DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„Trophic ecology and phylogeography of fairy shrimps (Anostraca), key species of temporary waters"

verfasst von / submitted by

Dunja Lukić

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien, 2019 / Vienna 2019

Table of content

Acknowledgements

Summary

Temporary ponds are widespread throughout the world and comprise diverse systems regarding size, shape, hydroregime and water chemistry. They contribute significantly to regional biodiversity and host several taxa specially adapted to these environments. One of the most conspicuous examples are fairy shrimps (Crustacea, Anostraca), a flagship group of temporary waters. The aims of the thesis were twofold: (1) we first studied the trophic role of this relatively little-known group, with *Branchinecta orientalis* as our model species (food spectrum of fairy shrimps in Chapter 1 and the effect of turbidity on feeding in Chapter 2); and (2) we revealed phylogeographic patterns in two closely related species (*Branchinecta orientalis* and *Branchinecta ferox*), investigating the role of historic and recent connectivity among their habitats of occurrence (Chapter 3).

We showed that fairy shrimps are capable of feeding on both phyto- and zooplankton, effectively ingesting prey particles ranging from 2 µm to 2 mm in size (Chapter 1). They therefore function as intraguild predators in the food web of temporary waters. Then we investigated whether inorganic turbidity has a direct effect on the feeding of fairy shrimps, causing changes in the strength of intraguild predation (Chapter 2). The results showed that the diet of fairy shrimps was more herbivorous in clear waters, while it was predominantly carnivorous in turbid waters. Turbidity may therefore have an important role in the shaping of plankton communities of temporary ponds by directly altering the strength of trophic interactions. In Chapter 3, we revealed the phylogeny of *B. orientalis* and *B. ferox*, covering a wide geographic area of their current distribution in the Palearctic realm. Even though the two species have overlapping distribution today, we found that most likely they survived the last glacial periods in separate refugia. Their genetic diversity was generally low which implied a relatively recent areal expansion, especially in *B. orientalis*. This indicates that migratory waterbirds probably play a crucial role in their passive dispersal.

Zusammenfassung

Temporäre Gewässer sind weit verbreitet auf der Erde. Sie sind sehr vielgestaltig hinsichtlich ihrer Größe, Form, Hydroperiode und Wasserchemie, und beherbergen viele Arten die speziell an diese Lebensräume angepasst sind. Diese Arbeit behandelt eine für diese Gewässer besonders charakteristische Gruppe, Feenkrebse (Anostraca), und gliedert sich in zwei Schwerpunkte: (1) zuerst wird die trophische Ökologie dieser bislang wenig untersuchten Gruppe anhand einer Modellart untersucht (*Branchinecta orientalis*; Größenspektrum des Futters in Kapitel 1 und Einfluss von Trübung auf die Futterwahl in Kapitel 2). Danach wird die Phylogeographie (2) zweier nah verwandter Arten (*Branchinecta orientalis* and *Branchinecta ferox*) unter Berücksichtigung der historischen und rezenten Vernetztheit ihrer Habitate untersucht.

Anhand experimenteller Daten wird gezeigt dass Feenkrebse sowohl Phyto- and Zooplankton fressen, und dabei ein Größenspektrum von 2 µm bis 2 mm abdecken (Kapitel 1). Sie fressen somit auf zwei trophischen Ebenen (‚intraguild-predators'). Daraufhin wird untersucht ob anorganische Trübung die Nahrungswahl und somit die trophische Ebene von *B.orientalis* beeinflusst (Kapitel 2). Die Ergebnisse zeigen dass B.orientalis in klarem Wasser weitgehend herbivor ist, und mit zunehmender Trübung zum karnivoren Prädator wird. Trübung kann somit unmittelbar die Stärke tropischer Interaktionen und somit die Nahrungsnetzstruktur temporärer Gewässer verändern. Im Kapitel 3 wird die Phylogenie von *B. orientalis* und *B. ferox* in einem großen geographischen Bereich ihres aktuellen Verbreitungsgebietes in der paläarktischen Region untersucht. Obwohl die Verbreitungsgebiete beider Arten heute überlappen, legen die Daten nahe dass sie im letzten Glazial unterschiedliche Rückzugsgebiete hatten. Die genetische Diversität war vor allem bei *B. orientalis* generell begrenzt, was eine rezente Ausbreitung nahelegt. Daher ist anzunehmen dass Wasservögel eine besondere Rolle für ihre Ausbreitung spielen.

Zusammenfassung

General Introduction

Introduction

Temporary ponds as unique freshwater habitats

Compared to lakes, ponds and temporary waters are underrepresented in traditional and contemporary limnology. Intensive research on ponds and pools only gained momentum in the 21th century, when ecologists recognised that their small size, ecological simplicity and frequent occurrence in the landscape makes them attractive model systems (De Meester *et al.*, 2005; Oertli *et al.*, 2009). Despite the fact that they are generally small and shallow, ponds host a unique flora and fauna. High local diversity and dissimilarity among habitats allow ponds to host a disproportionally high fraction of total regional biodiversity (Williams *et al.*, 2004; Oertli *et al.*, 2005; Downing, 2010; Boix *et al.*, 2012). Besides their biodiversity value, they also deliver a number of ecosystem services related to recreation, agriculture, water retention and purification and play a major role in elemental cycles (Downing, 2010; Hassall, 2014).

Temporary ponds comprise very diverse ecosystems regarding size, shape, hydroregime and water chemistry (Williams, 2006). Recurring dry phases make these habitats periodically unfavourable for aquatic organisms (Schwartz & Jenkins, 2000) (**Fig. 1A & B**). The dry phase is also the key factor preventing most fish from colonizing temporary habitats, which allows for a very unique biota in terms of macroinvertebrates. In extreme cases, they can remain dry for several years or even decades. They are widespread throughout the world (Jeffries *et al.*, 2016), but are especially prevalent in arid and semi-arid climatic regions (Brendonck, 1996). Due to their small size, short duration and absence of fish, the food webs of temporary ponds are generally expected to be simpler (with less trophic levels) than those of larger water bodies such as lakes (Begon, Townsend & Harper, 2006).

The most important local environmental factors that structure community composition include hydrology, habitat morphometry (e.g. depth, surface area), water quality (e.g. salinity, pH, nutrient concentrations) and biotic interactions (e.g. predation, competition). In addition, as temporary waters are generally very shallow, their sediment is easily stirred up by wind (Lahr *et al.*, 1999; Naganawa & Zagas, 2002) (**Fig. 1C**) or biotic agents such as large wallowing mammals and waterfowl (Vanschoenwinkel *et al.*, 2011). As a result, turbidity is generally high, but may vary dramatically among temporary habitats (from transparent to highly turbid conditions with transparency measurable only in centimetres; Boros *et al.*, 2017) (**Fig. 3D**).

While the community structuring effects of several environmental factors are generally appreciated (e.g. nutrient levels [Leibold, 1999], shading [Mokany, Wood & Cunningham, 2008], hydroregime [Waterkeyn et al., 2008; Vanschoenwinkel et al., 2013; Zokan and Drake, 2015], salinity [Waterkeyn *et al.*, 2008; Horváth *et al.*, 2014] and predation [Zokan & Drake, 2015]), the impact of turbidity caused by inorganic particles such as clay or silt received only limited attention.

In temporary ponds, the biomass of herbivorous consumers is often dominated by suspension-feeding crustaceans (cladocerans, anostracans, copepods) and rotifers. These organisms, particularly anostracans (Brendonck *et al.*, 2008), benefit from the general lack of fish in temporary waters. However, for cladocerans it is already known that they are at the same time adversely affected by the high concentration of inorganic suspended solids, which they ingest together with organic particles (e.g. bacteria, algae) due to non-selective filter-feeding (Levine, Zehrer & Burns, 2005). The obvious numeric importance of large cladocerans and anostracans in turbid temporary waters (Horváth *et al.*, 2014) is in clear conflict with this assumption. At present, it is not well known how zooplankton communities might be shaped by turbidity in temporary systems and whether there is a difference among the major groups in the ability of coping with (often extreme) turbid conditions.
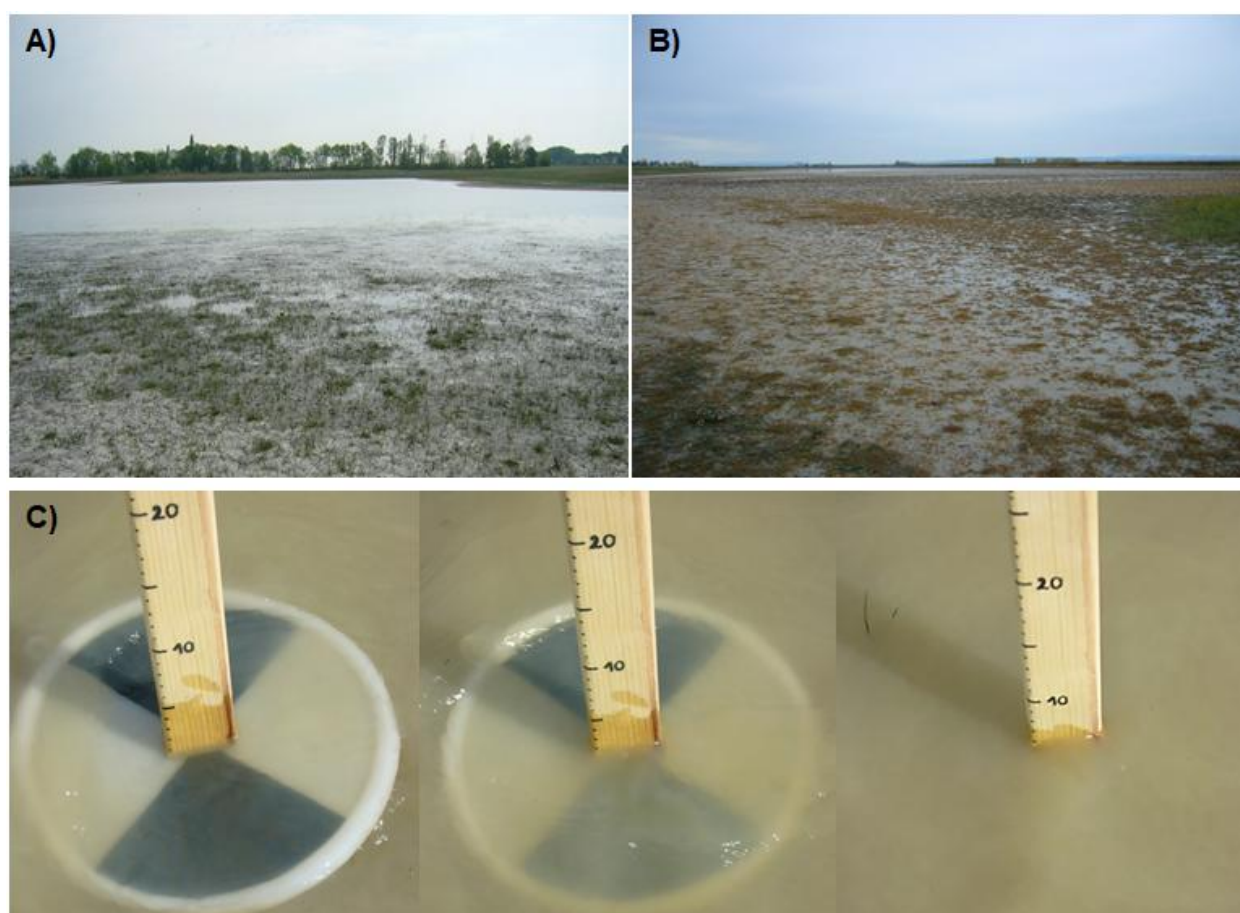
Figure 1. Some characteristics of temporary ponds on the example of my study systems, the soda pans, in Eastern Austria. A) Wet phase of a soda pan (Oberer Stinkersee); B) Dry phase of the same soda pan; C) Illustration of Secchi depth and turbidity of soda pans. Photos by Zsófia Horváth.

Anostracans and other crustaceans in temporary ponds

Crustacean zooplankton (branchiopods, copepods and ostracods) from temporary ponds evolved specific strategies to cope with the specific hydroregime of their environment. They can grow and reproduce rapidly during the favourable periods, and produce resistant dormant eggs

(**Fig. 2**) to bridge the unfavourable periods (Hildrew, 1985; Weeks, Marcus & Alvarez, 1997; Brendonck, De Meester & Riddoch, 2000a). Resting eggs are generally resistant to a wide range of environmental conditions (e.g. high solar radiation, extreme temperatures) (Brendonck & De Meester, 2003; Brock *et al.*, 2003; Havel & Shurin, 2004; Radzikowski, 2013). Zooplankton disperse passively and the dormant eggs act as propagules that can be picked up by vectors such as wind (e.g. Vanschoenwinkel et al., 2008), animals or humans (Bilton et al. 2001; Havel and Shurin 2004). The main animal vectors that are known to mediate passive dispersal of aquatic invertebrates are waterbirds (e.g. Figuerola and Green, 2002), mammals (Vanschoenwinkel *et al.*, 2008b, 2011), amphibians (e.g. Bohonak and Whiteman, 1999), and aquatic insects (e.g. Van de Meutter et al., 2008). The different dispersal vectors may drive landscape-scale patterns in community composition (Horváth, Vad & Ptacnik, 2016) or even continental-scale gene flow in zooplankton (Viana *et al.*, 2013).
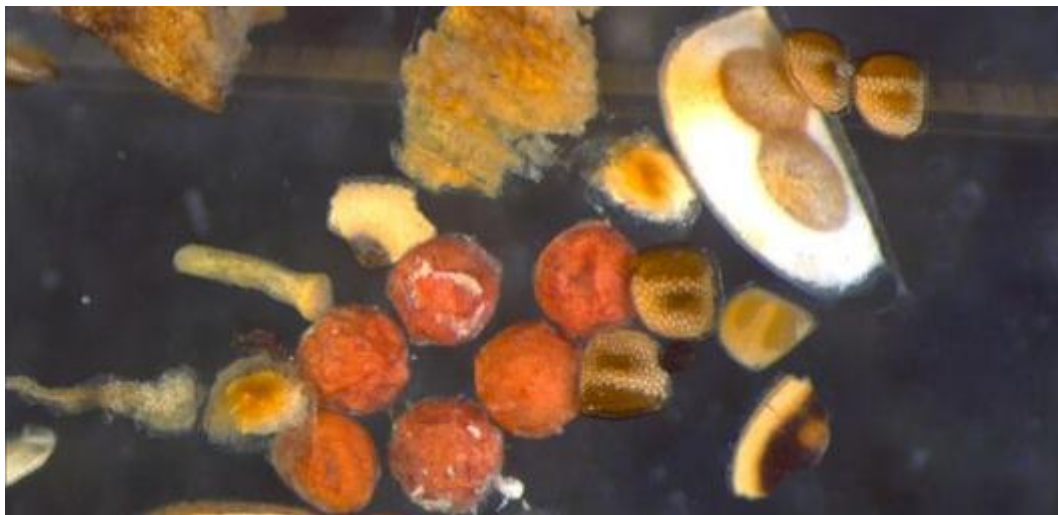
Figure 2. The egg bank of crustacean zooplankton from a temporary pond. Photo by Zsófia Horváth.

Large branchiopods (encompassing five recent orders: Anostraca, Notostraca, Spinicaudata, Laevicaudata and Cyclestherida) are a flagship group in temporary ponds. Being relatively large-bodied animals, they are conspicuous in these habitats. Due to their vulnerability to fish predation, they occur almost exclusively in temporary waters. The fossil remains of the group derive from Upper Cambrian (Waloßek, 1995) and large branchiopods are frequently referred to as living fossils due to their morphological stasis since that period (Williams, 2006). Some notostracan taxa are considered ecosystem-engineers, as they can increase water turbidity by the bioturbation of sediment (Yee, Willig & Moorhead, 2005; Croel & Kneitel, 2011; Waterkeyn, Grillas & Brendonck, 2016). Moreover, anostracans in soda pan systems of Central Europe represent an important food source for waterbirds, and even drive compositional changes in the visiting waterbird communities (Horváth *et al.*, 2013b).

The feeding ecology of **anostracans** is far from resolved. Together with cladocerans, they are generally considered to be filter-feeders, thereby representing an important role in ecosystem functioning since filter-feeders link energy transfer from phytoplankton and bacteria to vertebrates

(Persson *et al.*, 2007) and drive trophic cascades (Carpenter, Kitchell & Hodgson, 1985). However, the diet of anostracans is much more diverse compared to smaller filter-feeding zooplankters as it consists of organic and inorganic detritus, phytoplankton, microzooplankton, nematodes and larval crustaceans (summarized in Sanchez and Angeler, 2007). Besides, a rising number of studies challenged the view of anostracans as strictly filter-feeding organisms by providing first evidence of active predation on adult crustaceans such as cladocerans (Fryer, 1983; Sarma & Nandini, 2002), copepods (White, Fabris & Hartland-Rowe, 1969; Fryer, 1983) and even other anostracans (White *et al.*, 1969; Rogers *et al.*, 2006).

Phylogeny of large branchiopods in the Palaearctic

Modern **molecular methods** allow us to study diversity even at the intraspecific level. With the help of molecular methods, it is possible to detect historical and ongoing gene flow among populations and to follow dispersal events more precisely than with traditional methods based on the data on morphological features (Freeland, Kirk & Peterson, 2012). Moreover, molecular methods can be regarded as the only tools to track real dispersal events in microscopic species, as the collection and study of dispersing propagules only informs on potential dispersal, while molecular markers show effective gene flow which depends not only on dispersal, but also successful colonization events. They can inform on the impacts of environmental changes on individual species which enables us to better predict the effects of comparable local changes in the future. Finally, molecular methods help us to define evolutionary significant units for efficient biodiversity conservation (Moritz, 1994).

The Pleistocene period (2.5 mya - 11 kya) was characterised by extreme climate fluctuations. It involved repeated cold periods with broadly distributed ice cover and is therefore often referred to as the Ice Age. The cyclical periods included ice cover expansions alternating with the milder periods of glacial retreat. Glacial periods, especially for the temperate species, were associated with local population extinctions, southward range shifts (in the northern hemisphere) and genetic bottlenecks. In contrast, milder periods of glacial retreat were followed by range expansions from south to the north, in spite of the general difficulty of latitudinal range shifts in Western Palaearctic due to longitudinal barriers (the Alps, Pyrenees, the Mediterranean Sea). In Europe, the southern regions such as the Balkan, Apennine and Iberian Peninsula are recognized as valuable Ice Age refugia of many species and nowadays these regions consequently host high genetic diversity and local endemics (Habel *et al.*, 2010). Moreover, North Africa is also recognized as a refugium for many recent European animal groups that spread/returned to Europe after the ice retreated (Husemann *et al.*, 2014).

As in many other animal groups, the Pleistocene climate fluctuations had a strong impact on the current distribution and genetic diversity of large branchiopods in the Palaearctic. In the recent years, numerous phylogenetic studies addressed species or genus level (Ketmaier *et al.*,

2008; Vanschoenwinkel *et al.*, 2012; Reniers *et al.*, 2013; Lukić *et al.*, 2019). However, most of these studies were strictly limited, or predominantly referring, to the Western Palaearctic. Several studies found relatively high intraspecific genetic divergence in anostracans and suggested existence of morphologically cryptic species (Ketmaier *et al.*, 2008; Lukić *et al.*, 2019), exceeding the generally accepted threshold of 10% difference in the mitochondrial CO1 gene region for species delineation in branchiopods (Cox & Hebert, 2001; Pinceel *et al.*, 2013a). A number of explanations were proposed to explain the high genetic divergences between morphologically similar anostracan taxa such as high mutation incidents in halophilic taxa (Hebert *et al.*, 2002; Ketmaier *et al.*, 2008) or long-lasting isolation between populations persisting in multiple refugia during the Pleistocene (Lukić et al., 2019). On the other hand, relatively low genetic diversity was found in other large branchiopod species (Vanschoenwinkel *et al.*, 2012), which was particularly evident in regions of Northern Europe (Lukić *et al.*, 2019). This again stresses the strong impact of historic glaciations and southern refugia on the biodiversity of the currently temperate regions of Europe.

In anostracans, multiple evidence exist for long-distance dispersal events up to continental scales (Viana *et al.*, 2013). The majority of long-distance dispersal events in pond organisms are likely mediated by migrating waterbirds (Figuerola & Green, 2002; Brochet *et al.*, 2009). As a consequence, over larger geographic areas, the genetic diversity of anostracan populations can match well with the migration routes of waterbirds (Ketmaier *et al.*, 2008). The special role of birds as dispersing agents is also confirmed by finding dispersal limitation in other species inhabiting very small aquatic habitats such as rock pools, puddles on agricultural fields and wheel track pools that are rarely visited by birds (Brendonck *et al.*, 2000b; Lukić *et al.*, 2019). Compared to birds, less is known about dispersal via other animal groups, such as insects (Beladjal & Mertens, 2009), amphibians (Bohonak & Whiteman, 1999) or mammals (Vanschoenwinkel *et al.*, 2008b), as well as anthropogenic dispersal (on the tyres of motorized vehicles; Waterkeyn et al., 2010a) which might be of comparable importance in driving the distribution patterns of anostracans.

Study system – soda pans of the Central European Lowlands

The soda pans of the Central European Lowlands (Austria, Hungary and Serbia) are inland saline waters of non-marine origin (**Fig. 3 A-C**). Their ionic composition differs considerably from sea water (Boros, Ecsedi & Oláh, 2013). The pH of the pans ranges mainly from 7.5 to 10, their ionic composition is dominated by sodium salts (Na^+, CO_3^{2-} and HCO_3^- ; Boros *et al.*, 2013). The amount of total suspended solids (TSS) varies from 10 to over 30,000 mg L^{-1} (Boros et al., 2013, Horváth, unpubl. data) (**Fig. 3D**). Especially at the upper end of this gradient, total suspended solids (TSS) are almost exclusively comprised of abiotic and inorganic particles (Boros *et al.*, 2017). During their wet phase, soda pans and other temporary saline pools on the steppes of Central and Eastern Europe represent a considerable proportion of shallow water habitats in that area. For this reason, soda pans and sodic meadows are important resting sites for numerous

waterbird species during their seasonal migration on the north-south route in the Western Palearctic (Boros *et al.*, 2013; Horváth *et al.*, 2013b), and several of these habitats are listed as Important Bird Areas, Ramsar sites, or are under the protection of the network of Natura 2000 or UNESCO.

Figure 3. Soda pans in Eastern Austria. A) Map of the Seewinkel region (source: Google Earth); B) Östliche and Westliche Wörthenlacke, Auerlacke and Sechsmahdlacke; C) Weisser See (B & C photos by Attila Pellinger); D) Water samples from nine different Seewinkel soda pans collected on the same day (photo by Zsófia Horváth).

Study species – the fairy shrimps *Branchinecta ferox* and *Branchinecta orientalis*

The family Branchinectidae consists of two recent genera: *Branchinecta* and *Archaebranchinecta* (Rogers & Coronel, 2011). These genera likely diverged already 400 mya (Rogers & Coronel, 2011). Genus *Archaebranchinecta* comprises of only two recent species with a very limited distribution range in South America (Rogers & Coronel, 2011; Cohen, Marinone & Adamowicz, 2019). Genus **Branchinecta** includes around 50 species and inhabits all continents except Australia (Rogers, 2006; Marrone *et al.*, 2016). Many *Branchinecta* species are known from only a number of ponds and represent local endemics (Lindholm *et al.*, 2016a). This genus is the most diverse in North and South America with ~20 species on each of these continents (Belk & Brtek, 1995; Rogers, 2006). The high diversity of *Branchinecta* across the Nearctic is probably the consequence of repetitive merging of North and South America across their geological history (Rogers & Coronel, 2011). In the Palaearctic, six *Branchinecta* species have been described until now (Belk & Brtek, 1995). Some *Branchinecta* species, such as *B. gigas* and *B. raptor* (from the

Nearctic) are predators and, at the same time the largest recorded anostracans with up to 10 cm of body length (Fryer, 1966; Rogers *et al.*, 2006).

Branchinecta ferox inhabits temporary waters in steppes and steppe-like regions (Petkovski, 1991) (**Fig. 4A-B**). It has a circum-Mediterranean distribution spreading over three continents (**Fig. 4C**). In Europe, its range further extends toward northeast through the Pannonian Plain and South Ukraine to the southwest of Russia. It is also present in Southwest Asia (e.g. Israel and Syria) and North Africa (Morocco, Algeria and Tunisia; Marrone et al., 2016). *B. ferox* is the largest anostracan species in Palaearctic, reaching up to 7 cm in body length (Petkovski, 1993). During ontogeny, they lose fine setae on their feeding extremities which probably coincide with the shift from filter-feeding to predatorial feeding (Fryer, 1983). Their populations are usually of low densities (pers. obs.), which is rather common for large predatory species of this group (Daborn, 1975; Boudrias & Pires, 2002).
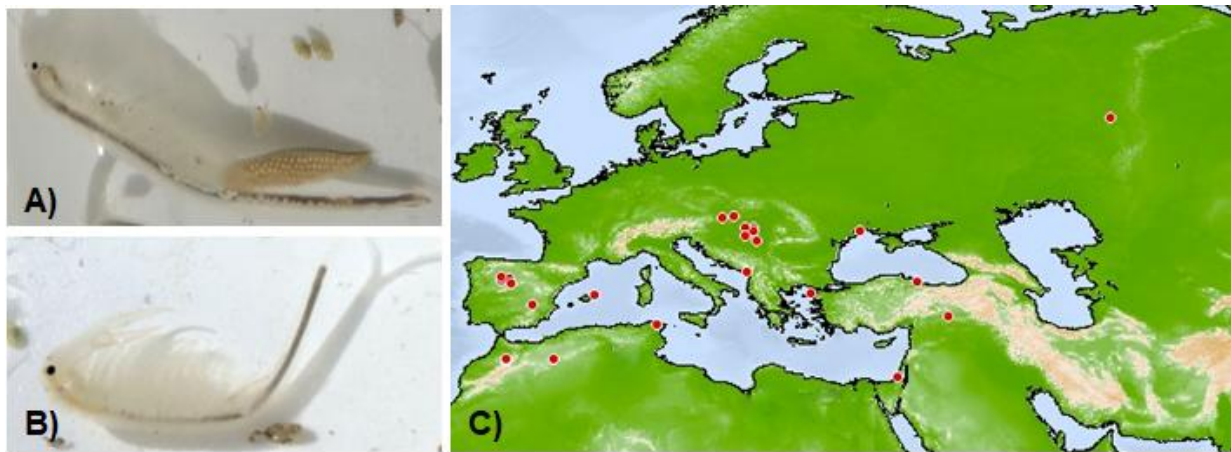
Figure 4. A) *Branchinecta ferox* female; B) *B. ferox* male; C) The currently known distribution of the anostracan *B. ferox* (Alonso, 1985; Petkovski, 1993; Horváth et al., 2013b; Rodríguez-Flores et al., 2017; Marrone et al., 2016; van den Broeck et al., 2015; Pretus, 1990; Mura et al., 2011; Marrone, pers. comm.). Records from the literature older than 50 years are not included. Photos by Zsófia Horváth.

Branchinecta orientalis inhabits mineral-rich temporary waters and occurs between 55° and 27° N in Europe and Asia (Mura & Takami, 2000; Padhye, Kulkarni & Dumont, 2017) (**Fig. 5 A-C**). According to its currently known distribution, this species inhabits five distinct regions in Eurasia: 1) Iberian peninsula, 2) Central Europe (Pannonian Plain), 3) Middle East (Turkey, Iran), and two in Central Asia 4) Himalayas and 5) Mongolia and Russia (Alonso, 1985; Petkovski, 1993; Manca & Mura, 1997; Mura & Takami, 2000; Mura *et al.*, 2011; Horváth *et al.*, 2013a; Atashbar *et al.*, 2014a; Marrone *et al.*, 2015; Naganawa *et al.*, 2019). Active populations of the species generally occur from March to June but exceptions have also been recorded in late autumn or winter (Petkovski, 1991; Eder et al., 1997; Atashbar et al., 2014; Šćiban et al., 2014). The discontinuous ('island') nature of this disjunct distribution range increases the potential for genetic isolation and differentiation between populations (Boileau & Hebert, 1991). *B. orientalis* is a mid-sized anostracan mostly reaching up to 3 cm of body length (pers. obs.). Compared to *B. ferox*, *B.*

orientalis has wider distribution (including more known populations) and can reach high densities at their habitats (Horváth *et al.*, 2013b).
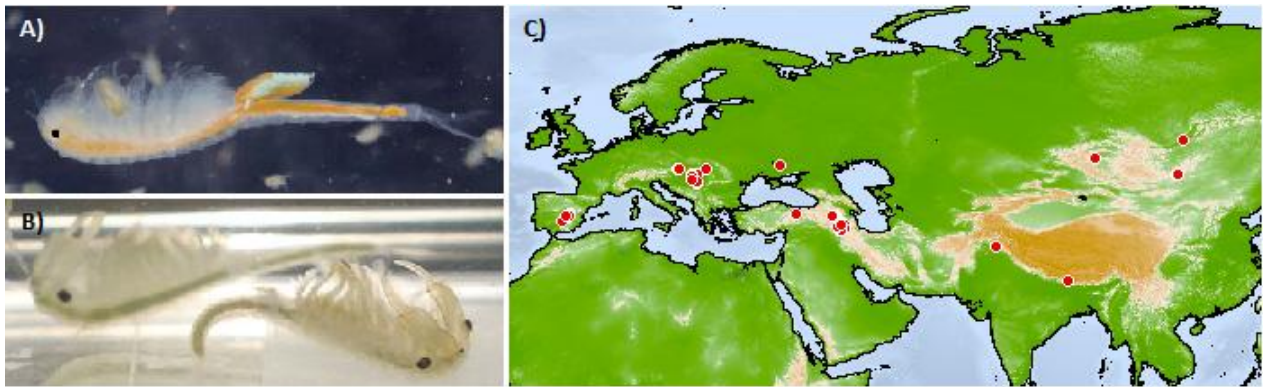
Figure 5. A) *Branchinecta orientalis* female; B) *B. orientalis* male; C) The currently known distribution of the anostracan *B. orientalis* (Alonso, 1985; Petkovski, 1993; Mura and Takami, 2000; Mura et al., 2011; Horváth et al., 2013b; Behroz Atashbar et al., 2014; Marrone et al., 2015; Angeler et al., 2008; Belk and Esparza, 1995; Ortells, pers. comm.). Records from the literature older than 50 years are not included. Photos by Imre Potyó (A) & Zsófia Horváth (B).

General research motivation

To date we have very limited information about anostracan taxonomy, phylogeny, ecology, and distribution (Brendonck, 1996; Brendonck *et al.*, 2008). A few local factors e.g. temperature, salinity or turbidity are already known to play an important role in their distribution (Boven et al., 2008; Horváth et al., 2013b; Lindholm et al., 2015; 2016b). Some species have a very broad geographic distribution, which makes them suitable objects to study historical distribution and dispersal patterns. Studies combining genetic and ecological information could offer both valuable insights into the evolutionary history of the studied groups and tools for delineating evolutionary significant units as focal points for conservation (Pinceel *et al.*, 2013b). Such overarching approaches would be essential since in large branchiopod crustaceans, as in many other groups, morphological variation does not adequately represent genetic diversity (Pinceel *et al.*, 2013b), and some species may harbour important cryptic adaptive variation that is essential to preserve in the context of current environmental changes (Pinceel *et al.*, 2013a). Anostracan (and, in general, large branchiopod) conservation is especially relevant as their habitats, temporary ponds, are threatened worldwide due to climate change and anthropogenic activities (Zacharias & Zamparas, 2010), and large branchiopods can be used as a flagship group for their conservation.

Judging from their distribution/habitat preference, several anostracan species, including *B. orientalis*, seem to be favoured by turbid conditions (Petkovski, 1991; 1993; Boudrias and Pires, 2002; Horváth et al., 2013b). Although high turbidity may protect them from visual predators (e.g. amphibians and birds; Petkovski, 1993; Boudrias and Pires, 2002; Boven et al., 2008) it likely also represents a feeding constraint. Furthermore, it may also influence the feeding success of predatory anostracans, as it probably reduces their ability of prey detection (Boudrias & Pires,

2002). Some predatory anostracans have well-developed chemical or mechanical sensory systems that could be an adaptation to life in turbid waters (Boudrias & Pires, 2002). The impact of turbidity on anostracan feeding (as a relatively large invertebrate in pond communities) may indirectly affect plankton community composition. It remains, however, largely unknown how the extreme amounts of suspended solids and highly turbid conditions affect the food uptake both via filter- and predatory feeding.

To the best of our knowledge, there are only two studies about genetic variation in *B. orientalis* populations, one from Iran (Atashbar et al., 2016) and one encompassing a population in Spain another in Hungary (Rodríguez-Flores *et al.*, 2017), while we lack genetic information from most of the current distribution range of this species.
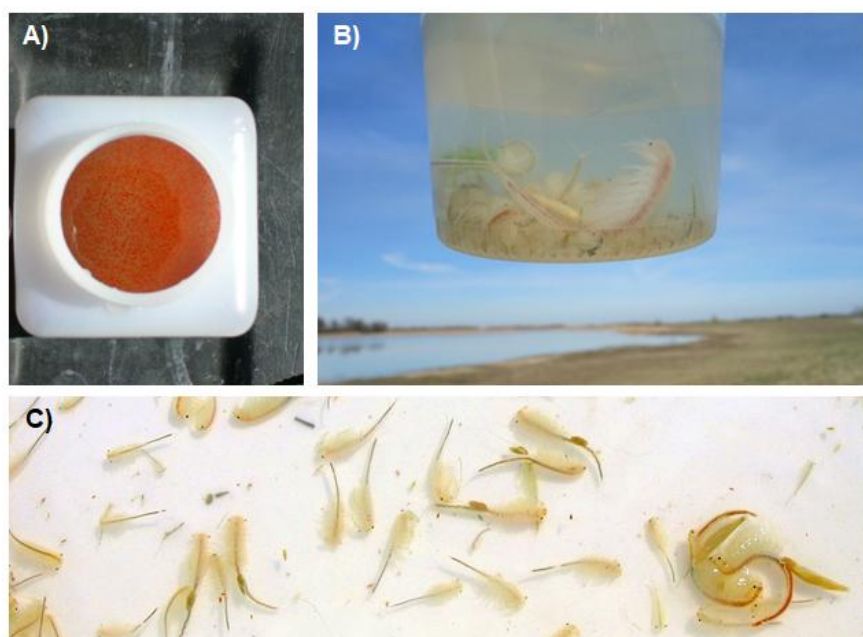
Figure 6. Study organisms. A) Calanoid copepods (*Arctodiaptomus spinosus*) used in feeding experiments as anostracan prey (Aim I); B) Sample with anostracans from one of the soda pans, with one *Branchinecta ferox* female in the focus; C) Anostracan sample with both studied species: *B. ferox* (two large specimens on the right with red intestines) and *Branchinecta orientalis* (the rest of the anostracans on the photo). Photos by Zsófia Horváth.

Specific aims

During my PhD research, I focused on a flagship group of organisms in temporary waters: fairy shrimps (Anostraca) (**Fig. 6 B-C** & **Fig. 7**). In the first part of my research, I investigate the trophic role of this group in turbid waters using *B. orientalis* as model species (**Aim I**). In the second part, I use two congeneric anostracan species, *B. ferox* and *B. orientalis*, with overlapping but overall distinct geographic distribution patterns to study the impact of historic and current connectivity among habitats on the genetic structure within each species (**Aim II**).
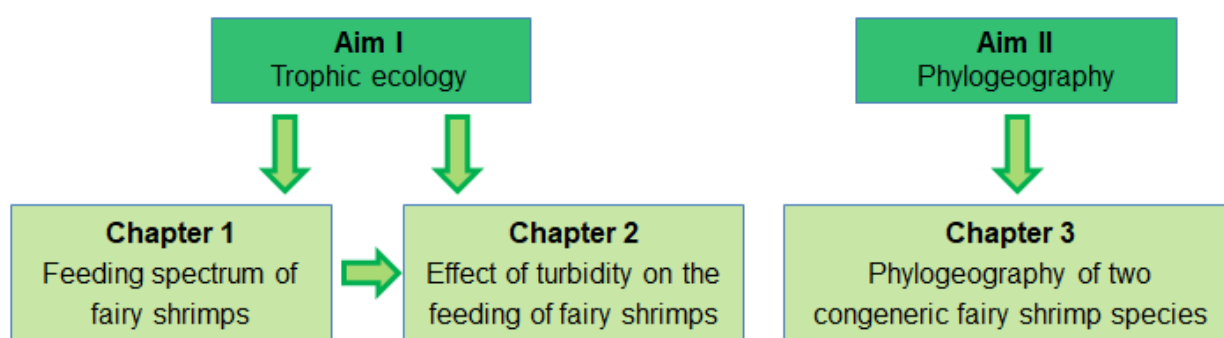
Figure 7. Schematic representation of the research aims and thesis content.

