

Morphological and life history changes of *Daphnia* during oligotrophication

Doctoral thesis for obtaining

the academic degree

Doctor of Natural Sciences

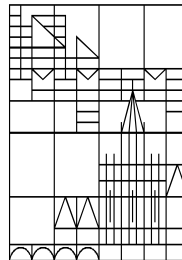
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*“Recite, and your Lord is the Most Generous.
Who taught by the pen. Taught man that which he knew not.”
(Al-’Alaq 96: 3–5)*

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Multiple abiotic and biotic factors, as well as evolutionary dynamics, play crucial roles for predator-prey interactions. Some of the best studied examples of predator-prey interactions in aquatic ecosystems are those involving the cladoceran herbivore *Daphnia*, both as a predator and prey species. Daphniids serve as vital links within food webs connecting higher and lower trophic levels. In lakes often various *Daphnia* species coexist, and these *Daphnia* spp. usually are exposed to several predator species.

The Lake Constance food web is home to three species of the *Daphnia longispina* species complex (*D. longispina*, *D. galeata* and *D. cucullata*) and the carnivorous cladocerans, *Bythotrephes longimanus* and *Leptodora kindtii*. Interactions between *Daphnia* and these predators are not well understood in Lake Constance. Furthermore, the trophic status of the lake changed strongly during the last decades from eutrophic conditions in the 1970s to oligotrophic conditions since the 2000s. It is unclear whether trophic change resulted in microevolution of *Daphnia* in respect to *Daphnia*'s response to invertebrate predators and changing food availability.

Based on this background, I aimed in my PhD theses to investigate the response of the cladoceran herbivore *Daphnia* to different environmental cues and pressures. The main focus was to examine the morphological and life history responses induced by two selection pressures, predators and food availability. I have structured my thesis around five chapters that investigate aspects of this topic through laboratory and mesocosm experiments, partially using a resurrection ecology approach.

The first chapter uses a mesocosm experiment to analyse the role of the genus *Daphnia* in the pelagic food web of Lake Constance. We show that two fish species strongly feed on the large *Daphnia longispina*, but less so on smaller *D. cucullata*. As *D. longispina* also showed the strongest ability to control phytoplankton, these results suggest that large *Daphnia* species are more likely compared to small *Daphnia* species to function as keystone species linking higher and lower trophic levels in lake food webs.

The subsequent three chapters analyse different aspects of morphological and life history changes induced by two invertebrate predators in Lake Constance. **Chapter II** shows that the morphological responses of *D. galeata* and their costs differ regarding the kairomones of two invertebrate predators, *Bythotrephes longimanus* versus *Leptodora kindtii*.

Chapter III expands **chapter II** to include two more *Daphnia* species, *D. longispina* and *D. cucullata*, and to include also the study of microdefenses. This chapter shows that morphological predator defences are also *Daphnia*-species specific, and that microdefenses are expressed independently from the usually studied macrodefenses such as the tail spine or helmets.

Chapter IV focusses again on *D. galeata* and uses a resurrection ecology approach to study microevolutionary changes in antipredator responses during oligotrophication of Lake Constance. This chapter shows that antipredator morphologies indeed showed microevolutionary change during oligotrophication, but we found no evidence for the evolution of plasticity. Finally, **chapter V** expands the resurrection approach of **chapter IV** to study the microevolutionary response of *D. galeata* to food quantity changes. This chapter did not find microevolutionary responses of filter comb dimensions such as filter areas and mesh sizes, but the evolution of phenotypic plasticity in respect to the timing of reproductive events: at low, but not a high food concentration, clones from oligotrophic periods reproduced later compared to clones from the eutrophic period. Combining the results of the last two chapters, my thesis shows that oligotrophication will result in microevolutionary changes in traits influencing, both the growth and mortality of *Daphnia*.

Zusammenfassung

Zahlreiche abiotische und biotische Faktoren sowie evolutionäre Dynamiken spielen eine wichtige Rolle bei Räuber-Beute-Interaktionen. Einige der am besten untersuchten Beispiele für Räuber-Beute-Interaktionen in aquatischen Ökosystemen sind diejenigen, an denen der Herbivor *Daphnia* beteiligt ist, sowohl als Räuber als auch als Beute. Daphnien sind wichtige Glieder in Nahrungsnetzen, die höhere und niedrigere trophische Ebenen verbinden. In Seen leben häufig mehrere Daphnienarten, und diese Daphnienarten sind in der Regel mehreren Räuberarten ausgesetzt.

Das Nahrungsnetz des Bodensees beherbergt drei Arten des *Daphnia longispina*-Artenkomplexes (*D. longispina*, *D. galeata* und *D. cucullata*) und die räuberischen Cladoceren *Bythotrephes longimanus* und *Leptodora kindtii*. Die Interaktionen zwischen Daphnien und diesen Räubern sind im Bodensee nicht gut bekannt. Außerdem hat sich der trophische Zustand des Sees in den letzten Jahrzehnten stark verändert, von eutrophen Bedingungen in den 1970er Jahren zu oligotrophen Bedingungen seit den 2000er Jahren. Es ist unklar, ob die trophischen Veränderungen zu einer Mikroevolution von Daphnien geführt haben in Bezug auf die Reaktion von Daphnien auf invertebrate Räuber und die veränderte Verfügbarkeit von Nahrung.

Vor diesem Hintergrund untersuchte ich in meiner Arbeit die Reaktion von *Daphnia* auf verschiedene Umweltstressoren. Das Hauptaugenmerk lag dabei auf der Untersuchung der morphologischen und Lebenszyklus Reaktionen, die durch die beiden Selektionsfaktoren Räuber und Nahrungsverfügbarkeit ausgelöst werden.

Ich habe meine Arbeit in fünf Manuskripte gegliedert, in denen verschiedene Aspekte dieses Themas anhand von Labor- und Mesokosmenexperimenten untersucht werden, wobei teilweise der Ansatz der «resurrection ecology» verwendet wurde.

Das erste Manuskript nutzt ein Mesokosmen-Experiment, um die Rolle der Gattung *Daphnia* im pelagischen Nahrungsnetz des Bodensees zu analysieren. Wir zeigen, dass zwei Fischarten sich stark von der großen *Daphnia longispina* ernähren, aber weniger von der kleineren *D. cucullata*. Da *D. longispina* auch die stärkste Fähigkeit zur Kontrolle des Phytoplanktons zeigte, deuten diese Ergebnisse darauf hin, dass große Daphnienarten im Vergleich zu kleinen Daphnienarten eher als Schlüsselarten fungieren, die höhere und niedrigere trophische Ebenen im Nahrungsnetz des Sees miteinander verbinden.

Die folgenden drei Manuskripte analysieren verschiedene Aspekte der morphologischen und Lebenszyklus Veränderungen, die durch zwei invertebrate Räuber im Bodensee verursacht werden. Manuskript II zeigt, dass sich die morphologischen Reaktionen von *D. galeata* und ihre Kosten in Bezug auf die Kairomone von zwei invertebraten Räubern, *Bythotrephes longimanus* versus *Leptodora kindtii*, unterscheiden.

Manuskript III erweitert Manuskript II um zwei weitere Daphnienarten, *D. longispina* und *D. cucullata*, und um die Untersuchung von Mikroabwehrstrukturen. In diesem Kapitel wird gezeigt, dass die morphologische Räuberabwehr ebenfalls Daphnien-Arten-spezifisch ist und dass die Mikrostrukturen unabhängig von den üblicherweise untersuchten Makrostrukturen wie dem Schwanzstachel oder den Helmen ausgeprägt werden.

Manuskript IV konzentriert sich wieder auf *D. galeata* und verwendet den Ansatz der «resurrection ecology», um mikroevolutive Veränderungen in der Räuberabwehr während der Oligotrophierung des Bodensees zu untersuchen. Dieses Kapitel zeigt, dass sich die Morphologie der Verteidigungsstrukturen während der Oligotrophierung tatsächlich mikroevolutiv verändert hat, aber wir haben keine Belege für die Evolution von Plastizität gefunden haben. Schließlich wird in Manuskript V der «resurrection ecology» Ansatz von Manuskript IV erweitert, um die mikroevolutionäre Reaktion von *D. galeata* auf Veränderungen der Nahrungsmenge zu untersuchen. In diesem Manuskript konnte keine Mikroevolution von Filterkammdimensionen wie Filterflächen und Maschengrößen nachgewiesen werden. Dagegen fanden wir Hinweise auf die Evolution von phänotypischer Plastizität in Bezug auf den Zeitpunkt der Reproduktionsereignisse: bei niedriger, aber nicht bei hoher Nahrungskonzentration reproduzierten Klone aus der oligotrophen Periode des Sees später als Klone aus der eutrophen Periode. Die Kombination der Ergebnisse der letzten beiden Manuskripte zeigt, dass die Oligotrophierung zu mikroevolutiven Veränderungen bei Merkmalen führt, die sowohl das Wachstum als auch die Sterblichkeit von Daphnien beeinflussen.

