formaldehyde	VK
Daphnia pulicaria	Yellowstone National Park, Wyoming, U.S.A.	44°33′40″N 110°22′23″W	Lake	17 August 1991	Formaldehyde	MC
Daphnia tibetana	Zorkul, Tajikistan	37°26′24″N 73°45′36″E	Saline pool near Zorkul lake	9 July 1971	Formaldehyde	AK

Abbreviations of collector names: AK, A. Khaitov; DB, D. Bovšková; ID, I. Dobiášovská; HK, H. Kling; JM, J. Mergeay; LK, L. Kohout; MB, M. Burgis; MC, M. Černý; MZ, M. Žofková; PDNH, P. D. N. Hebert; PJJ, P. J. Juračka; VS, V. Sacherová; VK, V. Kořínek; ZN, Z. Novosadová.

was separated to individual stained limbs, which were separated one by one from remaining remnants of the body and from the postabdomen.

Regardless of the dissection procedure, the whole specimen, filtering apparatus, or individual limbs were subsequently moved in a drop of ethanol to small Eppendorf tubes and subjected to dehydration in a graded series of acetone solutions in alcohol: 30, 50, 70, 80, 90, 95 and 97%, and twice in 100% acetone. These solutions were always exchanged in the tubes after 10 min using a small plastic pipette, avoiding physical contact of the pipette with the study objects. Finally, the acetone was replaced with HMDS and left for 45 min according to Laforsch & Tollrian (2000).

For mounting, the whole animals were positioned on aluminium stubs using a fine but stiff hair (e.g., eyelash, dog or paintbrush hair) glued to a dissecting needle, while the limbs or the whole filtering apparatus were moved in a drop of HMDS with a wide pipette directly to the surface of a microscopic cover glass. If needed, the objects were carefully positioned with a hair. When well positioned, the limbs were allowed to dry slowly in a flow box and their position was frequently checked while drying, and corrected as necessary. Once all visible HMDS had evaporated, the glass with the limbs or whole specimens mounted on the aluminium stubs were transferred to a desiccator or vacuum chamber with silica gel for approximately one day to finish the drying process.

Scanning electron microscopy and image processing

Completely dry material (both ephippia and limbs) mounted on aluminium stubs or microscopic glasses was gold-coated in BAL-TEC Sputter Coater SCD 050 for 5 min in argon plasma at 0.1 mbar vacuum. SEM analyses at standardized magnifications (1000×, 2000× and the whole ephippium) were subsequently done with a JEOL JSM-6380 LV scanning electron microscope at 15 kV. As the surface ultrastructure might be variable on different parts of the ephippium, a standardized area directly above the egg chamber was always photographed. The limbs were observed with the same microscope at the same settings, but under various magnifications according to the material size. For the purposes of presentation, subtle image noise was reduced and the heterogeneous background was replaced with solid black in Adobe Photoshop CC.

RESULTS

Ephippia

Sample dehydration with HMDS as suggested for daphnids by Laforsch & Tollrian (2000) worked well for both soft body structures and ephippia (fig. 1A).