Aim I: Trophic role of fairy shrimps in temporary waters and the role of turbidity

I investigated the feeding ecology and trophic position of *B. orientalis* along a wide turbidity gradient in soda pans of eastern Austria (Seewinkel region).

(a) First, I offered multiple prey types (phyto- and zooplankton; **Fig. 6A**) to anostracans and determined the feeding spectrum of anostracans. Then I compared filtration rates on the specific prey types (**Chapter 1**).

(b) I compared filtration rates in relation to anostracan body size and sex (**Chapter 1**).

(c) I measured filtration rates, studied prey selection, and investigated how they are affected by turbidity under laboratory conditions (**Chapter 2**).

(d) I carried out a stable isotope analysis based on field samples, to reveal the relative trophic position of anostracans within the soda pan food webs, and how it changes with turbidity (**Chapter 2**).

Aim II: Phylogeography of two congeneric fairy shrimp species

I investigated the gene flow in *B. ferox* and *B. orientalis* (see **Chapter 3**) populations based on an analysis of two molecular markers from field material sampled across a large spatial scale. For this, I used samples collected from soda pans of Central Europe (Austria, Hungary and Serbia) and from other similar habitats e.g. in Spain, Israel, Tunisia and Mongolia. I genotyped individuals from 51 populations, covering most regions where the species occur.

(a) First, I assessed the intra- and interspecific genetic diversity on mitochondrial COI and nuclear ITS2 DNA regions (**Chapter 3**).

(b) I ran different evolutionary models to reconstruct the phylogeographic trees of both species and phylogeny of the entire *Branchinecta* genus (including species where information on mitochondrial COI gene fragment was available) (**Chapter 3**).

(c) I identified the most likely Pleistocene refugia and range expansions after the glacial retreat for both species (**Chapter 3**).

References

Alonso M. (1985). A survey of the Spanish Euphyllopoda. *Miscellània Zoològica* **9**, 179–208.

Angeler D.G., Viedma O., Sánchez-Carrillo S. & Alvarez-Cobelas M. (2008). Conservation issues of temporary wetland Branchiopoda (Anostraca, Notostraca: Crustacea) in a semiarid agricultural landscape: What spatial scales are relevant? *Biological Conservation* **141**, 1224–1234.

Atashbar B., Agh N., Manaffar R., Van Stappen G., Mohamadyari A., Mertens J., *et al.* (2016). Morphometric and preliminary genetic characteristics of *Branchinecta orientalis* populations from Iran (Crustacea: Anostraca). *Zootaxa* **4109**, 31–45

Atashbar B., Agh N., Van Stappen G. & Beladjal L. (2014a). Diversity and distribution patterns of large branchiopods (Crustacea: Branchiopoda) in temporary pools (Iran). *Journal of Arid Environments* **111**, 27–34.

Atashbar B., Agh N., Van Stappen G., Mertens J. & Beladjal L. (2014b). Combined effect of temperature and salinity on hatching characteristics of three fairy shrimp species (Crustacea: Anostraca). **73**, 574–583.

Begon M., Townsend C.R. & Harper J.L. (2006). *Food webs. In Ecology: from individuals to ecosystems*, 4th edn. Blackwell Publishing, Malden, MA, pp 738.

Beladjal L. & Mertens J. (2009). Diaspore dispersal of Anostraca by flying insects. *Journal of Crustacean Biology* **29**, 266–268.

Belk D. & Brtek J. (1995). Checklist of the Anostraca. *Hydrobiologia* **298**, 315–353.

Belk D. & Esparza C.E. (1995). Anostraca of the Indian Subcontinent. *Hydrobiologia* **298**, 287–293.

Bilton D.T., Freeland J.R. & Okamura B. (2001). Dispersal in freshwater invertebrates. *Annual review of ecology and systematics* **32**, 159–181.

Bohonak A.J. & Whiteman H.H. (1999). Dispersal of the fairy shrimp *Branchinecta coloradensis* (Anostraca): Effects of hydroperiod and salamanders. *Limnology and Oceanography* **44**, 487–493.

Boileau M. & Hebert P. (1991). Genetic consequences of passive dispersal in pond-dwelling copepods. *Evolution* **45**, 721–733.

Boix D., Biggs J., Céréghino R., Hull A.P., Kalettka T. & Oertli B. (2012). Pond research and management in Europe: "Small is Beautiful." *Hydrobiologia* **689**, 1–9.

Boros E., Ecsedi Z. & Oláh J. (2013). *Ecology and management of soda pans in the Carpathian Basin*. Hortobágy Environmental Association.

Boros E., Katalin V.-B., Vörös L. & Horváth Z. (2017). Multiple extreme environmental conditions of intermittent soda pans in the Carpathian Basin (Central Europe). *Limnologica* **62**, 38–46.

Boudrias M.A. & Pires J. (2002). Unusual sensory setae of the raptorial *Branchinecta gigas* (Branchiopoda : Anostraca). *Hydrobiologia* **486**, 19–27.

Boven L., Vanschoenwinkel B., De Roeck E.R., Hulsmans A. & Brendonck L. (2008). Diversity and distribution of large branchiopods in Kiskunsag (Hungary) in relation to local habitat and spatial factors: implications for their conservation. *Marine and Freshwater Research* **59**, 940–950.

Brendonck L. (1996). Diapause, quiescence, hatching requirements: what we can learn from large freshwater branchiopods (Crustacea: Branchiopoda: Anostraca, Notostraca, Conchostraca). *Hydrobiologia* **320**, 85–97.

Brendonck L. & De Meester L. (2003). Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia* **491**, 65–84.

Brendonck L., De Meester L. & Riddoch B.J. (2000a). Regional structuring of genetic variation in short-lived rock pool populations of *Branchipodopsis wolfi* (Crustacea : Anostraca). *Oecologia* **123**, 506–515.

Brendonck L., De Meester L. & Riddoch B.J. (2000b). Regional structuring of genetic variation in short-lived rock pool populations of *Branchipodopsis wolfi* (Crustacea : Anostraca). *Oecologia* **123**, 506–515.

Brendonck L., Rogers D.C., Olesen J., Weeks S. & Hoeh W.R. (2008). Global diversity of large branchiopods (Crustacea : Branchiopoda) in freshwater. *Hydrobiologia* **595**, 167–176.

Brochet A.L., Gauthier-Clerc M., Guillemain M., Fritz H., Waterkeyn A., Baltanás Á., *et al.* (2009). Field evidence of dispersal of branchiopods, ostracods and bryozoans by teal (*Anas crecca*) in the Camargue (southern France). *Hydrobiologia* **637**, 255.

Brock M.A., Nielsen D.L., Shiel R.J., Green J.D. & Langey J.D. (2003). Drought and aquatic community resilience: the role of eggs and seeds in sediments of temporary wetlands. *Freshwater Biology* **48**, 1207–1218.

van den Broeck M., Waterkeyn A., Rhazi L. & Brendonck L. (2015). Distribution, coexistence, and decline of Moroccan large branchiopods. *Journal of Crustacean Biology* **35**, 355–365.

Carpenter S., Kitchell J. & Hodgson J. (1985). Cascading trophic interactions and lake productivity. *Bioscience* **35**, 634–639.

Cohen R.G., Marinone M.C. & Adamowicz S.J. (2019). A new species of *Archaebranchinecta* (Anostraca: Branchinectidae) from the South American Altiplano. *Zootaxa* **4683**, 55–83.

Cox A.J. & Hebert P.D.N. (2001). Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular Ecology* **10**, 371–386.

Croel R.C. & Kneitel J.M. (2011). Ecosystem-level effects of bioturbation by the tadpole shrimp Lepidurus packardi in temporary pond mesocosms. *Hydrobiologia* **665**, 169–181.

Daborn G. (1975). Life history and energy relations of giant fairy shrimp *Branchinecta gigas* Lynch 1937 (Crustacea-Anostraca). *Ecology* **56**, 1025–1039.

De Meester L., Declerck S., Stoks R., Louette G., Van De Meutter F., De Bie T., *et al.* (2005). Ponds and pools as model systems in conservation biology, ecology and evolutionary biology. *Aquatic Conservation: Marine and Freshwater Ecosystems* **15**, 715–725.

Downing J.A. (2010). Emerging global role of small lakes and ponds: little things mean a lot. In: *Limnetica.* pp. 0009–24.

Eder E., Hödl W. & Gottwald R. (1997). Distribution and phenology of large branchiopods in Austria. *Hydrobiologia* **359**, 13–22.

Figuerola J. & Green A.J. (2002). Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology* **47**, 483–494.

Freeland J.R., Kirk H. & Peterson S. (2012). *Molecular ecology.* 2nd ed, John Wiley & Sons, Ltd Oxford UK.

Fryer G. (1966). *Branchinecta gigas* Lynch, a non-filter-feeding raptatory anostracan, with notes on the feeding habits of certain other anostracans. *Proceedings Linnean Society London* **177**, 19–34.

Fryer G. (1983). Functional ontogenetic changes in *Branchinecta-Ferox* (Milne-Edwards) (Crustacea, Anostraca). *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **303**, 229-343.

Habel J.C., Drees C., Schmitt T. & Assmann T. (2010). Review refugial areas and postglacial colonizations in the Western Palearctic. In: *Relict Species.* (Eds J.C. Habel & T. Assmann), pp. 189–197. Springer Berlin Heidelberg.

Hassall C. (2014). The ecology and biodiversity of urban ponds. *Wiley Interdisciplinary Reviews: Water* **1**, 187–206.

Havel J.E. & Shurin J.B. (2004). Mechanisms, effects, and scales of dispersal in freshwater zooplankton. *Limnology and Oceanography* **49**, 1229–1238.

Hebert P.D.N., Remigio E.A., Colbourne J.K., Taylor D.J. & Wilson C.C. (2002). Accelerated molecular evolution in halophilic crustaceans. *Evolution* **56**, 909–926.

Hildrew A.G. (1985). A quantitative study of the life history of a fairy shrimp (Branchiopoda: Anostraca) in relation to the temporary nature of its habitat, a Kenyan rainpool. *Journal of Animal Ecology* **54**, 99–110.

Horváth Z., Vad C.F. & Ptacnik R. (2016). Wind dispersal results in a gradient of dispersal limitation and environmental match among discrete aquatic habitats. *Ecography* **39**, 726–732.

Horváth Z., Vad C.F., Tóth A., Zsuga K., Boros E., Vörös L., *et al.* (2014). Opposing patterns of zooplankton diversity and functioning along a natural stress gradient: when the going gets tough, the tough get going. *Oikos* **123**, 461–471.

Horváth Z., Vad C.F., Voeroes L. & Boros E. (2013a). Distribution and conservation status of fairy shrimps (Crustacea: Anostraca) in the astatic soda pans of the Carpathian basin: the role of local and spatial factors. *Journal of Limnology* **72**, 103–116.

Horváth Z., Vad C.F., Voeroes L. & Boros E. (2013b). The keystone role of anostracans and copepods in European soda pans during the spring migration of waterbirds. *Freshwater Biology* **58**, 430–440.

Husemann M., Schmitt T., Zachos F.E., Ulrich W. & Habel J.C. (2014). Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *Journal of Biogeography* **41**, 81–94.

Jeffries M.J., Epele L.B., Studinski J.M. & Vad C.F. (2016). Invertebrates in temporary wetland ponds of the temperate biomes. In: *Invertebrates in Freshwater Wetlands: An International Perspective on their Ecology*. (Eds D. Batzer & D. Boix), pp. 105–139. Springer International Publishing, Cham.

Ketmaier V., Pirollo D., De Matthaeis E., Tiedemann R. & Mura G. (2008). Large-scale mitochondrial phylogeography in the halophilic fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840) (Branchiopoda: Anostraca). *Aquatic Sciences* **70**, 65–76.

Lahr J., Diallo A.O., Ndour K.B., Badji A. & Diouf P.S. (1999). Phenology of invertebrates living in a sahelian temporary pond. *Hydrobiologia* **405**, 189–205.

Leibold M.A. (1999). Biodiversity and nutrient enrichment in pond plankton communities. *Evolutionary Ecology Research* **1**, 73–95.

Levine S.N., Zehrer R.F. & Burns C.W. (2005). Impact of resuspended sediment on zooplankton feeding in Lake Waihola, New Zealand. *Freshwater Biology* **50**, 1515–1536.

Lindholm M., d'Auriac M.A., Thaulow J. & Hobaek A. (2016a). Dancing around the pole: Holarctic phylogeography of the Arctic fairy shrimp *Branchinecta paludosa* (Anostraca, Branchiopoda). *Hydrobiologia* **772**, 189–205.

Lindholm M., Hessen D.O., Faerovig P.J., Rognerud B., Andersen T. & Stordal F. (2015). Is distribution of cold stenotherms constrained by temperature? The case of the Arctic fairy shrimp (*Branchinecta paludosa* OF Muller 1788). *Journal of Thermal Biology* **53**, 46–52.

Lindholm M., Wolf R., Finstad A. & Hessen D.O. (2016b). Water browning mediates predatory decimation of the Arctic fairy shrimp *Branchinecta paludosa*. *Freshwater Biology* **61**, 340–347.

Lukić D., Waterkeyn A., Rabet N., Mioduchowska M., Geudens B., Vanschoenwinkel B., *et al.* (2019). High genetic variation and phylogeographic relations among Palearctic fairy shrimp populations reflect persistence in multiple southern refugia during Pleistocene ice ages and postglacial colonisation. *Freshwater Biology* **64**, 1896-1907.

Manca M. & Mura G. (1997). On *Branchinecta orientalis* Sars (Anostraca) in the Himalayas. *Hydrobiologia* **356**, 111–116.

Marrone F., Alonso M., Pieri V., Augugliaro C. & Stoch F. (2015). The crustacean fauna of Bayan Onjuul area (Tov Province, Mongolia) (Crustacea: Branchiopoda, Copepoda, Ostracoda). *North-Western Journal of Zoology* **11**, 288–295.

Marrone F., Korn M., Stoch F., Naselli Flores L. & Turki S. (2016). Updated checklist and distribution of large branchiopods (Branchiopoda: Anostraca, Notostraca, Spinicaudata) in Tunisia. *Biogeographia – The Journal of Integrative Biogeography* **31**, 27–53.

Mokany A., Wood J.T. & Cunningham S.A. (2008). Effect of shade and shading history on species abundances and ecosystem processes in temporary ponds. *Freshwater Biology* **53**, 1917–1928.

Moritz C. (1994). Defining "Evolutionarily Significant Units" for conservation. *Trends in ecology & evolution* **9**, 373–375.

Mura G., Ozkutuk S.R., Aygen C. & Cottarelli V. (2011). New data on the taxonomy and distribution of anostracan fauna from Turkey. *Journal of Biological Research-Thessaloniki* **15**, 17–23.

Mura G. & Takami G.A. (2000). A contribution to the knowledge of the anostracan fauna of Iran. *Hydrobiologia* **441**, 117–121.

Naganawa H., Naumova E.Yu., Denikina N.N., Kondratov I.G., Dzyuba E.V. & Iwasawa A. (2019). Does the dispersal of fairy shrimps (Branchiopoda, Anostraca) reflect the shifting geographical distribution of freshwaters since the late Mesozoic? *Limnology*, 1-10.

Naganawa H. & Zagas B. (2002). General aspects of the large branchiopod crustacean fauna of Mongolia. *Limnology* **3**, 181–188.

Oertli B., Biggs J., Céréghino R., Grillas P., Joly P. & Lachavanne J.-B. (2005). Conservation and monitoring of pond biodiversity: introduction. *Aquatic Conservation: Marine and Freshwater Ecosystems* **15**, 535–540.

Oertli B., Céréghino R., Hull A. & Miracle R. (2009). Pond conservation: from science to practice. *Hydrobiologia* **634**, 1–9.

Padhye S.M., Kulkarni M.R. & Dumont H.J. (2017). Diversity and zoogeography of the fairy shrimps (Branchiopoda: Anostraca) on the Indian subcontinent. *Hydrobiologia* **801**, 117–128.

Persson J., Brett M.T., Vrede T. & Ravet J.L. (2007). Food quantity and quality regulation of trophic transfer between primary producers and a keystone grazer (*Daphnia*) in pelagic freshwater food webs. *Oikos* **116**, 1152–1163.

Petkovski S. (1991). On the presence of the genus Branchinecta Verrill, 1869 (Crustacea, Anostraca) in Yugoslavia. *Hydrobiologia* **226**, 17–27.

Petkovski S. (1993). *Taksonomsko-morfološka i zoogeografsko-ekološka studija Anostraca (Crustacea: Branchiopoda) jugoslovenskih zemalja*. PhD thesis. Prirodno-matematički fakultet, Novi Sad.

Pinceel T., Brendonck L., Larmuseau M.H.D., Vanhove M.P.M., Timms B.V. & Vanschoenwinkel B. (2013a). Environmental change as a driver of diversification in temporary aquatic habitats: does the genetic structure of extant fairy shrimp populations reflect historic aridification? *Freshwater Biology* **58**, 1556–1572.

Pinceel T., Vanschoenwinkel B., Waterkeyn A., Vanhove M.P.M., Pinder A., Timms B.V., *et al.* (2013b). Fairy shrimps in distress: a molecular taxonomic review of the diverse fairy shrimp genus *Branchinella* (Anostraca: Thamnocephalidae) in Australia in the light of ongoing environmental change. *Hydrobiologia* **700**, 313–327.

Pretus J.L. (1990). A commented check-list of the Balearic branchiopoda (Crustacea). *Limnetica* **6**, 157–164

Radzikowski J. (2013). Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *Journal of Plankton Research* **35**, 707–723.

Reniers J., Vanschoenwinkel B., Rabet N. & Brendonck L. (2013). Mitochondrial gene trees support persistence of cold tolerant fairy shrimp throughout the Pleistocene glaciations in both southern and more northerly refugia. *Hydrobiologia* **714**, 155–167.

Rodríguez-Flores P.C., Jiménez-Ruiz Y., Forró L., Vörös J. & García-París M. (2017). Non-congruent geographic patterns of genetic divergence across European species of Branchinecta (Anostraca: Branchinectidae). *Hydrobiologia* **801**, 47–57.

Rogers D.C. (2006). Three new species of *Branchinecta* (Crustacea : Branchiopoda : Anostraca) from the Nearctic. *Zootaxa* **1126**, 35–51.

Rogers D.C. & Coronel J.S. (2011). A redescription of *Branchinecta pollicifera* Harding, 1940, and its placement in a new genus (Branchiopoda: Anostraca: Branchinectidae). *Journal of Crustacean Biology* **31**, 717–724.

Rogers D.C., Quinney D.L., Weaver J. & Olesen J. (2006). A new giant species of predatory fairy shrimp from Idaho, USA (branchiopoda : anostraca). *Journal of Crustacean Biology* **26**, 1–12.

Sánchez B. & Angeler D.G. (2007). Can fairy shrimps (Crustacea : Anostraca) structure zooplankton communities in temporary ponds? *Marine and Freshwater Research* **58**, 827–834.

Sarma S.S.S. & Nandini S. (2002). Studies on functional response and prey selection using zooplankton in the anostracan *Chirocephalus diaphanus* Prevost, 1803. *Hydrobiologia* **486**, 169–174.

Schwartz S. & Jenkins D. (2000). Temporary aquatic habitats: constraints and opportunities. *Aquatic Eclogy* **34**, 3–8.

Šćiban M., Marković A., Lukić D. & Miličić D. (2014). Autumn populations of *Branchinecta orientalis* G. O. Sars, 1903 and *Chirocephalus diaphanus* Prevost, 1803 (Crustacea, Branchiopoda) in the Central European Lowlands (Pannonian Plain, Serbia). *North-Western Journal of Zoology* **10**, 435–437.

Van de Meutter F., Stoks R. & de Meester L. (2008). Size-selective dispersal of *Daphnia* resting eggs by backswimmers (*Notonecta maculata*). *Biology Letters* **4**, 494–496.

Vanschoenwinkel B., Gielen S., Seaman M. & Brendonck L. (2008a). Any way the wind blows - frequent wind dispersal drives species sorting in ephemeral aquatic communities. *Oikos* **117**, 125–134.

Vanschoenwinkel B., Pinceel T., Vanhove M.P.M., Denis C., Jocque M., Timms B.V., *et al.* (2012). Toward a global phylogeny of the "living fossil'" crustacean order of the Notostraca." *Plos One* **7**, e34998.

Vanschoenwinkel B., Waterkeyn A., Nhiwatiwa T., Pinceel T., Spooren E., Geerts A., *et al.* (2011). Passive external transport of freshwater invertebrates by elephant and other mud-wallowing mammals in an African savannah habitat. *Freshwater Biology* **56**, 1606–1619.

Vanschoenwinkel B., Waterkeyn A., Vandecaetsbeek T., Pineau O., Grillas P. & Brendonck L. (2008b). Dispersal of freshwater invertebrates by large terrestrial mammals: a case study with wild boar (*Sus scrofa*) in Mediterranean wetlands. *Freshwater Biology* **53**, 2264–2273.

Viana D.S., Santamaría L., Michot T.C. & Figuerola J. (2013). Migratory strategies of waterbirds shape the continental-scale dispersal of aquatic organisms. *Ecography* **36**, 430–438.

Waloßek D. (1995). The Upper Cambrian *Rehbachiella*, its larval development, morphology and significance for the phylogeny of Branchiopoda and Crustacea. In: *Studies on Large Branchiopod Biology and Aquaculture II*. Developments in Hydrobiology, (Eds D. Belk, H.J. Dumont & G. Maier), pp. 1–13. Springer Netherlands, Dordrecht.

Waterkeyn A., Grillas P. & Brendonck L. (2016). Experimental test of the ecosystem impacts of the keystone predator *Triops cancriformis* (Branchiopoda: Notostraca) in temporary ponds. *Freshwater Biology* **61**, 1392–1404.

Waterkeyn A., Grillas P., Vanschoenwinkel B. & Brendonck L. (2008). Invertebrate community patterns in Mediterranean temporary wetlands along hydroperiod and salinity gradients. *Freshwater Biology* **53**, 1808–1822.

Waterkeyn A., Vanschoenwinkel B., Elsen S., Anton-Pardo M., Grillas P. & Brendonck L. (2010). Unintentional dispersal of aquatic invertebrates via footwear and motor vehicles in a Mediterranean wetland area. *Aquatic Conservation: Marine and Freshwater Ecosystems* **20**, 580–587.

Weeks S.C., Marcus V. & Alvarez S. (1997). Notes on the life history of the clam shrimp, *Eulimnadia texana*. *Hydrobiologia* **359**, 191–197.

White G.E., Fabris G. & Hartland-Rowe R. (1969). The method of prey capture by *Branchinecta gigas* Lynch, 1937 (Anostraca). *Crustaceana* **16**, 158–160.

Williams D.D. (2006). *The biology of temporary waters*. Oxford University Press, Oxford UK.

Williams P., Whitfield M., Biggs J., Bray S., Fox G., Nicolet P., *et al.* (2004). Comparative biodiversity of rivers, streams, ditches and ponds in an agricultural landscape in Southern England. *Biological Conservation* **115**, 329–341.

Yee S.H., Willig M.R. & Moorhead D.L. (2005). Tadpole shrimp structure macroinvertebrate communities in playa lake microcosms. *Hydrobiologia* **541**, 139–148.

Zacharias I. & Zamparas M. (2010). Mediterranean temporary ponds. A disappearing ecosystem. *Biodiversity and Conservation* **19**, 3827–3834.

Zokan M. & Drake J.M. (2015). The effect of hydroperiod and predation on the diversity of temporary pond zooplankton communities. *Ecology and Evolution* **5**, 3066–3074.

Chapter 1 - Food spectrum of *Branchinecta orientalis*

Food spectrum of *Branchinecta orientalis* – are anostracans omnivorous top consumers of plankton in temporary waters?

Dunja Lukić, Zsófia Horváth, Csaba F Vad & Robert Ptacnik

Abstract

Anostracans are key elements of temporary ponds, due to the high abundance and importance as food for waterbirds. Except for a few large species, they are generally considered to be herbivorous filter feeders. However, this assumption is not supported by empirical data. In fact, there is a lack of quantitative experimental studies on their trophic role in the food webs of temporary waters. Here, we briefly revise the available data about the feeding spectrum of anostracans. Moreover, we present experimental data on the feeding behaviour of a key species of soda pans, *Branchinecta orientalis.* We show that *B. orientalis* is able to ingest a wide range of prey items, ranging from pico-sized algae, to motile prey such as copepods, with no significant differences in the ingested biomass from the different food types. We do not find evidence for sex and size-specific differences in the ingestion rates in adult animals. Our results clearly show that *B. orientalis* is an omnivorous planktivore. Based on our data and existing studies, we suggest refuting the general theorem that most anostracans are herbivorous filter feeders. More empirical data from the field are needed to fully appreciate the trophic role of these key consumers of temporary waters.

Introduction

Temporary ponds comprise very diverse ecosystems regarding size, shape, hydroperiod and water chemistry (Williams, 2006). They are widespread throughout the world, but are especially prevalent in arid and semi-arid climatic regions (Brendonck, 1996). Although the number of studies increased in the last decade, temporary ponds and their communities are still largely understudied compared to larger and more permanent water bodies (Céréghino *et al.*, 2008; Céréghino *et al.*, 2014; Marrone *et al.*, 2017). Our knowledge about their food webs is particularly limited (Boix *et al.*, 2016).

Anostracans are a group of branchiopod crustaceans present almost exclusively in fishless temporary waters. In temporary ponds, anostracans (as well as the other large branchiopod crustaceans) are key elements, reaching high densities (Horváth *et al.*, 2013a) and possibly having a strong impact on zooplankton communities through competition (Jocque *et al.*, 2010). Moreover, anostracans are important food for waterbirds along their migration routes (Malone, 1965; Silveira, 1998; Boros *et al.*, 2006; Sánchez *et al.*, 2007; Horváth *et al.*, 2013a) and during breeding seasons (Krapu, 1974).

The general biology and feeding ecology of anostracans are not well understood (Marrone *et al.*, 2017). Our knowledge on the diet of anostracans is mostly derived from anecdotal observations or correlative studies. There is a particular lack of quantitative data from controlled experiments. At present, anostracans are generally considered to be non-selective filter feeders (Brendonck, 1993). Filter-feeding organisms such as zooplankters have an important role in ecosystem functioning, since they are a key link from phytoplankton and bacteria to vertebrates (Persson *et al.*, 2007) and drive trophic cascades (Carpenter *et al.*, 1985). The majority of anostracans are predominantly considered herbivorous, feeding on algae in addition to organic and inorganic detritus which they filter from the water column or by mixing and scraping the bottom sediment. However, recent evidence suggests that they occasionally ingest nematodes and small zooplankters such as rotifers (see **Table I**). Only a few larger species were well documented to be predominantly predators, feeding on larger crustaceans such as copepods and other anostracans (White *et al.*, 1969; Rogers *et al.*, 2006; Rogers and Timms, 2017).

Table I. Literature review about the diet composition and feeding mode of anostracan species

Species	Size (cm)	Feeding mode	Diet	Method	Reference
Branchinecta ferox	~4.5	Filtration, predation	Dominantly calanoids, also *Daphnia*, algae, detritus	Feeding experiment, lab observation	(Fryer, 1983)
Branchinecta gaini	0.2-2	Mostly scraping	Algae, bryophyte, protozoa, rotifers, copepod remains, anostracans, chironomids	Intestinal content	(Paggi, 1996)
Branchinecta gigas	5-10	Predation	Dominantly copepods and anostracans	Lab observation, intestinal content	(Fryer, 1966; White et al., 1969; Daborn, 1975)
Branchinecta mackini	1.6-2.6	Filtration	Bacteria, organic particles	Unspecified literature source	(Daborn, 1977)
	2.1	Scraping	Bottom particles	Lab observation	(Fryer, 1966)
Branchinecta raptor	~5.5	Predation	Dominantly anostracans, also chironomid midges, mosquito larvae, cladocerans, copepods, and ostracods	Lab observation	(Rogers et al., 2006)
Branchinella occidentalis	2.2-5	Predation	Micrometazoans, algae, diatoms, detritus, chironomid midge larvae, anostracans	Lab observation, intestinal content	(Rogers and Timms, 2017)
Branchinella spinosa	1.3-4	Filtration, predation	Algae, cladocerans, copepods, anostracan eggs	Intestinal content	(Alonso, 1985)
Chirocephalus diaphanus	~1	Filtration, predation	Algae, rotifers, small cladocerans	Intestinal content, feeding experiment	(Sarma and Nandini, 2002)
Eubranchipus holmani	0.7-1	Filtration	Desmids, silt particles, *Protococcus*-like algae	Feeding experiment	(Modlin, 1982)
Eubranchipus vernalis	1.3-1.8	Bottom scraping	Benthic diatoms, filamentous algae, desmids, platyhelminth eggs, shells of *Arcella*	Material on the oral groove, in the gut and faeces	(Modlin, 1982)
Streptocephalus dichotomus	0.7-2.7	Filtration	Dominantly algae, also ciliates, rotifers, nematods, nauplii, remains of copepods and anostracans	Intestinal content	(Bernice, 1971)
Streptocephalus proboscideus	0.9-2.2	Filtration, predation	Algae, fungi, ciliates, rotifers, cladocerans, nauplii of copepods and anostracans	Feeding experiment	(Mertens et al., 1990; Brendonck, 1993; Dumont and Ali, 2004)

According to two studies performed with large anostracan species (*Branchinecta gigas* and *B. ferox*), anostracan nauplii feed on small organic particles and algae (Daborn, 1975; Fryer, 1983). As they grow, their diet presumably broadens towards larger particles. For small anostracan species (<4 cm in length), there is only scattered information on the diet of adults. These data were collected with diverse field and laboratory methods and in many cases without any standardized experimental test (**Table I**). According to the very few existing examples, ingestion rates are increasing with the size of adults within a given species (Daborn, 1975; Dumont and Ali, 2004). In large predatorial anostracan species (e.g. *Branchinecta ferox* and *B. raptor*), the morphology of thoracopods implies that they gradually lose the ability of filter feeding with growth, probably once they reach 4–5 cm in length (Fryer, 1983; Rogers *et al.*, 2006). Moreover, these large anostracan

species are considered of being able to actively capture motile animal prey (White *et al.*, 1969; Fryer, 1983; Rogers *et al.*, 2006). Taken together, with the exception of large predatory taxa, we currently lack a good understanding about the diet of anostracans in their environment, which would be needed to assess their trophic role in the food web of temporary waters.

Based on the existing data, size and feeding characteristics seem to lack a general pattern between different sexes of the same anostracan species. While differences in size were observed for some species (e.g. larger females in *B. gigas*; Daborn, 1975; larger males in *Streptocephalus proboscideus*; Brendonck, 1989), both sexes reach approximately the same size in others (Belk and Rogers, 2002; Miličić *et al.*, 2013; Horváth and Vad, 2015). Among the few studies that tested for sex-related differences in ingestion rates, Bernice (1971) found no differences in *Streptocephalus dichotomus*, while in some other *Streptocephalus* species, females ingested more food than males (Dierckens *et al.*, 1995; Ali *et al.*, 1996), probably due to the high energetic demand of egg production (Ali *et al.*, 1996).

The genus *Branchinecta* occurs on all continents except Australia (Obregón-Barboza *et al.*, 2002). It includes diverse species regarding body size and life history. Most species are smaller sized (up to 3 cm in length) and generally believed to be filter feeders and scrapers (Daborn, 1977; Paggi, 1996). This genus also contains the largest (predatory) anostracan species, such as *B. gigas* and *B. raptor*, that can grow up to 10 cm in body length (Daborn, 1975; Rogers *et al.*, 2006). The target species of our study, *Branchinecta orientalis*, inhabits mineral-rich temporary waters and is distributed between 55° and 30° N in Europe and Asia (Mura and Takami, 2000). In Central European populations, adult *B. orientalis* usually range from 1.5 to 3 cm (pers. obs.), but can grow up to 4.1 cm (Petkovski, 1991). This makes them comparable to other *Branchinecta* species that are generally considered filter feeders, while predatory *Branchinecta* have a larger adult size range of 5–10 cm.

Branchinecta orientalis is the most abundant anostracan species in Central European saline temporary waters, soda pans (Horváth *et al.*, 2013b). Soda pan communities lack fish and macrovegetation (except in some cases around the shoreline). Picoplanktonic algae are the key primary producers in these naturally hypertrophic systems (Vörös *et al.*, 2005, 2008; Somogyi *et al.*, 2009). Both phyto- and zooplankton communities can reach very high densities during the wet phase (Horváth *et al.*, 2014). *Branchinecta orientalis* reaches densities up to 13 ind L^{-1}, which makes it an important food source for waterbirds (Horváth *et al.*, 2013a). In spite of its ecological importance and high biomass, we are at present unaware about the diet of *B. orientalis* and its potential effects on the food web of soda pans.

We experimentally test here the adult diet of the anostracan species *B. orientalis* (of comparable body size to many anostracan species considered as filter feeders). By using this species as model organism for anostracan feeding, our aims are to (1) examine their diet and

compare the ingestion rates on different food types, ranging from pico-sized algae to large crustacean zooplankton; and (2) to determine whether ingestion rates depend on sex or body size.

Methods

Cultivation of *B. orientalis* and plankton for feeding experiments

All eggs and animals used in the experiments were collected in the Seewinkel region of Austria. To hatch animals, sediment containing resting eggs was collected from the soda pan Oberer Stinkersee (47.813722 N, 16.792889 E) and stored dry at 4°C in the dark for several months. After sieving and centrifuging the sediment by using the sugar flotation method (Onbe, 1978; Marcus, 1990), we incubated the eggs for hatching in a climate chamber, with a light regime 16:8 L:D and temperature of 18°C. Once animals started to hatch, we picked them out manually and transferred to larger plastic containers (25–35 adult animals in 21 L volume) filled with artificial soda water (NaHCO$_3$ solved in distilled water, with conductivity of 1 mS cm^{-1}) and constantly aerated. They were fed daily with a mix of algal food (*Cryptomonas* sp., *Scenedesmus* sp., *Chlamydomonas* sp.), which was at a later stage combined with zooplankton (rotifer *Brachionus asplanchnoidis*, cladoceran *Moina brachiata*, copepod *Arctodiaptomus spinosus*). Algal cultures were raised on WC medium, which was refreshed regularly (to keep them in exponential growth phase). Zooplankton was kept in 3 L volume jars in a medium of the same chemical composition as the anostracan medium, and fed regularly with a mix of algal food (*Cryptomonas* sp. and *Chlamydomonas* sp.). All phyto- and zooplankton cultures were kept under the same light regime and temperature conditions as anostracans.

For testing the size dependency of ingestion rates, live anostracans were collected from Mittlerer Stinkersee (47.807044 N, 16.788180 E). These animals were used as a larger size class in our experiment, because the anostracans raised in the lab did not reach the maximum body size of the individuals from the field. They were kept and maintained in the same manner as the population hatched in the climate chamber, and used in feeding experiments only several days after collection from the field.

Experimental design and data analyses

Experiments were carried out with adult individuals. Animals were considered adult once mating was observed (around 4-week-old in case of animals hatched in the lab). At the onset of an experiment, body length of 10 individuals from all groups involved was measured (males, females, small and big animals). Body length was measured from the tip of the head to the end of cercopods (by means of photographs taken of live animals placed in a narrow transparent tube above grid lined paper).