Ecosystems are interconnected systems with a complex web of interactions between their various components. One of these interactions include predator-prey system in which one is a prey and the other is a predator that extends far beyond from a simple "to eat or being eaten". This dynamic interaction between prey and predator is continually evolving over time and generations (Schmitz 2017), and is heavily influenced by the environment in which they coexist. Consequently, any changes on one side of the interaction can lead to cascading effects impacting other trophic levels in the ecosystem. Multiple abiotic and biotic factors, as well as evolutionary dynamics, play crucial roles in determining the success of this type of interaction.

Some of the best studied examples of predator - prey interactions in aquatic ecosystems are those involving the cladoceran herbivore *Daphnia*, acting both as a predator and a prey. These organisms serve as vital links within the food web, exerting a significant influence on the connections between high and low trophic levels. Moreover, *Daphnia* are known for their capacity to minimize the impact of predation and enhance the effectiveness of grazing. Through phenotypic and evolutionary adaptation of morphological, life history and behavioural traits, they can successfully cope with top-down and bottom-up pressures in their environment. However, in dynamic ecosystems where natural or anthropogenic factors may alter the nutrient levels and trophic status of lakes, the response of *Daphnia* is not fully understood.

***Daphniids* as a keystone herbivore.**

Daphnia are keystone species. Keystone species are predatory or consumer species with disproportionately large impacts on food web structure (Valls et al. 2015). The criteria for identifying keystone species include assessing centrality, whereby species with the highest degree of centrality having the most significant interactions are considered potential keystone species (Jiang & Zhang 2015; Sun et al. 2020). Highly central species have a significant impact on the ecosystem by exerting top-down control (Cagua et al. 2019; Fan et al. 2023), where their numerous interactions and behaviours influence a wide range of species, both species found in the higher and lower of the food chain.

Keystone species are crucial for food web dynamics and may cause cascading trophic interactions, where the effect of predation extends beyond the direct prey species creating indirect impacts on lower trophic levels (Pagnucco et al. 2016). *Daphniids* are

generally considered keystone species in pelagic food webs (Sarnelle 2005) as large *Daphnia* abundances can have a significant impact on phytoplankton by controlling their abundance and community structure (Cuenca Cambroneró et al. 2018) and *Daphnia* is a preferred prey of invertebrate and vertebrate predators (Ogorelec et al. 2021).

The subgenus *Hyalodaphnia* : *Daphnia longispina* species complex

Within the genus *Daphnia*, three closely related and hybridizing species from the *Daphnia longispina* species complex, *Daphnia longispina*, *Daphnia galeata* and *Daphnia cucullata* are important members of the zooplankton in many European lakes (Seda et al. 2007; Dlouhá et al. 2010; Nickel et al. 2021). *D. longispina* is often found in nutrient poor large lakes, whereas *D. galeata* and the smaller-bodied *D. cucullata* is commonly found in more nutrient rich lakes (Seda et al. 2007; Ma et al. 2019). In Lake Constance, all three species currently coexist with *D. longispina* performing diel vertical migration during the summer month, and *D. galeata* and *D. cucullata* residing in the epilimnion (Nickel et al. 2021; Ogorelec et al. 2021).

***Daphnia* response to predation pressure**

Predation can strongly influence prey population abundance (Tessier et al. 1992) and size structure (Manca et al. 2008), and thus, will have strong fitness consequences for prey populations (Lind & Cresswell 2005). Consequently, prey populations have evolved various means to mitigate or evade predation pressure. Such anti-predator strategies may include life history, morphological, chemical, or behavioral adaptations (Smith et al. 2016; Rojas & Burdfield-Steel 2017). *Daphnia* is a well-studied species, which has been shown to adapt its morphological traits, life-history characteristics, and behavioural patterns to increasing of predation pressure (Boersma et al. 1998; Carter et al. 2013; Santangelo et al. 2018; Hasnain & Arnott 2019).

Adaptation to increased predation pressure can be achieved via phenotypic plasticity or through microevolutionary changes. Phenotypic plasticity commonly refers to the capacity of individual genotype to produce different phenotypes (Dzialowski et al. 2003; Miner et al. 2005; West-Eberhard 2008; Fox et al. 2019), thereby allowing individuals to have a higher fitness in a new environment and broaden their tolerance to rapid environmental changes (Ghalambor et al. 2007).

Daphnia antipredator defenses are key examples of phenotypic plasticity. In *Daphnia*, plastic responses are usually induced via chemical cues released by the

predators which are called kairomones (Miyakawa et al. 2013; Carter et al. 2017). Daphniids exhibit a wide range of plasticity e.g. morphological plasticity via size adjustment in body length (Weber & Vesela 2002; Tanner & Branstrator 2006), head and tail spine elongation (Lüning 1992; Dzialowski et al. 2003) and body extensions such as the head spine (Bungartz & Branstrator 2003) or neck teeth (Lüning 1992; Sperfeld et al. 2020). Likewise, life history changes can also be induced by predator kairomones, for example, age at first reproduction (Weider & Pijanowska 1993) clutch size (Gliwicz & Boavida 1996) and eggs number (Sakamoto et al. 2015). Furthermore, changes in behavior such as diel vertical migration can also be induced by predators (Stich & Lampert 1981; Pangle & Peacor 2006).

Daphnia responds differently to kairomones released by fish versus invertebrate predators (Boeing et al. 2006) due to differences in size selectivity between these two predators (Miehls et al. 2014). Fish usually select for larger *Daphnia* species and larger individuals within a species, whereas invertebrate predators often do have a higher predation rate on smaller species. However, there is also an evidence that *Daphnia* can respond specifically to different invertebrate predators (Laforsch & Tollrian 2004; Tanner & Branstrator 2006; Pietrzak et al. 2020; Ritschar et al. 2020).

Two closely related cladoceran predators

Bythotrepehes and *Leptodora* are important invertebrate predators of *Daphnia* and can have strong effects on planktonic food webs (Branstrator & Lehman 1991; Lehman & Cáceres 1993; Burkhardt & Lehman 1994). Both are typical components of the zooplankton communities of the deep peri-Alpine lakes in Europe (Molinero et al. 2007; Manca et al. 2008; Straile 2015; Horváth et al. 2017). In these lakes both predators show similar spatio-temporal patterns, i.e., they occur at rather similar water depths and show similar seasonal dynamics with peak abundances around the same time of the year (Molinero et al. 2007; Seebens et al. 2013). However, the defensive responses of *Daphnia* species in European peri-Alpine lakes between these two predator species is not known, and thus, need to be studied.

***Daphnia* response to changes in food availability and quality**

Similar to predation pressure, *Daphnia* has been shown to respond phenotypically and evolutionarily to changes in food availability and food quality. *Daphnia* are able to adapt on low food condition and adjust their morphology to optimize food gathering

(Ghadouani & Pinel-Alloul 2002; Bednarska 2006). Changes in the size and structure of *Daphnia*'s filter screen are an important response to low food availability (Brendelberger & Geller 1985; Pop 1991; Lampert 1994) in an aquatic environment with small-food-particle-size (Gophen & Geller 1984) or in an environment with high abundances of inedible or harmful algae (Ghadouani & Pinel-Alloul 2002; Bednarska & Dawidowicz 2007). To increase efficiency in food gathering *Daphnia* are able to modify its filter area and its filter setae in terms of its size, diameter and number, as well as its filter mesh size (Repka et al. 1999; Macháček & Seda 2016; Wejnerowski et al. 2017) during ontogenetic growth. Beside its morphological traits, *Daphnia* may also adjust the size of its neonates to food availability (Gliwicz & Guisande 1992). Furthermore, *Daphnia* has been demonstrated to adapt to increased content of toxic cyanobacteria (Isanta-Navarro et al. 2021).

Prey and predator relationship under trophic changes

The biological productivity of a lake is strongly influenced by nutrients, especially its phosphorus concentrations. Increases (eutrophication) or declines of (oligotrophication) of nutrient concentrations may strongly modify planktonic communities and food web interaction (Manca & Ruggiu 1998; Manca et al. 2000; Krol et al. 2019; Rose et al. 2021). Presumably, such changes will alter both food availability and predation pressure on *Daphnia*. Consequently, eutrophication and oligotrophication have changed the relative importance of the two *Daphnia* species, *D. longispina* and *D. galeata* (Straile 2015) and the genetic composition of *D. galeata* (Weider et al. 1997; Jankowski & Straile 2004; Brede et al. 2009). Furthermore, there is evidence that eutrophication resulted in microevolutionary changes of *D. galeata* during eutrophication and oligotrophication. For one, they evolved to reduce growth rate losses due to cyanobacteria during eutrophication (Hairston et al. 1999), but lost this adaptation again during oligotrophication (Isanta-Navarro et al. 2021). However, evolutionary responses to changes in food quantity or changes in predator pressure during eutrophication have not been studied yet.