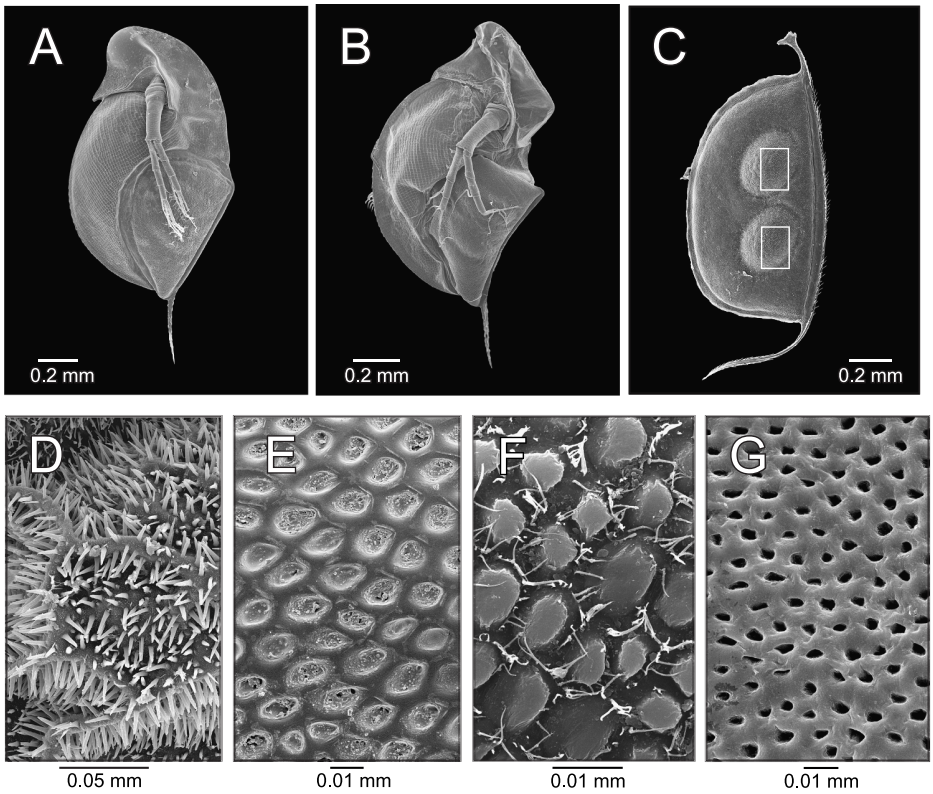

Fig. 1. A, Ephippial female of *Daphnia longispina* (O. F. Müller, 1776), dehydrated using hexamethyldisilazane (HMDS; see Methods). All body structures are well preserved; B, Ephippial female from same sample left to desiccate naturally. Note the crumpled body, while the ephippium is well preserved; C, Free ephippium of *Daphnia magna* Straus, 1820 collected from pool mud. The ephippium was fixed with formaldehyde, cleaned in potassium hydroxide and left to desiccate naturally. Rectangles show standard areas used to compare different ephippia and methods in this study; D-G, variation in the ephippial ultrastructure of selected *Daphnia* species. D, *Daphnia australis* (Sergeev & Williams, 1985); E, *D. middendorffiana* Fisher, 1851; F, *D. dolichocephala* Sars, 1895; G, *D. pulicaria* Forbes, 1893.

When ephippial females were left to desiccate naturally in the air without any special treatments, soft parts of the body were usually crumpled but ephippia remained well preserved within the female body (fig. 1B), similarly to free ephippia (fig. 1C-G). The observed ephippial surfaces varied substantially, even among ephippia of a single species sampled from one locality on the same date (fig. 2A-D). These probably differed in age, and some were covered with a massive layer of detritus. We succeeded in removing unwanted biofilm from the ephippial surface using both potassium hydroxide (fig. 2E, F) and lactic acid (fig. 3E, F). However, neither cleaning agent made a substantial difference in some cases