In our experiments, we incubated two female *B. orientalis* per replicate (except when we tested for sex differences, where we used two females vs. two males). For medium, we used 70 mL of artificial soda water at a conductivity of 1 mS cm^{-1} (same as in the cultures). The number of replicates in algal feeding tests (three) was usually lower than in the predatory tests (four to five), as we noticed an overall low variation between replicates in case of algal food. Controls (without *B. orientalis*) for all food types were run in parallel. To avoid algal sedimentation, medium was gently mixed in the algal feeding tests (both controls and vials with *B. orientalis*) with regular intervals (30 min) and immediately before sampling phytoplankton for quantification.

To quantify filter feeding on phytoplankton, we offered two algae with different sizes as food in separate experiments (in concentrations equal to 2.5 mg L^{-1} dry weight, 5× higher than the concentration considered as saturating food abundance for *Daphnia magna*; Porter *et al.*, 1982). A coccoid green algae *Mychonastes* sp. (Sphaeropleales; diameter 2–3 µm) was used as picoplankton (mean concentration 384 400 cells mL^{-1}), and the green flagellate *Chlamydomonas* sp. (Chlamydomonadales) represented a larger unicellular food (7-18 µm length; mean concentration 33 600 cells mL^{-1}). In the predatory feeding test, two zooplankters representative for soda pan communities were used (Horváth *et al.*, 2014; Tóth *et al.*, 2014): a copepod (*Arctodiaptomus spinosus*, 20 per vial, 3.8 mg L^{-1} dry weight) and a rotifer (*Brachionus asplanchnoidis*, 450 per vial, 3.2. mg L^{-1} dry weight). The length of adulty *A. spinosus* is 0.65–1.16 mm (Bottrell *et al.*, 1976), while it is between 0.185 and 0.510 mm for *B. asplanchnoidis* (Michaloudi *et al.*, 2017). They were collected from soda pans in the Seewinkel region, and cultivated in the lab during the experiments. We offered a comparable biomass to *B. orientalis* in all tested food types (2.5–3.8 mg dry weight biomass L^{-1}). Experiments were run for 40 min for *B. asplanchnoidis*, 1 hour for *A. spinosus*, 2 hours for *Chlamydomonas* sp. and 4 hours for *Mychonastes* sp. The length of experiments was decided for each food type based on ingestion rates observed in pre-experimental trials, where we counted the remaining algal cells or zooplankton individuals at multiple time points, until the effect of anostracans feeding became evident.

For comparing differences between sex and size groups, experiments were set the same way as already described, with only a few minor differences in density and variety of food types tested. We used *Chlamydomonas* sp. (concentration 2.5 mg L^{-1} dry weight) as food in the filter feeding and *B. asplanchnoidis* (100 per vial) in the predatory feeding test to compare ingestion rates between males and females. To compare different adult size groups, we used *Mychonastes* sp. (concentration 2.5 mg L^{-1} dry weight) and *Chlamydomonas* sp. (concentration 7.5 mg L^{-1} dry weight) in filter feeding and *A. spinosus* (20 per vial, 3.8 mg L^{-1} dry weight) in predatory feeding tests.

In an additional test, we offered two species of cladocerans to *B. orientalis*, *M. brachiata* (40 per vial) and *D. magna* (20 per vial, separately tested on two different age classes). These

cladocerans are frequent members of zooplankton communities in soda pans and, therefore, it is relevant to check if they also represent a prey of *B. orientalis*. Besides, adults of *D. magna* are the largest representatives of crustacean zooplankton in these habitats and offering them as food allows for conclusions on the dietary size-spectrum of *B. orientalis*. Here, we only recorded whether these species were consumed by *B. orientalis*, without comparing ingestion rates with other food types. Body length of a cladoceran *M. brachiata* is around 1 mm, while *D. magna* ranges between 1 and 2 mm as juvenile and 2–5 mm as adult (pers.obs.). Per replicate, we offered cladocerans to two female anostracans for 20 min in 70 mL volume of medium (prepared the same way as explained above).

Calculation of biomass and ingestion rates

Biovolume of the algal food was approximated by measuring cellular dimensions and approximating them to simple geometrical bodies (sphere for *Mychonastes* sp. and depressed ellipsoid for *Chlamydomonas* sp.). Furthermore, we calculated the dry weight biomass per cell using the approximation that carbon biomass (~14% of biovolume) comprises 40% of total dry weight (Bowie *et al.*, 1985). In zooplankton, *B. asplanchnoidis* and *A. spinosus* biomass per individual were calculated from the average weight of dried individuals (0.5 µg per individual for *B. asplanchnoidis* and 13.5 µg per individual for *A. spinosus*).

Biomass ingestion rate per anostracan in the experiments was calculated based on the equations from Marin *et al.* (1986), with assumption that food concentration at the beginning of the experiment was below saturating concentration:

$$M = \frac{g C_0 V m}{N}$$

with *M* being the ingested biomass per animal and time (in µg h^{-1}); *g* is the grazing coefficient; C_0 is the prey concentration (phytoplankton: cells mL^{-1}; zooplankton: individuals mL^{-1}) offered at the beginning of the experiment; *V* is the volume of medium (in mL); *m* is the average biomass (in µg) per phytoplankton cell or zooplankton individual; *N* is the number of anostracans per vial. The grazing coefficient (*g*) was calculated for all food types according to the following formula:

$$g = k - \frac{\ln(C_t) - \ln(C_0)}{t}$$

where C_0 is the cell concentration of phytoplankton or concentration of zooplankton per unit volume offered as food at the beginning of experiment; C_t is the cell concentration of phytoplankton or concentration of zooplankton offered as food at the end of experiment, *k* is the growth rate based on the change of algal concentration in controls (applicable for phytoplankton); *t* is the duration of the experiment (expressed in hours).

Statistical analyses

One-way ANOVA was used to test for differences in biomass ingestion rates of the two algal and zooplankton groups in *B. orientalis* (normality and homogeneity of variances were met according to Shapiro–Wilk and Levene's tests). Tukey's *post hoc* test was applied to identify significant differences among different food types.

In parallel with testing the differences in ingestion rates between males and females, we also tested for size difference in relation to sex. We first tested if the data on *B. orientalis* body length and ingestion rates for different food items follow a normal distribution with Shapiro–Wilk tests (separately for males and females). Length data were not normally distributed, so we applied Kruskal–Wallis rank sum test to test for differences in length between males and females. Afterwards, we checked whether there were any sex-related differences in ingestion rates. In the experiments with *B. asplanchnoidis*, we excluded one replicate in both the male and female treatments, where all rotifers were eaten before the end of the experiment. As data were normally distributed (ingestion rates on *Chlamydomonas* sp. and *B. asplanchnoidis*) and the assumption of homogeneity of variances was not violated (based on *F* test), we applied Student's *t*-test to test for significant differences.

The same procedure was applied to test length and feeding differences between two size classes of adult animals (mean ± SD: small: 1.44 ± 0.13 cm; big: 2.46 ± 0.22 cm). We checked for normality and homogeneity of variances before performing each test. The length differences between the two size classes were checked with Student's *t*-test. Then, ingestion rates for feeding on *Mychonastes* sp., *Chlamydomonas* sp. and *A. spinosus* were compared between the two size classes. We used Kruskal–Wallis rank sum test for *Mychonastes* sp. and *A. spinosus* and Student's test for *Chlamydomonas* sp. All data were analysed in R (R Core Team, 2014).

Results

Anostracan diet width and ingestion rates

Anostracans ingested all food types (small and large algae, rotifers and copepods) (**Fig. 1a**). Biomass ingestion rates on the picoalgae *Mychonastes* sp. (mean ± SD: 10.38 ± 7.26 µg h^{-1} or 20.8 ± 14.5 × 10^5 cells per hour), the larger algae *Chlamydomonas* sp. (30.55 ± 14.35 µg h^{-1}; or 49.8 ± 23.4 × 10^4 cells per hour), the rotifer *B. asplanchnoidis* (123.37 ± 55.77 µg h^{-1}; or 246.73 ± 111.55 individuals per hour) and the copepod *A. spinosus* (101.50 ± 123.68 µg h^{-1}; or 7.54 ± 9.18 individuals per hour) were not significantly different from each other (ANOVA: $F_{(3,11)} = 1.62$, $P = 0.24$; **Fig. 1b**). However, once an outlier from the *A. spinosus* treatment was removed (ingestion rate was above 300 µg h^{-1}, while all others were ≤200 µg h^{-1}; see Fig. 1b) we found significant difference between food types (ANOVA: $F_{(3,10)} = 5.3$, $P = 0.02$), where ingestion rates on *B.*

asplanchnoidis were significantly higher than those observed on *Mychonastes* sp. (Tukey's *post hoc* test: $P = 0.02$).
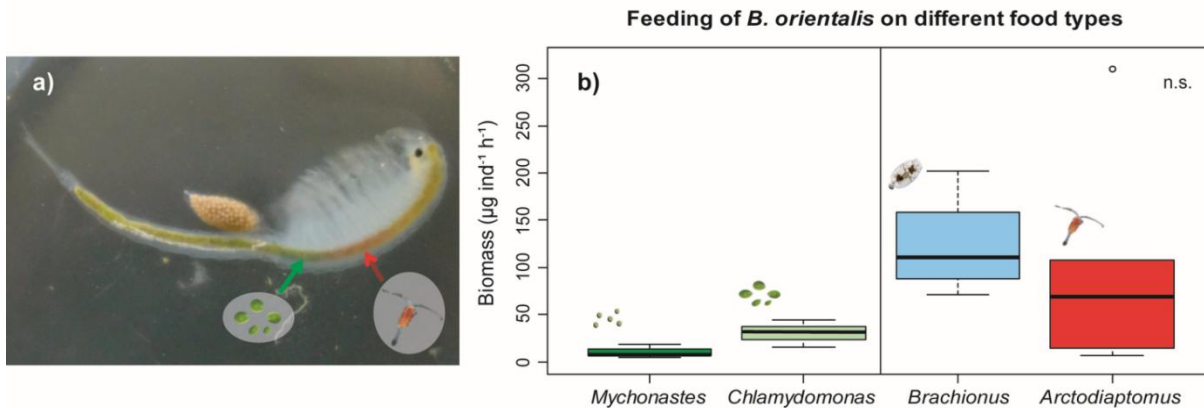
Figure 1. (a) Colouration of *B. orientalis* intestine after feeding the first with sole algal (*Chlamydomonas* sp.) and then zooplankton (*Arctodiaptomus spinosus*) food. (**b**) Biomass ingestion rates on different food types. ANOVA showed no significant difference (n.s.) between the four groups ($F_{(3,11)} = 1.62$, $P = 0.24$). When an outlier for the *A. spinosus* feeding test result was removed (ingestion rate above 300 μg h^{-1}), ANOVA showed significant variance between the groups ($F_{(3,10)} = 5.30$, $P = 0.02$), with significant difference between *Mychonastes* sp. and *B. asplanchnoidis* (Tukey's *post hoc* test: $P = 0.02$). Box gives the interquartile range and whiskers give the approximate 95% confidence intervals.

The two cladocerans *M. brachiata* and juvenile *Daphnia magna* were both efficiently ingested by *B. orientalis* (they were all removed within 20 min). However, fairy shrimps were not feeding on adult *D. magna* (~5 days and older, which meant >1.5 mm in body length), which represented the only food type that was not ingested.

Sex differences

There was no significant difference in body length between males and females (mean ± SD: 1.41 ± 0.13 cm for males and 1.44 ± 0.13 cm for females; Kruskal–Wallis rank sum test: $\chi^2 = 0.40$, $P = 0.525$). Differences in food ingestion rates were also not significant, neither in case of filter feeding on *Chlamydomonas* sp. (males: 12.95 ± 8.70 μg h^{-1}, females: 15.88 ± 8.50 μg h^{-1}; *t*-test: $t = 0.54$, $P = 0.60$) nor in predatory feeding on *B. asplanchnoidis* (males: 95.20 ± 54.33 μg h^{-1}, females: 92.32 ± 38.44 μg h^{-1}; *t*-test: $t = -0.20$, $P = 0.85$) (**Fig. 2**).
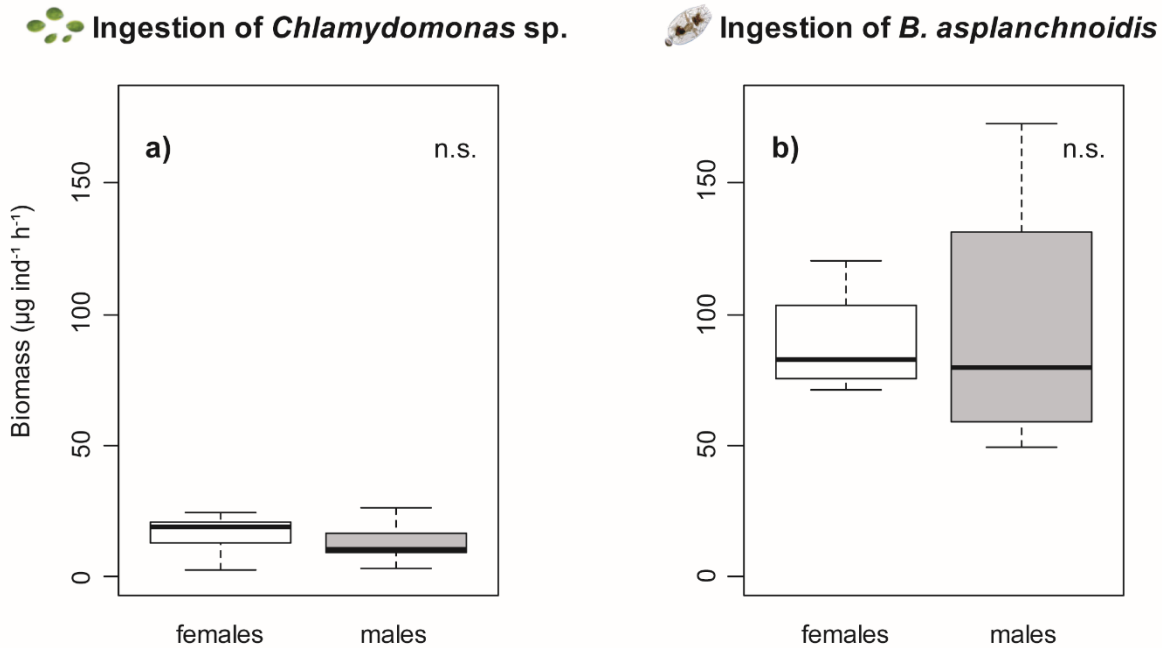
Figure 2. Comparison of biomass ingestion rates between males and females on (**a**) algal food *Chlamydomonas* sp. (*t*-test, *t* = 0.54, *P* = 0.60); and (**b**) zooplankton *Brachionus asplanchnoidis* (*t*-test, *t* = −0.20, *P* = 0.85). Box gives the interquartile range and whiskers give the approximate 95% confidence intervals.

Size differences

Adults raised in the lab from eggs were significantly smaller (mean ± SD: 1.44 ± 0.13 cm) than the adult animals collected on the field (mean ± SD: 2.46 ± 0.22 cm; *t*-test: *t* = 12.839, *P* < 0.001). However, we did not observe any significant difference in biomass ingestion rates, neither for filter feeding on *Mychonastes* sp. (small: 10.38 ± 7.26 µg h^{-1}, big: 21.12 ± 8.22 µg h^{-1}; Kruskal–Wallis rank sum test: χ^2 = 2.33, *P* = 0.13) and *Chlamydomonas* sp. (small: 63.63 ± 11.38 µg h^{-1}, big: 126.47 ± 61.84 µg h^{-1}; *t*-test: *t* = 1.73, *P* = 0.22), nor for predatorial feeding on *A. spinosus* (small: 101.50 ± 123.68 µg h^{-1}, big: 57.25 ± 49.64 µg h^{-1}; Kruskal–Wallis rank sum test: χ^2 = 0.06, *P* = 0.80) (**Fig. 3**).

Figure 3. Comparison of biomass ingestion rates in two size groups of adult animals on (**a**) picoplanktonic algae *Mychonastes* sp. (Kruskal–Wallis rank sum test: $\chi^2 = 2.33$, $P = 0.13$); (**b**) algae *Chlamydomonas* sp. (*t*-test: $t = 1.73$, $P = 0.22$); and (**c**) zooplankton *Arctodiaptomus spinosus* (Kruskal–Wallis rank sum test: $\chi^2 = 0.06$, $P = 0.80$). Box gives the interquartile range and whiskers give the approximate 95% confidence intervals.

Discussion

In our feeding experiments, *B. orientalis* was capable of capturing and ingesting a high variety of food types, differing both in size and in trophic level (phyto- and zooplankton). The results thus clearly show that *B. orientalis* is an omnivorous predator. Adult *D. magna* was the only food item *B. orientalis* was not able to ingest. As *B. orientalis* effectively preyed on juvenile *D. magna*, this indicates an upper prey size limit. It appears that anostracans in our experiments consumed slightly more zooplankton than phytoplankton biomass when offered in comparable amounts (Fig. 1B). It was more expressed in the rotifer *B. asplanchnoidis*, probably due to limited motility of this species, which makes it an easy prey for anostracans compared to the motile copepod. However, the estimated feeding rates on algae should be seen as conservative estimates, as food concentrations were below saturating levels (see **Supplement 1**).

Although Petkovski (1991) noted that females were generally larger than males in *B. orientalis*, we did not find significant sex-specific size differences. Moreover, we did not find significant differences in the ingestion rates between males and females, in spite of the fact that females were producing eggs at the time of the experiment. Daborn (1975) noted for *B. gigas* that assimilation rates in males were lower than in females, while motion of extremities (and consequently filtration rates) were slightly higher than in females suggesting that this way males

ingest more food and compensate for lower assimilation efficiency. Bernice (1971) found no difference in ingestion rates between males and females of *Streptocephalus dichotomus*, species of comparable size to *B. orientalis*. Males probably spend more energy in swimming by searching for females, while females use energy mostly for egg production, which would explain similar food consumption (Daborn, 1975).

Food ingestion rates between the two studied size classes of adult anostracans showed no significant difference in any of the tested food types. It suggests that once adult and capable of predation, the filtering ability of *B. orientalis* does not change considerably with growth. It is possible that in long-term experiments, the effect of body size on feeding rates would be easier to observe, due to higher metabolic demands (Daborn, 1975). In our experiments, we tested ingestion rates in short-term experiments (2–4 hours). From this, we could conclude that there is no change in the ability of filter feeding with growth. Size-dependent decrease in ingestion rates on phytoplankton was not observed in our experiments, not even with *Mychonastes* sp. as smaller sized algal food. On the contrary, ingestion rates for both algae types were slightly higher in animals of larger body size. Considering that there was no significant difference between feeding on algae (true filter feeding) and on copepods (motile animals, which could be regarded as some form of an active predation mode of feeding), our results show that *B. orientalis* is capable of employing two alternative feeding modes in its adult life stage with similar efficiency.

In rock pools, the anostracan *Branchipodopsis* were observed to feed both by filtering water and scraping benthic particles. This flexible feeding behaviour is probably an adaptation to low-nutrient content (Brendonck *et al.*, 2000). In general, being omnivorous and having a broad diet spectrum might be an advantage in temporary habitats with short inundation phase. The omnivorous diet of *B. orientalis* is probably a good example of adaptation to the short inundation phase of anostracan habitats and the fact that they need to grow and reach maturity very fast (Beladjal *et al.*, 2003; Sánchez and Angeler, 2007).

In many cases, filter feeding is not in conflict with the ingestion of small-sized zooplankton such as rotifers and small cladocerans together with phytoplankton (Table I). However, in some studies, deductions on anostracan diet were based on indirect evidence, e.g. on trunk limb morphology in *Branchinecta gaini* (Paggi, 1996) or mouth orientation in *S. dichotomus* (Bernice, 1971). Diverse crustacean remains were found in the gut content of both species, but it was assumed that anostracans ingested them only when the prey was already dead. *Branchinecta gaini* individuals reached 2 cm in body length (Paggi, 1996), while *S. dichotomus* reached up to 2.7 cm (Bernice, 1971), which is comparable with our experimental *B. orientalis* animals, as well as with most anostracan species (ranging between 1 and 3 cm in length; Sánchez *et al.*, 2007). For studying diet composition, gut content studies have some limitations, because food groups have different resistance to gut digestion (Mertens *et al.*, 1990). Moreover, gut content cannot provide information on whether the prey was actively captured alive or was picked up as part of detritus,

which results in different interpretations of the trophic role of anostracans. On the other hand, our experimental tests with single species offered as food only prove that *B. orientalis* was able to ingest diverse members of phyto- and zooplankton and that it actively predates on zooplankton. These experiments do not inform about possible preferences for a certain food types in the field. In numerous zooplankton taxa, it was shown that omnivorous feeding enhances growth and reproduction (Kleppel *et al.*, 1998; Breteler *et al.*, 1999). Anostracans fed with a mixed diet (algae + zooplankton) grew faster coupled with higher fecundity than animals fed on pure algal diet (Dumont and Ali, 2004). Hence, it is possible that the omnivorous feeding described here overall enhances food quality for anostracans. Therefore, we need both more *in situ* studies and empirical tests, resulting in a critical re-evaluation of the existing knowledge about anostracans.

The community structuring role of anostracans in temporary ponds is still little studied (Sánchez and Angeler, 2007). We found that the diet of *B. orientalis* is very diverse, comprising of most phyto- and zooplankton community members, making this species a top consumer of soda pans. During spring, anostracans and copepods both reach very high densities in soda pans, with a maximum dry-weight biomass of 23 mg L^{-1} of for *Arctodiaptomus* spp. and 7 mg L^{-1} for anostracans (Horváth *et al.*, 2013a). Calculating with the mean consumption rate in our experiment (2.4 mg of *Arctodiaptomus* per day) and the maximum density of *B. orientalis* from the field (13 ind L^{-1}), this implies strong top-down effect of *B. orientalis* on zooplankton. Anostracans occur only in spring, while *Arctodiaptomus* stays until the pans dry out. It is possible that the short life span of *B. orientalis* enables coexistence of the two groups even due to interactions through competition and predation.

Competition and predation effects can be difficult to discriminate. Negative correlations between *B. orientalis* and some cladoceran species were recorded previously (Sánchez *et al.*, 2007). It is in agreement with the implications of our findings, while it does not clarify the direct effects of *B. orientalis* on the zooplankton community. A predator–prey interaction between anostracans and copepods beside competition was suggested earlier, but not tested experimentally (Pociecha and Dumont, 2008). Waterkeyn *et al.* (2011) found that anostracans have a strong negative effect on the population size of diverse zooplankton groups, probably due to both types of interactions. Our study complements these results, by showing that anostracans can act as intraguild predators of a diverse array of zooplankton taxa (rotifers, cladocerans and copepods).

Conclusions

Contrary to assumption, anostracans seem to be omnivorous predators, capable of ingesting a wide range of food particles ranging from picoplankton to medium-sized zooplankton. Studies are needed to verify if the results shown for *B. orientalis* can be regarded a common trait in other (small-sized) anostracan species. Our results imply a more complex trophic role of anostracans

than previously assumed, suggesting to further study possible food selection and related trade-offs of omnivorous feeding such as food quality and quantity at different trophic levels in the habitats of anostracans. Finally, more empirical data from the field are needed, to fully appreciate the trophic role of these key consumers of temporary waters.

Acknowledgments

References

Ali, A.J., Sarma, S. S. S., Murugan, G. and Dumont, H. J. (1996) Effect of zooplankton type and abundance on prey consumption by the fairy shrimp, *Streptocephalus proboscideus* (Anostraca: Crustacea). *Hydrobiologia*, 319, 191–202.

Alonso, M. (1985) A survey of the Spanish Euphyllopoda. *Misc. Zool.*, 9, 179–208.

Beladjal, L., Peiren, N., Vandekerckhove, T. T. M. and Mertens, J. (2003) Different life histories of the co-occuring fairy shrimp *Branchipus schaefferi* and *Streptocephalus torvicornis* (Anostraca). *J. Crustac. Biol.*, 23, 300-307.

Belk, D. and Rogers, D. C. (2002) A confusing trio of *Branchinecta* (Crustacea: Anostraca) from the western North America with a description of a new species. *Hydrobiologia*, 486, 49–55.

Bernice, R. (1971) Food, feeding and digestion in *Streptocephalus dichotomus* Baird (Crustacea: Anostraca). *Hydrobiologia*, 38, 507–520.

Boix, D., Kneitel, J., Robson, B. J., Duchet, C., Zúñiga, L., Day, J., Gascón, S., Sala, J. et al. (2016) Intvertebrates of freshwater temporary ponds in Mediterranean climate. In Batzer, D. and Boix, D. (eds), *Invertebrates in Freshwater Wetlands*. Spinger, Cham, pp. 141-189.

Boros, E., Bánfi, S. and Forró, L. (2006) Anostracans and microcrustaceans as potential food sources of waterbirds on sodic pans of the Hungarian plain. *Hydrobiologia*, 567, 341–349.

Bottrell, H., Duncan, A., Gliwicz, Z., Grygierek, E., Herzig, A., Hillbricht-Ilkowska, A., Kurasawa, H., Larsson, P. et al. (1976) Review of some problems in zooplankton production studies. *Norwegian J. Zool.*, 24, 419–456.

Bowie, G. L., Mills, W. B., Porcella, D. B., Campbell, C. C., Pagenkopf, J. R., Rupp, G. L., Johnson, K. M., Chan, R. W. H. et al. (1985) Rates, constants and kinetics formulations in surface water quality modeling, 2nd edn. Environment Research Laboratory, Environmental Protection Authority EPA/600/3-85/040, p. 338.

Brendonck, L. (1989) Redescription of the fairy shrimp Streptocephalus proboscideus (Frauenfeld, 1873) (Crustacea, Anostraca). *Bull. Inst. R. Sc. N. B.-B*, 59, 49–57.

Brendonck, L. (1993) Feeding in the fairy shrimp *Streptocephalus proboscideus* (Frauenfeld) (Branchiopoda, Anostraca) .1. Aspects of the feeding biology. *J. Crustac. Biol.*, 13, 235–244.

Brendonck, L. (1996) Diapause, quiescence, hatching requirements: what we can learn from large freshwater branchiopods (Crustacea: Branchiopoda: Anostraca, Notostraca, Conchostraca). *Hydrobiologia*, 320, 85–97.

Brendonck, L., Hamer, M. L., Riddoch, B. J. and Seaman, M. (2000) *Branchipodopsis* species—specialists of ephemeral rock pools. *Afr. J. Aquat. Sci.*, 25, 98–104.

Breteler, W. C. M. K., Schogt, N., Baas, M., Schouten, S. and Kraay, G.W. (1999) Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. *Mar. Biol.*, 135, 191–198.

Carpenter, S., Kitchell, J. and Hodgson, J. (1985) Cascading trophic interactions and lake productivity. *Bioscience*, 35, 634–639.

Céréghino, R., Biggs, J., Oertli, B. and Declerck, S. (2008) The ecology of European ponds: defining the characteristics of a neglected freshwater habitat. *Hydrobiologia*, 597, 1–6.

Céréghino, R., Boix, D., Cauchie, H.-M., Martens, K. and Oertli, B. (2014) The ecological role of ponds in a changing world. *Hydrobiologia*, 723, 1–6.

Daborn, G. (1975) Life-history and energy relations of giant fairy shrimp *Branchinecta gigas* Lynch 1937 (Crustacea, Anostraca). *Ecology*, 56, 1025–1039.

Daborn, G.(1977) Life history of *Branchinecta mackini* Dexter (Crustacea, Anostraca) in an argillotrophic lake of Alberta. *Can. Zool.*, 55, 161–168.

Dierckens, K., Sarma, S. S. S., Mertens, J. and Dumont, H. (1995) Feeding the fairy shrimp *Streptocephalus* (Anostraca-Crustacea) with the rotifer *Anuraeopsis. Hydrobiologia*, 308, 29-33.

Dumont, H. J. and Ali, A. J. (2004) Stage-specific cannibalism and spontaneous cyst hatching in the freshwater fairy shrimps *Streptocephalus proboscideus* Frauenfeld. Hydrobiologia, 524, 103–113.

Fryer, G. (1966) *Branchinecta gigas* Lynch, a non-filter-feeding raptatory anostracan, with notes on the feeding habits of certain other anostracans. *Proc. Linn. Soc. Lond.*, 177, 19–34.

Fryer, G. (1983) Functional ontogenetic changes in *Branchinecta ferox* (Milne-Edwards) (Crustacea, Anostraca). *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.*, 303, 229–343.

Horváth, Zs. and Vad, Cs. F. (2015) Life history and current distribution of the fairy shrimp *Chirocephalus carnuntanus* (Brauer, 1877) (Crustacea: Anostraca). *North-West. J. Zool.*, 11, 102–109.

Horváth, Zs., Vad, Cs. F., Tóth, A., Zsuga, K., Boros, E., Vörös, L. and Ptacnik, R. (2014) Opposing patterns of zooplankton diversity and functioning along a natural stress gradient: when the going gets tough, the tough get going. *Oikos*, 123, 461–471.

Horváth, Zs., Vad, Cs. F., Vörös, L. and Boros, E. (2013a) The keystone role of anostracans and copepods in European soda pans during the spring migration of waterbirds. *Freshw. Biol.*, 58, 430–440.

Horváth, Zs., Vad, Cs. F., Vörös, L. and Boros, E. (2013b) Distribution and conservation status of fairy shrimps (Crustacea: Anostraca) in the astatic soda pans of the Carpathian basin: the role of local and spatial factors. *J. Limnol.*, 72, 103–116.

Jocque, M., Vanschoenwinkel, B. and Brendonck, L. (2010) Anostracan monopolisation of early successional phases in temporary waters? *Fundam. Appl. Limnol.*, 176, 127–132.

Kleppel, G. S., Burkart, C. A. and Houchin, L. (1998) Nutrition and the regulation of egg production in the Calanoid copepod Acartia tonsa. *Limnol. Oceanogr.*, 43, 1000–1007.

Krapu, G. (1974) Foods of breeding pintails in North Dakota. *J. Wildl. Manage.*, 38, 408–417.

Malone, C. R. (1965) Dispersal of plankton: rate of food passage in mallard ducks. *J. Wildlife Manage.*, 29, 529–533.

Marcus, N. (1990) Calanoid copepod, cladoceran, and rotifer eggs in sea-bottom sediments of northern Californian coastal waters: identification, occurrence and hatching. *Mar. Biol.*, 105, 413–418.

Marin, V., Huntley, M. E. and Frost, B. (1986) Measuring feeding rates of pelagic herbivores: analysis of experimental design and methods. *Mar. Biol.*, 93, 49–58.

Marrone, F., Rogers, D. C., Zarattini, P. and Naselli-Flores, L. (2017) New challenges in anostracan research: old issues, new perspectives and hot topics. *Hydrobiologia*, 801, 179-185.

Mertens, J., Munuswamy, N., Dewalsche, C. and Dumont, H. J. (1990) On predatory tendencies in the feeding ecology of the fairy shrimp *Streptocephalus proboscideus* (Frauenfeld, 1873) (Crustacea, Anostraca). *Hydrobiologia*, 198, 119–123.

Michaloudi, E., Mills, S., Papakostas, S., Stelzer, C.-P., Triantafyllidis, A., Kappas, I., Vasileiadou, K., Proios, K. et al. (2017) Morphological and taxonomic demarcation of *Brachionus*

asplanchnoidis Charin within the *Brachionus plicatilis* cryptic species complex (Rotifera, Monogononta). *Hydrobiologia*, 796, 19–37.

Miličić, D. Đorđević, S., Tomović, Lj. and Pavković-Lučić, S. (2013) Sexual dimorphism in *Branchipus schaefferi* Fischer, 1834 (Anostraca: Branchiopoda: Crustacea) from Serbia. *North-West. J. Zool.*, 9, 425–428.

Modlin, R. (1982) A comparison of 2 *Eubranchipus* species (Crustacea, Anostraca). *Am. Midl. Nat.*, 107, 107–113.

Mura, G. and Takami, G. A. (2000) A contribution to the knowledge of the anostracan fauna of Iran. *Hydrobiologia*, 441, 117–121.

Obregón-Barboza, H., Maeda-Martinez, A. M., Garcia-Velazco, H. and Dumont, H. J. (2002) *Branchinecta oterosanvicentei* n. sp (Branchiopoda: Anostraca), a new fairy shrimp from the Chihuahuan desert, with a proposal for the conservation of the Branchinectidae of Mexico. *Hydrobiologia*, 467, 45–56.

Onbe, T. (1978) Sugar flotation method for sorting the resting eggs of marine cladocerans and copepods from sea-bottom sediment. *Bull. Jap. Soc. Sci. Fish.*, 44, 1411–1411.

Paggi, J. C. (1996) Feeding ecology of *Branchinecta gaini* (Crustacea: Anostraca) in ponds of South Shetland Islands, Antarctica. *Polar Biol.*, 16, 13–18.

Persson, J., Brett, M. T., Vrede, T. and Ravet, J. L. (2007) Food quantity and quality regulation of trophic transfer between primary producers and a keystone grazer (*Daphnia*) in pelagic freshwater food webs. *Oikos*, 116, 1152–1163.

Petkovski, S. (1991) On the presence of the genus Branchinecta Verrill, 1869 (Crustacea, Anostraca) in Yugoslavia. Hydrobiologia, 226, 17–27.

Pociecha, A. and Dumont, H. J. (2008) Life cycle of *Boeckella poppei* Mrazek and *Branchinecta gaini* Daday (King George Island, South Shetlands). *Polar Biol.*, 31, 245–248.

Porter, K. G., Gerritsen, J. and Orcutt, J. D. (1982) The effect of food concentration on swimming patterns, feeding behavior, ingestion, assimilation, and respiration by *Daphnia. Limnol. Oceanogr.*, 27, 935–949.

R Core Team (2014) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/, URL.

Rogers, D. C., Quinney, D. L., Weaver, J. and Olesen, J. (2006) A new giant species of predatory fairy shrimp from Idaho, USA (Branchiopoda: Anostraca). *J. Crustac. Biol.*, 26, 1–12.

Rogers, D. C. and Timms, B. V. (2017) Predatory morphology and behaviour in *Branchinella occidentalis* (Dakin, 1914) (Branchiopoda: Anostraca: Thamnocephalidae). *Proc. Linn. Soc. N.S.W.*, 139, 1–8.

Sánchez, B. and Angeler, D. G. (2007) Can fairy shrimps (Crustacea: Anostraca) structure zooplankton communities in temporary ponds? *Mar. Freshw. Res.*, 58, 827–834.

Sánchez, M. I., Green, A. J., Amat, F. and Castellanos, E. M. (2007) Transport of brine shrimps via the digestive system of migratory waders: dispersal probabilities depend on diet and season. *Mar. Biol.*, 151, 1407–1415.

Sarma, S. S. S. and Nandini, S. (2002) Studies on functional response and prey selection using zooplankton in the anostracan *Chirocephalus diaphanus* Prevost, 1803. *Hydrobiologia*, 486, 169–174.

Silveira, J. G. (1998) Avian uses of vernal pools and implications for conservation practice. In Witham, C. W., Bauder, E. T., Belk, D., Ferren, W. R., Jr. and Ornduff, R. (eds), *Ecology, Conservation and Management of Vernal Pool Ecosystems.* California Native Plant Society, Sacramento, CA, pp. 92–106.

Somogyi, B., Felföldi, T., Vanyovszki, J., Ágyi, A., Márialigeti, K. and Vörös, L. (2009) Winter bloom of picoeukaryotes in Hungarian shallow turbid soda pans and the role of light and temperature. *Aquat. Ecol.*, 43, 735–744.

Tóth, A., Horváth, Zs., Vad, Cs. F., Zsuga, K., Nagy, S. A. and Boros, E. (2014) Zooplankton of the European soda pans: fauna and conservation of a unique habitat type. *Int. Rev. Hydrobiol.*, 99, 255–276.

Vörös, L., Balogh, K. V. and Boros, E. (2005) Picoplankton predominance in soda lakes (in Hungarian with English summary). *Hidrológiai Közlöny*, 85, 166–168.

Vörös, L., Somogyi, B. and Boros, E. (2008) Birds cause net heterotrophy in shallow lakes. *Acta Zool. Acad. Sci. Hung.*, 54, 23–34.