Resurrection Ecology

Resting stages of organisms can be sampled from the sediment layers of known age, hatched in the laboratory, and can be used for common garden experiments. This research approach is used to study microevolutionary adaptation and is known as

“Resurrection ecology” (Kerfoot et al. 1999; Pauwels et al. 2014; Stoks et al. 2016). For *Daphnia*, this approach has already been used to study microevolutionary changes in growth rate declines due to cyanobacteria, phototactic behaviour and life history changes in response to fish predator pressure (Stoks et al. 2016), and critical thermal maxima in response to global warming (Yousey et al. 2018; Tachibana et al. 2019).

Knowledge gaps and research questions

Multiple *Daphnia* species frequently co-exist in time and space in aquatic environments. While *Daphnia* play keystone roles with substantial ecosystem impacts, it is not fully understood whether ecologically similar co-occurring species share identical functions in connecting trophic levels and enable energy transfer. Each *Daphnia* species in a community can have considerable interspecific differences that lead to varied control over primary producers (Barnett et al., 2007). Therefore, it is unclear whether every co-existing *Daphnia* should be considered a keystone species with similar cascading effects. Lake Constance provides a good opportunity to investigate the role of coexisting *Daphnia* species in the food web, given that it is occupied by several herbivorous zooplankton taxa including the *D. longispina* species complex (*D. longispina*, *D. galeata* and *D. cucullata*). Grouping these potential keystone taxa may mask specific role of each species, hence, evaluating individual species contributions is key to reveal crucial drivers of deep pelagic aquatic food webs.

Daphnia demonstrate well-documented capacity to express suitable specific antipredator defenses based on unique chemical signatures of predators' kairomones (Boeing et al. 2006; Reger et al. 2018; Tams et al. 2018). However, facing multi-predator environment while phenotypic plasticity is limited and costly, *Daphnia* might either evolve broader or flexible responses that work against multiple common predators (generalist), or very narrow defenses aimed at just the specific predator around (specialist) (Palaima & Spitze 2004). Measuring daphniids' antipredator responses to closely related and co-occurring cladoceran predators *Bythotrephes* and *Leptodora* from peri-Alpine lake has not been experimentally studied yet. Examining their varying defensive responses between the two related predators with different hunting techniques will elicit deeper understanding on how well *Daphnia* match defenses to their threats.

The defensive responses of *Daphnia* are reported to be specific to different predators and between *Daphnia* species (intraspecific). In nature, *Daphnia* thrive in a

multi-predator environment, and they also co-exist with other *Daphnia* species. Both *Bythotrephes* and *Leptodora* are gape-limited predators, hence, size matters in predation. The question still lingers whether a general response exists among *Daphnia* species against two closely related predators.

Trophic state is proven to regulate the planktonic community and drive evolutionary changes. *Daphnia* have developed strategies to adapt to stresses like predation and food limitation. However, it is difficult to distinguish whether the traits are a result of phenotypic plasticity or if it indicates possible microevolution from long-term trophic shifts. Laboratory experiments on testing interclonal response to predator and food pressure is commonly done, but studying using the ancient and recent clones of one species which experienced long trophic state shift is rare. Many questions still remain around whether trait differences seen between recent *Daphnia* clones and their ancestors from different trophic state are just random variation or if they are connected to oligotrophication. Comparing resurrected clones spanning across trophic gradients would help determine if consistent divergences in traits signify microevolutionary adaptations, providing insights into enduring impacts of shifting lake states.

Thesis outline and aims

The aim of my PhD thesis is to investigate the response of the cladoceran herbivore *Daphnia* to environmental cues. I focus on examining the morphological and life history changes induced by two selective pressures, predation and food availability. I have structured my thesis around five manuscripts that investigate different aspects of this topic through laboratory and mesocosm experiments, partially using a resurrection ecology approach.

Chapter I explores the multi-level cascading effect of fish predation in Lake Constance pelagic food web in a mesocosm experiment, with special regards to the two *Daphnia* species, *D. cucullata* and *D. longispina*. The remaining chapters examine the effects of top-down and bottom-up control on *Daphnia* traits from Lake Constance in laboratory experiments. **Chapter II** and **III** study the morphological and life history changes of *Daphnia spp.* to two invertebrate predators in order to test whether phenotypic responses are predator- and/or species-specific. Finally, **chapter IV** and **V** use a resurrection ecology approach to test whether *Daphnia galeata* in Lake Constance during oligotrophication evolved different phenotypic responses to predator kairomones

(**chapter IV**) as well as phenotypic and life history responses to changes in food availability (**chapter V**). A short overview of chapters **I - V** is given below.

Chapter I explores the relative importance of two *Daphnia* species, *D. longispina* and *D. cucullata*, for cascading interactions in a deep oligotrophic lake using a mesocosm experiment. I participated in conducting the mesocosm experiment in the field, preparing water samples for water quality analysis in the laboratory and measuring the body size of different zooplankton species. The results of the mesocosm experiment revealed that only large *Daphnia* species play an important role in linking trophic levels - with *D. longispina* being the keystone species exerting cascade effects in Lake Constance's pelagic food web. We published the manuscript titled "*Large daphniids are keystone species that link fish predation and phytoplankton in a trophic cascade*" in the journal *Fundamental and Applied Limnology* (<https://doi.org/10.1127/fal/2020/1344>).

Chapter II investigates the response of *D. galeata* clones from *Lake Constance* to invertebrate predators. The eight *D. galeata* clones were exposed to two cladoceran predators that are co-occurring in Lake Constance, *Bythotrephes longimanus* and *Leptodora kindtii*. We quantified five morphological traits - body size, spina length, head size, eye size, and body width. Additionally, two life history traits were examined: age at maturity and neonate numbers. Despite their relatedness, *Bythotrephes* and *Leptodora* kairomones induced predator-specific morphological and life history changes across the *D. galeata* clones. We published the manuscript *Morphological defences and defence-cost trade-offs in Daphnia in response to two co-occurring invertebrate predators* in the journal *Freshwater Biology* (<https://doi.org/10.1111/fwb.13888>).

Chapter III expands **chapter II** to investigate induced morphological defenses in the three members of the *Daphnia longispina* species complex from Lake Constance, *D. longispina*, *D. galeata* and *D. cucullata*. The results reveal species-specific antipredator phenotypes. Notably, *D. galeata* and *D. cucullata* formed distinctive helmets while only *D. galeata* and *D. longispina* developed microdefenses (smaller-scale structures). This demonstrates that the expression of micro- and macrodefenses across species was unrelated, highlighting the possible independent evolution of microstructures as defensive modules in *Daphnia*'s anti-predator strategies. We published the manuscript "*Antipredator responses of three Daphnia species within the D. longispina species complex to two invertebrate predators*" in the journal *Ecology and Evolution* (<https://doi.org/10.1002/ece3.10841>).

In **chapter IV and V**, I investigated the morphological and life history changes in *D. galeata* under top-down (predation) and bottom-up (food availability) controls during oligotrophication using a resurrection ecology approach. Thirty different *D. galeata* clones were hatched, of which 15 were isolated from sediment layers deposited prior to the mid-1990s (“eutrophic clones”) and 15 clones from sediment layers deposited after the mid-1990s (“oligotrophic clones”). The predation and food level experiments were conducted separately. The kairomone experiment (top-down control) results are discussed in **chapter IV**, while the food level experiment (bottom-up control) results are detailed in **chapter V**. Both chapters include discussions of morphological and life history responses across ontogenetic stages. The result in **chapter IV** revealed ontogenetic variation on anti-predator responses where *D. galeata* tend to be generalist in early stage and become specialist during the adulthood. The result in **chapter V** showed starvation leading to a smaller body size and delayed maturity. We further investigated the potential effect of oligotrophication on trait shifts over time. The goal was to investigate the possibility of microevolution occurring in *D. galeata* morphological and life history changes induced by kairomones and food resources. The possibility of microevolution was shown in the results of **chapter IV and V** where oligotrophication lead to the main morphology traits such as spina length and head length.

Large daphniids are keystone species that link fish predation and phytoplankton in a trophic cascade

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1.1. Abstract

Daphniids act as keystone species in lake ecosystems by controlling phytoplankton biomass and experiencing intense fish predation. However, the importance of single daphniid species as trophic links between phytoplankton and fish remains unclear, especially compared with other zooplankton taxa. To disentangle the role of individual zooplankton taxa in the food web of a large lake, we performed an in-situ mesocosm experiment with natural phytoplankton and zooplankton communities in three treatments with native, invasive or no fish predators, respectively. A large daphniid, *Daphnia longispina*, was the zooplankton most strongly predated by both fish species, and also had the highest top-down effects on phytoplankton. All other zooplankton taxa, including a small daphniid species, had minor roles in terms of both predation by fish and grazing on phytoplankton. We suggest that daphniid species with large body sizes can strongly link higher and lower trophic levels in lake food webs, and thus function as keystone species in trophic cascades from fish to phytoplankton.