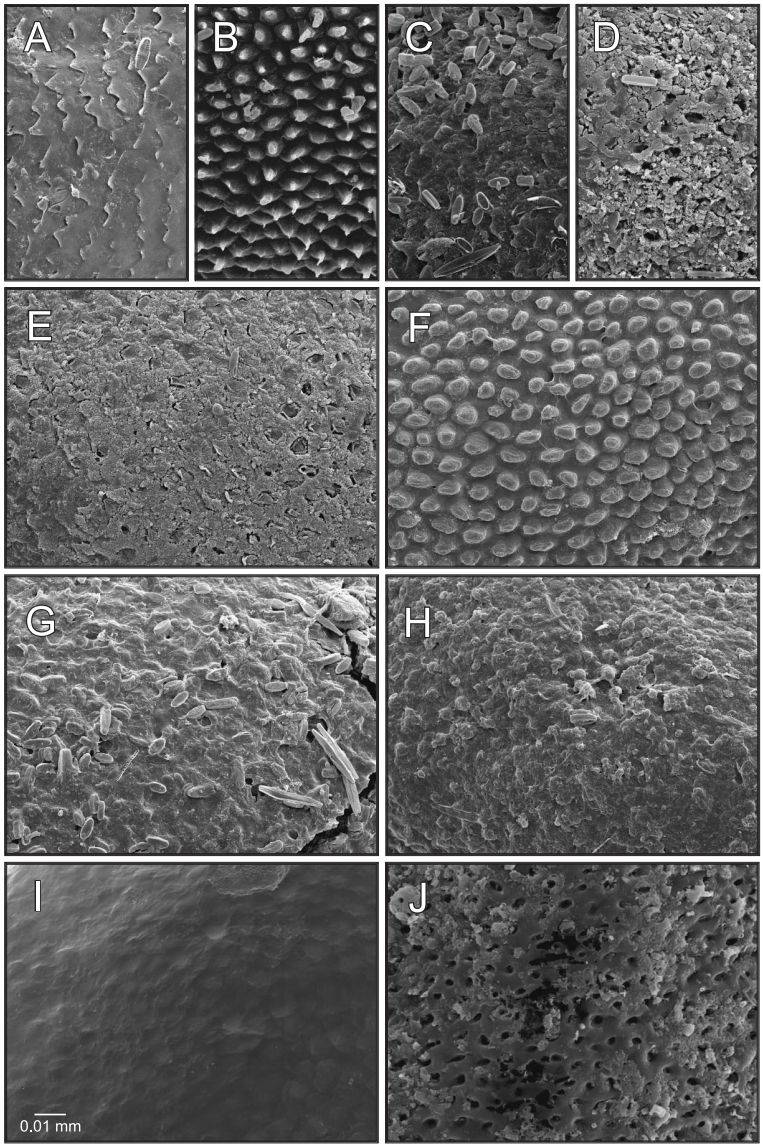

Fig. 2. A-D, Variability in the surface ultrastructure of *Daphnia magna* ephippia sampled from the same fishless pool. A, B, variation arising from ontogenetic development; C, D, ephippial surfaces covered with particles. None of the ephippia were either fixed or treated with chemicals, and were left to desiccate naturally; E-J, comparison of results gained with chemical cleaning of the ephippial surface. Ultrastructure of *Daphnia magna* ephippium without any treatment is covered with a massive layer of debris (E), while the opposite half of the same ephippium treated with potassium hydroxide was well cleaned (F). The same treatment of another ephippium from the same sample (G) did not have significant effect (H). Potassium hydroxide treatment may alter the ephippial ultrastructure: halves of a *D. pulex* ephippium without (I) and with (J) treatment.

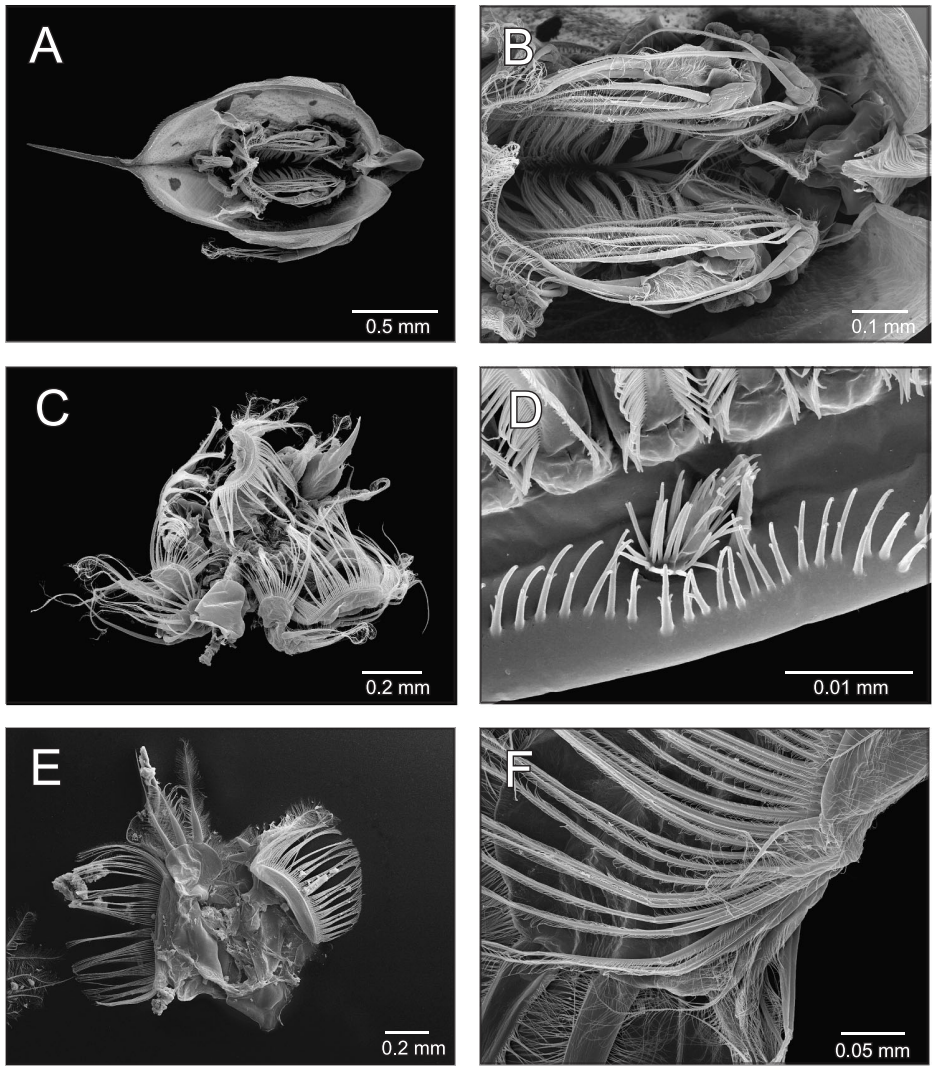

Fig. 3. Microphotographs of *Daphnia* filtering limbs. A, B, adult female of *Daphnia pulicaria* dehydrated in HMDS, and slightly opened with needles when mounted on an aluminium stub; C, D, dissected limbs of *D. magna* treated with lactic acid and dehydrated with HMDS; the whole filtering apparatus was dissected from the animal prior to dehydration; E, F, thoracopods of *Daphnia tibetana* (Sars, 1903) washed in lactic acid; E, endo- and exopod of the third filtering limb (left) and endopod of the fourth limb (right) dissected from the animal prior to dehydration; F, limb setation in detail.