Waterkeyn, A., Grillas, P., Anton-Pardo, M., Vanschoenwinkel, B. and Brendonck, L. (2011) Can large branchiopods shape microcrustacean communities in Mediterranean temporary wetlands? *Mar. Freshw. Res.*, 62, 46–53.

White, G. E., Fabria, G. and Hartland-Rowe, R. (1969) The method of prey capture by *Branchinecta gigas* Lynch, 1937 (Anostraca). *Crustaceana* , 16, 158–160.

Williams, D. D. (2006) Introduction to temporary waters. In Williams, D. D. (ed.), *The Biology of Temporary Waters.* Oxford University Press, Oxford, pp. 1–4.

Supplement

Methods

We set separate experiments to detect the saturation value of food ingestion for *Chlamydomonas* sp. (3 replicates; 2 hours) and *Brachionus asplanchnoidis* (3 replicates, apart from the three highest food concentrations, where we used 4 replicates; 40 min). We used the same medium, environmental conditions and animals of *Branchinecta orientalis* as described in the Methods of the main text (see experimental design to test ingestion rates between different prey types). For *Chlamydomonas* sp., we had algal concentrations equivalent to 0.5; 1; 2; 3; 4 and 5 mg C L^{-1}. For *B. asplanchnoidis*, we had densities of 0.71; 1.43; 2.14; 2.86; 3.57; 4.29; 5; 5.71 and 6.43 ind mL^{-1}. To assess the number of algal cells or rotifers eaten (per hour), we calculated ingestion rates obtained with the same equations as in the main text (see calculation of ingestion rates in the Methods). We estimated the attack rate (or searching and attacking efficiency of the predator; a') and handling time (the time spent processing each prey item in hours; T_h) using the standard equations for functional response of type II most commonly observed in other animals (Holling, 1959; Begon et al., 1996):

$$N_i = \frac{a'NT}{1 + aT_h N}$$

with N_i – ingested prey; a' – attack rate; T_h – handling time; N – prey concentration; T – incubation time. All data analyses were performed in R (R Core Team, 2014).

Results and Discussion

Numbers of algal cells or zooplankton individuals remaining after feeding of *B. orientalis* are given in **Table S1** for *Chlamydomonas* sp. and **Table S2** for *B. asplanchnoidis*. With *Chlamydomonas sp.* food (a' estimate ± SE: 0.15±0.08, *p*=0.06; T_h estimate ± SE: 1.5*10^{-6} ± 0.43*10^{-6}, *p*=0.003), a threshold of feeding rate increase was reached at a food concentration of approx. 3–4 mg C L^{-1} (**Fig. S1a**). With *B. asplanchnoidis* (a' estimate ± SE: 13.25 ± 9.95, *p*=0.19; T_h estimate ± SE: 2.06*10^{-3} ± 0.29*10^{-3}, *p*<0.001), the saturation occurred at a food concentration of approx. 4–5 ind mL^{-1} (**Fig. S1b**). Interestingly, in one case, more than 150 rotifers were ingested per *B. orentalis* in 40 min. Attack rate (a') was marginally significant for *Chlamydomonas* sp. (*p*=0.06) as food and not significant for *B. asplanchnoidis* (*p*=0.19) which is probably due to the high variation among the replicates with the same prey density. Feeding of *B. orientalis* (for both food types) corresponds to the functional response of type II, which was most commonly observed in filter feeding zooplankton (Lampert and Sommer, 2007) and also found in other anostracan species (Sarma and Nandini, 2002).
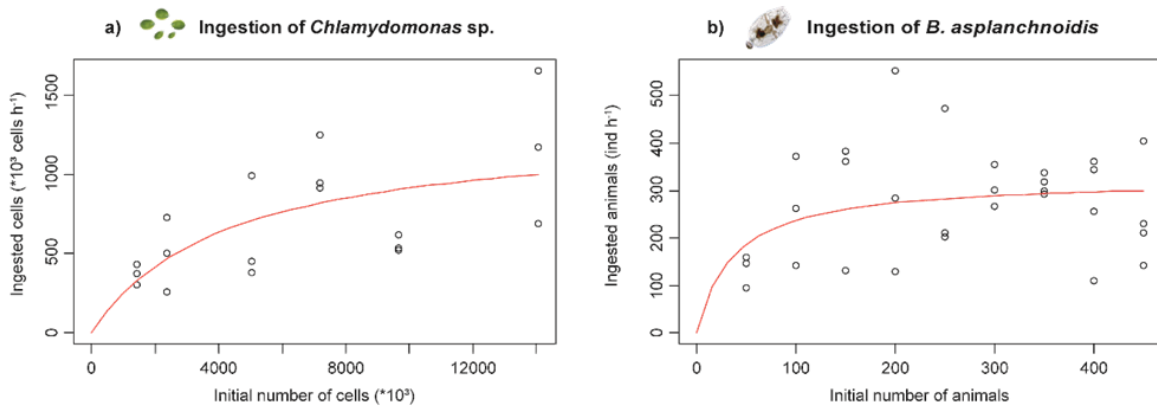
Fig. S1. Ingestion rates of *B. orientalis* on (a) *Chlamydomonas* sp. and (b) *Brachionus asplanchnoidis* as food at different prey concentrations. a) attack rate a' estimate ± SE: 0.15±0.08, *p*=0.06; handling time T_h estimate ± SE: $1.5*10^{-6}±0.43*10^{-6}$, *p*=0.003; b): a' estimate ± SE: 13.25 ± 9.95, *p*=0.19; T_h estimate ± SE: $2.06*10^{-3} ± 0.29*10^{-3}$, *p*<0.001.

Table SI. Feeding of *B. orientalis* (with two anostracans per vial) on *Chlamydomonas* sp. at different concentrations. C_0 – algal concentration at the beginning of experiment (in mg C L^{-1}); N_0 – initial number of algal cells at the beginning of experiment (in 10^5); N_t –number of algal cells at the end of experiment (in 10^5; mean ± SD).

C_0	0.5	1	2	3	4	5
N_0	14.1	23.6	50.3	71.9	96.5	140.6
N_t	2.7±0.5	4.1±1.6	9.3±2.3	23.6±2.3	34.8±0.8	77.7±10.7

Table SII. Feeding of *B. orientalis* (with two anostracans per vial) on rotifer *B. asplanchnoidis* at different concentrations. C_0 – concentration of rotifers at the beginning of experiment (in ind mL^{-1}); N_0 – initial number of rotifers at the beginning of experiment (mean ± SD); N_t –number of rotifers at the end of experiment (mean ± SD).

C_0	0.71	1.43	2.14	2.86	3.57	4.29	5	5.71	6.43
N_0	50	100	150	200	250	300	350	400	450
N_t	2±2	6±8	19±24	40±40	62±36	78±15	107±8	173±72	225±66

References

Begon, M., Harper, J.L., and Townsend, C.R. (eds) (1996) *Ecology; individuals, populations and communities*, 3rd edition. Blackwell Science Ltd., Cambridge, MA.

Holling, C.S. (1959) Some characteristics of simple types of predation and parasitism. *Can. Entomol.*, 91, 385–398.

Lampert, W., and Sommer, U. (eds) (2007) *Limnoecology*. Oxford Univeristy Press, New York.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Sarma, S.S.S., and Nandini, S. (2002) Studies on functional response and prey selection using zooplankton in the anostracan *Chirocephalus diaphanus* Prevost, 1803. *Hydrobiologia*, 486, 169–174.

Chapter 2 - Effect of turbidity on the feeding of fairy shrimps

Environmental constraint of intraguild predation: Inorganic turbidity modulates omnivory in fairy shrimps

Dunja Lukić, Robert Ptacnik, Csaba F Vad, Csenge Póda & Zsófia Horváth

Abstract

1. Omnivory is widespread in food webs, with an important stabilising effect. The strength of omnivorous trophic interactions may change considerably with changes in the local environment.

2. Shallow temporary waters are often characterised by high levels of inorganic turbidity that may directly limit the food uptake of filter-feeding organisms, but there is little evidence on how it might affect omnivorous species. Anostracans are key species of temporary waters and recent evidence suggests that these organisms are omnivorous consumers of both phyto- and zooplankton.

3. Using *Branchinecta orientalis* as a model species, our aim was to test how turbidity affects the feeding of an omnivorous anostracan. To do this, we used short-term feeding experiments and stable isotope analyses, with animals collected from soda pans in eastern Austria. In the feeding experiments, algae and zooplankton were offered as food either separately or in combination. The prey type treatments were crossed with turbidity levels in a factorial design.

4. There was a pronounced decrease in the ingested algal biomass with increasing turbidity. Conversely, ingestion rates on zooplankton were less affected by turbidity. Stable isotope analyses from field material supported our experimental results by showing a positive relationship of the trophic position of anostracans and the trophic niche of the communities with turbidity.

5. Our results show that turbidity modulates the intraguild trophic relationship between anostracans and their prey by shifting the diet of anostracans from more herbivorous in transparent to more carnivorous in turbid waters. Thus, inorganic turbidity might also have a community-shaping role in plankton communities of temporary waters through altering trophic relationships.

Introduction

Omnivory, that is feeding on multiple trophic levels (Coll & Guershon, 2002; Pimm & Lawton, 1978), is a very frequent component of all food webs (Holt & Polis, 1997; Kratina, LeCraw, Ingram, & Anholt, 2012; Thompson, Hemberg, Starzomski, & Shurin, 2007). Intraguild predation (IGP) is one type of omnivory, referring to predation on a potential competitor (Arim & Marquet 2004; Polis, Myers, & Holt, 1989). In an IGP system, two predators share a food source (shared prey), thereby acting as competitors. One of them furthermore acts as a predator for the other, being an intraguild predator (IG predator), whereas the preyed competitor is the intraguild prey (IG prey). The feeding of an IG predator may have important influence on community persistence (Stouffer & Bascompte, 2010) and stability (Neutel et al., 2007; Wootton, 2017).

Intraguild predation interactions are not static and their strength varies over time (Wootton, 2017). Besides changes in community composition (e.g. due to dispersal; Amarasekare, 2006) or habitat structure (Anderson & Semlitsch, 2016; Janssen, Sabelis, Magalhaes, Montserrat, & Van der Hammen, 2007), changes in the local environment (Sentis, Hemptinne, & Brodeur, 2014) can alter the strength of the links in IGP systems, as was shown in the case of temperature (Boersma et al., 2016) and productivity (Diehl & Feissel, 2000, 2001). In general, factors that decrease the strength of omnivorous trophic interactions (i.e. the omnivore feeds predominantly on one trophic level) will favour coexistence and increase the stability of food webs (Wootton, 2017). This suggests that the presence of a refuge that decreases the foraging ability of the IG predator on some prey groups will promote species coexistence (Janssen et al., 2007), which has been shown due to the presence and the structuring effect of vegetation (Bell, McCoy, & Mushinsky, 1991), but decreased visibility can also decrease the susceptibility of zooplankton to visual predators (Nurminen & Horppila, 2006; Vinyard & O'Brien, 1976).

Temporary waters are generally shallow and strongly exposed to the mixing effect of wind (Lahr, Diallo, Ndour, Badji, & Diouf, 1999; Naganawa & Zagas, 2002). Besides, they are often visited by large wallowing mammals and waterfowl with obvious consequences for turbidity and mixing (Vanschoenwinkel et al., 2011). As a result, inorganic turbidity is generally high, but may vary considerably among temporary habitats due to differences in depth and macrovegetation cover (Boros, Katalin, Vörös, & Horváth, 2017; Boven, Stoks, Forró, & Brendonck, 2008). As temporary ponds are often dominated by suspension-feeding crustaceans (cladocerans, copepods, anostracans) and rotifers, it raises the question of how these organisms cope with turbid conditions. As suspended inorganic particles (e.g. clay, silt; Eiler et al., 2003) interfere with the filter-feeding process (Kirk & Gilbert, 1990), their high concentration in the water is expected to have a negative effect on zooplankters (Dejen, Vijverberg, Nagelkerke, & Sibbing, 2004; Teffera et al., 2018; Zhou, Qin, & Han, 2018). There is, however, a difference among suspension feeders in their ability of coping with (often extreme) turbid conditions. Non-selective filter feeders such as cladocerans are generally believed to be adversely affected by the high concentration of inorganic suspended

particles (but see Hart, 1992), which they ingest together with their food (e.g. bacteria, algae) (Levine, Zehrer, & Burns, 2005; McCabe & O'Brien, 1983). Conversely, rotifers and copepods are more flexible in their feeding mode and can selectively feed on suitable particles, hence they are less affected by inorganic suspended solids than cladocerans (Hart, 1986, 1988; Kirk, 1991a; Kirk & Gilbert, 1990). Compared to cladocerans and copepods, we know much less about the feeding mechanism of anostracans. These flagship species of temporary waters play several key roles in their ecosystems, for example, it is suggested that they interact strongly with zooplankton with consequences for the trophic structure (Waterkeyn, Grillas, Anton-Pardo, Vanschoenwinkel, & Brendonck, 2011), and are also important as food source for waterbirds (Horváth, Vad, Vörös, & Boros, 2013b; Sánchez, Green, & Castellanos, 2006). Recent findings suggest that several anostracan species might act as IG predators in their habitats by preying on zooplankton and at the same time, competing for e.g. algal food (Lukić, Horváth, Vad, & Ptacnik, 2018). Based on their occurrence and habitat preferences, several anostracan species seem to be favoured by turbid conditions (Boudrias & Pires, 2002; Horváth, Vad, Vörös, et al., 2013b; Petkovski, 1991, 1993). For example, the three fairy shrimp species inhabiting Central European soda pans (including our study species *Branchinecta orientalis*) all seem to prefer highly turbid habitats according to field data (Horváth, Vad, Vörös, & Boros, 2013a). Although high turbidity may protect them from visual predators (e.g. amphibians and certain bird species; Boudrias & Pires, 2002; Boven, Vanschoenwinkel, De Roeck, Hulsmans, & Brendonck, 2008; Petkovski, 1993), it could at the same time be a constraint for food uptake by filter-feeding. It therefore seems unclear whether there is a causal relationship beyond predator avoidance behind the occurrence of anostracans in turbid waters, or whether turbidity merely coincides with other factors supporting them.

To date, there is little evidence on how inorganic turbidity affects trophic interactions, and particularly IGP relationships, in temporary ponds. To study whether herbivorous (on algae; shared prey) and carnivorous feeding (on zooplankton; IG prey) of anostracans (IG predator) is affected by increasing turbidity in a similar manner, we first performed controlled laboratory experiments and then compared our results to empirical data from the field. The experimental turbidity gradient in our feeding experiments covered the typical range of turbidity found in our study systems, soda pans. We crossed the turbidity gradient with a treatment of prey type (algae, zooplankton, algae, and zooplankton combined) to study the effect of increasing turbidity on ingestion rates for different food types. We then performed a field test to find empirical support for whether the trophic position of anostracans and the trophic niche of communities (which are expected to shift with changes in the dominant prey type) change along a natural gradient of turbidity in nine soda pans based on carbon and nitrogen stable isotope analysis.

Methods

Study system—soda pans of the Central European Lowlands

The soda pans of the Central European lowlands (Austria, Hungary, and Serbia) are inland saline waters of non-marine origin. Their ionic composition is dominated by Na^+, CO_3^{2-} and HCO_3^- that is considerably different from sea water (Boros, Ecsedi, & Oláh, 2013). Due to their high surface-to-volume ratio their sediment can easily be stirred up by the mixing effect of winds, consequently the amount of total suspended solids (TSS) can be extremely high, varying from 10 to over 30,000 mg/L (Boros et al., 2013). Particularly at the upper end of this gradient, TSS comprises almost exclusively abiotic and inorganic particles (e.g. clay, silt, sand) (Boros et al., 2017; Somogyi, Pálffy, Balogh, Botta-Dukót, & Vörös, 2017). The systems are naturally hypertrophic with total phosphorus concentrations (TP) ranging between 67 and 58,772 μg/L (measured between 2009 and 2010; Boros et al., 2013). During their wet phase, soda pans and other temporary saline pools on the steppes of Central and Eastern Europe represent a considerable proportion of shallow water habitats. For this reason, soda pans and sodic meadows are important resting sites for numerous waterbirds during their seasonal migration on the north-south route in the Western Palaearctic (Boros et al., 2013; Horváth, Vad, Vörös, et al., 2013b). In general, these systems are fishless, while vegetation is only occasionally present around the shore. The algal and zooplankton density generally increases with TSS in the pans, reaching extremely high abundances (Horváth et al., 2014). Picosized unicellular algae are the dominant group of phytoplankton in these systems (Somogyi et al., 2009, 2017). The dominant species of zooplankton are the calanoid copepod *Arctodiaptomus spinosus* and the cladoceran *Moina brachiata*, especially in turbid and saline pans (Horváth et al., 2014).

Study species—the fairy shrimp *Branchinecta orientalis*

Branchinecta orientalis inhabits mineral-rich temporary waters and is distributed between 27° and 55°N in Europe and Asia (Mura & Takami, 2000; Padhye, Kulkarni, & Dumont, 2017). Active populations of the species generally occur from March to June but exceptions have also been recorded in late autumn or winter (Atashbar, Agh, Van Stappen, & Beladjal, 2014; Eder, Hödl, & Gottwald, 1997; Petkovski, 1991; Šćiban, Marković, Lukić, & Miličić, 2014). In Central European soda pans, they occur within a wide turbidity range and represent the most frequent and numerous anostracan species (Horváth, Vad, Vörös, et al., 2013a). *B. orientalis* is an omnivore, feeding on diverse groups of both phyto- and zooplankton (Lukić et al., 2018).

Feeding experiments under laboratory conditions

Cultivation of B. orientalis *for feeding experiments: laboratory population*

To hatch animals, sediment containing resting eggs was collected from a soda pan, Oberer Stinkersee (47°48′49.4″N 16°47′34.4″E), and stored dry at 4°C in the dark for several months. After sieving and centrifuging the sediment according to the sugar flotation method (Marcus, 1990; Onbe, 1978), we incubated the eggs for hatching in a climate chamber, with a light regime 16L:8D and a temperature of 18°C. Resting eggs were incubated in a medium prepared from sodium hydrogen carbonate and distilled water (i.e. artificial soda water, 0.5 g $NaHCO_3$ per L water; conductivity 0.5 mS/cm). Once the animals started to hatch, we picked them out manually and transferred them to larger plastic containers (21 L) filled with artificial soda water and with constant aeration. A small amount of dry sediment from their natural habitat was added to the medium, to promote bacterial growth (that can serve as food for anostracan nauplii; final conductivity of medium around 1 mS/cm). We fed anostracans daily with a mix of algal food (*Cryptomonas* sp., *Scenedesmus* sp., *Chlamydomonas* sp.), which was at a later stage combined with zooplankton (the rotifer *Brachionus asplanchnoidis*, the cladoceran *M. brachiata*, and the copepod *A. spinosus*), constantly maintaining a sufficient amount of food for animals in the culture. Animals were considered adults once mating was observed (around 4 weeks old in the case of the laboratory population).

Collection of B. orientalis *for feeding experiments: field population*

The anostracans raised in the laboratory (mean ± *SD* of the body length 1.44 ± 0.13 cm) were smaller compared to those collected in the field (2.46 ± 0.22 cm); therefore, in a second set of experiments, we collected full-sized adults in the field to verify data obtained with laboratory animals. For these experiments, live animals were collected from Mittlerer Stinkersee (47°48′27.5″N 16°47′19.5″E) during spring 2017. After collection from the field, the population was kept and maintained the same way as the population hatched in the climate chamber.

Experimental design and food types

The zooplankton groups used in the experiments as food were also collected live on the field in the Seewinkel area (47°48′49.4″N 16°47′34.4″E) and cultivated in the laboratory during the experiments. In the predatory feeding tests, two typical members of soda pan zooplankton communities were used (Horváth et al., 2014; Tóth et al., 2014): a copepod (*A. spinosus,* 20 individuals in 70 ml) and a rotifer (*B. asplanchnoidis*, 100 individuals in 70 ml). The zooplankton prey concentrations were comparable to the zooplankton densities in their natural habitats in previous seasons (data not shown).

We offered two different sized algae as food in the herbivory feeding tests. A coccoid green algae *Mychonastes* sp. (Sphaeropleales; diameter 2–3 μm) was used as picoplankton (the

dominant size group of phytoplankton in soda pans; Felföldi, Somogyi, Márialigeti & Vörös, 2009; Vörös, Balogh & Boros, 2005), and the green flagellate *Chlamydomonas* sp. (Chlamydomonadales), which represented a larger unicellular food (7–18 µm length). The algal food concentration was at least 4 × higher than the concentration considered as saturating food abundance for *Daphnia magna* (Porter, Gerritsen & Orcutt, 1982), that is 2 mg/L dry weight in the feeding experiments with the laboratory animals and 7 mg/L with the anostracan population from the field.

We performed short-term feeding experiments at multiple turbidity levels (from 40 min to 4 hr depending on the prey type and anostracan population such as in Lukić et al., 2018). We applied a logarithmic scale covering the entire range of turbidity levels in soda pans during spring (Horváth, Vad, Vörös, et al., 2013a), when *B. orientalis* occurs (1–10,000 mg/L TSS concentrations; for duration, food concentration, number of replicates and turbidity levels see Table S1). The number of replicates was generally higher in the carnivory feeding experiments compared to the herbivory feeding experiments, because of the previously noticed higher variation between replicates (Lukić et al., 2018).

To create *turbidity*, we sieved the fine sediment (<250 µm) from a soda pan, Oberer Stinkersee (this is the same pan that was the source of the anostracan eggs used for the laboratory-raised population). The chosen soda pan is turbid and the characteristic greyish-white colour is caused by the high amount of suspended mineral particles in the water column (Boros et al., 2013). The sediment was dried and sterilised in an oven at 90°C (to avoid the growth of bacteria or algae). We added the selected amount of sediment (1–10,000 mg/L TSS; Table S1) to distilled water. Adding different amounts of dry sediment can result in slightly different salinity and pH levels in the treatments. To keep them the same at all turbidity levels, we added sodium hydrogen carbonate ($NaHCO_3$, one of the most dominant compounds in the natural habitats of *B. orientalis*; Boros, Horváth, Wolfram, & Vörös, 2014; Horváth, Vad, Vörös, & Boros, 2013a) to the lower turbidity treatments until salinity was equal to the treatment with the highest concentration of TSS (10,000 mg/L; final conductivity 1.6 mS and pH 8.4). As experimental units, transparent plastic vials of 100 mL total volume were used, filled up with 70 mL of the medium. In all experiments, two adult *B. orientalis* females were used per replicate. All experiments were run in a climate chamber during daytime under the same conditions used to maintain the algal and animal cultures (18°C). For all treatments and food items, controls (without *B. orientalis*) were run in parallel. To avoid algal sedimentation, medium was gently mixed in the algal feeding experiments (both controls and vials with *B. orientalis*) at regular intervals (30 min) and immediately before sampling phytoplankton for quantification.

In the first set of experiments, we tested how turbidity affects the ingestion rates of anostracans for all four prey types separately (*Mychonastes, Chlamydomonas, Brachionus,* and *Arctodiaptomus*) along an experimental turbidity gradient represented by five levels (1, 10, 100,

1,000, and 10,000 mg/L TSS). We then performed a second set of experiments employing *B. orientalis* collected as adults on the field, in order to test whether the effect of turbidity depends on adult size (animals from the field were considerably larger, see above). Here, we repeated the feeding experiments with three prey types individually (*Mychonastes, Chlamydomonas,* and *Arctodiaptomus*) and then tested for selective feeding using two food items (*Chlamydomonas* and *Arctodiaptomus*) provided in a mixture. In the second set of experiments, we used three turbidity levels (1, 100, and 10,000 mg/L TSS) except for *Arctodiaptomus* (five levels; see Table S1).

Calculation of biomass and ingestion rates

We calculated the dry weight biomass per cell from biovolume based on (Bowie et al., 1985; Vadstein, Jensen, Olsen, & Reinertsen, 1988). Biovolume of the algal food was approximated by measuring cellular dimensions and approximating them to simple geometrical bodies (sphere for *Mychonastes* sp. and depressed ellipsoid for *Chlamydomonas* sp). In zooplankton, *B. asplanchnoidis* and *A. spinosus* biomass per individual were calculated from the average weight of dried individuals (0.5 µg per individual for *B. asplanchnoidis* and 13.5 µg per individual for *A. spinosus*).

Biomass ingestion rate per anostracan in the experiments was calculated based on the equations from Frost (1972) and Marin, Huntley, and Frost (1986), assuming that food concentrations were below saturating concentration:

$$M = \frac{g C_0 V m}{N}$$

where *M* is ingested biomass per animal and time (in µg/h); *g*, grazing coefficient; C_0, phytoplankton cell concentration or concentration of zooplankton offered as food at the beginning of experiment (in cells/ml); *V*, volume of medium (in ml); *m*, average biomass (in µg) per phytoplankton cell or zooplankton individual; and *N*, number of anostracans per vial. The grazing coefficient (*g*) was calculated for all food types according to the formula:

$$g = k - \frac{ln(C_t) - ln⁡(C_0)}{t}$$

where C_0 is initial cell concentration of phytoplankton or initial concentration of zooplankton offered as food at the beginning of the experiment; C_t, final cell concentration of phytoplankton or final concentration of zooplankton offered as food at the end of the experiment, *k*, growth rate based on the change of algal concentration in controls (applicable for phytoplankton); and *t*, duration of the experiment (in hr) (Marin et al., 1986).

Trophic position in the natural environment: field sampling and sample analyses

Field sampling and laboratory measurements

In April 2018, zooplankton and fully adult anostracans were collected in nine soda pans to determine the stable isotope (δ^{15}N and δ^{13}C) values of *A. spinosus* and *B. orientalis* in relation to turbidity. The anostracans were collected with a push net (mesh size of 2 mm), while water was collected with a plastic beaker and sieved through a plankton net (mesh size of 100 µm) for zooplankton. Samples of *B. orientalis* and zooplankton were rinsed with distilled water (to remove the sediment and other particles suspended in the water column) and immediately frozen on dry ice and, after being transported to the laboratory, stored at −80°C. We also collected additional zooplankton samples for species identification and for enumerating zooplankton density. For this, 20 L of water was sieved through the 100-µm plankton mesh and the sample was fixed in 70% ethanol. Animals were identified and quantified under stereo and light microscope in the laboratory.

Environmental parameters (water depth, conductivity, pH, TSS, chlorophyll *a* concentration, total nitrogen [TN], and TP) and zooplankton density were analysed 2 weeks prior to the collection of animals and again on the day of anostracan collection (except for zooplankton density). To measure the amount of TSS in soda pans, water samples were collected and for each pond 1–50 mL water (depending on turbidity) was filtered through a pre-weighted GF/F filter immediately after returning from the field. Filters were dried in the oven at 60°C for overnight and measured again. The TSS concentration was the difference between the dry weight of the filters before and after filtering, divided by the volume of water filtered through the filters.

Stable isotope analysis

We used *B. orientalis* individuals (both males and females) of 2.0–2.7 cm body length, which was the largest anostracan size group found in soda pans during the sampling. We decided to use one of the zooplankton prey groups as a baseline (Jardine, Kidd, & Fisk, 2006), because in a previous sampling campaign we found the δ^{15}N and δ^{13}C values of seston unreliable (due to the high variation among the replicates; data not shown). *Arctodiaptomus spinosus*, the IG prey of anostracans, was one of the dominant zooplankton taxa, being present in all of the nine soda pans. Therefore, we isolated *Arctodiaptomus* under a stereo microscope from the thawed zooplankton samples in the laboratory and used them as a baseline for the relative trophic position of anostracans. Calanoid copepods are mostly feeding on algae, protozoans and rotifers (Kleppel, 1993; Lapesa, Snell, Fields, & Serra, 2004). Since *Arctodiaptomus* is an omnivore, the relative trophic position of *Branchinecta* should range from −0.5 (being purely herbivorous) to 0.5 (being a pure primary predator). Isolated *Arctodiaptomus* and *Branchinecta* specimens were rinsed once again with distilled water, then freeze-dried and 0.3 mg of a homogenised sample was placed in tin caps (three replicates for each animal group and soda pan) as part of standard sample preparation procedure to analyse nitrogen stable isotope composition by using the Elemental Analysis Isotope

Ratio Mass Spectrometry (EA-IRMS; EA—Thermo Scientific™ FLASH 2000 HT™; IRMS—Thermo Scientific™ Delta V™ Advantage). For both *Arctodiaptomus* and *Branchinecta*, the whole body of the animals was used in the analyses, which is a regularly used method for both zooplankton (reviewed in Feuchtmayr & Grey, 2003) and anostracans (Sánchez et al., 2013). Relative trophic position of *Branchinecta* was determined based on the following equation (modified from Hobson & Welch, 1992; Jardine et al., 2006):

$$RTP_{B.orientalis} = \frac{\delta^{15}N_{B.orientalis} - \delta^{15}N_{A.spinosus}}{\Delta^{15}N}$$

where $\delta^{15}N$ is the stable isotope ratio of nitrogen and $\Delta^{15}N$ is the *enrichment factor* representing the increase in $\delta^{15}N$ from one trophic position to the next, for which we used the general value of 3.4‰ (Fry, 2006; Jardine et al., 2006).

Statistical analyses

Negative biomass ingestion rates were set to zero prior to statistical analysis of grazing rates (Boersma et al., 2016; Nejstgaard, Naustvoll, & Sazhin, 2001). Moreover, in a few cases with *Brachionus* as food, all offered rotifers were ingested, setting the final food concentration at the end of the experiments to zero. Since the final concentrations are log-transformed in our equation to calculate the grazing coefficients, this would not have resulted in a meaningful value (Legendre & Legendre, 1998). Therefore, in these cases we decided to use a small non-zero value of 0.25 rotifer per experimental vial (mean between 0 and 0.5, a range that should be rounded to 0). TSS values were also log-transformed for the analyses. We fitted linear regressions (LR) to analyse feeding efficiency (expressed in biomass ingestion rates) as a function of turbidity (log-transformed TSS). We used a standardised regression coefficient (*lm.beta* package; Behrendt, 2014) to compare the effect of turbidity on different food types. As variation in the experimental data tended to scale with turbidity, we tested for heteroscedasticity (Breusch–Pagan test, *lmtest* package; Zeileis & Hothorn, 2002). In cases where this test was significant (*Mychonastes* prey with laboratory-raised anostracans, *Chlamydomonas* prey with field anostracans, and *Chlamydomonas* and *Chlamydomonas* + *Arctodiaptomus* prey in the mixed feeding of field anostracans, Table 1), we performed the Box–Cox (BC) transformation implemented with the *caret* package (Kuhn, 2008) on the biomass ingestion rate data set and then repeated the LR test. In every case, heteroscedasticity was non-significant after transformation.

To compare the differences between the slopes of two selected prey types (*Chlamydomonas* and *Arctodiaptomus* in all the three experiments) we fitted LR including two treatments and their interaction (turbidity*prey) in each of the three sets of experiments. If the Breusch–Pagan test was significant in this step, a robust LR (lm_robust() function in the *estimatr* package; Blair, Cooper, Coppock, Humphreys, & Sonnet, 2019) was chosen over a regular LR (this was necessary in the

experiments with laboratory-raised anostracans and the mixed food experiments with field anostracans).

For stable isotope analyses, we first checked the effect of turbidity on the $\delta^{15}N$ values of *Arctodiaptomus* and *Branchinecta* in two separate LR models. Then to study the relationship between the relative trophic position of anostracans and environmental parameters, we selected the best linear regression model explaining relative trophic position. For that, we used a stepwise regression with forward selection implemented with the *caret*, *leaps* (Lumley & Miller, 2017) and *MASS* (Venables & Ripley, 2002) packages. As predictors in the null model of the stepwise regression, we included TSS, conductivity, water depth, zooplankton density, and TN, including both measurements 2 weeks prior and on the day of the anostracan sampling (except zooplankton density). All predictors except conductivity and TN were log-transformed. Total phosphorus concentration and chlorophyll *a* were not included in the initial model because they both showed strong significant positive correlation with TSS at both time points, which is a general pattern in these habitats (see also Horváth et al., 2014). Model selection was based on mean absolute error and root mean squared error criteria. We tested the effect of the resulting best predictor (TSS) on the relative trophic position with an LR.

We used $\delta^{15}N$ and $\delta^{13}C$ values to calculate the area of the convex hull (i.e. trophic niche, as in Layman, Quattrochi, Peyer, & Allgeier, 2007) of the soda pan communities (*Arctodiaptomus* + *Branchinecta,* altogether 6 samples per soda pan) using the *SIBER* package (Jackson, Inger, Parnell, & Bearhop, 2016). We log-transformed the obtained trophic niche values and then tested their relationship with turbidity (log-transformed TSS measured 2 weeks prior anostracan sampling) with an LR model. All data were analysed in R (R Core Team, 2014).

Results

Feeding experiments

In the algal feeding experiments (for the ingestion rates see Table S2), turbidity had a similar significant negative effect on the ingestion rates of both anostracan populations, the laboratory-raised anostracans (Table 1 and Figure 1a,b) and the ones from the field (Table 1 and Figure 2a). *Mychonastes* was ingested at rates similar to those found in the case of the laboratory population (consisting of somewhat smaller animals), while ingestion rates on *Chlamydomonas* appeared to be higher in the anostracans from the field. Ingestion rates on *Chlamydomonas* were overall higher (with close to one order of magnitude in a few treatments) than on *Mychonastes* for both the population from the field and the laboratory-raised anostracans.

Table 1. Linear regressions fitted for the feeding experiments: individual prey offered to the laboratory-raised population of *Branchinecta*, individual prey offered to the population of *Branchinecta* from the field, and mixed prey (*Chlamydomonas + Arctodiaptomus*) offered to the population of *Branchinecta* from the field.

	Food	No transformation			Box-Cox transformation		
		β	R^2	p	β	R^2	p
Lab population of *Branchinecta;* individual prey	*Mychonastes*	-0.63	0.35	0.012	-0.72	0.48	0.002
	Chlamydomonas	-0.84	0.69	<0.001	-	-	-
	Brachionus	-0.47	0.19	0.017	-	-	-
	*Arctodiaptomus**	-0.18	-0.01	0.386	-0.17	-0.01	0.403
Population of *Branchinecta* from the field; individual prey	*Mychonastes*	-0.72	0.45	0.028	-	-	-
	Chlamydomonas	-0.51	0.23	0.004	-0.52	0.25	0.003
	Arctodiaptomus	-0.14	-0.00	0.327	-	-	-
Population of *Branchinecta* from the field; mixed prey	*Chlamydomonas*	-0.53	0.25	0.003	-0.44	0.16	0.015
	*Arctodiaptomus**	-0.37	0.10	0.046	-0.29	0.05	0.123
	Chlamydomonas + Arctodiaptomus	-0.72	0.50	<0.001	-0.75	0.54	<0.001

β is the standardised regression coefficient. Missing values ('–') indicate that no Box–Cox transformation was performed for the data due to non-significant outcomes of the Breusch–Pagan test which indicated a lack of heteroscedasticity. Asterisks indicate experiments where the Breusch–Pagan test was marginally significant ($0.05 < p < 0.1$).