Keywords: Cladocera, *Daphnia*, food chain, ecosystem dynamics, oligotrophic lake, pelagic, trophic cascading

1.2. Introduction

Understanding the ecological roles of individual species and their interactions within food webs is essential for the characterisation of ecosystem dynamics. This is particularly true for keystone species, which have a strong influence on ecosystem functioning (Cottee-Jones & Whittaker 2012). For example, predator-mediated reductions in the abundance of keystone prey species can have indirect effects on lower

trophic levels, resulting in trophic cascades (Carpenter et al. 1985; Moyle & Light 1996; Pagnucco et al. 2016). However, the influence of keystone prey species on food webs may change if the predation pressure they experience shifts, for example, due to the invasion of a new predator.

The development of strong trophic cascading effects depends on multiple factors, and disentangling top-down and bottom-up regulation within food webs may therefore be difficult. In general, bottom-up control is more pronounced at the base of food webs, whereas top-down control is stronger at higher trophic levels (Brett & Goldman 1997). Therefore, fish have a greater role in regulating zooplankton biomass and abundance than nutrients, but have less influence on phytoplankton (Post & McQueen 1987; Hansson et al. 2004; Vakkilainen et al. 2004). Multi-level cascades often occur in aquatic ecosystems (Jürgens 1994; Östman et al. 2016), especially when fish predation affects species with key roles in linking different trophic levels and transferring energy through food chains (Hansson et al. 2004).

Although zooplankton represent a link between primary producers and fish in freshwater ecosystems (Brooks & Dodson 1965; Hansson et al. 2007), not all zooplankters are keystone species that link trophic levels. Substantial differences in functional traits between species lead to their different effects on phytoplankton (Cyr & Curtis 1999; Sommer et al. 2003; Barnett et al. 2007), and conceptualising zooplankton as a homogeneous functional group thus masks species-specific roles in within food webs. Hence, studies considering the effects of total zooplankton biomass on phytoplankton have typically found no or only weak top-down effects (McQueen et al. 1989; Mehner et al. 2008). More pronounced cascading effects have been observed for zooplankton of the genus *Daphnia* (McQueen et al. 1986; 1989), which are considered as keystone taxa that link primary and tertiary production due to their effective grazing on a broad size spectrum of edible phytoplankton (Lampert 1987). As well as influencing phytoplankton from green food webs, *Daphnia* can cause complex cascading effects also in detritivore-based brown food webs by consuming microorganisms through non-selective grazing (Zöllner et al. 2003).

Body size is among the most important traits influencing both zooplankton filtration rates and therefore top-down control of lower trophic levels (Gianuca et al. 2016). Many species of the genus *Daphnia* are large zooplankters and are therefore keystone grazers with a high capacity to reduce phytoplankton biomass (Vakkilainen et

al. 2004). Larger-bodied daphniids can outcompete smaller species due to consuming wider range of phytoplankton, and the growth and reproduction of larger bodied daphniids is greater when phytoplankton densities are low (Gliwicz 1990a; 1990b). Body size also influences fish predation, because most fish are size-selective predators that prefer larger and less evasive prey (Brooks & Dodson 1965). *Daphnia* densities can be strongly reduced by specialised planktivorous fish, which typically leads to an increase in phytoplankton biomass (Williams & Moss 2003).

Although many studies have considered fish effects on zooplankton and consequently on phytoplankton (Helminen & Sarvala 1997; Bertolo et al. 2000; Williams & Moss 2003; Vakkilainen et al. 2004), the importance of individual zooplankton species within trophic cascades is still poorly understood, even for potential keystone species such as daphniids. The role of individual zooplankton species in trophic cascades is challenging to investigate within natural ecosystems because species-specific effects are difficult to isolate. Equally, small-scale ex-situ top-down experiments designed to compare the feeding impacts of individual species on phytoplankton (Adrian & Schneider-Olt 1999; Gianuca et al. 2016) lack a natural zooplankton community structure as well as predation by fish. In-situ mesocosm experiments, which allow examination of natural zooplankton communities exposed to natural predation, rarely focus on the effects of single zooplankton species on phytoplankton (Lynch 1979; Bertolo et al. 2000; Hansson et al. 2004). The role of individual zooplankton species in lake food webs thus remains unclear, especially in deeper pelagic and oligotrophic environments.

To investigate the trophic roles of individual zooplankton species, we performed a mesocosm experiment in an offshore area of Upper Lake Constance, central Europe. This area allowed us to represent the pelagic environment using natural phytoplankton and zooplankton communities and the two most abundant pelagic fish species, the native whitefish (*Coregonus wartmanni*; hereafter, whitefish), and the invasive three-spined stickleback (*Gasterosteus aculeatus*; hereafter, stickleback). Our aim was to determine which zooplankters function as keystone species that represent strong links between fish predation and phytoplankton biomass, and which zooplankters are susceptible to changes in top-down predation in natural environments. We tested four hypotheses: 1) smaller and more evasive zooplankton species and combined groups of multiple zooplankton species are less susceptible to fish predation than large daphniid species; 2) as specialised planktivores, native whitefish consume more zooplankton than sticklebacks, which are

generalist feeders; 3) large daphniids cause greater reductions in edible and total phytoplankton biovolumes than other zooplankton taxa; and 4) fish stimulate increases in phytoplankton biovolumes by decreasing large daphniid densities.

1.3. Methods

Study sites

Lake Constance is a large (536 km²), deep (maximum 251 m) pre-Alpine lake situated at the border of Germany, Switzerland and Austria. It consists of two basins, the Lower Lake Constance and the larger Upper Lake Constance, in which our study was performed. Upper Lake Constance experienced several decades of anthropogenic eutrophication during the 20th century with phosphorus concentrations peaking in 1979, but has since returned to an oligotrophic state due to restoration efforts (IGKB 2013; Schotzko 2018). The most abundant zooplankton taxa in Upper Lake Constance are cyclopoid copepods, the calanoid copepod *Eudiaptomus gracilis*, and the cladocerans *Diaphanosoma brachyurum*, *Bosmina* spp., *Daphnia longispina* (formerly known as *D. hyalina*), *Daphnia galeata*, *Leptodora kindtii* and *Bythotrephes longimanus* (Straile & Geller 1998). Recently, a small daphniid species, *Daphnia cucullata*, has been recorded at high densities (IGKB 2018, Isanta Navarro et al. 2019). The fish fauna comprises approx. 30 species, of which three are common in the pelagic zone: the native lake char (*Salvelinus umbla*), the whitefish and the stickleback. The latter is an invasive, non-native species that has occurred at high densities in the pelagic zone of Upper Lake Constance since 2012–2013 (Rösch et al. 2018; Eckmann & Engesser 2019), and in 2014 represented 96% of the total abundance and 28% of the biomass of pelagic fish (Alexander & Vonlanthen 2016).

Experimental design

The experiment was conducted in the Upper Guell bay of Upper Lake Constance between 27 June and 25 July 2019. It comprised 12 plastic cylindrical mesocosms made of transparent polyethylene, each with a 15-m depth, a 96-cm diameter and a 10 m³ volume. Mesocosms were heat-sealed at the base and open to the atmosphere above the water surface. Mesocosms were attached to pontoons, freely hanging in open water, with their bases approx. 2 m above the sediment. Each pontoon accommodated four mesocosms in a row at 25-cm intervals and in an east-west direction to prevent shading

by adjacent mesocosms. Each mesocosm was covered with a transparent acrylic glass plate, to protect it from bird excrement and other external disturbances.

On June 19, the mesocosms were filled with filtered ($< 300 \mu\text{m}$) lake water and then inoculated with lake zooplankton. Zooplankton were sampled using vertical net tows ($300 \mu\text{m}$) from a depth of 15 m to the surface, filtering the same volume of water as in the 12 mesocosms, to ensure inoculation of zooplankton at natural densities at these depths. Samples were filtered through a $630\text{-}\mu\text{m}$ sieve to remove predatory zooplankton such as *L. kindtii* and *B. longimanus* as well as fish larvae; this process also removed the largest adult daphniids and copepods. The sampled zooplankton volume was then divided into 12 equal parts and one part added to each mesocosm. Zooplankton were then left to acclimatise within the mesocosms for eight days, allowing conditions to stabilise and zooplankton to develop to adult sizes (Schwartz 1984; Peterson 2001). The experiment lasted four weeks, to provide enough time for phytoplankton and zooplankton reproduction, community development and response to experimental conditions (Riebesell et al. 2011). To enable characterisation of well-pronounced top-down effects, the experimental period coincided with the lake's phytoplankton spring peak, which occurred unusually late in the study year i.e. around 1 July (unpublished data).