(fig. 2G, H), and they can even substantially damage the ephippial surface, which may get crumpled or even entirely removed (fig. 2I, J).

Limbs

We prepared filtering limbs of 5 *Daphnia* species with the new simplified method, which repeatedly enabled the dehydration and direct observation of individual limbs under the scanning electron microscope (fig. 3), with very low risk of limb damage or loss. The limbs kept their natural look without any unwanted artefacts. Biofilm and unwanted particles covering sensory organs and other limb details were successfully removed using both lactic acid and potassium hydroxide.

DISCUSSION

In contrast to taxonomic studies based mostly on fresh or recently collected material from which suitable objects can be more easily selected, paleoecological studies need to work with large amount of ephippia stored in the sediments for a long time, which are therefore usually covered with a layer of debris. Hence, there is a need to process the material safely and quickly. For ephippia, natural dehydration by simply leaving them in the air is easy and suitable in most cases, while sophisticated dehydration methods, such as critical-point drying, are time consuming and generally do not provide better results.

However, both for taxonomic and ecological studies it is important to compare sufficient numbers of ephippia from each population and sample, because the observed ephippial surface ultrastructure may vary substantially (fig. 2A-D). We explain this variation through two main effects: (1) changes to the physical state of the ephippium as it ages; and (2) differences arising from ontogenetic development, as the ultrastructure might differ substantially during the formation of the ephippium (Hiruta & Tochinai, 2014). When evaluating the suitability of the two strong chemical agents for cleaning the ephippial surface, we often obtained excellent results (fig. 2F) but also observed some unwanted impacts that may affect the results, such as the upper layer of the ephipipum being damaged, or even destroyed (fig. 2J).

Ephippia desiccate naturally, so it is possible to observe them under the scanning electron microscope with minimal preparation. This is of great advantage, as it is usually impossible to observe biological material under the SEM without fixation and dehydration, which may both induce unwanted artefacts. Therefore, we would recommend first studying ephippial ultrastructures without any treatment. If there is a massive layer of unwanted particles covering the surface and therefore preventing observation of the ultrastructure, it might be useful to try cleaning the surface with both chemicals on a small subsample to test which of these two agents, if any, provides suitable results. Under the most common circumstances,

we suggest to apply these chemicals for 5 min at 80°C, and increase the incubation time if no effect is observed. As mentioned above, the ephippial ultrastructure might differ during the ontogenetic development (Hiruta & Tochinai, 2014); therefore, we suggest analysing free ephippia rather than ephippial females whenever possible. If only ephippial females are available, it is preferable to select those carrying ephippia with well-developed melanization, which should be more mature than less pigmented ones (Hiruta & Tochinai, 2014).

Both potassium hydroxide and lactic acid can be used to clean filtering limbs as well; this is often useful, as organic or inorganic particles of various chemical composition tend to attach to limb setae. As every manipulation with the filtering limbs introduces a serious risk of material damage or even loss, it is important to minimize handling; in the workflow suggested by us, it is only necessary during initial sample preparation. Our approach also allows fine adjustment of the limb position, without the undesirable artefacts associated with physical manipulation. To remove debris, we recommend first washing a well-stained cladoceran specimen in lactic acid at 80°C for 10 min, then dissecting it and removing all filtering limbs in one pack, followed by dehydration by the graded acetone series. If necessary, separation of individual limbs may be performed either before dehydration or at its final stage, in a drop of HDMS. To avoid damaging fragile dehydrated limbs, it is best to transfer them in a drop of HMDS using a wide pipette to a microscope slide, and leaving them to desiccate.

This suggested workflow provides a fast, easy and low-cost method of ephippia and limb preparation for SEM analyses, allowing a relatively large amount of material to be studied. This is particularly suitable for ecological and palaeoecological surveys, especially for species that can be reliably identified by the ephippia structure. One emerging example of such species are the phenotypically similar North American *Daphnia* species *D. ambigua* and *D. parvula*, which have recently spread across Central Europe and often co-occur in invaded regions (Žofková et al., 2002). These can be particularly well-differentiated by their ephippial ultrastructure (Juračka & Kořínek, unpubl. data). Their ephippia found in lake sediments can be identified to species level, allowing more precise analyses of their local invasion histories. Another example where such an approach may be useful is screening for the presence of the recently described *Daphnia hrbaceki* (Juračka et al., 2010), a species with an unknown distribution area and origin that differs in the ephippial ultrastructure from its closest relative, *D. curvirostris*.

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