The effect of turbidity on the carnivory of *Branchinecta* was not detectable in most cases. We did not find a significant effect of turbidity on feeding on *Arctodiaptomus* either in the laboratory-raised (Figure 1d and Table 1) or in the field population (Figure 2b and Table 1). Turbidity only had a significant negative impact on the ingestion of *Brachionus* (Figure 1c and Table 1).
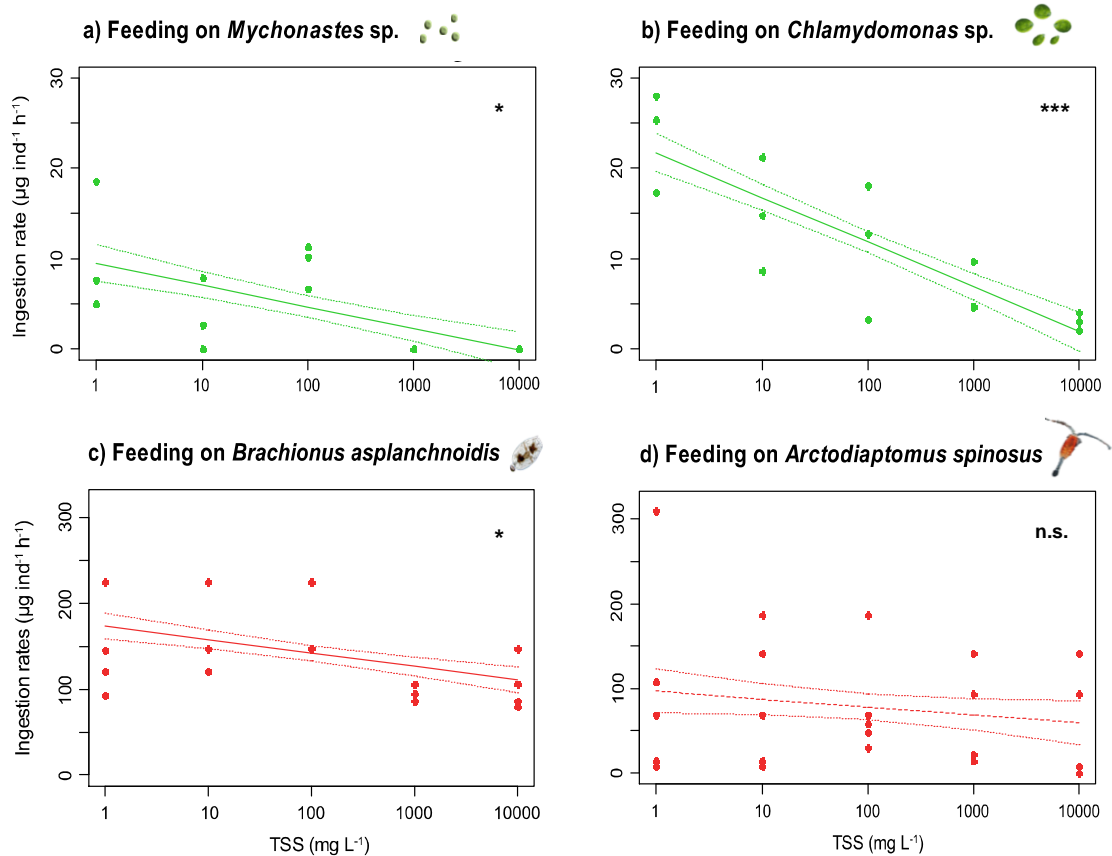
Figure 1. Biomass ingestion rates of *Branchinecta orientalis* (lab-raised specimens) on the four different food groups. Effect of turbidity was significantly negative for herbivorous feeding on a) *Mychonastes* sp. (LR: $R^2=$ 0.35, *p*=0.012), b) *Chlamydomonas* sp. (LR: $R^2=$ 0.69, *p*<0.001) and carnivorous feeding on rotifer c) *Brachionus asplanchnoidis* (LR: $R^2=$ 0.19, *p*=0.017) but not significant for carnivorous feeding on calanoid copepod d) *Arctodiaptomus spinosus* (LR: $R^2=$ -0.01, *p*=0.386).

In the feeding experiments with mixed food (algae + zooplankton offered simultaneously to the field population), results were similar to the individual experiments with both anostracan populations (Table S2). Turbidity had a significant negative effect both on herbivorous and carnivorous feeding without BC transformation (Figure 3a), but after BC transformation, only the effect on herbivorous feeding stayed significant (Table 1). Overall, total biomass ingestion rates (sum of all algae + zooplankton biomass consumed per experimental vial) were also decreasing with increasing turbidity (Figure 3b; Table 1). At the same time, the ratio of ingested food types (based on biomass) reversed with increasing turbidity, with a higher ratio of algal food ingested in clear compared to a higher ratio of animal food in turbid water (Figure S1).
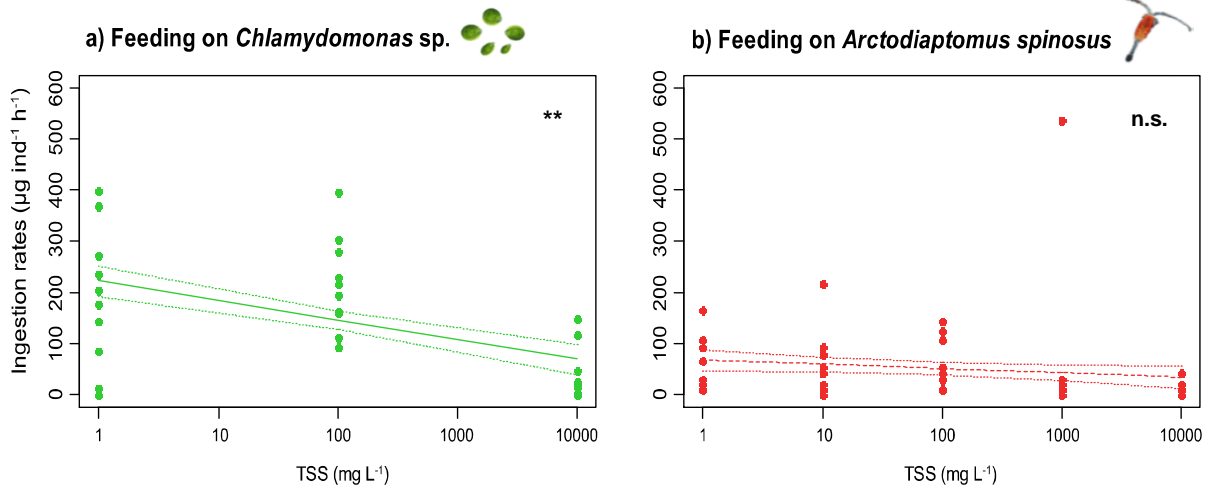
Figure 2. Biomass ingestion rates of *Branchinecta orientalis* (specimens collected from the field) in the single-food experiments. In case of a) herbivorous feeding on the algae *Chlamydomonas* sp. biomass ingestion rates decreased significantly along the turbidity gradient (linear regressions, LR: R^2=0.23, p=0.004), while in b) carnivorous feeding on the zooplankter *Arctodiaptomus spinosus* no significant pattern was detected (LR: R^2=-0.0004, p=0.327).
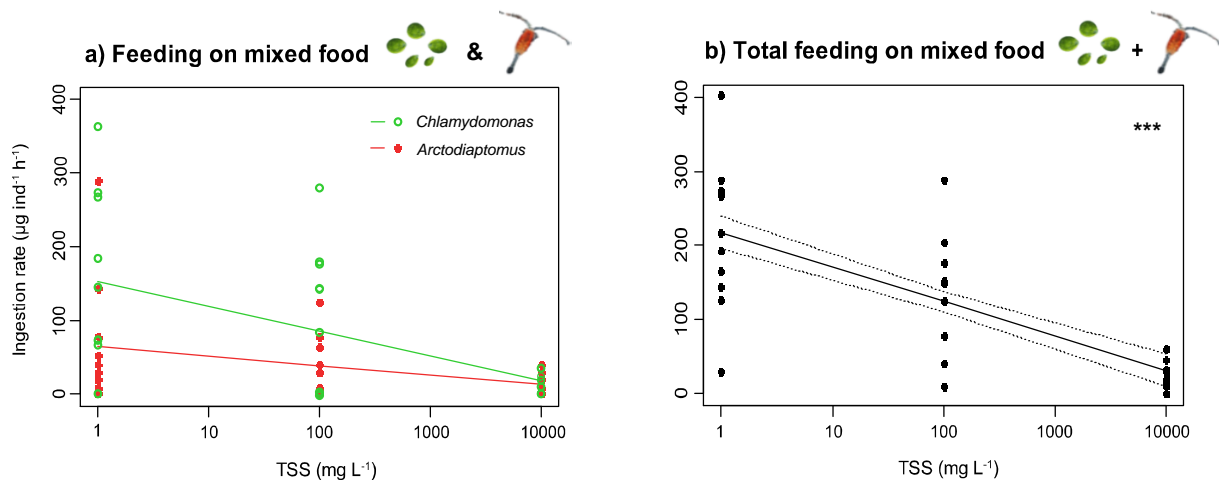
Figure 3. Biomass ingestion rates of *Branchinecta orientalis* (specimens collected from the field) in the mixed-food experiment along the turbidity gradient. a) Ingestion rates for *Chlamydomonas* sp. (linear regressions, LR: R^2=0.25, p=0.003; green empty circles and green regression line) and *Arctodiaptomus spinosus* (LR: R^2=0.10, p=0.046; red filled circles and red regression line). b) Total ingestion rate in the same experiment (algae + zooplankton; LR: R^2=0.50, p<0.001).

By directly comparing the regression slopes for three experimental groups (feeding experiments with the laboratory-raised anostracans, individual and mixed feeding experiments with anostracans from the field) across food type treatments, we found significant differences between the slopes of *Chlamydomonas* and *Arctodiaptomus* prey in the individual feeding experiments of anostracans from the field (the turbidity*prey interaction in LR: t_{76} = −2.11, p = 0.038). This

interaction was not significant in the laboratory- raised population (t_{36} = 0.35, p = 0.724) or the mixed feeding experiment with the anostracans from the field (t_{56} = −1.68, p = 0.097).

Trophic position of anostracans in their natural habitats

There was a significant correlation between the δ^{15}N values of anostracans and turbidity, while this was not the case for copepods (Figure 4a). The relative trophic position of *Branchinecta* relative to *Arctodiaptomus* (omnivore) ranged from −0.39 (equivalent to the position of primary consumers, implying the dominance of herbivorous feeding on phytoplankton) to 0.68 (position of secondary consumers, implying the dominance of carnivorous feeding on primary consumers). The relative trophic position of anostracans was best explained by the mean turbidity of their habitats measured 2 weeks before sampling for the stable isotope analysis (Table S3), although the relationship in the following LR was not significant (Figure 4b, p = 0.152).

The log-transformed trophic niche values of soda pan communities (*Arctodiaptomus* + *Branchinecta*) showed a significant positive correlation with the same measurement of turbidity (Figure 4c), indicating that the trophic role of anostracans became more distinct from the copepods as turbidity increased, which widened the isotopic niche of the community.
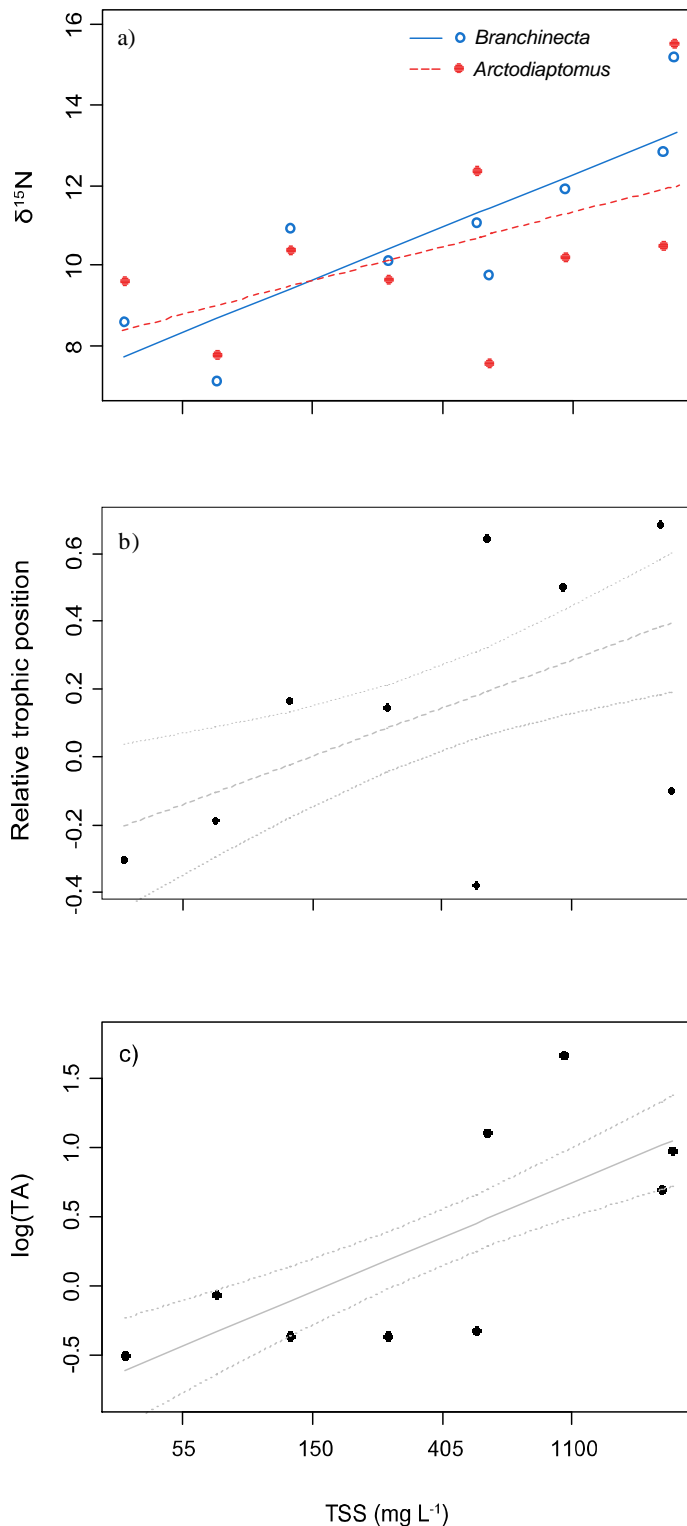
Figure 4. Stable isotope data of calanoid copepod *Arctodiaptomus spinosus* and anostracan *Branchinecta orientalis* from nine soda pans. a) $\delta^{15}N$ values of *A. spinosus* (linear regressions, LR: R^2=0.19, *p*=0.133; red filled circles and red regression line) and *B. orientalis* (LR: R^2=0.69, *p*=0.003; blue empty circles and blue regression line) along the turbidity gradient (represented by log-transformed total suspended solids [TSS]). TSS in the soda pans measured 2 weeks prior to animal sampling for stable isotope analysis. b) Relative trophic position of *B. orientalis* (compared to *A. spinosus*) along the turbidity gradient (log-transformed TSS; LR: R^2=0.16, *p*=0.152). Trophic position is calculated in relation to *A. spinosus* (intraguild [IG] prey), where 0 indicates when they occupy the same position (competition between IG predator and IG prey) whereas 1 indicates that the position of *B. orientalis* is one level higher (implying the predominance of a predator-prey relationship between IG predator and IG prey); c) Convex hull area (i.e. trophic niche) of the soda pan communities (*B. orientalis* + *A. spinosus*) along the turbidity gradient (both variables were log-transformed; LR: R^2=0.46, *p*=0.026).

Discussion

Turbidity reduced the feeding rates of *Branchinecta* on both algal prey types (*Mychonastes* and *Chlamydomonas*) in all experimental setups. Although the ingestion rates on *Chlamydomonas* in the clear water treatment were much higher in the large anostracan population (compared to the smaller population raised in the laboratory; see Figures 1b and 2a), the overall response to the

turbidity treatment was the same. In contrast, turbidity had no significant to weak effect on the ingestion of zooplankton. This altogether implied a shift in the IGP system, with an increasing relative importance of carnivory towards high turbidity. The observed increase in anostracan δ^{15}N values and relative trophic position, as well as the community trophic niche, match the experimental results. Thus, our results showed that inorganic turbidity modulated the strength of trophic relationships between an IG predator and its prey, which implies an important community shaping role of turbidity in aquatic systems.

Effect of turbidity on anostracan feeding

Up to now, very little is known on the possible environmental effects on the ingestion rates in anostracans. Herbivorous feeding (i.e. filter-feeding on algae) have been shown to be affected by salinity (Sánchez, Paredes, Lebouvier, & Green, 2016) and water quality (Brendonck, 1993), but the effect of inorganic turbidity was so far unknown. For non-selective filter feeders such as cladocerans, high amounts of TSS usually lead to the ingestion of increased amounts of inorganic particles and consequently a reduction in ingested algae (Arruda, Marzolf, & Faulk, 1983; Hart, 1988). Another possible effect is the mechanical inhibition of food collection by inorganic particles, which has also been documented for filter feeders (Kirk, 1991b). Our observations on live anostracans collected from the field and used in our feeding experiments rather suggested the first effect (ingestion of high amounts of inorganic particles), as the intestines of animals feeding in turbid environments were greyer (D. Lukić, personal observation). In addition to a lower ingestion of algae, we also recorded a decrease in the ingestion rate on the rotifer *Brachionus*. *Brachionus* is a relatively large rotifer species (180–500 µm body length), but a very slow swimmer. Therefore, some authors already considered feeding on rotifers as one way of filter-feeding (as in feeding on algae) rather than true predatorial behaviour (Dumont, Ali, Sarma, & Mertens, 1994), which is also supported by our results.

Many anostracan species live in turbid waters (Daborn, 1977; Hancock & Timms, 2002; Horváth, Vad, Vörös, et al., 2013a). Based on the existing evidence, anostracans are presumably not visual predators (Boudrias & Pires, 2002; Rogers & Timms, 2017) and rely on other senses instead to locate their prey. Their antennae and other extremities are densely covered by setae (Boudrias & Pires, 2002; Rogers, Quinney, Weaver, & Olesen, 2006; Rogers & Timms, 2017), which are suggested to play an important role in tactile (as suggested for *Branchinella occidentalis*; Rogers & Timms, 2017) or chemosensory detection of prey (suggested for *Branchinecta* species; Boudrias & Pires, 2002). These are in agreement with our findings and could explain the weak to no effect of increased turbidity on carnivorous feeding on the largest and most motile prey (*Arctodiaptomus*) offered in our experiments. This implies that feeding on *Arctodiaptomus* probably happens via another mechanism of feeding (different from the filtration of algae and rotifers), which

suggests that anostracans are able to use different feeding mechanisms—filter and predatory feeding—depending on the prey type.

In this study, the whole body of anostracans was used for stable isotope analysis, which integrates the information on food assimilated during the majority of the adult life of the studied organisms (Zanden, Clayton, Moody, Solomon, & Weidel, 2015). For anostracans, it would probably be around 4 weeks prior to our sampling (judged based on the size of anostracans and the time of ice break). However, local values of TSS can show extreme temporal changes in soda pans (see Table S4). For that reason, we included data on TSS (and the other available environmental parameters) from 2 weeks before the stable isotope sampling, and the 2-week prior TSS values were indeed the best predictors of the trophic position of *Branchinecta*. The results of the field test were in accordance with our laboratory experiments—the relative trophic position of *Branchinecta* rose with TSS, with an increase of one entire level from the most transparent to the most turbid habitat—implying that increased turbidity induced a shift in their feeding (indicating zooplankton as major prey in these cases). This suggests that turbidity acts as an important determinant of the strength of trophic relationships between anostracans and their prey (Figure 5). Moreover, the increasing amount of available food, algae, and zooplankton, along the turbidity gradient (which is a general pattern in our study systems, Horváth et al., 2014) might compensate for the overall decrease in total ingested biomass with increasing turbidity we observed in our experimental setup, where food levels were kept constant. This can eventually explain the high abundances of anostracans even in very turbid habitats (Boudrias & Pires, 2002; Boven, Vanschoenwinkel et al., 2008; Horváth, Vad, Vörös, et al., 2013a).

Effect of anostracans on the community as an IG predator

Our results have further implications regarding the importance of anostracans as IG predators for the entire community. Anostracans can reach high densities in their habitats (Daborn, 1977; Horváth, Vad, Vörös, et al., 2013b; Vanschoenwinkel, Brendonck, Pinceel, Dupriez, & Waterkeyn, 2013; Vanschoenwinkel, Seaman, & Brendonck, 2010) and they act both as competitors and predators of smaller zooplankton (Jocque, Vanschoenwinkel, & Brendonck, 2010; Lukić et al., 2018; Waterkeyn et al., 2011), which suggests a high impact on the zooplankton community. Even though *B. orientalis* hatches very early after inundation/ice break (Lukić, Vad, & Horváth, 2016; Petkovski, 1991), it is probably predominantly herbivorous in the early stages of its life (similar to other anostracans; Daborn, 1975; Fryer, 1983), which gives some time for zooplankton communities to establish at the beginning of the wet phase of their habitats. Moreover, anostracans disappear before temporary ponds dry out, while many zooplankton species occur throughout the whole wet season (Horváth, Vad, Vörös, et al., 2013a; Jocqué, Riddoch, & Brendonck, 2007; Tóth et al., 2014; Vanschoenwinkel, Waterkeyn, et al., 2010), which overall provides a temporal refuge for zooplankton (Kratina et al., 2012). In addition, anostracans do not

feed on the largest zooplankton such as adult *D. magna* (they are only susceptible to anostracan predation in the earliest stages of their life, Lukić et al., 2018). In this way, anostracans could even promote the maintenance of larger *Daphnia* in the communities through decreasing the densities of their competitors, that is smaller zooplankton taxa.

Influence of turbidity on the IGP system

Changes in inorganic turbidity can lead to significant shifts in community composition (Teffera et al., 2018; Zhou et al., 2018). According to our results, suspended inorganic particles create a refuge for smaller prey of anostracans (algae and small zooplankton), comparable to structured habitats providing a refuge for IG prey (although the direct mechanism is different) (Janssen et al., 2007). Similar phenomena were shown in case of visual predators (planktivorous fish) and their prey (zooplankton), where increased levels of turbidity or the deeper dark strata of lakes provided a refuge for zooplankton (the latter via diurnal vertical migration of zooplankton) (Gardner, 1981; Vinyard & O'Brien, 1976). Furthermore, turbidity might also affect trophic relationships beyond the boundaries of aquatic habitats as experimental evidence showed that increased amounts of detritus particles can inhibit the filtration rate of filter-feeding waterbirds such as shovelers (Gurd, 2007).

At the same time, the effect of turbidity on the IGP system of temporary ponds is more complex. Our results show that turbidity probably does not affect the predation efficiency of anostracans on the IG prey (zooplankton) considerably, but it rather decreases competition for the shared resource (phytoplankton). We found that grazing on phytoplankton was very close to 0 in the most turbid treatments in our experiments, and the relative trophic position of anostracans similarly implied a predominantly carnivorous feeding in turbid habitats. As chlorophyll *a* concentrations significantly increase along the turbidity gradient in soda pans (Horváth et al., 2014), we can exclude that the reason for the predominantly carnivorous feeding of anostracans was resulting from limited amounts of phytoplankton available as food. In temporary pond systems with anostracans as IG predators, turbidity does not provide efficient refuge for zooplankton (due to probably non-visual predation of anostracans), but it decreases their grazing pressure on phytoplankton (Figure 5), which can indirectly benefit zooplankton due to the higher phytoplankton (food) abundance in turbid ponds (Horváth et al., 2014). In hypertrophic systems such as soda pans (TP 67–4,177 µg/L in spring 2018), the IG prey would otherwise be prone to extinction (Diehl & Feissel, 2001). Turbidity and productivity are typically correlated in soda pans (this study; Horváth et al., 2014), which suggests the importance of turbidity as a stabilising factor in this part of the food web.
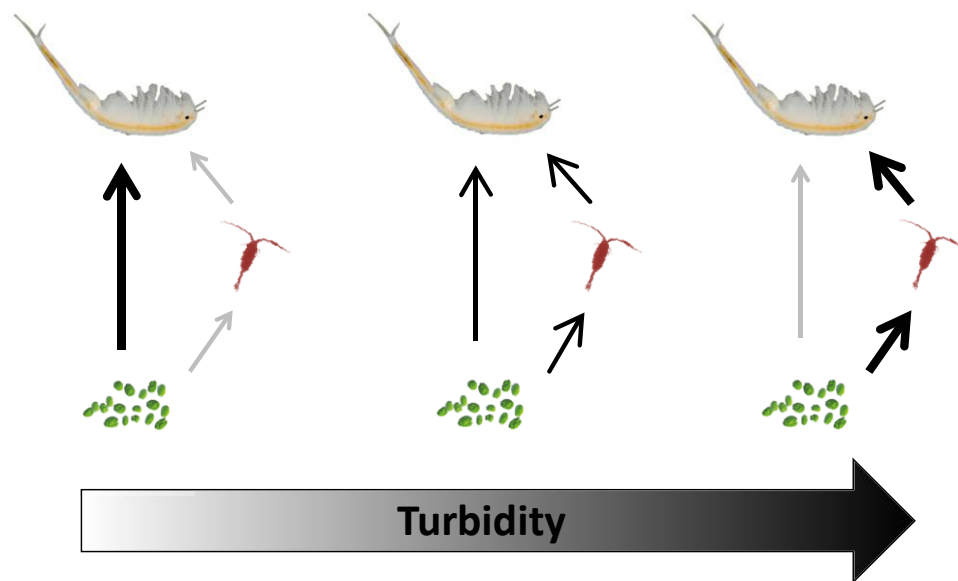
Figure 5. A schematic representation of the changes in the relative strength of interactions in the studied intraguild predation system along the turbidity gradient, with anostracans as intraguild predators, zooplankton as intraguild prey, and phytoplankton as shared prey.

Conclusions

Our results show that inorganic turbidity can alter trophic relationships through the feeding of an IG predator (anostracans in this study). This also implies a possible community shaping role of turbidity on plankton communities, with the strength of top-down control on the different groups varying with turbidity. In addition to the effect on the feeding of anostracans, other taxonomic groups are also likely to be directly affected, depending on their feeding type. For example, the ability of copepods to cope with increased turbidity better than cladocerans (Dejen et al., 2004) could shape competitive interactions within the zooplankton communities and help to sustain copepod dominance in highly turbid soda pans (Horváth et al., 2014). The global presence of anostracans in turbid ponds could also at least partly stem from to their ability to feed on different prey types and to switch between multiple feeding mechanisms. Furthermore, amphibians and waterbirds feeding from temporary ponds could also be affected by changes in inorganic turbidity and subsequent changes in prey availability (Gurd, 2007). Therefore, the consequences of increased turbidity are expected to be complex, acting on multiple trophic levels.

Acknowledgments

References

Amarasekare, P. (2006). Productivity, dispersal and the coexistence of intraguild predators and prey. *Journal of Theoretical Biology*, *243*, 121–133.

Anderson, T. L., & Semlitsch, R. D. (2016). Top predators and habitat complexity alter an intraguild predation module in pond communities. *Journal of Animal Ecology*, *85*, 548–558.

Arim, M., & Marquet, P. A. (2004). Intraguild predation: a widespread interaction related to species biology. *Ecology Letters*, *7*, 557–564.

Arruda, J. A., Marzolf, G. R., & Faulk, R. T. (1983). The role of suspended sediments in the nutrition of zooplankton in turbid reservoirs. *Ecology*, *64*, 1225–1235.

Atashbar, B., Agh, N., Van Stappen, G., & Beladjal, L. (2014). Diversity and distribution patterns of large branchiopods (Crustacea: Branchiopoda) in temporary pools (Iran). *Journal of Arid Environments*, *111*, 27–34.

Behrendt, S. (2014). *lm.beta: Add standardized regression coefficients to lmobjects*. R package version 1.5-1. Retrieved from http://cran.r-project.org/packa ge=lm.beta

Bell, S. S., McCoy, E. D., & Mushinsky, H. R. (1991). *Habitat Structure: The physical arrangement of objects in space*. Dordrecht, the Netherlands: Springer Science & Business Media.

Blair, G., Cooper, J., Coppock, A., Humphreys, M., & Sonnet, L. (2019). *estimatr: Fast Estimators for Design-Based Inference*. R package. Retrieved from https://cran.r-proje ct.org/web/packa ges/estim atr/index.html

Boersma, M., Mathew, K. A., Niehoff, B., Schoo, K. L., Franco-Santos, R. M., & Meunier, C. L. (2016). Temperature driven changes in the diet preference of omnivorous copepods: No more meat when it's hot? *Ecology Letters*, *19*, 45–53.

Boros, E., Ecsedi, Z., & Oláh, J. (2013). *Ecology and management of soda pans in the Carpathian Basin*. Balmazújváros, Hungary: Hortobágy Environmental Association.

Boros, E., Horváth, Z., Wolfram, G., & Vörös, L. (2014). Salinity and ionic composition of the shallow astatic soda pans in the Carpathian Basin. *Annales de Limnologie-International Journal of Limnology*, *50*, 59–69.

Boros, E., Katalin, V.-B., Vörös, L., & Horváth, Z. (2017). Multiple extreme environmental conditions of intermittent soda pans in the Carpathian Basin (Central Europe). *Limnologica (Online)*, *62*, 38–46.

Boudrias, M. A., & Pires, J. (2002). Unusual sensory setae of the raptorial *Branchinecta gigas* (Branchiopoda : Anostraca). *Hydrobiologia*, *486*, 19–27.

Boven, L., Stoks, R., Forró, L., & Brendonck, L. (2008). Seasonal dynamics in water quality and vegetation cover in temporary pools with variable hydroperiods in Kiskunság (Hungary). *Wetlands*, *28*, 401.

Boven, L., Vanschoenwinkel, B., De Roeck, E. R., Hulsmans, A., & Brendonck, L. (2008). Diversity and distribution of large branchiopods in Kiskunsag (Hungary) in relation to local habitat and spatial factors: Implications for their conservation. *Marine and Freshwater Research*, *59*, 940–950.

Bowie, G. L., Mills, W. B., Porcella, D. B., Campbell, C. C., Pagenkopf, J. R., Rupp, G. L., et al. (1985). *Rates, constants and kinetics formulations in surface water quality modeling* (2nd edn). *Environment Research Laboratory, Environmental Protection Authority EPA/600/3-85/040*.

Brendonck, L. (1993). Feeding in the fairy shrimp *Streptocephalus proboscideus* (Frauenfeld) (Branchiopoda: Anostraca). II. Influence of environmental conditions on feeding rate. *Journal of Crustacean Biology*, *13*, 245–255.

Coll, M., & Guershon, M. (2002). Omnivory in terrestrial arthropods: Mixing plant and prey diets. *Annual Review of Entomology*, *47*, 267–297.

Daborn, G. (1975). Life history and energy relations of giant fairy shrimp *Branchinecta gigas* Lynch 1937 (Crustacea-Anostraca). *Ecology*, *56*, 1025–1039.

Daborn, G. R. (1977). The life history of *Branchinecta mackini* Dexter (Crustacea: Anostraca) in an argillotrophic lake of Alberta. *Canadian Journal of Zoology*, *55*, 161–168.

Dejen, E., Vijverberg, J., Nagelkerke, L. A. J., & Sibbing, F. A. (2004). Temporal and spatial distribution of microcrustacean zooplankton in relation to turbidity and other environmental factors in a large tropical lake (L. Tana, Ethiopia). *Hydrobiologia*, *513*, 39–49.

Diehl, S., & Feissel, M. (2000). Effects of enrichment on three-level food chains with omnivory. *The American Naturalist*, *155*, 200–218.

Diehl, S., & Feissel, M. (2001). Intraguild prey suffer from enrichment of their resources: A microcosm experiment with ciliates. *Ecology*, *82*, 2977–2983.

Dumont, H. J., Ali, A. J., Sarma, S. S. S., & Mertens, J. (1994). Predatory filter-feeding in fairy shrimps: Functional response of *Streptocephalus proboscideus* (Crustacea: Anostraca) fed Anuraeopsis fissa (Rotifera). *Internationale Revue der Gesamten Hydrobiologie und Hydrographie*, *79*, 511–519.

Eder, E., Hödl, W., & Gottwald, R. (1997). Distribution and phenology of large branchiopods in Austria. *Hydrobiologia*, *359*, 13–22.

Eiler, A., Farnleitner, A. H., Zechmeister, T. C., Herzig, A., Hurban, C., Wesner, W., Krachler, R., Velimirov, B., & Kirschner, A. K. T. (2003). Factors controlling extremely productive heterotrophic bacterial communities in shallow soda pools. *Microbial Ecology*, *46*, 43–54.

Felföldi, T., Somogyi, B., Márialigeti, K., & Vörös, L. (2009). Characterization of photoautotrophic picoplankton assemblages in turbid, alkaline lakes of the Carpathian Basin (Central Europe). *Journal of Limnology*, *68*, 385–395.

Feuchtmayr, H., & Grey, J. (2003). Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determinations from zooplankton. *Rapid Communications in Mass Spectrometry*, *17*, 2605–2610.

Frost, B. W. (1972). Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. *Limnology and Oceanography*, *17*, 805–815.

Fry, B. (Ed.) (2006). Isotope Chi. In *Stable isotope ecology* (pp. 76–118). New York, NY: Springer.

Fryer, G. (1983). Functional ontogenetic changes in *Branchinecta ferox* (Milne-Edwards) (Crustacea, Anostraca). *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, *303*, 229-343.

Gardner, M. B. (1981). Effects of turbidity on feeding rates and selectivity of bluegills. *Transactions of the American Fisheries Society*, *110*, 446–450.

Gurd, D. B. (2007). Predicting resource partitioning and community organization of filter-feeding dabbling ducks from functional morphology. *The American Naturalist*, *169*, 334–343.

Hancock, M. A., & Timms, B. V. (2002). Ecology of four turbid clay pans during a filling-drying cycle in the Paroo, semi-arid Australia. *Hydrobiologia*, *479*, 95–107.

Hart, R. C. (1986). Zooplankton abundance, community structure and dynamics in relation to inorganic turbidity, and their implications for a potential fishery in subtropical Lake le Roux, South Africa. *Freshwater Biology*, *16*, 351–371.

Hart, R. C. (1988). Zooplankton feeding rates in relation to suspended sediment content: Potential influences on community structure in a turbid reservoir. *Freshwater Biology*, *19*, 123–139.

Hart, R. (1992). Experimental studies of food and suspended sediment effects on growth and reproduction of 6 planktonic cladocerans. *Journal of Plankton Research*, *14*, 1425–1448.

Hobson, K. A., & Welch, H. E. (1992). Determination of trophic relationships within a high Arctic marine food web using $\delta^{13}C$ and $\delta^{15}N$ analysis. *Marine Ecology Progress Series*, *84*, 9–18.

Holt, R. D., & Polis, G. A. (1997). A theoretical framework for intraguild predation. *The American Naturalist*, *149*, 745–764.

Horváth, Zs., Vad, Cs. F., Tóth, A., Zsuga, K., Boros, E., Vörös, L., & Ptacnik, R. (2014). Opposing patterns of zooplankton diversity and functioning along a natural stress gradient: When the going gets tough, the tough get going. *Oikos, 123*, 461–471.

Horváth, Zs., Vad, Cs. F., Vörös, L., & Boros, E. (2013a). Distribution and conservation status of fairy shrimps (Crustacea: Anostraca) in the astatic soda pans of the Carpathian basin: The role of local and spatial factors. *Journal of Limnology*, *72*, 103–116.

Horváth, Zs., Vad, Cs. F., Vörös, L., & Boros, E. (2013b). The keystone role of anostracans and copepods in European soda pans during the spring migration of waterbirds. *Freshwater Biology*, *58*, 430–440.

Jackson, A. L., Inger, R., Parnell, A. C., & Bearhop, S. (2016). Comparing isotopic niche widths among and within communities: SIBER – Stable Isotope Bayesian Ellipses in R. *Journal of Ecology*, *80*, 595–602.

Janssen, A., Sabelis, M. W., Magalhaes, S., Montserrat, M., & Van der Hammen, T. (2007). Habitat structure affects intraguild predation. *Ecology*, *88*, 2713–2719.

Jardine, T. D., Kidd, K. A., & Fisk, A. T. (2006). Applications, considerations, and sources of uncertainty when using stable isotope analysis in ecotoxicology. *Environmental Science & Technology*, *40*, 7501–7511.

Jocqué, M., Riddoch, B. J., & Brendonck, L. (2007). Successional phases and species replacements in freshwater rock pools: Towards a biological definition of ephemeral systems. *Freshwater Biology*, *52*, 1734–1744.

Jocque, M., Vanschoenwinkel, B., & Brendonck, L. (2010). Anostracan monopolisation of early successional phases in temporary waters? *Fundamental and Applied Limnology*, *176*, 127–132.

Kirk, K. (1991a). Inorganic particles alter competition in grazing plankton - the role of selective feeding. *Ecology*, *72*, 915–923.