Treatments were randomly assigned to mesocosms, ensuring that each pontoon had at least one replicate of each treatment and that the treatments were placed in different orders within the pontoons. After the acclimatisation period ended (i.e. day 0, 27 June), the first samples were collected from all mesocosms (see below), and predatory zooplankton and fish were then added to treatments. Due to the patchy distribution of larger predatory zooplankton species and their potentially important roles in pelagic food webs, 200 *L. kindtii* and 40 *B. longimanus* were counted and added separately to each mesocosm, representing $1/4$ and $1/5$ of their typical maximum Lake Constance densities, respectively. These densities were limited by unusually low numbers of these two species in the lake during the study period. A treatment containing only zooplankton (including predatory taxa) acted as the control.

Two fish treatments received either four sticklebacks (mean \pm SD, 0.31 ± 0.048 g) or four whitefish (0.32 ± 0.093 g). The body length of whitefish was generally greater than that of sticklebacks with the same biomass, although exact lengths were not measured. Total fish densities in mesocosms were 1.71 ± 0.096 g m^{-2} SD for sticklebacks and 1.77 ± 0.094 g m^{-2} SD for whitefish, representing natural densities in Lake Constance.

Because fish densities in the lake are difficult to estimate precisely, annual fishery yields of adult whitefish were used as a reference, with 2007–2016 yields corresponding to 0.58 g m⁻² (Kugler & Friedl 2018). Because adult whitefish dominate pelagic fish biomass (Alexander & Vonlanthen 2016) and because they are usually harvested soon after reaching their adult size (Eckmann & Rösch 1998), densities 2–3 times higher than annual whitefish yields might adequately represent actual densities in the lake.

Sampling and sample processing

Starting on day 0, abiotic parameters were measured and both phytoplankton and zooplankton samples were taken from mesocosms. Sampling frequency was different for each parameter. Twice a week, dissolved oxygen concentrations, pH and temperature were measured in each mesocosm and in the lake with a multiparameter probe (CTD-90 M; Sea&Sun Technology), and chlorophyll concentrations were measured with a fluorescence probe (bbe Moldaenke, TS 17-09). These abiotic measurements were taken by lowering probes from the surface to a depth of 12 m in each mesocosm, prior to disturbance of the water column during biotic sample collection. Zooplankton were sampled using vertical net hauls (16.5-cm opening, 100-cm length and 100- μ m mesh) from a depth of 12 m to the mesocosm surface, on days 0, 14, 21 and 28. Phytoplankton were sampled on days 0, 14 and 28 by performing a vertical tow with an integrated water sampler across the same depth range, which collected 2 L of water, from which 100-mL subsamples were taken for analysis.

Collected zooplankton samples were preserved at -20°C until processing. After thawing, samples were divided into aliquots of ≥ 300 zooplankters, identified to the lowest taxonomic resolution possible (order to species), counted, and values used to back-calculate densities of each taxon. Although hybrids of the three *Daphnia* species probably occurred in our samples (Isanta Navarro et al. 2019), we designated each as only one of three species based on its morphological features. All copepod life stages were included except nauplii, due to their inconsistent sampling by a 100- μ m mesh and identification difficulties. The five most abundant taxa were used for further analyses: *Bosmina* spp. (3.6 %), Cyclopoida (20.5 %), *E. gracilis* (10.1 %), *D. cucullata* (44.9 %) and *D. longispina* (20.8 %). In addition, *D. longispina* and *D. cucullata* were combined as a total daphniid fraction. All other zooplankton taxa (including *D. galeata*) represented

< 0.2 % of total zooplankton abundance and were therefore excluded from further analysis.

Phytoplankton samples were fixed in Lugol's acid solution + sodium acetate, identified to genus level using an inverted light microscope and counted following Utermöhl (1958). Biovolumes were calculated using taxon-specific body volumes established for Lake Constance (Pauli 1989; Kümmerlin & Bürgi 1989; Müller et al. 1991). Phytoplankton species were divided into two groups according to their edibility to large daphniids. Unicellular taxa < 40 µm in cell length were classified as edible, whereas those > 40 µm in cell length, colony-forming and mucilaginous species were classified as inedible (Lampert 1987); cell lengths were based on Rimet & Druart (2018).

Data analysis

Shapiro-Wilk tests confirmed that all zooplankton and phytoplankton data had normal distributions. Initial (day 0) total zooplankton densities were compared with a one-way ANOVA, to ensure comparability among treatments. To compare densities of different zooplankton species among treatments (hypothesis 1) including identification of differences between the whitefish and stickleback treatments (hypothesis 2), we performed a two-way repeated-measures (RM) ANOVA with treatment (control, whitefish, stickleback) as the between-subjects factor and day (0, 14, 21, 28) as the within-subjects factor. Mauchly's tests were used to test the RM ANOVA assumption of sphericity, with deviations from sphericity addressed using Greenhouse-Geisser corrections. Pairwise t-tests with Bonferroni corrections were used to identify differences between treatments on each day. Linear regression models (LM) were used to identify relationships between the densities of zooplankton taxa (as predictor variables) and total, edible and inedible fractions of phytoplankton biovolumes (as dependent variables; hypothesis 3). To test hypothesis 4, a second RM ANOVA and pairwise tests (as described above) were done to compare phytoplankton biovolumes in each treatment. All analyses were done in the statistical software R (R Core Team 2018), using the package *afex* (Singmann et al. 2020) to perform RM ANOVAs and Mauchly's tests, the basic R function *lm()* to run LM, and the package *ggplot2* (Wickham 2016) to plot graphics.

1.4. Results

Abiotic conditions were stable during the experiment: dissolved oxygen ranged from 10–12 mg L⁻¹ at the surface to 12–15 mg L⁻¹ at a depth of 12 m, pH varied between 8.5–9.5 at all depths, and temperatures ranged from 22–25°C at the surface to 10–12°C at 12 m.

Table 1. Results of two-way repeated-measures ANOVAs describing differences in zooplankton densities and total phytoplankton biovolumes among treatments, days and their interaction. Significant results ($p < 0.05$) are indicated in bold.

Group	Effect	df	F	p
<i>Bosmina</i> spp.	Treatment	2	1.19	0.367
	Day	3	5.36	0.025
	Treatment × day	6	0.96	0.462
Cyclopoida	Treatment	2	0.82	0.484
	Day	3	11.78	<0.001
	Treatment × day	6	1.25	0.336
<i>Daphnia cucullata</i>	Treatment	2	1.64	0.26
	Day	3	5.17	0.036
	Treatment × day	6	1.21	0.353
<i>Daphnia longispina</i>	Treatment	2	28.60	<0.001
	Day	3	2.46	0.133
	Treatment × day	6	3.84	0.037
<i>Eudiaptomus gracilis</i>	Treatment	2	2.48	0.164
	Day	3	9.70	0.03
	Treatment × day	6	2.06	0.152
Total daphniids	Treatment	2	11.39	0.006
	Day	3	1.28	0.306
	Treatment × day	6	1.96	0.184
Total zooplankton	Treatment	2	6.69	0.024
	Day	3	0.22	0.751
	Treatment × day	6	1.77	0.212
Total phytoplankton	Treatment	2	11.28	0.005
	Day	2	147.73	<0.001
	Treatment × day	4	8.546	0.006

Zooplankton densities were comparable in all treatments at the start of the experiment (i.e. on day 0; one-way ANOVA $F_{2,6} = 0.03$, $p = 0.969$). Total zooplankton and total daphniid densities differed among treatments but not days, and no interactions were identified between day and treatment (RM ANOVA, Table 1). Both total

zooplankton densities (pairwise t-test, $p = 0.012$) and total daphniid densities ($p = 0.004$) were higher in the control than in the whitefish treatment but not the stickleback treatment ($p > 0.05$). The large daphniid *D. longispina* was the only zooplankton taxon whose densities responded to fish, including a significant treatment effect and a significant treatment–day interaction (Table 1, Fig. 1): while *D. longispina* densities were comparable on all days in both fish treatments, densities increased over time in the control treatment (Table 1, Fig. 2). *Daphnia longispina* densities were lower in the whitefish treatment compared to the control treatment on days 14 (pairwise t-test, $p = 0.005$), 21 ($p = 0.012$) and 28 ($p = 0.009$). Densities of this taxon were also higher in the stickleback treatment compared to the control on days 21 ($p = 0.038$) and 28 ($p = 0.013$). Densities of all other zooplankton taxa changed over time but did not differ among treatments, and no interactions were identified between treatment and time (Table 1, Fig. 1). *Bosmina spp.* (pairwise t-test, $p = 0.013$) and *Cyclopoida* ($p < 0.001$) densities increased whereas *E. gracilis* and *D. cucullata* densities decreased ($p < 0.001$) between day 0 and day 28.

Total phytoplankton biovolumes were negatively related to *D. longispina* densities on both day 14 (LM; $p = 0.049$) and day 28 ($p = 0.01$), and also to total daphniid densities on day 28 ($p = 0.03$; Table 2). Increasing *D. longispina* densities were related to a decline in edible phytoplankton biovolumes on day 14 (LM, $R^2 = 0.41$, $p = 0.025$; Fig. 3a) and day 28 ($R^2 = 0.56$, $p = 0.005$; Fig. 3b), whereas biovolumes of the inedible fraction did not respond to *D. longispina* densities (Fig. 3c–d). Phytoplankton biovolumes were not related to densities of total zooplankton ($p = 0.07$) or of any other individual taxon ($p > 0.1$; Table 2).

Phytoplankton biovolumes differed between days and treatments and a significant interaction was observed between treatment and date (Table 1). In all treatments, total phytoplankton biovolumes were higher on day 14 compared to day 0 (pairwise t-test, $p < 0.001$) then declined between days 14 and 28 ($p < 0.001$). Compared to the control, biovolumes were higher in the whitefish treatment on days 14 (pairwise t-test, $p = 0.001$) and 28 ($p < 0.001$) and in the stickleback treatment on day 28 ($p < 0.001$; Fig. 4). Biovolumes were higher in the whitefish treatment compared to the stickleback treatment on day 14 ($p = 0.002$; Fig. 4).

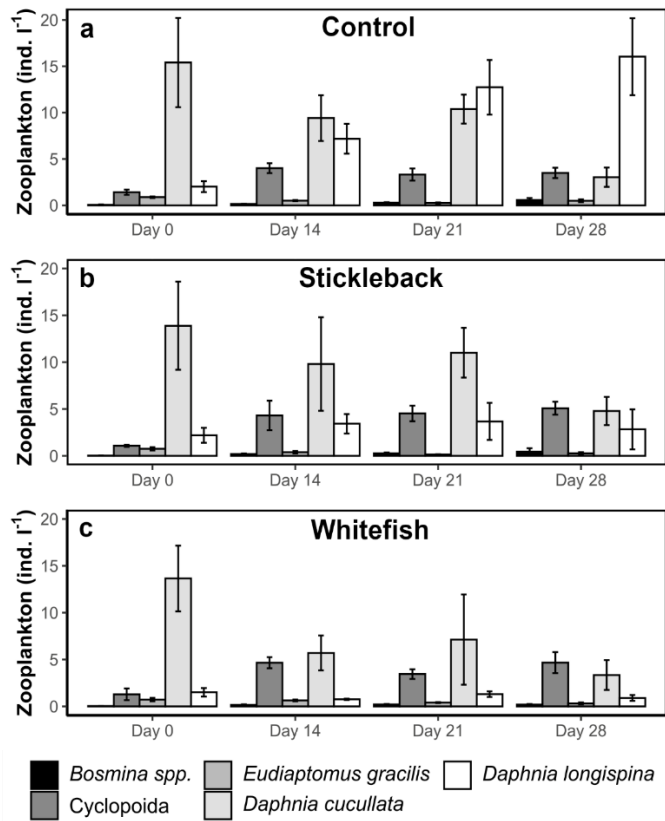


Figure 1. Mean (\pm 1 SD) zooplankton taxa densities (individuals [ind.] l⁻¹) in control (a), stickleback (b) and whitefish (c) treatments on each sampling day.

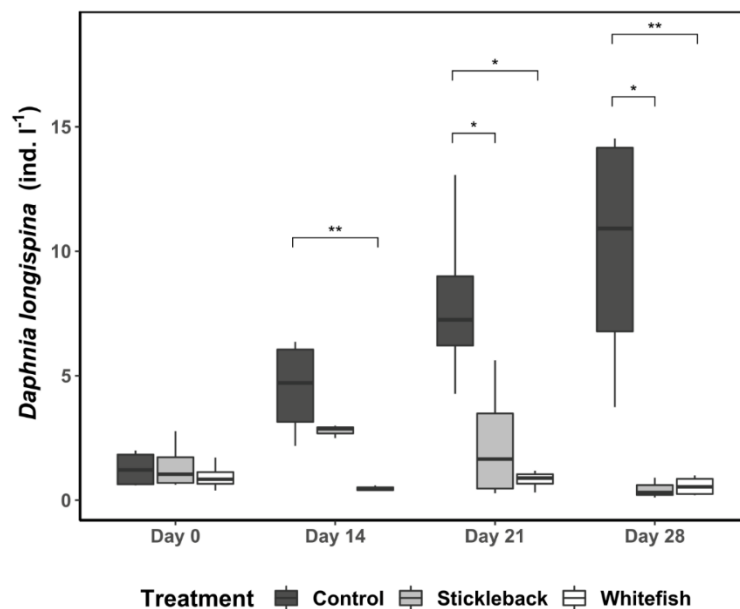


Figure 2. *Daphnia longispina* densities (individuals [ind.] l⁻¹) in each treatment on each sampling day. The results of the pairwise t-tests comparing treatments are indicated by brackets and asterisks (* p < 0.05, ** p < 0.01).

Phytoplankton biovolumes differed between days and treatments and a significant interaction was observed between treatment and date (Table 1). In all treatments, total phytoplankton biovolumes were higher on day 14 compared to day 0 (pairwise t-test, $p < 0.001$) then declined between days 14 and 28 ($p < 0.001$). Compared to the control, biovolumes were higher in the whitefish treatment on days 14 (pairwise t-test, $p = 0.001$) and 28 ($p < 0.001$) and in the stickleback treatment on day 28 ($p < 0.001$; Fig. 4). Biovolumes were higher in the whitefish treatment compared to the stickleback treatment on day 14 ($p = 0.002$; Fig. 4).

1.5. Discussion

Zooplankton of the genus *Daphnia* are typically described as keystone grazers that link primary production and higher trophic levels in lake ecosystems, but the roles of single daphniid species remain poorly known. Supporting our first hypothesis, densities of the largest-sized daphniid studied, *D. longispina*, decreased in response to fish predation. In contrast, densities of other zooplankters, including the small daphniid *D. cucullata*, did not decline. These findings demonstrate that considering the genus *Daphnia* as a keystone taxon is a generalisation that overlooks size-mediated differences between species. Whitefish and sticklebacks had comparable effects on zooplankton, which refutes our second hypothesis, that whitefish – as specialised planktivores – have a greater impact on zooplankton communities. Supporting our third hypothesis, phytoplankton biovolumes decreased mostly in response to *D. longispina* densities, likely their large body size enables filtration of a wider spectrum of phytoplankton taxa and also growth at lower phytoplankton densities (Gliwicz 1990b).

Therefore, evidence of cascading effects was seen as higher phytoplankton biovolumes in fish treatments, caused by fish-mediated decreases in the densities of large daphniids (Fig. 5), supporting our fourth hypothesis. Both fish treatments were linked to comparable increases in phytoplankton densities, which our results suggest were mediated by fish predation on zooplankton, in particular *D. longispina*. We thus suggest *D. longispina* regulated trophic cascade by mediating top down effects in pelagic food webs including fish with both specialist and generalist feeding modes.

Table 2. Linear model results describing relationships between total phytoplankton biovolumes and densities of different zooplankton taxa and groups. Significant results ($p < 0.05$) are indicated in bold.

Zooplankton taxon/group	Day 14		Day 28	
	R ²	p	R ²	p
<i>Bosmina</i> spp.	0.04	0.54	0.06	0.45
Cyclopoida	0.00	0.89	0.24	0.11
<i>Daphnia cucullata</i>	0.12	0.27	0.00	0.98
<i>Daphnia longispina</i>	0.33	0.049	0.51	0.01
<i>Eudiaptomus gracilis</i>	0.20	0.14	0.18	0.17
Total daphniids	0.14	0.23	0.39	0.03
Total zooplankton	0.12	0.28	0.28	0.07

Fish predation on zooplankton

Compared to the initial lake conditions, *D. longispina* densities increased only in the fishless control treatments reflecting release from vertebrate predation. Densities of other zooplankton taxa changed over time but not between treatments, suggesting that changes were not predation mediated. The increases in *Bosmina* spp. and cyclopoid *Copepods* might be seasonal, because comparable increases were observed in the lake during the study. In addition, cyclopoid copepod densities might still have been increasing following inoculation, since their densities were lower on day 0 than on any other date.