Kirk, K. (1991b). Suspended clay reduces *Daphnia* feeding rate – behavioral mechanisms. *Freshwater Biology*, *25*, 357–365.

Kirk, K. L., & Gilbert, J. J. (1990). Suspended clay and the population dynamics of planktonic rotifers and cladocerans. *Ecology*, *71*, 1741–1755.

Kleppel, G. (1993). On the diets of calanoid copepods. *Marine Ecology Progress Series*, *99*, 183–195.

Kratina, P., LeCraw, R. M., Ingram, T., & Anholt, B. R. (2012). Stability and persistence of food webs with omnivory: Is there a general pattern? *Ecosphere*, *3*, art50.

Kuhn, M. (2008). Building predictive models in R using the caret package. *Journal of statistical software*, *28*, 1–26.

Lahr, J., Diallo, A. O., Ndour, K. B., Badji, A., & Diouf, P. S. (1999). Phenology of invertebrates living in a sahelian temporary pond. *Hydrobiologia*, *405*, 189–205.

Lapesa, S., Snell, T. W., Fields, D. M., & Serra, M. (2004). Selective feeding of *Arctodiaptomus salinus* (Copepoda, Calanoida) on co-occurring sibling rotifer species. *Freshwater Biology*, *49*, 1053–1061.

Layman, C. A., Quattrochi, J. P., Peyer, C. M., & Allgeier, J. E. (2007). Niche width collapse in a resilient top predator following ecosystem fragmentation. *Ecology Letters*, *10*, 937–944.

Legendre, P., & Legendre, L. (1998). *Numerical ecology* (2nd ed.). Amsterdam, the Netherlands: Elsevier Science B.V.

Levine, S. N., Zehrer, R. F., & Burns, C. W. (2005). Impact of resuspended sediment on zooplankton feeding in Lake Waihola, New Zealand. *Freshwater Biology*, *50*, 1515–1536.

Lukić, D., Horváth, Z., Vad, C. F., & Ptacnik, R. (2018). Food spectrum of *Branchinecta orientalis*—are anostracans omnivorous top consumers of plankton in temporary waters? *Journal of Plankton Research*, *40*, 436–445.

Lukić, D., Vad, C. F., & Horváth, Z. (2016). Isolation by sugar flotation has no direct effect on the hatching success of zooplankton resting eggs. *Journal of Limnology*, *75*, 415–421.

Lumley, T., & Miller, A. (2017). *Leaps: Regression Subset Selection*. R package version 3.0. https://CRAN.R-project.org/package=leaps

Marcus, N. (1990). Calanoid copepod, cladoceran, and rotifer eggs in sea-bottom sediments. *Marine Biology*, *105*, 413–418.

Marin, V., Huntley, M. E., & Frost, B. (1986). Measuring feeding rates of pelagic herbivores: Analysis of experimental design and methods. *Marine Biology*, *93*, 49–58.

McCabe, G., & O'Brien, W. (1983). The effects of suspended silt on feeding and reproduction of *Daphnia pulex*. *American Midland Naturalist*, *110*, 324–337.

Mura, G., & Takami, G. A. (2000). A contribution to the knowledge of the anostracan fauna of Iran. *Hydrobiologia*, *441*, 117–121.

Naganawa, H., & Zagas, B. (2002). General aspects of the large branchiopod crustacean fauna of Mongolia. *Limnology*, *3*, 181–188.

Nejstgaard, J. C., Naustvoll, L.-J., & Sazhin, A. (2001). Correcting for underestimation of microzooplankton grazing in bottle incubation experiments with mesozooplankton. *Marine Ecology Progress Series*, *221*, 59–75.

Neutel, A.-M., Heesterbeek, J. A. P., van de Koppel, J., Hoenderboom, G., Vos, A., Kaldeway, C., Berendse, F., & de Ruiter, P. C. (2007). Reconciling complexity with stability in naturally assembling food webs. *Nature*, *449*, 599–602.

Nurminen, L., & Horppila, J. (2006). Efficiency of fish feeding on plantattached prey: Effects of inorganic turbidity and plant-mediated changes in the light environment. *Limnology and Oceanography*, *51*, 1550–1555.

Onbe, T. (1978). Sugar flotation method for sorting the resting eggs of marine cladocerans and copepods from sea-bottom sediment. *Bulletin of the Japanese Society of Scientific Fisheries*, *44*, 1411.

Padhye, S. M., Kulkarni, M. R., & Dumont, H. J. (2017). Diversity and zoogeography of the fairy shrimps (Branchiopoda: Anostraca) on the Indian subcontinent. *Hydrobiologia*, *801*, 117–128.

Petkovski, S. (1991). On the presence of the genus *Branchinecta* Verrill, 1869 (Crustacea, Anostraca) in Yugoslavia. *Hydrobiologia*, *226*, 17–27.

Petkovski, S. (1993). *Taksonomsko-morfološka i zoogeografsko-ekološka studija Anostraca (Crustacea: Branchiopoda) jugoslovenskih zemalja*. Prirodno-matematički fakultet. PhD dissertation, University of Novi Sad, Novi Sad, Serbia.

Pimm, S. L., & Lawton, J. H. (1978). On feeding on more than one trophic level. *Nature*, *275*, 542–544.

Polis, G. A., Myers, C. A., & Holt, R. D. (1989). The ecology and evolution of intraguild predation: potential competitors that eat each other. *Annual review of ecology and systematics*, *20*(1), 297–330.

Porter, K. G., Gerritsen, J., & Orcutt, J. D. (1982). The effect of food concentration on swimming patterns, feeding behavior, ingestion, assimilation, and respiration by *Daphnia. Limnology and Oceanography*, *27*, 935–949.

R Core Team (2014). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-proje ct.org/

Rogers, D. C., Quinney, D. L., Weaver, J., & Olesen, J. (2006). A new giant species of predatory fairy shrimp from Idaho, USA (Branchiopoda: Anostraca). *Journal of Crustacean Biology*, *26*, 1–12.

Rogers, D. C., & Timms, B. V. (2017). Predatory morphology and behavior in *Branchinella occidentalis* (Dakin, 1914) (Branchiopoda: Anostraca: Thamnocephalidae). *Linnean Society of New South Wales*, *139*, 1–8.

Sánchez, M. I., Green, A. J., & Castellanos, E. M. (2006). Temporal and spatial variation of an aquatic invertebrate community subjected to avian predation at the Odiel salt pans (SW Spain). *Archiv für Hydrobiologie*, *166*, 199–223.

Sánchez, M. I., Paredes, I., Lebouvier, M., & Green, A. J. (2016). Functional role of native and invasive filter-feeders, and the effect of parasites: Learning from hypersaline ecosystems. *PLoS ONE, 11*, e0161478.

Sánchez, M. I., Varo, N., Matesanz, C., Ramo, C., Amat, J. A., & Green, A. J. (2013). Cestodes change the isotopic signature of brine shrimp, *Artemia*, hosts: Implications for aquatic food webs. *International Journal for Parasitology*, *43*, 73–80.

Šćiban, M., Marković, A., Lukić, D., & Miličić, D. (2014). Autumn populations of *Branchinecta orientalis* G. O. Sars, 1903 and *Chirocephalus diaphanus* Prevost, 1803 (Crustacea, Branchiopoda) in the Central European Lowlands (Pannonian Plain, Serbia). *North-Western Journal of Zoology*, *10*, 435–437.

Sentis, A., Hemptinne, J.-L., & Brodeur, J. (2014). Towards a mechanistic understanding of temperature and enrichment effects on species interaction strength, omnivory and food-web structure. *Ecology Letters*, *17*, 785–793.

Somogyi, B., Felföldi, T., Vanyovszki, J., Ágyi, Á., Márialigeti, K., & Vörös, L. (2009). Winter bloom of picoeukaryotes in Hungarian shallow turbid soda pans and the role of light and temperature. *Aquatic Ecology*, *43*, 735–744.

Somogyi, B., Pálffy, K., Balogh, K. V., Botta-Dukát, Z., & Vörös, L. (2017). Unusual behaviour of phototrophic picoplankton in turbid waters. *PLoS ONE, 12*, e0174316.

Stouffer, D. B., & Bascompte, J. (2010). Understanding food-web persistence from local to global scales. *Ecology Letters, 13*, 154–161.

Teffera, F. E., Lemmens, P., Deriemaecker, A., Deckers, J., Bauer, H., Gamo, F. W., Brendonck, L., & De Meester, L. (2018). Why are Lake Abaya and Lake Chamo so different? A limnological comparison of two neighboring major Ethiopian Rift Valley lakes. *Hydrobiologia*, *829*, 113–124.

Thompson, R. M., Hemberg, M., Starzomski, B. M., & Shurin, J. B. (2007). Trophic levels and trophic tangles: The prevalence of omnivory in real food webs. *Ecology*, *88*, 612–617.

Tóth, A., Horváth, Z., Vad, C. F., Zsuga, K., Nagy, S. A., & Boros, E. (2014). Zooplankton of the European soda pans: Fauna and conservation of a unique habitat type. *International Review of Hydrobiology*, *99*, 255–276.

Vadstein, O., Jensen, A., Olsen, Y., & Reinertsen, H. (1988). Growth and phosphorus status of limnetic phytoplankton and bacteria. *Limnology and Oceanography*, *33*, 489–503.

Vanschoenwinkel, B., Brendonck, L., Pinceel, T., Dupriez, P., & Waterkeyn, A. (2013). Rediscovery of *Branchipus schaefferi* (Branchiopoda: Anostraca) in Belgium - notes on habitat requirements and conservation management. *Belgian Journal of Zoology*, *143*, 3–14.

Vanschoenwinkel, B., Seaman, M., & Brendonck, L. (2010). Hatching phenology, life history and egg bank size of fairy shrimp *Branchipodopsis* spp. (Branchiopoda, Crustacea) in relation to the ephemerality of their rock pool habitat. *Aquatic Ecology*, *44*, 771–780.

Vanschoenwinkel, B., Waterkeyn, A., Jocqué, M., Boven, L., Seaman, M., & Brendonck, L. (2010). Species sorting in space and time—the impact of disturbance regime on community assembly in a temporary pool metacommunity. *Journal of the North American Benthological Society*, *29*, 1267–1278.

Vanschoenwinkel, B., Waterkeyn, A., Nhiwatiwa, T., Pinceel, T., Spooren, E., Geerts, A., Clegg, B., & Brendonck, L. (2011). Passive external transport of freshwater invertebrates by elephant and other mudwallowing mammals in an African savannah habitat. *Freshwater Biology*, *56*, 1606–1619.

Venables, W. N., & Ripley, B. D. (2002). *Modern applied statistics with S* (4th ed.). New York, NY: Springer.

Vinyard, G. L., & O'Brien, W. J. (1976). Effects of light and turbidity on the reactive distance of bluegill (*Lepomis macrochirus*). *Journal of the Fisheries Research Board of Canada*, *33*, 2845–2849.

Vörös, L., Balogh, K. V., & Boros, E. (2005). Picoplankton predominance in soda lakes (in Hungarian with English summary). *Hidrológiai*, *85*, 166–168.

Waterkeyn, A., Grillas, P., Anton-Pardo, M., Vanschoenwinkel, B., & Brendonck, L. (2011). Can large branchiopods shape microcrustacean communities in Mediterranean temporary wetlands? *Marine and Freshwater Research*, *62*, 46–53.

Wootton, K. L. (2017). Omnivory and stability in freshwater habitats: Does theory match reality? *Freshwater Biology*, *62*, 821–832.

Zanden, M. J. V., Clayton, M. K., Moody, E. K., Solomon, C. T., & Weidel, B. C. (2015). Stable isotope turnover and half-life in animal tissues: A literature synthesis. *PLoS ONE*, *10*, e0116182.

Zeileis, A., & Hothorn, T. (2002). Diagnostic Checking in Regression Relationships. *R News*, *2*, 7–10. https://CRAN.R-project.org/doc/Rnews/

Zhou, J., Qin, B., & Han, X. (2018). The synergetic effects of turbulenceand turbidity on the zooplankton community structure in large, shallow Lake Taihu. *Environmental Science and Pollution Research*, *25*, 1168–1175.

Supplement

Table S1. Overview of the experimental setup of all performed experiments. The duration of the experiments (t) differed in relation to the size of anostracans (population from the field was larger than the lab-raised population) and food type, to avoid food depletion. C_0 is food concentration at the beginning of the experiment. In setups using three turbidity levels, TSS concentrations were 1, 100 and 10 000 mg L^{-1}; whereas the five levels were 1, 10, 100, 1 000 and 10 000 mg L^{-1}.

	Food		C_o (ind mL^{-1})	t (h)	Number of replicates	Turbidity levels
Lab population of *Branchinecta*	phytoplankton	*Mychonastes*	$3*10^5$	4	3	5
		Chlamydomonas	$2.7*10^4$	2	3	5
	zooplankton	*Brachionus*	1.43	0.75	5	5
		Arctodiaptomus	0.29	1	5	5
Population of *Branchinecta* from the field	phytoplankton	*Mychonastes*	$5*10^5$	2	3	3
		Chlamydomonas	$5*10^4$	0.75	10	3
	zooplankton	*Arctodiaptomus*	0.29	0.75	10	5
	mixed	*Chlamydomonas*	$5.2*10^4$	0.75	10	3
		Arctodiaptomus	0.29			

Table S2. Ingestion rates (biomass µg h^{-1}; mean ± SD of the replicates) of *Branchinecta orientalis* in the experiments. In the mixed food treatment, we present rates for the two food types (*Chlamydomonas* sp., *Arctodiaptomus spinosus*) both separately and as total ingested biomass.

	Food		Ingestion rates (biomass µg h^{-1}) at different levels of turbidity (TSS)				
			1 mg L^{-1} TSS	10 mg L^{-1} TSS	100 mg L^{-1} TSS	1000 mg L^{-1} TSS	10000 mg L^{-1} TSS
Lab population of *Branchinecta*	phyto-plankton	*Mychonastes*	10.4±7.3	2.4±5.5	9.3±2.5	0	0
		Chlamydomonas	23.6 ± 5.5	14.9 ± 6.3	11.3 ± 7.5	6.3 ± 2.9	3.0 ± 0.9
	zoo-plankton	*Brachionus*	141.5±33.9	146.7±18.4	157.1±14.2	99.5±8.7	112.9±32.2
		Arctodiaptomus	101.5±123.7	83.6±78.9	78.3±62.2	58.5±56.4	66.9±61.3
Population of *Branchinecta* from the field	phyto-plankton	*Mychonastes*	20.9 ± 8.2	-	25.0 ± 4.8	-	0
		Chlamydomonas	181.3±150.3	-	214.2±92.1	-	26.6±61.5
	zoo-plankton	*Arctodiaptomus*	57.2±49.6	53.3±64.8	65.1±50.4	66.2±166.1	8.7±12.8
	mixed	*Chlamydomonas*	140.9±129.6	-	92.1±107.5	-	0
		Arctodiaptomus	65.9±89.6	-	37.2±40.2	-	13.5±13.4
		Chlamydomonas + Arctodiaptomus	210.6±104.2	-	137.1±80.9	-	24.1±17.9

Table S3. Linear regression model selection based on Mean Absolute Error (MAE) and Root Mean Squared Error (RMSE) criteria. TSS - total suspended solids (TSS) on the day of the animal sampling; TSS* - total suspended solids two weeks before animal sampling; TN - total nitrogen; cond - conductivity; Zs - Secchi depth.

Number of predictors	Predictors	MAE	RMSE
1	log(TSS*)	0.37	0.39
2	log(TSS*)+log(TSS)	0.62	0.63
3	log(TSS*)+log(TSS)+TN	0.75	0.79
4	log(TSS*)+log(TSS)+TN+cond	1.03	1.09
5	log(TSS*)+log(TSS)+TN+cond+log(Zs)	1.34	1.48

Table S4. Summary table of the temporal changes (difference between the values measured at two occasions, 2nd and 18th April 2018, in a given habitat) in the abiotic environmental parameters during the development of the field population of *Branchinecta orientalis* used in the trophic position study. Data from nine soda pans indicate that TSS within a habitat is highly variable over the season which might weaken the relationship between the trophic position of *B. orientalis* and a single measurement of TSS.

Environmental factor (unit)	2nd occasion (18th April) - 1st occasion (2nd April)					
	Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
TSS (mg L^{-1})	-194.00	70.0	333.1	526.3	977.4	1905.00
TN (mg L^{-1})	0.55	0.84	1.63	1.68	2.09	3.27
TP (mg L^{-1})	0.03	0.23	0.71	0.65	0.87	1.52
Conductivity (mS cm^{-1})	0.52	0.64	0.80	0.90	1.19	1.44
Depth (cm)	-8	-6	-5	-4	-2	2
pH	-0.25	-0.02	0.05	0.05	0.18	0.34
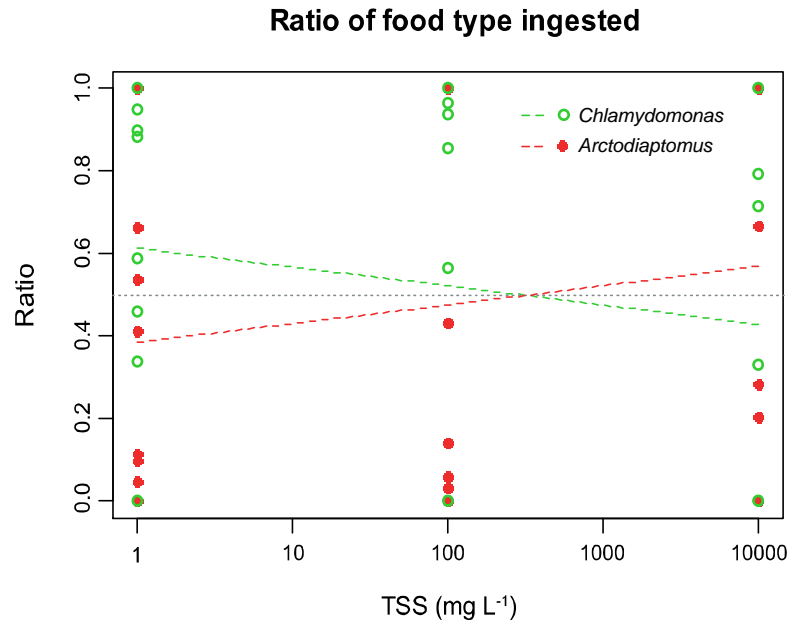
Figure S1. Biomass ratio of algae (*Chlamydomonas* sp., green empty circles and green regression line) and animal prey (*Arctodiaptomus spinosuδs*, red filled circles and red regression line) ingested by large anostracans (from the field) at different turbidity levels (for both food types; LR: R^2=-0.004; p=0.357). One replicate was removed from 10 000 mg L^{-1} TSS treatment due to resulting in NA (animals were not feeding on any food type). Grey dashed line indicates 1:1 ingestion rate of algae and zooplankton.

Chapter 3 - Phylogeography of two *Branchinecta* species

Global phylogeography of congeneric fairy shrimps reveal distinct evolutionary histories in the Palaearctic

Dunja Lukić, Tom Pinceel, Federico Marrone, Robert Ptacnik & Zsófia Horváth

Unpublished manuscript

Abstract

Temporary ponds are ubiquitous habitats, representing excellent model systems to understand biodiversity pattern from landscape to continental levels. Anostracans are an ancient group specialized to these often extreme habitats, and play an important role in ecosystem functioning. Here we aim to reconstruct the phylogeography of two closely related anostracan species, *Branchinecta ferox* and *Branchinecta orientalis*. To this end we analyse the mitochondrial COI and nuclear ITS2 DNA regions of *B. ferox* (48 specimens) and *B. orientalis* (114 specimens) originating from 51 populations across their range of occurrence. Genetic distances for the mitochondrial COI gene fragment among populations ranged between 0 - 9.0 % in *B. ferox* and 0 - 5.8 % in *B. orientalis*, while for the nuclear ITS2 DNA region, genetic distance ranged between 0 - 5.0 % in *B. ferox* and 0 - 3.1 % in *B. orientalis*. Biogeographic patterns of haplotype diversities showed clearly different evolutionary histories of the two species with different glacial refugia during the Pleistocene. *B. ferox* carried a clear geographic signal with populations clustering in a Middle Eastern, two African and two European haplogroups, indicating a glacial refugium in the southernmost regions of its current distribution. In contrast, the populations of *B. orientalis* mostly belonged to two widely distributed haplogroups. The Pleistocene refugium of this species was most likely in the Balkan Peninsula or the Pannonian Plain and rapid recolonization from these regions resulted in the currently observed phylogeographic patterns. Finally, we found evidence for multiple long-distance dispersal events, most likely via migrating waterbirds. In conclusion, the phylogeography of two *Branchinecta* species shows a strong geographical pattern which calls for an adequate protection of these species on the regional (metapopulation) level, especially considering that many of them are the resting sites for migrating waterbirds.

Introduction

Temporary ponds are widespread throughout the world (being prevalent in arid and semi-arid regions; Brendonck, 1996). They encompass very diverse ecosystems in regards to size, shape, hydroregime and water chemistry (Williams, 2006). They can sustain a high fraction of total regional biodiversity due to high local diversity and high compositional turnover among habitats (Williams *et al.*, 2004; Oertli *et al.*, 2005; Downing, 2010; Vad *et al.*, 2017). Moreover, they are often inhabited by a unique aquatic fauna and flora (Williams, 2006). However, they are often exposed to strong anthropogenic influence (e.g. agriculture, infrastructure, pollution) and are particularly vulnerable to climate change due to their small size and shallowness (Moss, 2012; Tuytens *et al.*, 2014; Stoks, Geerts & Meester, 2014), which has led to severe losses in many regions worldwide (Rhazi *et al.*, 2012; Horváth *et al.*, 2019).

Large branchiopods (including five extant orders: Anostraca, Notostraca, Spinicaudata, Laevicaudata and Cyclestherida) are well adapted and almost exclusively limited to temporary ponds. They are considered to play important roles in ponds communities, such as community-shaping (anostracans and notostracans; Jocque, Vanschoenwinkel & Brendonck, 2010; Waterkeyn *et al.*, 2011; Lukić *et al.*, 2018) and ecosystem engineering (notostracans; Waterkeyn *et al.*, 2011). To bridge the unfavorable parts of the year in their local habitats, they produce resting eggs, which can remain viable buried in the sediment for years before hatching (Hairston *et al.*, 1995; Brendonck & De Meester, 2003). Resting eggs also play an important role in the passive dispersal of this group, being carried by wind (Brendonck & Riddoch, 1999) and animal vectors, such as mammals (Vanschoenwinkel *et al.*, 2008), birds (Figuerola & Green, 2002; Brochet *et al.*, 2009), amphibians (Bohonak & Whiteman, 1999) and even insects (Beladjal & Mertens, 2009).

The anostracan genus *Branchinecta* comprises around 50 species. The genus is found on all continents except Australia (Belk & Brtek, 1995; Rogers, 2006; Marrone *et al.*, 2016). There are six species in the genus that are present in Eurasia, with only two being present in temperate Europe: *Branchinecta ferox* and *Branchinecta orientalis*.

B. ferox has a circum-Mediterranean distribution (Fig. 4C in General Introduction). It is the only *Branchinecta* species in Africa, present in the north-western part of the continent (Morocco, Algeria and Tunisia; Marrone et al., 2016). In Europe, it occurs in Spain and Central Europe (Pannonian Plain), while its range extends further toward the east across South Ukraine to the southwest of Russia. This species has also been reported in the Middle-East (e.g. Israel and Syria). Active populations occur in late winter and early spring (Eder, Hödl & Gottwald, 1997; Horváth *et al.*, 2013a; Marrone *et al.*, 2016).

B. orientalis inhabits mineral-rich temporary waters and is distributed between 27° and 55° N in Europe and Asia (Mura & Takami, 2000; Padhye, Kulkarni & Dumont, 2017). According to its currently known distribution (excluding some occasional pre-1950s records), this species inhabits

distinct regions in Eurasia, including the Iberian peninsula (Alonso, 1985), Central Europe (Austria, Hungary and Serbia; Petkovski, 1993; Horváth *et al.*, 2013a), the Middle East (Turkey, Iran; Mura & Takami, 2000; Mura *et al.*, 2011; Atashbar *et al.*, 2014a), and the area of Lake Baikal (Russia; Naganawa *et al.*, 2019), Himalayas (Nepal; Manca & Mura, 1997) and Mongolia (Marrone *et al.*, 2015) in Central Asia (Fig. 5C in General Introduction). Active populations of *B. orientalis* generally occur from March to June, but exceptions have also been recorded in late autumn or winter (Eder *et al.*, 1997; Atashbar *et al.*, 2014b; Šćiban *et al.*, 2014). The geographic distribution of these two *Branchinecta* species overlaps only in the Pannonian Plain and Iberian Peninsula.

Genetic information and phylogenetic reconstructions provide efficient tools to assess species diversity and evolution beyond the resolution of classical morphology. In the era of strong environmental changes and extinction, conservationists need to consider morphologically cryptic genetic lineages as they may represent unique evolutionary potential (evolutionary significant unit [ESU]). Moreover, molecular methods are valuable tools to study propagule dispersal. To date, phylogenetic reconstructions of anostracans were only performed on some common large branchiopod species in Palaearctic. In notostracans, Vanschoenwinkel *et al.* (2012) found very low genetic diversity in the European *Triops cancriformis* populations, while slightly higher variation (7 % average variation at COI) was found in *Triops mauritanicus* from the Iberian Peninsula and North Africa. In anostracans, very high genetic variation (mostly on mitochondrial COI) was found in *Branchipus schaefferi* (in Europe and North Africa; Lukić *et al.*, 2019), *Phallocryptus spinosus* (Ketmaier *et al.*, 2008), *Tanymastix stagnalis* (Ketmaier *et al.*, 2005) and *Chirocephalus* species (Ketmaier *et al.*, 2012; Reniers *et al.*, 2013), in some cases even indicating the presence of morphologically cryptic species. Moreover, genetic studies revealed a low gene flow between populations of *Phallocryptus spinosus* on a large scale (with some exceptions though; Ketmaier *et al.*, 2008) and of *Branchipodopsis wolfi* (Brendonck, De Meester & Riddoch, 2000) even on a small geographical scale. In other cases, indications of past long-distance dispersal events were found, which were likely mediated by waterbirds as dispersal vectors (Lukić *et al.*, 2019).

Here we study the genetic diversity on mitochondrial COI and nuclear ITS2 DNA regions of the two *Branchinecta* species in a total of 51 populations across the Palaearctic. Firstly, our aim is to determine whether these two species share the history of other large branchiopod groups, which typically survived the Pleistocene glaciations in southern refugia (Vanschoenwinkel *et al.*, 2012; Reniers *et al.*, 2013; Lukić *et al.*, 2019). Second, we aim to assess the global genetic diversification of these two species. The discontinuous (island-like) distribution of *B. ferox* and *B. orientalis* increases the potential for genetic isolation and differentiation between populations (Boileau & Hebert, 1991). As a consequence, we expect to find a rather high genetic variation in both species on a global scale, especially in some geographically isolated regions as Mongolia and Nepal, while we expect that populations along the main migration routes of waterbirds (such as North Africa, Iberian Peninsula and Central Europe) will share haplotypes.

Methods

Sampling procedure

We collected *Branchinecta orientalis* specimens from 31 temporary ponds and soda pans in Europe and Asia (Table 1 & Fig. 1). *Branchinecta ferox* specimens were collected from 16 temporary ponds in Europe, North Africa and Asia (Table 1 & Fig. 1). Specimens were collected between 1971 and 2018 and fixed in ethanol (of various dilution rates). Once the samples arrived at the lab, they were all immediately transferred to absolute ethanol until further processing. All samples were dissected to obtain phyllopod tissue for DNA extraction.

Table 1. Overview of all specimens of *Branchinecta ferox* and *Branchinecta orientalis* used in the analyses, with details on the sequence IDs, collection localities and number of specimens.

Nr.	ID	Country	Region, locality	Latitude	Longitude	COI sequence Nr.	ITS2 sequence Nr.
	Branchinecta ferox						
1	AB1	Austria	Apetlon, Birnbaumlacke	47.817715	16.864911	1	1
2	AN1-2	Austria	Apetlon, Kühbrunnlacke	47.792736	16.878626	2	2
3	AL6	Austria	Apetlon, Lange Lacke	47.757475	16.878765	1	0
4	AS6-7	Austria	Apetlon, Sechsmahdlacke	47.783789	16.884112	2	0
5	AW6	Austria	Apetlon, West. Wörthenlacke	47.770922	16.870777	1	1
6	AK5-10	Austria	Illmitz, Kirchsee	47.758679	16.785489	6	4
7	HO1-2	Hungary	Csongrád, Kis-sóstó	46.74096	19.99218	0	2
8	HK6,8	Hungary	Fülöpszállás, Kelemen-szék	46.797356	19.183097	0	2
9	HP1-3	Hungary	Pusztaszer, Büdös-szék	46.546253	20.03282	3	3
10	HS1-3	Hungary	Solt, Bogárzó	46.808029	19.141267	3	2
11	HU6-9	Hungary	Szabadszállás, Büdös-szék	46.866044	19.169286	4	2
12	HZ2-5	Hungary	Szabadszállás, Zab-szék	46.837517	19.16978	4	1
13	IS1, 686 Is	Israel	Ashdod, Tel Ashdod	31.75361	34.6525	2	1
14	M1	Morocco	Ifran, Daya Mertissiliouine	33.29503	-5.1791	1	1
15	SB1-5	Serbia	Stanišić, Bela Bara	45.94707	19.090246	5	3
16	EA1-5	Spain	Albacete, Navas del Bonillo	38.93533	-2.47305	5	5
17	EG1-3	Spain	Segovia, Laguna de la Iglesia	41.203861	-4.56927	3	2

18	688 Tun	Tunisia	Kelaat El Andaluus, El Hisiane	36.995056	10.158451	1	0

Branchinecta orientalis

19	AP1-3	Austria	Apetlon, Apetloner Meierhoflacke	47.721576	16.82404	3	1
20	AG1-5	Austria	Apetlon, Grosse Neubruchlacke	47.786121	16.842198	5	2
21	AL1-5	Austria	Apetlon, Lange Lacke	47.757475	16.878765	5	3
22	AE1-2	Austria	Apetlon, Öst. Fuchslochlacke	47.790441	16.866186	2	1
23	AS1-5	Austria	Apetlon, Sechsmahdlacke	47.783789	16.884112	5	1
24	AF1-5	Austria	Apetlon, West. Fuchslochlacke	47.790077	16.852349	3	4
25	AW1-5	Austria	Apetlon, West. Wörthenlacke	47.770922	16.870777	4	2
26	AA1-5	Austria	Illmitz, Albersee	47.775136	16.770119	5	2
27	AK1-4	Austria	Illmitz, Kirchsee	47.758679	16.785489	0	4
28	AM1-5	Austria	Illmitz, Mittlerer Stinkersee	47.806759	16.787522	5	2
29	AH1-5	Austria	Illmitz, Obere Höllacke	47.827119	16.807932	5	1
30	AO1-5	Austria	Illmitz, Oberer Stinkersee	47.813611	16.792517	5	4
31	AR1-5	Austria	Illmitz, Runde Lacke	47.785745	16.792744	5	3
32	AZ1-4	Austria	Illmitz, Zicklacke	47.766964	16.784752	4	0
33	HT1-4	Hungary	Bácsalmás, Sóstó	47.005063	18.491114	1	3
34	HB1	Hungary	Dunatetétlen, Böddi-szék south pool	46.764764	19.147753	1	0
35	HD1	Hungary	Dunatetétlen, Böddi-szék southeast pool	46.756864	19.156653	0	1
36	HK1-4	Hungary	Fülöpszállás, Kelemen-szék	46.797356	19.183097	2	4
37	HG1-4	Hungary	Kiskunság, Böddi-szék	46.768361	19.147694	4	4
38	HS6	Hungary	Solt, Bogárzó	46.808029	19.141267	1	1
39	HU1-3	Hungary	Szabadszállás, Büdös-szék	46.866044	19.169286	3	3
40	HZ1	Hungary	Szabadszállás, Zab-szék	46.837517	19.16978	0	1
41	HN1-3	Hungary	Újfehértó, Nagy-Vadas-tó	47.858719	21.656685	1	0
42	MB1	Mongolia	Baraatiin toirom	46.81072	106.29568	1	1
43	MK1-3	Mongolia	Khakzangiin goliin toirom	47.08083	105.955	3	2
44	MS1-6	Mongolia	Shiliin nuuriin toirom	47.0075	106.12972	5	4
45	HY1	Nepal	Khimbu Valley	27.9433	86.7738	1	0
46	SO1-2,4	Serbia	Elemir, Okanj	45.467165	20.300073	3	3
47	SR2-4	Serbia	Melenci, Mala Rusanda	45.512444	20.302639	2	2
48	SS1,3-5	Serbia	Novi Bečej, Slano	45.625224	20.210024	4	1

			Kopovo				
49	SM1-2	Serbia	Riđica, Medura	45.992088	19.133211	2	1
50	ES1-5	Spain	Cuenca, Laguna de la Dehesilla	39.421788	-2.84055	3	4
51	EC1-5	Spain	Cuenca, Laguna del Hito	39.863944	-2.693361	5	5

DNA extraction, Polymerase Chain Reaction (PCR), DNA purification and sequencing

Genomic DNA was extracted from tissue using the NucleoSpin® extraction kit for individual samples (Macherey-Nagel, Düren, Germany). The PCR volume of 25 µL contained 2 µL of template DNA, 12.5 µl of 2x My Taq HS mix (Bioline, London, UK) and 1 µL of primer mix (forward and reverse, 20 µM). Reaction volumes were supplemented with 9.5 µL of sterile deionized water (Sigma-Aldrich, Missouri, USA). The mitochondrial COI DNA region of *B. orientalis* and *B. ferox* was amplified using universal invertebrate forward (5' GGTCAACAAATCATAAAGATATTGG 3') and reverse (5' TAAACTTCAGGGTGACCAAAAAATCA 3') primers (Folmer *et al.*, 1994). When amplification was unsuccessful with these two primers, we changed the reverse primer (and combined it with the universal forward primer). Our first alternative was reverse COI-H (5' TCAGGGTGACCAAAAAATCA 3') primer (Machordom *et al.*, 2003), also used by (Rodríguez-Flores *et al.*, 2017), but eventually we also designed a new reverse primer COI_ORIENFER_R648 (5' TGGTAAAGYATAGGATCTCCRCC 3') for a few of the remaining samples. Eventually, to obtain short DNA sequence of the specimen from Nepal (HY1, ~300 bp), we used a combination of two primers specifically designed for *B. orientalis* COI_ORIEN_F367 (5' TGCAGGACCTTCAGTAGATCT 3') and COI_ORIENFER_R648. To amplify nuclear ITS2 DNA region, we used forward CAS5p8sFt (5' TGAACATCGACATTTYGAACGCATAT 3') and reverse CAS28sB1d (5' TTCTTTTCCTCCSCTTAYTRATATGCTTAA 3') primers (Ji, Zhang & He, 2003). To amplify both genetic markers, the cycle settings were modified from Adamowicz, Hebert & Marinone (2004), with an initial denaturation of 1 min at 95 °C, followed by 5 liberal amplification cycles (denaturation for 15 s at 94 °C, annealing for 15 s at 45 °C and elongation for 10 s at 72 °C) and 35 more rigid cycles (denaturation for 15 s at 95 °C, annealing for 15 s at 50 °C and elongation for 10 s at 72 °C) and a final elongation of 6 min at 72 °C.