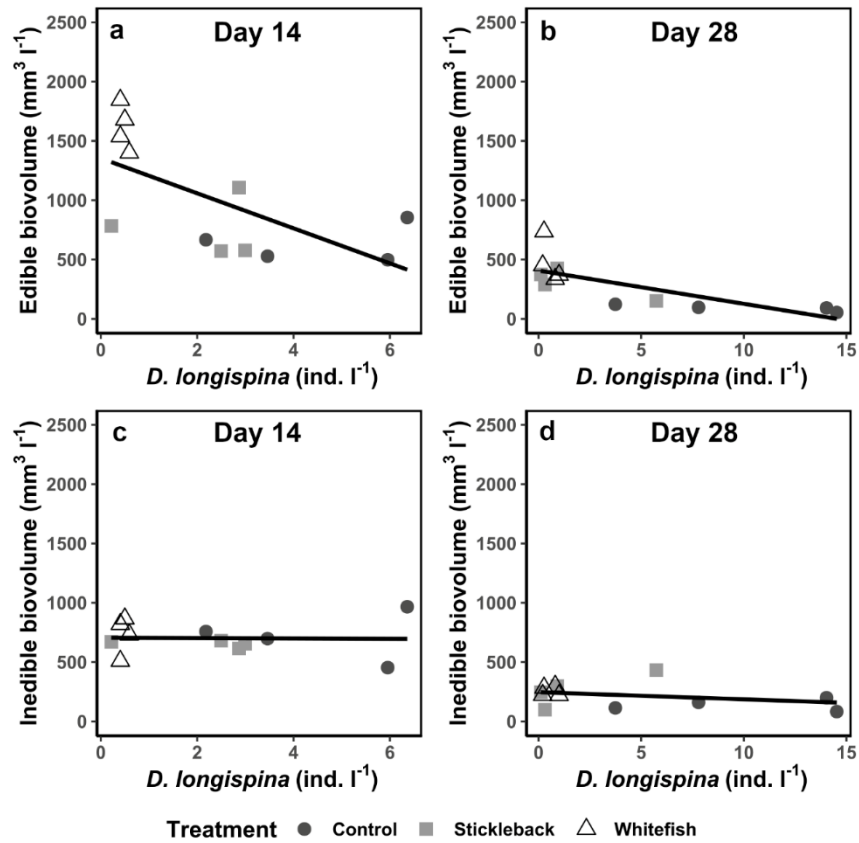


Figure 3. Linear model ($y = b \cdot x$) showing the relationship between *Daphnia longispina* densities (individuals [ind.] l⁻¹) and biovolumes (mm³ l⁻¹) of edible (< 40 μm) and inedible (> 40 μm) phytoplankton on day 14 (a, c) and day 28 (b, d).

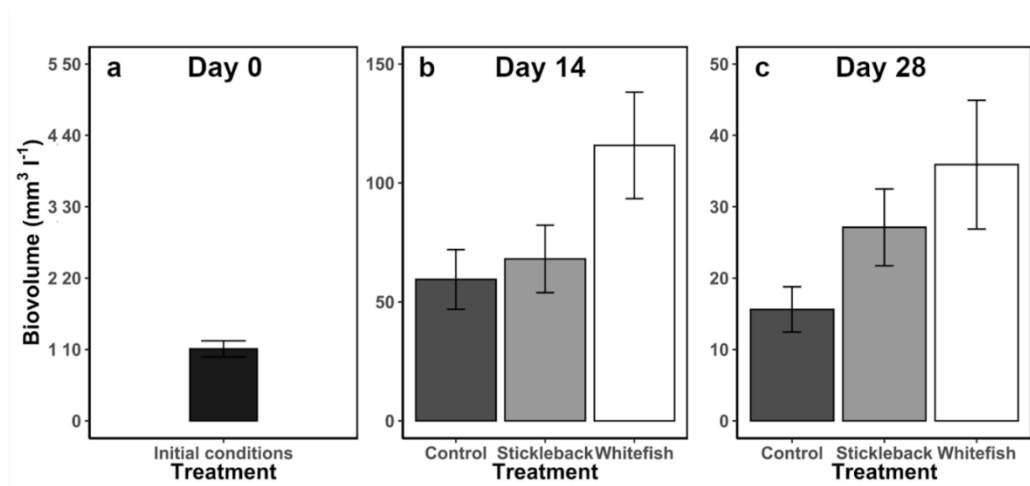


Figure 4. Mean \pm 1 SD phytoplankton biovolumes (mm³ l⁻¹) in control and fish treatments on sampling days 0 (a), 14 (b) and 28 (c). Identical phytoplankton biovolumes at day 0 are joined in one bar as initial conditions (a).

The drivers of temporal decreases in *D. cucullata* and *E. gracilis* are unclear but might reflect specific conditions within the closed mesocosm environment. Although densities of microcrustaceans other than large daphniids can be reduced when fish predation is high (Post & McQueen 1987; Bertolo et al. 2000), we did not observe such reductions, probably because fish densities were low, as is natural in oligotrophic Lake Constance. Mehner et al. (2008) also found no negative relationship between the biomass of planktivorous coregonid fish and zooplankton species other than *Daphnia* in an oligotrophic lake.

Various factors contribute to reduced fish predation on zooplankton other than large daphniids. The contrasting movement pattern of cyclopoid and calanoid copepods makes them harder to capture compared to cladocerans such as *Daphnia* (Visser 2007; Peterka & Matěna 2011). Furthermore, due to their small size, taxa such as the genus *Bosmina* are less conspicuous and therefore less predated than larger zooplankters (Brooks & Dodson 1965). Although fish predate small and evasive zooplankton, densities of some zooplankters can increase at low fish densities due to the removal of their competitors by selective fish predation on larger and less evasive species (Brooks & Dodson 1965; Christoffersen et al. 1993). Comparable densities of the small daphniid *D. cucullata* in control and fish treatments may reflect its small size and also its morphology: it is slimmer and therefore less conspicuous than *D. longispina* of the same body length (Gliwicz 2001). The reactive distance of roach for detecting *D. longispina* is double that for *D. cucullata* of the same body length, resulting in a near-tenfold larger reactive field volume and thus higher fish predation on *D. longispina* (Gliwicz 2001). The dominance of small or large-sized zooplankton communities can thus largely reflect predation pressure in lakes (Jeppesen et al. 1997; Iglesias et al. 2017; Ersoy et al. 2017), as indicated in our study by the dominance of *D. cucullata* and cyclopoid copepods in fish treatments and *D. longispina* in non-fish treatments.

The natural zooplankton community represented in our experiments included two invertebrate predators, *B. longimanus* and *L. kindtii*. Although fish probably influenced their densities, we suggest that invertebrate predators did not cause pronounced differences in prey zooplankton composition between treatments. Invertebrate predation on zooplankton is typically much lower compared to vertebrate (fish) predation (Gliwicz & Pijanowska 1989; Šorf et al. 2014). In addition, due to their low densities, larger invertebrate predators were rarely observed in our zooplankton samples, and densities of

smaller invertebrate predators (i.e. cyclopoid copepods) did not differ between treatments.

Cascading effects of fish species

Whitefish did not cause greater reductions in any zooplankton species compared to sticklebacks. However, cascading effects on phytoplankton via consumption of zooplankton (especially *D. longispina*) were more pronounced for whitefish than sticklebacks, as indicated by faster increases in phytoplankton biovolumes (i.e. by day 14). Whitefish are specialised zooplankton feeders with adaptations to pelagic environments whereas sticklebacks are generalist feeders (Morrow 1980; Becker & Eckmann 1992; Kottelat & Freyhof 2007), which probably enabled higher whitefish consumption of phytoplankton grazers. Although differences in zooplankton densities between fish treatments were not significant, moderately lower densities in the whitefish treatment might have been enough to cause significant differences in phytoplankton biovolume. Over time, differences in zooplankton densities and phytoplankton biovolumes between whitefish and sticklebacks became less pronounced, showing that within 28 days, sticklebacks and whitefish trigger similar trophic cascades. Other mesocosm experiments have indicated greater species-specific differences between fish, with specialised planktivores affecting zooplankton and consequently phytoplankton communities more strongly than facultative planktivorous fish, at least in shallow (<1 m) eutrophic littoral environments (Williams & Moss 2003; Des Roches et al. 2013). However, we are not aware of any previous studies with similarly low fish densities and/or sufficiently deep mesocosms to develop a thermocline and thus to represent cascading in pelagic and oligotrophic environments; these factors may explain the contrasting patterns observed in our study.

The effects of large daphniids on phytoplankton biomass

Many studies indicate that the genus *Daphnia* causes greater reductions in edible phytoplankton biovolumes than other zooplankton taxa (McQueen & Post 1988; Adrian & Schneider-Olt 1999; Mehner et al. 2008). In particular, McQueen et al. (1986; 1989) studied how body size influences the effects of *Daphnia* on phytoplankton biomass and linked large-sized daphniids to reduced biomass in mesoeutrophic and eutrophic environments. Our results build on such research by comparing the effects of different-

sized daphniids and suggest that larger daphniids, such as *D. longispina*, reduce phytoplankton biovolumes in natural lake communities.