Reaction contaminants were removed from the samples using CleanPCR beads (GC Biotech, Waddinxveen, Netherlands). Purified products were again amplified with the Big Dye Terminator 3.1 kit (Applied Biosystems, Gent, Belgium), following a 1/8 dilution of the Big Dye Terminator sequencing protocol and the corresponding primers that were specified above (3.2 µM; forward and reverse primers were used separately). The PCR program entailed one initial denaturation step of 95 °C for 1 min, followed by 30 amplification cycles (denaturation for 10 s at 95 °C, annealing for 5s at 50 °C and elongation for 4 min at 60 °C). The extension products were once again purified using the CleanDTR beads (GC Biotech, Waddinxveen, Netherlands). Finally, the

products were run on an ABI PRISM 3500 Avant Genetic Analyser automated sequencer (Applied Biosystems, Gent, Belgium).
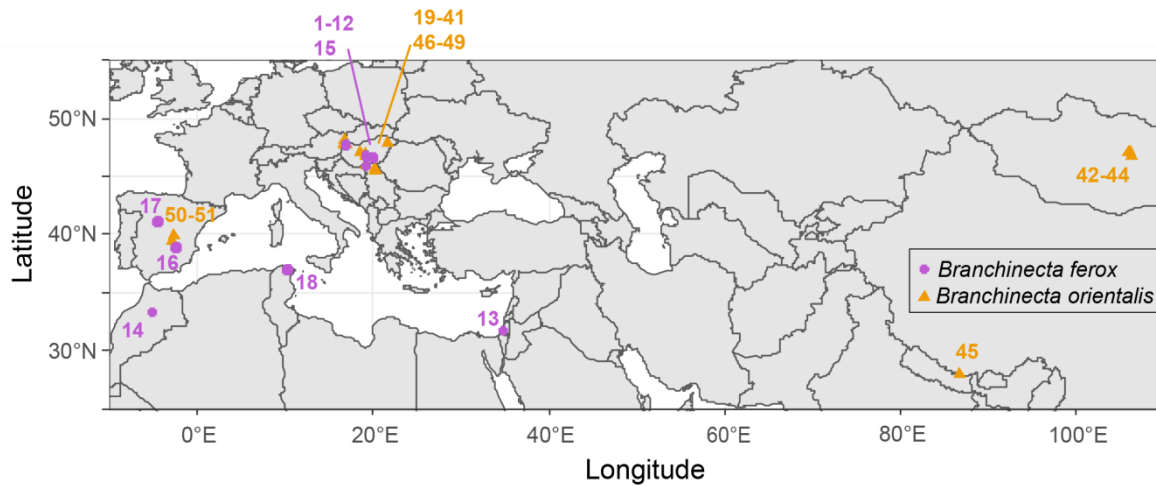

Figure 1. Location of the studied *Branchinecta* populations. Numbers correspond to populations listed in Table 1.

Reconstructions of phylogeny based on mitochondrial COI and nuclear ITS2 region

All generated *B. ferox and B. orientalis* sequences were assembled and visually checked for quality in SeqScape v3. We checked the COI alignment for indels which would indicate unintentional amplification of nuclear pseudogenes (Song *et al.*, 2008). The produced sequences were edited in BioEdit (Hall, 1999). The newly produced sequences were aligned together with the existing sequences in GenBank (Rodríguez-Flores *et al.*, 2017) and one outgroup taxon (for COI, we used *Branchipus schaefferi* MK449416 and for ITS2, *Chirocephalus diaphanus* LT860206) by using CLUSTALW multiple alignment tool in BioEdit. The most likely evolutionary model for both markers was determined in PhyML (Lefort, Longueville & Gascuel, 2017) and MEGA X (Kumar *et al.*, 2018) based on the Akaike information criterion (AIC). For *B. ferox*, the AIC selected a Tamura-Nei evolutionary substitution model (TN93; Tamura & Nei, 1993) with proportion of invariant sites (+I; I=0.650) which was used to assemble COI maximum likelihood (ML) tree. The General Time Reversible model (GTR) with the proportion of invariant sites (which scored only slightly higher than TN93+I) was used to assemble the Bayesian inference (BI) tree since the TN93 model is not embedded within the MrBayes software package. For *B. orientalis*, the AIC (in MEGA X) selected the GTR model with a gamma shape parameter (+G, γ=4.55) and the proportion of invariable sites (I=0.660) which was used for construct the BI and ML tree. Both species were analysed together for the phylogeny of the ITS2 marker due to the lower intraspecific differentiation of the marker and lower number of produced sequences. The AIC selected for GTR model with a gamma shape parameter (+G, γ=0.54) which was used to assemble ML and BI tree. TN93+G model was used to assemble the ITS2 neighbor joining (NJ) tree (γ=0.54 for ITS2).

Substitution saturation was tested in DAMBE v. 7.0.28 (Xia & Kumar, 2018), using the default settings and including all sites. The index of substitution saturation (Iss) was significantly smaller than the critical index of substitution saturation (Iss c), indicating little saturation (Xia *et al.*, 2003; Xia & Lemey, 2009) for both markers. Pairwise genetic K2P distances between all generated sequences and the mean genetic distances within and among the main groups in the phylogeny of *B. ferox and B. orientalis* were calculated in MEGA X (Kimura, 1980) with partial deletion of 90% (395 positions in the final data set for COI and 609 positions for ITS2). The haplotype number was determined based on the calculated pairwise distances between the sequences.

The consensus phylogeny was constructed based on COI sequences by comparing phylogenetic trees obtained with two different methods of inference, ML and BI. ML analyses were performed in MEGA X with 1000 bootstrap replicates. Bayesian inference was performed in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist *et al.*, 2012) for up to $3x10^6$ generations depending on the species and genetic marker (until standard deviation of split frequencies reached <0.01) while the trees were sampled every 1000 generations. Initial 25% of produced trees were discarded as burn-in. The maximum parsimony (MP) and NJ analyses were also performed for ITS2 DNA region. The MP analyses were performed in PAUP* v. 4.0 (Swofford, 2001). The heuristic search included 100 replications with space for 1000 saved trees and Tree-Bisection-Reconnection (TBR) search strategy. NJ analyses were performed in MEGA X according to 1000 bootstrap replicates and partial deletion of 90% (less than 10% alignment gaps, missing data and ambiguous bases were allowed at any position).

For the *B. ferox* and *B. orientalis* COI gene fragments, we built, for each species, a median-joining haplotype network (ε=0; Bandelt, Forster & Röhl, 1999) using PopART v 1.7 (Leigh & Bryant, 2015; http://popart.otago.ac.nz). First, this program recognized the sequences that contained significantly more ambiguous bases than the others and excluded them from the further analysis (HU9 & HZ3 from Hungary in *B. ferox*; AF5 & AA5 from Austria and HK4 & HN3 from Hungary in *B. orientalis*). Then in the leftover sequences, the remaining sites containing ambiguous bases were masked (2 sites in *B. ferox* and 4 sites in *B. orientalis*) leaving 601 (*B. ferox*) and 654 (*B. orientalis*) sites for further network and statistical analysis. We conducted Tajima´s D neutrality test (Tajima, 1989) to examine the patterns related to population expansions and bottlenecks.

Results

We generated 125 COI sequences of *Branchinecta* spp. (36 sequences of *B. ferox* and 89 sequences of *B. orientalis*) from 9 countries and 23 regions (altogether 16 populations of *B. ferox* and 31 populations of *B. orientalis*; see sequence information in Table 1). Generated COI sequences ranged from 443 to 659 bp. Combined with the COI sequences already present in

GenBank (LT821325-LT821341; Rodríguez-Flores *et al.*, 2017), we assembled a COI alignment of 142 sequences (44 sequences of *B. ferox* and 98 sequences of *B. orientalis*).

For the nuclear ITS2 region, we generated 86 *Branchinecta* sequences (25 *B. ferox* and 61 *B. orientalis*). Generated ITS2 sequences ranged from 370 to 626 bp. Combined with the ITS2 sequences already present in GenBank (LT821342-LT821357; Rodríguez-Flores *et al.*, 2017), we assembled an ITS2 alignment of 102 sequences (32 sequences of *B. ferox* and 70 sequences of *B. orientalis*).

Genetic diversity

Mitochondrial COI DNA region

The mean interspecific divergence between *B. ferox* and *B. orientalis* was 10.30 % (range among individuals: 8.17 - 13.61 %) based on the mitochondrial COI gene region (based on K2P distance with partial deletion of 90 %). A total of 66 (+ one outgroup) lineages were included in the phylogenetic reconstructions (for two species combined), with multiple identical sequences of one locality considered as a single lineage. Of 215 polymorphic sites, 117 were parsimony informative (the constant character proportion was 68.0%).

For *B. ferox*, we identified a total of 17 unique COI haplotypes. The overall mean intraspecific genetic variation was 2.6 % considering 29 lineages (a single lineage represents all identical sequences of one population; outgroup was excluded). One haplotype was relatively common in Central Europe and present in 11 populations (in Austria, Hungary and Serbia). All other haplotypes, except one from Hungary (in three soda pans), were present in only one population each. Among individuals, genetic differentiation ranged between 0 - 9.0 %. The highest difference was found between a Moroccan specimen (M1) and an individual from Israel (IS1).

For *B. orientalis*, we identified a total of 24 unique COI haplotypes. The overall mean intraspecific genetic variation was 2.5 % considering 57 lineages (a single lineage represents all identical sequences of one population; outgroup was excluded). Two haplotypes were relatively common in Central Europe, with one present in 13 populations (12 populations in Austria and one in Serbia) and another in likely 13 populations as well (due to ambiguous bases; in Austria, Hungary and Serbia). Other haplotypes were only recorded from one to three populations. Among individuals, genetic differentiation ranged between 0 - 5.8 %. The highest pairwise distance was found between one Hungarian specimen (HK4) and individuals from three populations in Austria (AM5, AP1, AR4).

Nuclear ITS2 DNA region

Based on the nuclear ITS2 DNA region, the mean interspecific distance between *B. ferox* and *B. orientalis* was 9.96 % (with a range between 9.65 - 12.77 % among individuals). A total of

47 (+ one outgroup) lineages were included in phylogenetic reconstructions as multiple identical sequences of one locality were considered as a single lineage. Of 238 polymorphic sites, 76 were parsimony informative (the constant character proportion was 62.7 %).

For *B. ferox*, we identified a total of four unique ITS2 haplotypes (based on K2P distances with partial deletion of 90 %). The overall mean intraspecific genetic differentiation was 0.9 % considering 15 lineages. Genetic differentiation among individuals ranged between 0 - 5.0 %. The highest genetic distance was found between a specimen from Israel and the majority of other specimens (excluding specimens from the Albacete region in Spain). One haplotype was widely distributed across Central Europe and one locality in Spain (Segovia region). Three haplotypes were found in only one population each: one in Israel, one in Morocco and one in Spain.

For *B. orientalis*, we identified a total of six unique ITS2 haplotypes (based on K2P distances with partial deletion of 90 %). The overall mean intraspecific genetic differentiation was 0.8 % considering 32 lineages. Genetic differentiation among individuals ranged between 0 - 3.1 %. The highest genetic difference was found between one specimen from Mongolia (MB1) and one Serbian specimen (SO4). Three haplotypes were found in Central Europe, two in Spain, and one in Mongolia. Two (out of three) haplotypes in Central Europe were rare and each was present in only one population (one in Serbia and one in Hungary).

Phylogenetic analyses

Mitochondrial COI DNA region

The two different methods of phylogenetic inference ML and BI produced trees with highly similar topologies for the populations of both *B. ferox* and *B. orientalis*. *B. ferox* (Fig. 2A) populations can be subdivided into five main haplogroups, corresponding to distinct geographical regions. These haplogroups are present in the Middle East, two regions in Northern Africa (Tunisia and Morocco) and two in Europe (Spain and Central Europe). One group in Europe included specimens from the Albacete region in Spain and the second included populations from Segovia in Spain and all populations from Central Europe. The haplotype network of *B. ferox* (Fig. 2B) had 84 segregating sites, of which 57 were recognized as parsimony informative. Tajima's D was -1.42 (p=0.93) for the entire data set. The phylogenetic search methods (ML and BI) grouped the studied *B. orientalis* haplotypes in two larger haplogroups (Fig. 3A), except for one specimen (SS4) from Serbia. Populations from Central Europe (Austria, Hungary and Serbia) were present in both haplogroups. Mongolian populations all belonged to the first haplogroup forming a separate branch within it. The partial fragment of the Nepalese specimen (HY1) was placed in the second haplogroup and was very similar to one Hungarian specimen (HG4). Most of the Spanish individuals (except from one specimen from Cuenca region) also belonged to the second haplogroup. Haplotype network analysis for *B. orientalis* (Fig. 3B) identified 44 segregating sites, of

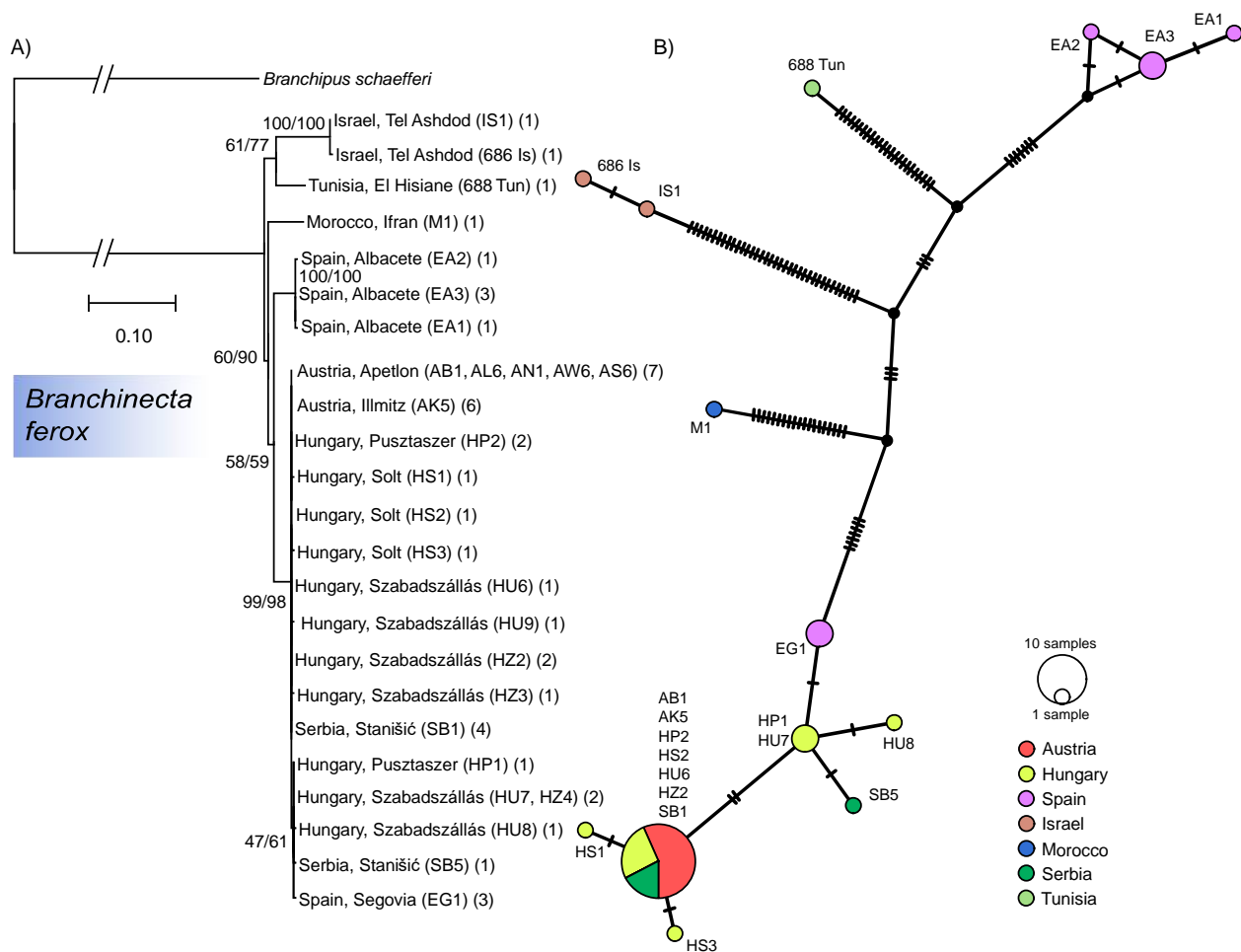which 32 were recognized as parsimony informative. Tajima's D was 1.94 (*p*=0.03) for the entire data set.

Figure 2. Phylogeography of *Branchinecta ferox* based on the mitochondrial COI gene fragment. A) Consensus phylogeographic tree for *B. ferox*, based on the mitochondrial COI gene fragment (maximum likelihood and Bayesian inference). The supporting values based on maximum likelihood and Bayesian inference models (ML/BI) are included close to the nodes. B) Haplotype network based on the median joining network. Black circles represent missing haplotypes. Short vertical bars indicate the number of mutations between haplotypes. Tajima's D was -1.42 (*p*=0.93).

Nuclear ITS2 DNA region

All four phylogenetic reconstructions first differentiated *B. ferox* from *B. orientalis* species (Fig. 4). Within *B. ferox*, it was possible to differentiate between three groups, one in the Middle East (Israel), another in North Africa (Morocco) and Iberian Peninsula (Albacete), and a third from the Iberian Peninsula (Segovia) and Central Europe. Unfortunately, no ITS2 sequences were obtained from Tunisia.

For *B. orientalis*, reconstructions based on the ITS2 region suggested slightly different phylogenetic relationships compared to the COI DNA sequences. The sequences from Spain and Mongolia were grouped together, but further branching in two distinctive haplogroups matching

with the geographic origin of the samples, while all populations from Central Europe were in another haplogroup (although the genetic distance was probably too low for reliable branching). Mean within group distances (for Spanish, Mongolian and Central European groups) were all lower than 0.1 %. Mean between group (K2P) distances were 2.1 % for Spain and Central Europe, 2.6 % for Spain and Mongolia, and 2.7 % for Mongolia and Central Europe.
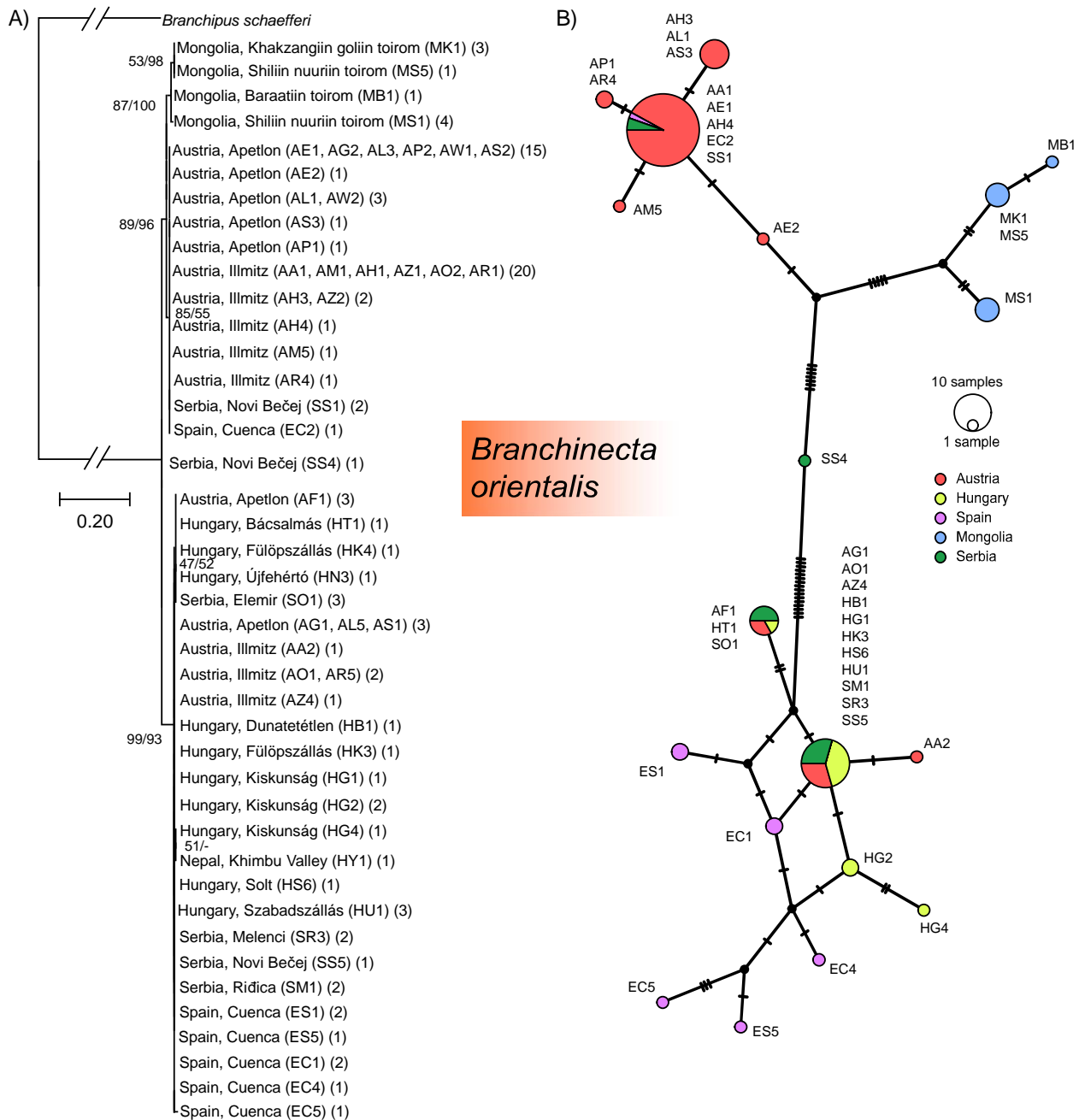

Figure 3. Phylogeography of *Branchinecta orientalis* based on the mitochondrial COI gene fragment. A) Maximum likelihood phylogeographic tree. The supporting values based on maximum likelihood and Bayesian inference models (ML/BI) are included close to the nodes. Localities are specified in the first and the number of specimens in the second brackets. The unsupported groupings are indicated with '-'. B) Haplotype network based on the median joining network. Black circles represent missing haplotypes. Short vertical bars indicate the number of mutations between haplotypes. Tajima's D was 1.94 (*p*=0.03).
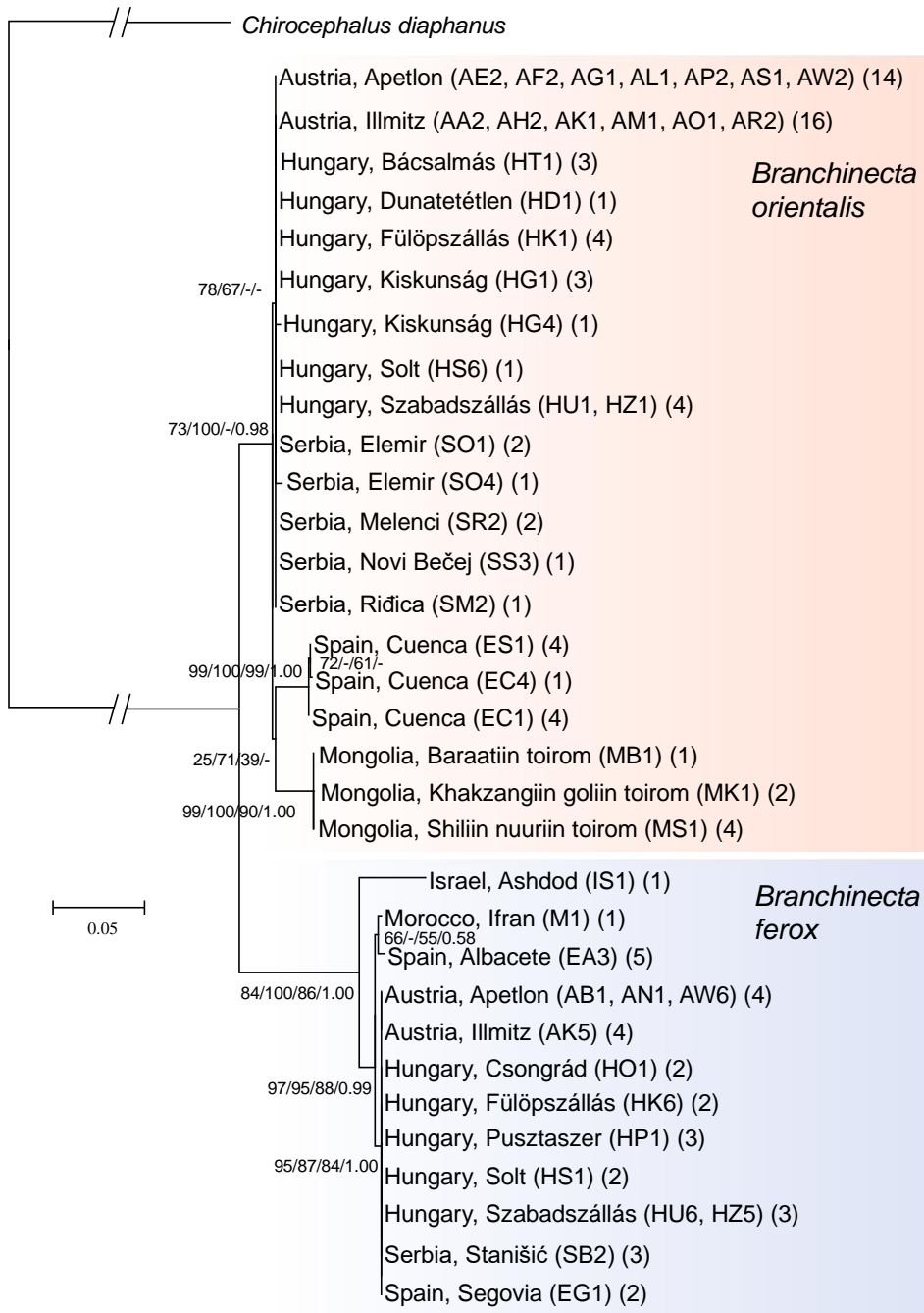
Figure 4. Consensus phylogeographic tree for both *Branchinecta ferox* and *Branchinecta orientalis*, based on the nuclear ITS2 DNA region. The supporting values based on all four models (maximum likelihood/ maximum parsimony/neighbor joining/Bayesian inference) are included close to the nodes. The unsupported groupings are indicated with '-'.

Discussion

Phylogenetic reconstruction of the two investigated *Branchinecta* species from across the Palaearctic yielded similar results in terms of within-species differentiation, which was relatively low in both *B. ferox* and *B. orientalis* for both genetic COI and ITS2. While intraspecific differentiation was higher on COI than ITS2 in both species, interspecific distances were very similar between COI and ITS2 sequences. The geographic distribution could visually explain a good portion of

genetic diversity. Central Europe features particularly high genetic diversity in *B. orientalis* while in *B. ferox* this was rather the case in the southernmost regions of its distribution. These regions of high diversity are of particular interest for conservation of this threatened group and their habitats.

In *B. ferox*, we found the highest genetic differentiation between the populations from geographically distant localities around the Mediterranean, with higher levels of diversity in the southern populations. This pattern was consistent for both genetic markers, mitochondrial COI and nuclear ITS2. In case of *B. orientalis*, all COI haplotypes (except one from Serbia) were classified in two haplogroups. The phylogeny carried a visible geographic signal. For instance, all Mongolian specimens were grouped into one haplogroup and most specimens from Spain were in another. In the ITS2 tree, there were three geographically distinguished haplogroups, from Central Europe, Spain and Mongolia, although divergence was low in general. The slightly different tree topologies found for these two markers (especially in Central Europe that holds a large number of local populations of *B. orientalis*) could be explained by the different inheritance mechanisms. While mitochondrial genes are inherited from the mothers (but see exceptions to that rule; Lindholm *et al.*, 2016), nuclear genes are typically inherited from both parents. Finally, based on the mitochondrial COI DNA region, it was reconfirmed that both species are very closely related (with an inter-species genetic distance of 8.33 - 12.32 %) while most of the species from the same genus are more distant relatives (with 9.66 - 20.60 % genetic differentiation, with the exception of pairwise genetic distances between *B. hiberna* and *B. orientalis* with 7.82 - 10.06 % genetic differentiation; see Supplement Table S1 and Fig. S1) (see also Rodríguez-Flores *et al.*, 2017).

Pleistocene refugia and the phylogeography of *Branchinecta*

The Pleistocene glaciations (2.5 mya - 11 kya) were characterised by extreme climatic fluctuations, such as extended periods of ice cover alternating with periods of milder temperatures (Paillard, 1998). The glacial expansion had a strong effect on most species from temperate regions, leading to the local extinction of populations and severe bottleneck effects, as well as range shifts to more southern regions. These long-lasting climatic changes played an important role in the species distributions visible today, especially in Europe, which was largely covered by ice or tundra vegetation during the glaciation periods.

Our phylogeographic analysis strongly indicates that the refugium of *B. ferox* was in the southern parts of its current distribution (Northern Africa and the Middle East) where we found generally higher diversity of both COI and ITS2 DNA fragments compared to the other regions. The Mediterranean areas of Africa and Asia were not covered by ice, and provided suitable habitats to serve as refugia for many species (Habel *et al.*, 2010; Husemann *et al.*, 2014) including temporary pond-dwellers, for instance copepods (Marrone, Brutto & Arculeo, 2010) and other anostracan species (Lukić *et al.*, 2019). After the ice sheets retreated, *B. ferox* could (re)colonize the north, possibly arriving first to the Iberian Peninsula (where we found two haplogroups for both genetic

markers) and then Central Europe (where we can only find one haplogroup for both genetic markers).

For *B. orientalis*, evidence was less conclusive, leaving three regions as possible refugia during the Pleistocene. The first and most likely explanation is that the *B. orientalis* refugium was in the Balkan Peninsula or a bit more north, in the Pannonian Plain (where it is commonly present today). This could be partially supported by relatively high diversity on both genetic markers in Central Europe and the topology of the constructed haplotype network. The species likely spread to the Iberian Peninsula and Asia (and to Central Europe, in case the refugium was on the Balkan). The Balkan Peninsula (together with other peninsulas in Southern Europe) is known to have acted as a refugium for many species during the Pleistocene glaciations (Ursenbacher *et al.*, 2006; Previšić *et al.*, 2009; Pabijan *et al.*, 2015). The Pannonian Plain is also recognised as an area of refuge in some aquatic groups (e.g. amphibians; Vörös *et al.*, 2016). The low genetic distance between *B. orientalis* and *B. ferox* implies that geographic isolation of the two ancestral *Branchinecta* populations started ± 2.5 mya (based on the average 2 % divergence rate of mtDNA per my; Reniers *et al.*, 2013) likely due to the existence of at least two isolated refugia for their populations during the Pleistocene, which could be the reason for eventually diverging into these two different species.

The second of the three possibilities could be that *B. orientalis* was restricted to the region of Iran or nearby regions in the Middle East. Some parts of this area were found to be refugia for many invertebrate species, such as other crustaceans (Parvizi *et al.*, 2018) and insects (Rajaei Sh *et al.*, 2013). This hypothesis is based mainly on the high genetic diversity of COI haplotypes in local populations in Iran, with pairwise genetic differences of up to 6 % at a limited geographic scale (maximum distance between two sites: 251 km) according to Atashbar *et al.* (2016). Unfortunately, the generated COI sequences from that study were not made publicly available to verify this hypothesis through comparing *B. orientalis* populations from Iran with our sequences. However, considering that we generated sequences from populations from Mongolia and Nepal, which are even further east, and that the haplotypes from these populations generally resembled those from Europe more closely, this level of genetic differentiation in Iran needs to be revisited. A possible explanation for such a high genetic diversity could be strong environmental differences between the studied populations in Iran, driving a rapid evolution within individual populations. But even so, the similarities between the Central European and Middle Asian populations make a historic expansion from the Middle East very unlikely.

A third option could be that the refugium was in Northern Africa, as for *B. ferox*. It would mean that this species later spread to the Balkans (and successively, Central Europe), the Iberian Peninsula and the Middle East once the climate became more suitable. In the meantime, it either disappeared from Africa or was simply not yet recorded there. At the same time, the much rarer species *B. ferox* still inhabits this continent along with numerous other large branchiopods (van den

Broeck *et al.*, 2015; Marrone *et al.*, 2016). Given that the area is rather well studied in terms of anostracans, it seems unlikely that the generally more common *B. orientalis* was overlooked in one of these targeted surveys.

Dispersal of Palaearctic *Branchinecta*

The dispersal of *B. ferox* was most likely northward from Northern Africa to the south of Europe. Moreover, it is possible that multiple colonization events to the Iberian and Balkan Peninsula occurred from the southern refugia (in pincer-like dispersion movements, as suggested for other anostracans; Dumont, Mertens & Maeda-Martinez, 1995). We mostly lack evidence for later dispersal events happening in the opposite direction which is generally in accordance with previous studies on other animal groups (Husemann *et al.*, 2014). The only exception to this pattern in *B. ferox* is a single population from Segovia in Spain.

On the other hand, the refugium of *B. orientalis* was likely in the Pannonian Plain (or this region was the one colonized first, once the expansion from more southern regions in the Balkans started). Except for Central Europe, *B. orientalis* is present in the Iberian Peninsula and several regions of Asia. On the Iberian Peninsula, most samples belong to one of two identified COI haplogroups (except for one specimen – EC2). In Asia, we found that the Nepalese specimen and specimens from Mongolia belong to two different haplogroups. If we assume that all current populations originated from the Balkans, it would mean that colonization of both Iberian Peninsula and Asia happened as at least two separate events.

Our results suggest a strong bottleneck effect of the Pleistocene glaciation on both studied species. The both species were probably introduced to the majority of their current habitats (from the refugia) after the ice retreat. Long-distance dispersal via migratory waterbirds is well documented in many branchiopod species including anostracans (Green *et al.*, 2005; Brochet *et al.*, 2009; Rogers, 2014) which are an important food source for the waterbirds (Horváth *et al.*, 2013b). Moreover, the recent distribution of both studied species (in the Western Palaearctic at least) match well with the seasonal migration routes of waterbirds (Svensson, 2009). However, there could be more than one animal vector group playing a role in anostracan dispersal. Although the dispersal range of these groups is considered to be much lower compared to the birds, mammal and insect migrations could be additional stepping stone links in explaining the dispersal and distribution of *B. orientalis*, especially if we consider that dispersal to these far places happened in several steps.

Implications for conservation of genetic diversity

Temporary aquatic habitats are disappearing at an alarming rate, mostly due to human interference and ongoing climate change. This is especially notable in the Mediterranean region (Zacharias & Zamparas, 2010; van den Broeck *et al.*, 2015) but also in e.g. the Pannonian Plain of

Central Europe (Horváth *et al.*, 2019). Not only is this of concern in light of the conservation of the fairy shrimp species in this study, but the habitats of *Branchinecta* also act as important resting sites for waterbirds on their seasonal migration routes. Anostracans, such as *Branchinecta* spp. represent important food sources of waterbirds (Horváth *et al.*, 2013b). Our data clearly show a recent gene flow among *Branchinecta* populations in these habitats suggesting high dispersal frequency of anostracans (possibly via waterbirds). This contrasts with findings for other anostracan species such as *Chirocephalus diaphanus* (Reniers *et al.*, 2013) and *Branchipus schaefferi* (Lukić *et al.*, 2019), where only few events of long-distance dispersal via waterbirds could be suggested. These could be due to the fact that these species inhabit rather small waterbodies, unattractive to the waterbirds.

Acknowledgements

References

Adamowicz S.J., Hebert P.D.N. & Marinone M.C. (2004). Species diversity and endemism in the *Daphnia* of Argentina: a genetic investigation. *Zoological Journal of the Linnean Society* **140**, 171–205.

Alonso M. (1985). A survey of the Spanish Euphyllopoda. *Miscellania Zoologica* **9**, 179–208

Atashbar B., Agh N., Manaffar R., Van Stappen G., Mohamadyari A., Mertens J., *et al.* (2016). Morphometric and preliminary genetic characteristics of *Branchinecta orientalis* populations from Iran (Crustacea: Anostraca). *Zootaxa* **4109**, 31–45

Atashbar B., Agh N., Van Stappen G. & Beladjal L. (2014a). Diversity and distribution patterns of large branchiopods (Crustacea: Branchiopoda) in temporary pools (Iran). *Journal of Arid Environments* **111**, 27–34.

Atashbar B., Agh N., Van Stappen G., Mertens J. & Beladjal L. (2014b). Combined effect of temperature and salinity on hatching characteristics of three fairy shrimp species (Crustacea: Anostraca). *Journal of Limnology* **73**, 574–583.

Bandelt H.J., Forster P. & Röhl A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**, 37–48.

Beladjal L. & Mertens J. (2009). Diaspore dispersal of anostraca by flying insects. *Journal of Crustacean Biology* **29**, 266–268.

Belk D. & Brtek J. (1995). Checklist of the Anostraca. *Hydrobiologia* **298**, 315–353.

Bohonak A.J. & Whiteman H.H. (1999). Dispersal of the fairy shrimp *Branchinecta coloradensis* (Anostraca): Effects of hydroperiod and salamanders. *Limnology and Oceanography* **44**, 487–493

Boileau M. & Hebert P. (1991). Genetic consequences of passive dispersal in pond-dwelling copepods. *Evolution* **45**, 721–733.

Brendonck L. (1996). Diapause, quiescence, hatching requirements: what we can learn from large freshwater branchiopods (Crustacea: Branchiopoda: Anostraca, Notostraca, Conchostraca). *Hydrobiologia* **320**, 85–97.

Brendonck L. & De Meester L. (2003). Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. *Hydrobiologia* **491**, 65–84

Brendonck L., De Meester L. & Riddoch B.J. (2000). Regional structuring of genetic variation in short-lived rock pool populations of *Branchipodopsis wolfi* (Crustacea : Anostraca). *Oecologia* **123**, 506–515.

Brendonck L. & Riddoch B.J. (1999). Wind-borne short-range egg dispersal in anostracans (Crustacea: Branchiopoda). *Biological Journal of the Linnean Society* **67**, 87–95.

Brochet A.L., Gauthier-Clerc M., Guillemain M., Fritz H., Waterkeyn A., Baltanás Á., *et al.* (2009). Field evidence of dispersal of branchiopods, ostracods and bryozoans by teal (*Anas crecca*) in the Camargue (southern France). *Hydrobiologia* **637**, 255.

van den Broeck M., Waterkeyn A., Rhazi L. & Brendonck L. (2015). Distribution, coexistence, and decline of Moroccan large branchiopods. *Journal of Crustacean Biology* **35**, 355–365.

Downing J.A. (2010). Emerging global role of small lakes and ponds: little things mean a lot. *Limnetica*. **29**, 0009–24.

Dumont H.J., Mertens J. & Maeda-Martinez A.M. (1995). Historical biogeography and morphological differentiation of *Streptocephalus torvicornis* (Waga) since the Würm III-glaciation. *Hydrobiologia* **298**, 281–286.

Eder E., Hödl W. & Gottwald R. (1997). Distribution and phenology of large branchiopods in Austria. *Hydrobiologia* **359**, 13–22.

Figuerola J. & Green A.J. (2002). Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology* **47**, 483–494.

Folmer O., Black M., Hoeh W., Lutz R. & Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology* **3**, 294–299

Green A.J., Sánchez M.I., Amat F., Figuerola J., Hontoria F., Ruiz O., *et al.* (2005). Dispersal of invasive and native brine shrimps *Artemia* (Anostraca) via waterbirds. *Limnology and Oceanography* **50**, 737–742.

Habel J.C., Drees C., Schmitt T. & Assmann T. (2010). Review refugial areas and postglacial colonizations in the Western Palearctic. In: *Relict Species.* (Eds J.C. Habel & T. Assmann), pp. 189–197. Springer Berlin Heidelberg.

Hairston N.G., Brunt R.A.V., Kearns C.M. & Engstrom D.R. (1995). Age and survivorship of diapausing eggs in a sediment egg bank. *Ecology* **76**, 1706–1711.

Horváth Z., Ptacnik R., Vad C.F. & Chase J.M. (2019). Habitat loss over six decades accelerates regional and local biodiversity loss via changing landscape connectance. *Ecology Letters* **22**, 1019–1027.

Horváth Z., Vad C.F., Voeroes L. & Boros E. (2013a). Distribution and conservation status of fairy shrimps (Crustacea: Anostraca) in the astatic soda pans of the Carpathian basin: the role of local and spatial factors. *Journal of Limnology* **72**, 103–116.

Horváth Z., Vad Cs.F., Vörös L. & Boros E. (2013b). The keystone role of anostracans and copepods in European soda pans during the spring migration of waterbirds. *Freshwater Biology* **58**, 430–440

Huelsenbeck J.P. & Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Husemann M., Schmitt T., Zachos F.E., Ulrich W. & Habel J.C. (2014). Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *Journal of Biogeography* **41**, 81–94.

Ji Y.-J., Zhang D.-X. & He L.-J. (2003). Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Molecular Ecology Notes* **3**, 581–585.

Jocque M., Vanschoenwinkel B. & Brendonck L. (2010). Anostracan monopolisation of early successional phases in temporary waters? *Fundamental and Applied Limnology* **176**, 127–132.

Ketmaier V., Mandatori R., Matthaeis E.D. & Mura G. (2005). Molecular systematics and phylogeography in the fairy shrimp *Tanymastix stagnalis* based on mitochondrial DNA. *Journal of Zoology* **266**, 401–410.

Ketmaier V., Marrone F., Alfonso G., Paulus K., Wiemann A., Tiedemann R., *et al.* (2012). Mitochondrial DNA regionalism and historical demography in the extant populations of *Chirocephalus kerkyrensis* (Branchiopoda: Anostraca). *PLOS ONE* **7**, e30082.

Ketmaier V., Pirollo D., De Matthaeis E., Tiedemann R. & Mura G. (2008). Large-scale mitochondrial phylogeography in the halophilic fairy shrimp *Phallocryptus spinosa* (Milne-Edwards, 1840) (Branchiopoda: Anostraca). *Aquatic Sciences* **70**, 65–76.

Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution* **35**, 1547–1549.

Lefort V., Longueville J.-E. & Gascuel O. (2017). SMS: Smart Model Selection in PhyML. *Molecular Biology and Evolution* **34**, 2422–2424.

Leigh J.W. & Bryant D. (2015). PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110–1116.

Lindholm M., d'Auriac M.A., Thaulow J. & Hobaek A. (2016). Dancing around the pole: Holarctic phylogeography of the Arctic fairy shrimp *Branchinecta paludosa* (Anostraca, Branchiopoda). *Hydrobiologia* **772**, 189–205.

Lukić D., Horváth Z., Vad C.F. & Ptacnik R. (2018). Food spectrum of *Branchinecta orientalis*—are anostracans omnivorous top consumers of plankton in temporary waters? *Journal of Plankton Research* **40**, 436–445.

Lukić D., Waterkeyn A., Rabet N., Mioduchowska M., Geudens B., Vanschoenwinkel B., *et al.* (2019). High genetic variation and phylogeographic relations among Palearctic fairy shrimp populations reflect persistence in multiple southern refugia during Pleistocene ice ages and postglacial colonisation. *Freshwater Biology* **64**, 1896-1907.

Machordom A., Araujo R., Erpenbeck D. & Ramos M.-Á. (2003). Phylogeography and conservation genetics of endangered European Margaritiferidae (Bivalvia: Unionoidea). *Biological Journal of the Linnean Society* **78**, 235–252.

Manca M. & Mura G. (1997). On *Branchinecta orientalis* Sars (Anostraca) in the Himalayas. *Hydrobiologia* **356**, 111–116.

Marrone F., Alonso M., Pieri V., Augugliaro C. & Stoch F. (2015). The crustacean fauna of Bayan Onjuul area (Tov Province, Mongolia) (Crustacea: Branchiopoda, Copepoda, Ostracoda). *North-Western Journal of Zoology* **11**, 288–295

Marrone F., Brutto S.L. & Arculeo M. (2010). Molecular evidence for the presence of cryptic evolutionary lineages in the freshwater copepod genus *Hemidiaptomus* G.O. Sars, 1903 (Calanoida, Diaptomidae). *Hydrobiologia* **644**, 115–125.

Marrone F., Korn M., Stoch F., Naselli Flores L. & Turki S. (2016). Updated checklist and distribution of large branchiopods (Branchiopoda: Anostraca, Notostraca, Spinicaudata) in Tunisia. **31**, 27–53.

Moss B. (2012). Cogs in the endless machine: Lakes, climate change and nutrient cycles: A review. *Science of the Total Environment* **434**, 130–142.

Mura G., Ozkutuk S.R., Aygen C. & Cottarelli V. (2011). New data on the taxonomy and distribution of anostracan fauna from Turkey. *Journal of Biological Research-Thessaloniki* **15**, 17–23

Mura G. & Takami G.A. (2000). A contribution to the knowledge of the anostracan fauna of Iran. *Hydrobiologia* **441**, 117–121.

Naganawa H., Naumova E.Yu., Denikina N.N., Kondratov I.G., Dzyuba E.V. & Iwasawa A. (2019). Does the dispersal of fairy shrimps (Branchiopoda, Anostraca) reflect the shifting geographical distribution of freshwaters since the late Mesozoic? *Limnology,* 1-10.

Oertli B., Biggs J., Céréghino R., Grillas P., Joly P. & Lachavanne J.-B. (2005). Conservation and monitoring of pond biodiversity: introduction. *Aquatic Conservation: Marine and Freshwater Ecosystems* **15**, 535–540.

Pabijan M., Zieliński P., Dudek K., Chloupek M., Sotiropoulos K., Liana M., *et al.* (2015). The dissection of a Pleistocene refugium: phylogeography of the smooth newt, *Lissotriton vulgaris*, in the Balkans. *Journal of Biogeography* **42**, 671–683.

Padhye S.M., Kulkarni M.R. & Dumont H.J. (2017). Diversity and zoogeography of the fairy shrimps (Branchiopoda: Anostraca) on the Indian subcontinent. *Hydrobiologia* **801**, 117–128.

Paillard D. (1998). The timing of Pleistocene glaciations from a simple multiple-state climate model. *Nature* **391**, 378–381.

Parvizi E., Naderloo R., Keikhosravi A., Solhjouy-Fard S. & Schubart C.D. (2018). Multiple Pleistocene refugia and repeated phylogeographic breaks in the southern Caspian Sea region: Insights from the freshwater crab *Potamon ibericum*. *Journal of Biogeography* **45**, 1234–1245.

Petkovski S. (1993). Taksonomsko-morfološka i zoogeografsko-ekološka studija Anostraca (Crustacea: Branchiopoda) jugoslovenskih zemalja. PhD Thesis. Prirodno-matematički fakultet, Novi Sad.

Previšić A., Walton C., Kučinić M., Mitrikeski P.T. & Kerovec M. (2009). Pleistocene divergence of Dinaric *Drusus endemics* (Trichoptera, Limnephilidae) in multiple microrefugia within the Balkan Peninsula. *Molecular Ecology* **18**, 634–647.

Rajaei Sh H., Rödder D., Weigand A.M., Dambach J., Raupach M.J. & Wägele J.W. (2013). Quaternary refugia in southwestern Iran: insights from two sympatric moth species (Insecta, Lepidoptera). *Organisms Diversity & Evolution* **13**, 409–423.

Reniers J., Vanschoenwinkel B., Rabet N. & Brendonck L. (2013). Mitochondrial gene trees support persistence of cold tolerant fairy shrimp throughout the Pleistocene glaciations in both southern and more northerly refugia. *Hydrobiologia* **714**, 155–167

Rhazi L., Grillas P., Saber E.-R., Rhazi M., Brendonck L. & Waterkeyn A. (2012). Vegetation of Mediterranean temporary pools: a fading jewel? *Hydrobiologia* **689**, 23–36.

Rodríguez-Flores P.C., Jiménez-Ruiz Y., Forró L., Vörös J. & García-París M. (2017). Non-congruent geographic patterns of genetic divergence across European species of *Branchinecta* (Anostraca: Branchinectidae). *Hydrobiologia* **801**, 47–57.

Rogers D.C. (2014). Larger hatching fractions in avian dispersed anostracan eggs (Branchiopoda). *Journal of Crustacean Biology* **34**, 135–143.

Rogers D.C. (2006). Three new species of *Branchinecta* (Crustacea : Branchiopoda : Anostraca) from the nearctic. *Zootaxa* **1126**, 35–51.

Ronquist F. & Huelsenbeck J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Hohna S., *et al.* (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

Šćiban M., Marković A., Lukić D. & Miličić D. (2014). Autumn populations of *Branchinecta orientalis* G. O. Sars, 1903 and *Chirocephalus diaphanus* Prevost, 1803 (Crustacea, Branchiopoda) in the Central European Lowlands (Pannonian Plain, Serbia). *North-Western Journal of Zoology* **10**, 435–437.

Song H., Buhay J.E., Whiting M.F. & Crandall K.A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences* **105**, 13486–13491.

Stoks R., Geerts A.N. & Meester L.D. (2014). Evolutionary and plastic responses of freshwater invertebrates to climate change: realized patterns and future potential. *Evolutionary Applications* **7**, 42–55.

Svensson L. (2009). *Collins Bird Guide. 2nd edition*. HarperCollins Publishers Ltd. London.

Swofford D.L. (2001). PAUP*: Phylogenetic Analysis Using Parsimony (and other methods) 4.0.b5.

Tajima F. (1989). The effect of change in population size on DNA polymorphism. *Genetics* **123**, 597–601

Tamura K. & Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526.

Tuytens K., Vanschoenwinkel B., Waterkeyn A. & Brendonck L. (2014). Predictions of climate change infer increased environmental harshness and altered connectivity in a cluster of temporary pools. *Freshwater Biology* **59**, 955–968.

Ursenbacher S., Carlsson M., Helfer V., Tegelström H. & Fumagalli L. (2006). Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. *Molecular Ecology* **15**, 3425–3437.

Vad C.F., Péntek A.L., Cozma N.J., Földi A., Tóth A., Tóth B., *et al.* (2017). Wartime scars or reservoirs of biodiversity? The value of bomb crater ponds in aquatic conservation. *Biological conservation* **209**, 253–262.

Vanschoenwinkel B., Pinceel T., Vanhove M.P.M., Denis C., Jocque M., Timms B.V., *et al.* (2012). Toward a global phylogeny of the "living fossil'" crustacean order of the Notostraca. *Plos One* **7**, e34998.

Vanschoenwinkel B., Waterkeyn A., Vandecaetsbeek T., Pineau O., Grillas P. & Brendonck L. (2008). Dispersal of freshwater invertebrates by large terrestrial mammals: a case study with wild boar (*Sus scrofa*) in Mediterranean wetlands. *Freshwater Biology* **53**, 2264–2273.

Vörös J., Mikulíček P., Major Á., Recuero E. & Arntzen J.W. (2016). Phylogeographic analysis reveals northerly refugia for the riverine amphibian *Triturus dobrogicus* (Caudata: Salamandridae). *Biological Journal of the Linnean Society* **119**, 974–991.

Waterkeyn A., Grillas P., Anton-Pardo M., Vanschoenwinkel B. & Brendonck L. (2011). Can large branchiopods shape microcrustacean communities in Mediterranean temporary wetlands? *Marine and Freshwater Research* **62**, 46–53.

Williams D.D. (2006). *The biology of temporary waters*. Oxford University Press, Oxford.

Williams P., Whitfield M., Biggs J., Bray S., Fox G., Nicolet P., *et al.* (2004). Comparative biodiversity of rivers, streams, ditches and ponds in an agricultural landscape in Southern England. *Biological Conservation* **115**, 329–341.

Xia X. & Kumar S. (2018). DAMBE7: New and Improved Tools for Data Analysis in Molecular Biology and Evolution. *Molecular Biology and Evolution* **35**, 1550–1552.

Xia X. & Lemey P. (2009). Assessing substitution saturation with DAMBE. In: *The phylogenetic handbook*, 2nd edn. pp. 615–630. Cambridge University Press, Cambridge.

Xia X., Xie Z., Salemi M., Chen L. & Wang Y. (2003). An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution* **26**, 1–7.

Zacharias I. & Zamparas M. (2010). Mediterranean temporary ponds. A disappearing ecosystem. *Biodiversity and Conservation* **19**, 3827–3834.

Supplement

Table S1. Divergence between species within *Branchinecta* genus (involving 53 sequences). The lower triangle indicates the mean genetic divergence between species in %; the upper triangle indicates the minimum and maximum genetic distances between species (in %). The number of base substitutions per site, averaged over all sequence pairs between groups, represents the mean distances. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). Fewer than 10 % alignment gaps, missing data, and ambiguous bases were allowed at any position. Sequences were 503 base pairs in length in the final alignment.

| Species	B. ferox	B. hiberna	B. lindahli	B. lynchi	B. mackini	B. orientalis	B. paludosa	B. sandiegonensis
Branchinecta ferox		10.77 - 14.34	11.66 - 14.34	11.03 - 14.57	10.52 - 15.72	8. 33 - 12.32	14.58 - 19.87	13.52 - 17.91
Branchinecta hiberna	12.16		9.29 - 10.84	8.44 - 10.32	11.36 - 12.5	7.82 - 10.06	13.21 - 17.86	12.51 - 13.95
Branchinecta lindahli	12.72	10.02		8.68 - 11.79	10.09 - 11.57	9.66 - 13.21	11.06 - 18.03	12.20 - 15.32
Branchinecta lynchi	12.24	9.47	10.21		11.1 - 12.86	10.22 - 12.79	14.20 - 18.82	11.93 - 13.56
Branchinecta mackini	12.47	11.89	10.97	11.79		13.44 - 15.25	13.62 - 17.98	13.29 - 14.94
Branchinecta orientalis	9.93	9.17	11.4	11.28	14.05		14.39 - 20.60	14.30 - 17.76
Branchinecta paludosa	17.3	15.55	15.9	16.65	15.77	17.53		15.31 - 21.35
Branchinecta sandiegonensis	15.12	13.33	13.28	12.76	14.08	15.6	18.36	
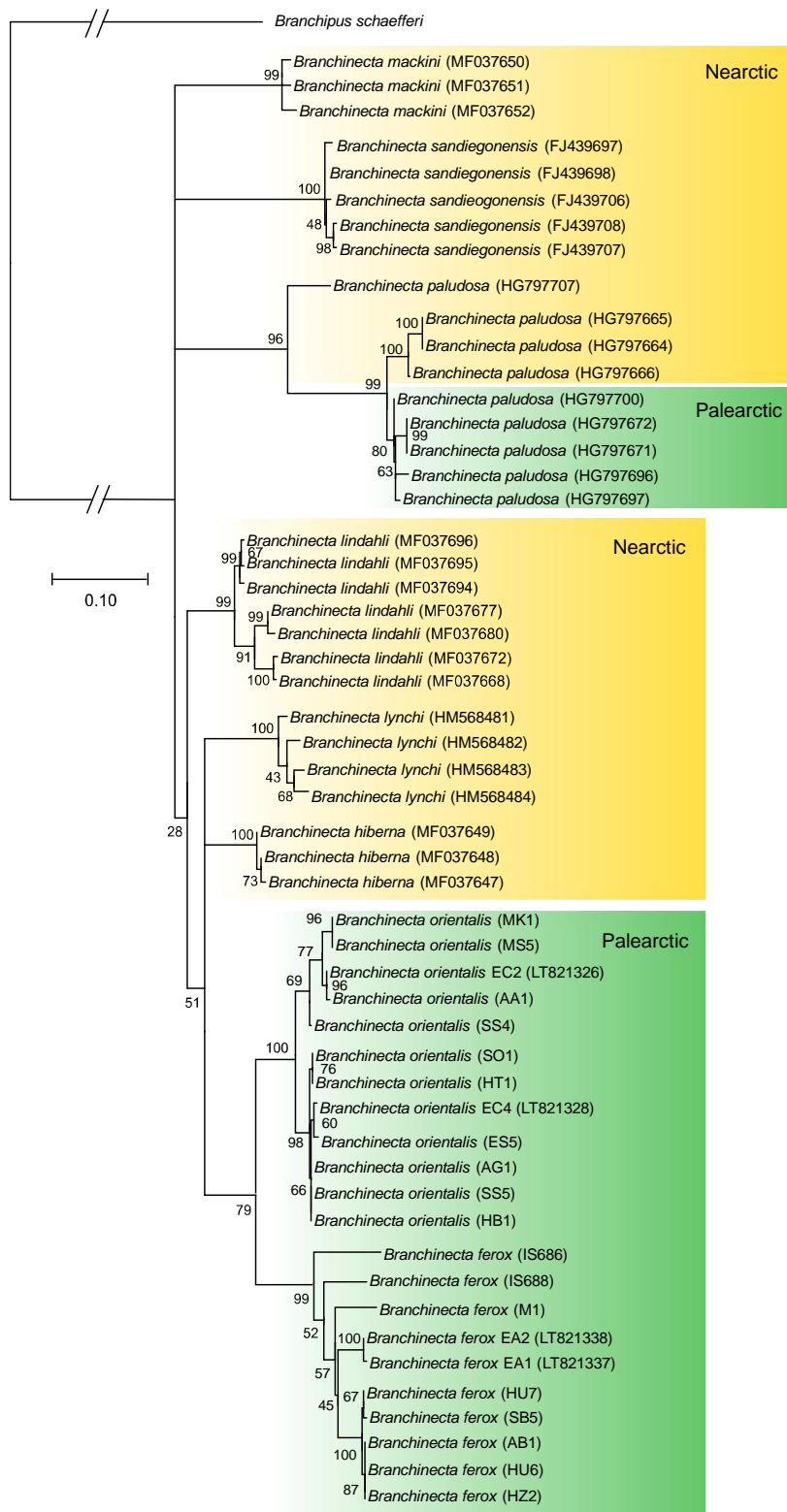
Figure S1. Maximum likelihood phylogenetic tree for genus *Branchinecta*, based on the mitochondrial CO1 gene fragment. All studied species, except *B. paludosa*, inhabit either Nearctic (yellow) or Palearctic (green). The supporting values based on maximum likelihood models are included close to the nodes. The most probable evolutionary model was determined in MEGA X (Kumar *et al.*, 2018) based on the Akaike information criterion (AIC), which selected for the General Time Reversible (GTR) model with a gamma shape parameter (+G, γ=1.31) and proportion of invariable sites (I=0.57).

References:

Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution* **35**, 1547–1549.

General Discussion & Conclusions

I experimentally studied the feeding ecology and trophic position of *B. orientalis* with numerous representative prey types that were selected from its natural habitats. My results revealed a hitherto overlooked role of anostracans as intraguild predators, and thus shed new light on the biotic interactions between plankton and anostracans, which were also confirmed by stable isotope analysis of field material. In a molecular study involving *B. orientalis* and a closely related species, *B ferox*, I analysed phylogenetic pattern in these two species and reconstructed their postglacial recolonization in Europe. These novel results contribute to a better understanding of these vulnerable taxa and their habitats, and gives foundation for further new research topics with large branchiopods and communities of temporary waters.

Main findings

With my model species, the medium-sized anostracan *Branchinecta orientalis*, I provided evidence of anostracan omnivory and showed that the feeding spectrum of anostracans is much wider than previously assumed (**Chapter 1**). Moreover, I documented that the ingestion rate of anostracans could reach extremely high values compared to other planktonic filter-feeders (up to 0.5 mg h^{-1}). I did not find any difference in the feeding rates related to size or sex of the anostracans. My study implied that in these systems, anostracans represent both competitors and predators for smaller zooplankton, suggesting a strong top-down structuring effect on the plankton communities in these systems.

Chapter 2 is a continuation on the feeding ecology of anostracans. As I have shown in **Chapter 1**, my model anostracan species (*B. orientalis*) is an intraguild predator in its habitats. In **Chapter 2**, I examined the effect of a prominent environmental factor, inorganic turbidity, on this intraguild relationship. I found contrasting effects of turbidity on herbivorous and carnivorous feeding. While herbivorous feeding was negatively affected by increased turbidity, carnivorous feeding on larger zooplankton prey was in most cases unaffected. This suggested that anostracans were able to employ different feeding mechanisms - filter and predatory feeding - depending on the available prey type and potential interference with suspended solids. My results from the laboratory experiments were confirmed in the original habitats, where stable isotope analyses suggested a stable difference in the main food source of local anostracans among habitats, linked to local levels of inorganic turbidity. Thus, through interference with the feeding of an intraguild predator, environment may also play an important role in structuring plankton communities.

Finally in **Chapter 3**, I revealed the global phylogeography of two congeneric anostracan species: *Branchinecta ferox* and *B. orientalis*, covering most of their currently known distribution in the Palearctic. For that, I sequenced two DNA regions, mitochondrial CO1 and nuclear ITS2, which have different evolutionary rates. I found relatively low divergence on both genetic markers in both species. Interestingly, the genetic analyses revealed different refugial regions and post-glacial colonization pattern for these two closely related *Branchinecta* species. While the Pleistocene refugia of *B. ferox* were likely in the North Africa and the Middle East (**Fig. 1**), those of *B. orientalis*

were more likely in the Balkans and/or the Pannonian Plain (**Fig. 2**), suggesting spatial separation and independent evolution for most of the Pleistocene. After the last glacial retreat, *B. ferox* expanded towards north, colonizing the southern and central parts of Europe (**Fig. 1**), while *B. orientalis* expanded both to the west (to the Iberian Peninsula) and to the east (as far as Mongolia) (**Fig. 2**).
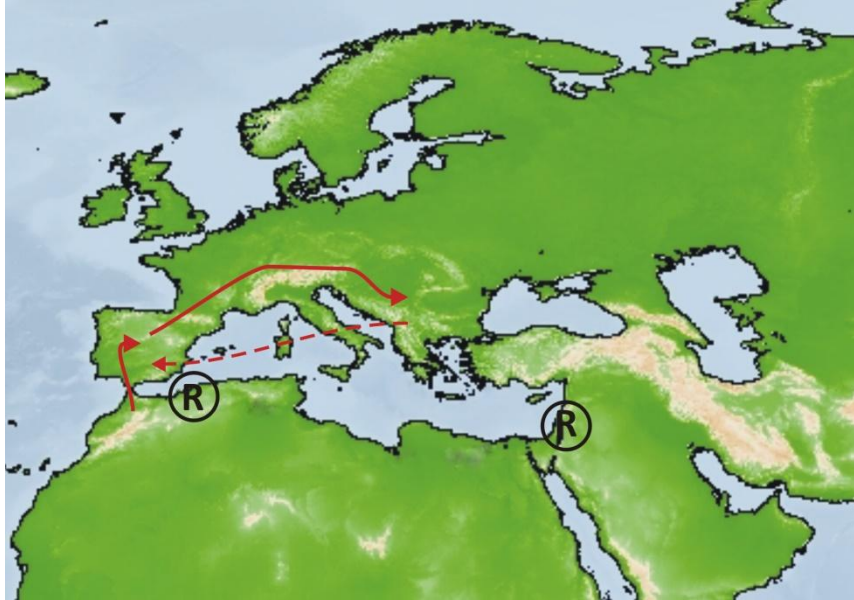

Figure 1. Glacial refugia and postglacial range expansion of *Branchinecta ferox* based on our results. "R" indicates the Pleistocene refugium, while arrows indicate range expansion after the latest glacial retreat. Dashed arrow indicates a single recent dispersal event.
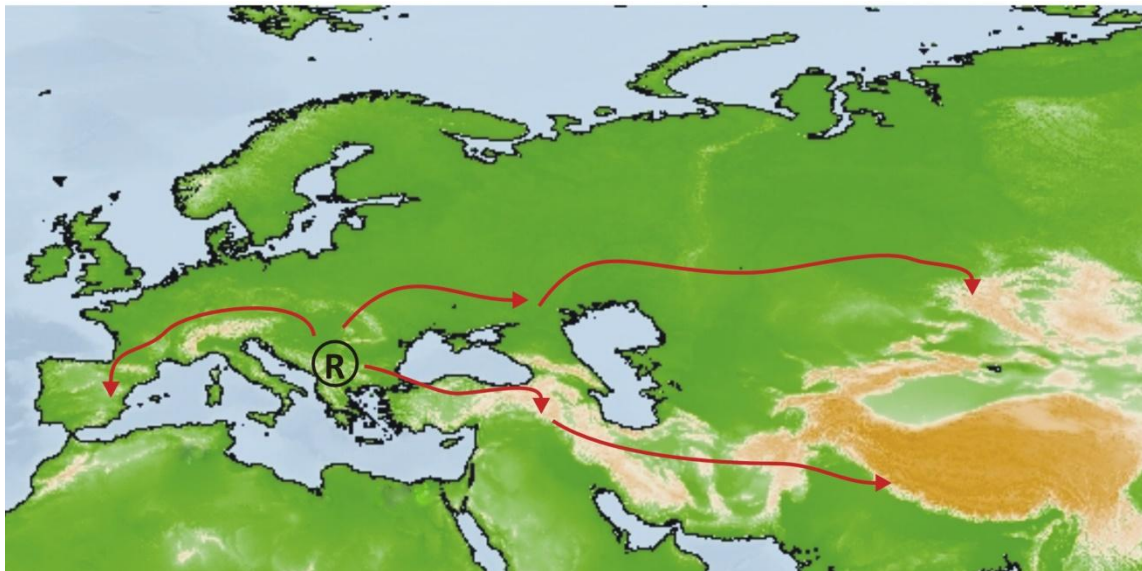

Figure 2. Glacial refugium and postglacial range expansion of *Branchinecta orientalis* based on our results. "R" indicates the Pleistocene refugium, while arrows indicate range expansion after the latest glacial retreat.

Future research questions

- **Food spectrum of anostracans** – My results show that the medium sized anostracan *B. orientalis* (approx. 2.5 cm of body length) can feed efficiently on a wide range of prey types,

covering an effective prey size range between 2 µm – 2 mm in length. Importantly, the upper end encompasses highly mobile crustacean zooplankton taxa (with adult *Daphnia magna* as the only exception). A logical next step would be to test the generality of this observation among similar-sized or even smaller anostracans, also beyond the genus *Branchinecta*.

- **Community structuring effects of anostracans** – My results imply that anostracans might have a significant role in structuring plankton communities. Their high capacity in efficient filter-feeding (Jocque, Vanschoenwinkel & Brendonck, 2010) represents a strong competitive force for other zooplankton. My findings about omnivorous feeding in anostracans highlight their additional effect as predators (Waterkeyn *et al.*, 2011). My results furthermore showed that the level of anostracan omnivory can also be modulated by the local environment. Disentangling these complex relationships and the role of anostracans in temporary pond communities (based on explicit experiments with and without anostracans) could be very informative on the possible community shaping role of this group, with implications for further omnivores of similar aquatic systems.

- **The role of intraguild predators (IGP) in aquatic communities** – Omnivory is a common feature in most food webs (Holt & Polis, 1997; Thompson *et al.*, 2007). The actual role of omnivory in terms of (de)stabilizing food web dynamics is under debate. While some studies suggest a stabilizing effect of omnivory, others argue that this is not universally true (reviewed in Wootton, 2017). Intraguild predation should affect the intraguild prey (smaller zooplankton, in this case) especially at high productivity, as here the double effect of the IG predator on its prey as competitor and predator is maximized (Diehl & Feissel, 2000). By showing coexistence of an omnivore with its intra-guild prey in a hypertrophic system, my results provide an interesting exception to this hypothesis. An open question remains whether omnivorous feeding in anostracans serves only to maximise uptake of food, or if biochemical aspects are involved, similar to the trophic upgrading which is known from copepods that optimize the quality of their diet by combining herbivory with selective feeding on ciliates and other microzooplankton (Calbet & Saiz, 2005; Wollrab & Diehl, 2015). Albeit I could clarify an important aspect in these systems by better understanding the feeding ecology of a key consumer, the food web structure of soda pans is still poorly characterized. Calanoid copepods, which are otherwise found especially in oligotrophic systems, are another key player in soda pans. Though they seem to thrive in these systems which are often dominated by picoalgae, they are likely not directly relying on picophytoplankton as main food source. Instead, picoalgae – protozoa – copepods seem to represent another triangular food web in these systems which is hardly explored.

- **Importance of connectivity for diversity** – Local genetic diversity was especially high in the soda pans of the Seewinkel region (Austria), where almost all sites hosted individuals from both mitochondrial COI haplogroups (see **Chapter 3**, **Fig. 3A**). Movements and migration of waterbird probably contributed substantially to the high gene flow I detected not only within this smaller region, but also at continental scale (as waterbirds can carry anostracan resting eggs over

large distances especially in their digestive system; Green *et al.*, 2005; Sánchez *et al.*, 2012). The high local genetic diversity could be maintained by the high environmental diversity, number and proximity of the soda pans in the Seewinkel region, in addition to potential seasonal differences. Thus, this region provides an excellent playground to study historical and current dispersal events and the importance of connectivity for the gene flow and maintenance of genetic diversity. Eggs could be extracted from different depths of the pond sediment (with older eggs stored in the deeper layers) to reveal if genetic composition within each individual population and the entire metapopulation (the Seewinkel region) changed over time and whether the ongoing loss of habitats have an impact on genetic diversity (as seen for communities, Horváth *et al.*, 2019). This might also help approximating the time when these two different genetic lines colonized the area.

- **The vectors for propagule dispersal at the local scale** – Landscape genetic patterns of the *Branchinecta* populations within the Seewinkel region would be another interesting spinoff (by using more variable markers, e.g. microsatellites). Here, I could examine recent dispersal events and possibly identify dominant dispersal vectors (especially wind versus waterbirds). One possible outcome could be that dispersal is predominantly mediated by wind as it was shown in these habitats that the prevailing wind direction has an imprint on zooplankton community composition (the area is characterised by strong north-western winds; Horváth, Vad & Ptacnik, 2016). Alternatively, animal vectors, such as waterbirds, may play an important role in propagule dispersal via daily movements between neighbouring soda pans and bringing in new genetic lines from other regions.

- **Phylogeny in the *Branchinecta* genus and historic events** – The *Branchinecta* genus is an ancient, diverse, and widely distributed anostracan genus. Therefore, this genus is an excellent model to study speciation, dispersal limitation and historical range shifts and expansions. However, the genetic studies so far were predominantly focused only on one genetic marker – mitochondrial COI gene region – and encompassed eight rather common *Branchinecta* species in the Ne- and Palaearctic (out of ~50 described species; counting only studies for which sequences were deposited at the GenBank). The genetic information on species with more restricted distribution is particularly lacking. Moreover, studies on gene markers other than the mitochondrial COI gene region are still scarce which would be crucial to disentangle the phylogeny of the entire *Branchinecta* genus.

References

Calbet A. & Saiz E. (2005). The ciliate-copepod link in marine ecosystems. *Aquatic Microbial Ecology* **38**, 157–167.

Diehl S. & Feissel M. (2000). Effects of enrichment on three-level food chains with omnivory. *American Naturalist* **155**, 200–218.

Green A.J., Sánchez M.I., Amat F., Figuerola J., Hontoria F., Ruiz O., *et al.* (2005). Dispersal of invasive and native brine shrimps *Artemia* (Anostraca) via waterbirds. *Limnology and Oceanography* **50**, 737–742.

Holt R.D. & Polis G.A. (1997). A theoretical framework for intraguild predation. *American Naturalist* **149**, 745–764.

Horváth Z., Ptacnik R., Vad C.F. & Chase J.M. (2019). Habitat loss over six decades accelerates regional and local biodiversity loss via changing landscape connectance. *Ecology Letters* **22**, 1019–1027.

Horváth Z., Vad C.F. & Ptacnik R. (2016). Wind dispersal results in a gradient of dispersal limitation and environmental match among discrete aquatic habitats. *Ecography* **39**, 726–732.

Jocque M., Vanschoenwinkel B. & Brendonck L. (2010). Anostracan monopolisation of early successional phases in temporary waters? *Fundamental and Applied Limnology* **176**, 127–132.

Sánchez M.I., Hortas F., Figuerola J. & Green A.J. (2012). Comparing the potential for dispersal via waterbirds of a native and an invasive brine shrimp. *Freshwater Biology* **57**, 1896–1903.

Thompson R.M., Hemberg M., Starzomski B.M. & Shurin J.B. (2007). Trophic levels and trophic tangles: The revalence of omnivory in real food webs. *Ecology* **88**, 612–617

Waterkeyn A., Grillas P., Anton-Pardo M., Vanschoenwinkel B. & Brendonck L. (2011). Can large branchiopods shape microcrustacean communities in Mediterranean temporary wetlands? *Marine and Freshwater Research* **62**, 46–53.

Wollrab S. & Diehl S. (2015). Bottom-up responses of the lower oceanic food web are sensitive to copepod mortality and feeding behavior. *Limnology and Oceanography* **60**, 641–656.

Wootton K.L. (2017). Omnivory and stability in freshwater habitats: Does theory match reality? *Freshwater Biology* **62**, 821–832.