Although other zooplankton species, such as copepods, can have complementary effect to daphniids by grazing on different-sized phytoplankton species, thus contributing to greater overall reductions in biomass (Sommer et al. 2001; 2003), they did not alter phytoplankton biovolumes at the natural densities represented in our communities. Total zooplankton densities had no significant effect on phytoplankton biovolume, due to the pronounced declines caused by *D. longispina* being offset by the limited effects of other, smaller taxa. Equally, the linear relationship between densities of the genus *Daphnia* and phytoplankton biovolumes on day 28 reflected only the pronounced effect of *D. longispina*, whereas *D. cucullata* had no effect on biovolumes. These results indicate that previous research may have overstated the importance of small *Daphnia* species, if genus-level responses are caused solely by larger, co-occurring congeners. Larger *D. longispina* also have higher potential than *D. cucullata* to increase their population densities due to higher birth and biomass production rates (Vijverberg & Richter 1982). This greater fecundity might have contributed to increasing *D. longispina* dominance in our fishless mesocosms, where increasing densities enabled greater control of phytoplankton.

Our results indicate that *D. longispina* was a keystone species that linked both planktivorous and generalist fish predators to phytoplankton communities (Fig. 5). No other zooplankton or daphniid taxa or groups were so greatly predated by fish or showed such strong phytoplankton control, and multi-taxon groups therefore formed much weaker cascading links between fish and primary producers. Although smaller zooplankton species can also contribute to trophic cascades from fish to phytoplankton if large cladocerans are absent (Christoffersen et al. 1993; Helminen & Sarvala 1997), most studies indicate that the genus *Daphnia* – if present – is the taxon most negatively affected by fish predation, and has also the strongest effects on phytoplankton densities (Post & McQueen 1987; Mehner et al. 2008). However, variability in the roles of different *Daphnia* species in trophic cascades remains poorly studied.

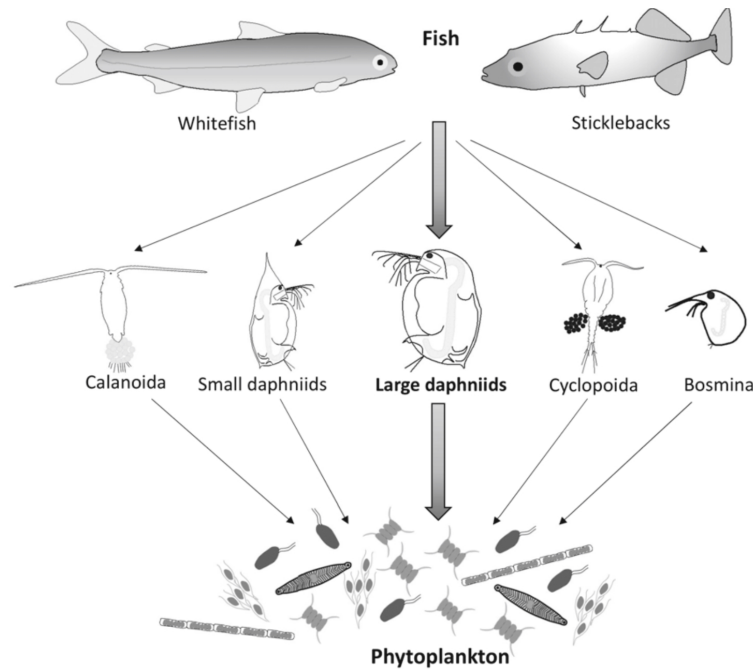


Figure 5. A conceptual model of trophic cascading from fish (represented by a stickleback and a whitefish) and primary producers (phytoplankton) within a pelagic ecosystem. Thick arrows represent reductions in densities at lower trophic levels. Of all zooplankton taxa, only densities of the large daphniid *were* reduced by fish predation and thus cause declines in phytoplankton biovolumes, indicating their role as keystone species.

Although fish can also have a positive effect on phytoplankton growth by remineralising nutrients, this effect is mostly limited to non-edible phytoplankton, whereas edible phytoplankton is more affected by zooplankton grazing (Vanni 2002). In our study, biovolumes of non-edible phytoplankton did not differ among treatments, whereas edible phytoplankton biovolumes decreased in response to *D. longispina* densities. These results suggest that phytoplankton biovolumes were strongly subjected to direct zooplankton (especially *D. longispina*) grazing, whereas fish effects such as remineralisation had a minor role.

Our results provide evidence that zooplankton taxa – including small *D. cucullate* might have a more limited influence on phytoplankton biomass in large oligotrophic lakes than larger *D. longispina*. Global warming is altering pelagic food webs by shrinking animal body sizes and shifting community compositions towards a higher proportion of small-sized species and individuals (Daufresne et al. 2009; Geerts et al. 2015). As such, increasing temperatures that cause intraspecific or interspecific decreases in zooplankton

body sizes could alter the functioning of green and brown food webs in oligotrophic freshwaters. Indeed, *D. cucullata* which was rarely observed in Upper Lake Constance before 2015, became a summer-dominant cladoceran in 2017 (IGKB 2018). How recent and ongoing changes in *Daphnia* composition are altering ecosystem dynamics is unknown. Further research is needed to determine how body size and other functional traits interact to determine the roles of different daphniid species in trophic cascading and energy transfer from phytoplankton to fish.

In conclusion our study advances our understanding of species-specific roles of zooplankton in food webs and trophic cascades in oligotrophic pelagic environments. We demonstrate that considering both zooplankton and daphniids as uniform taxonomic groups, as it is common praxis, might limit understanding of links between multiple trophic levels in aquatic ecosystems. We suggest that the genus *Daphnia* is not a taxon, but rather that large *Daphnia* species such as *D. longispina* can control food web structure at trophic levels from phytoplankton to fish. Our observation of comparable top-down predation on *D. longispina* by fish with specialist and generalist feeding modes suggests that the keystone role of large daphniids in oligotrophic lake food webs could extend across ecosystems with different fish predation regimes.

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Morphological defences and defence–cost trade-offs in *Daphnia* in response to two co-occurring invertebrate predators

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2.1. Abstract

1. Inducible morphological defenses are crucial for understanding predator-prey interactions. Such defenses have been mostly studied in a single-predator context, ignoring the fact that prey organisms are often exposed to multiple predators. In deep peri-Alpine European lakes, the keystone grazer *Daphnia* coexists with two cladoceran predators, *Bythotrephes longimanus* and *Leptodora kindtii*. Up to now, life history and morphological responses of *Daphnia* to these two predators have not been analysed systematically.
2. We studied the responses of two life history (age at first reproduction, and offspring production) and five morphological traits (body size, body width, head size, spina size, and eye diameter) of eight *Daphnia galeata* clones to the presence of *Bythotrephes* and *Leptodora* in a common garden experiment. We compared each response trait between treatments using linear mixed models, and investigated the covariation between defense traits and demographic costs (neonate production) for the two predators.
- 3: Our results show that the responses of *Daphnia* are predator- and trait-specific. *Daphnia* developed a typical helmet and a larger eye only in the presence of *Bythotrephes*, not in the presence of *Leptodora*. In contrast, both predators induced larger body sizes and longer spines. Age at first reproduction was latest and demographic costs were highest in the *Bythotrephes* treatment, suggesting that the development of a helmet exceeds the costs of spina elongation. The responses of *Daphnia* clones revealed a defense-cost trade-off for helmet formation in the *Bythotrephes*, and for spina elongation in the *Leptodora* treatment. Hence, despite *Bythotrephes* and *Leptodora* are closely related co-occurring predators, *Daphnia*

responds with a unique combination of trait changes and defense-cost trade-offs to the two predators.

4. The presence of predator-specific clonal defense-cost trade-offs suggests that the presence of these invertebrate predators can drive different evolutionary processes in natural zooplankton communities. Disentangling the evolutionary ecology of phenotypic responses of prey species to co-occurring predators will require multi-trait, multi-clone studies of induced antipredator defenses.

Key words: allocation costs, life history traits, morphological defences, multi-predator environment, clonal variability

2.2. Introduction

Induced prey defences are important components of predator prey interactions (Verschoor *et al.* 2004; Boeing & Ramcharan 2010) and may have ecosystem consequences exceeding the direct effects of predation (Miner *et al.* 2005). Inducible prey defences include morphological (Tanner & Branstrator 2006; Sperfeld *et al.* 2020), behavioural (Pangle & Peacor 2006), and life history responses (Weider & Pijanowska 1993; Sakamoto *et al.* 2015).

Prey species are typically confronted with various predators in their natural environment. Consequently, prey species evolved the ability to differentiate between predators and to show predator-specific defences (Crane *et al.* 2016; Diel *et al.* 2020; Dunn & Hovel 2020). Crustaceans of the genus *Daphnia* respond differently to vertebrate and invertebrate predators (Boeing *et al.* 2006b; Gélinas *et al.* 2007; Engel *et al.* 2014) and can differentiate even between various invertebrate predators (Laforsch & Tollrian 2004; Tanner & Branstrator 2006). Most studies conducted so far did investigate predator-specific responses by comparing *Daphnia* from different habitats in which one type or species of predator dominates, e.g., ponds with and without fish or ponds with and without larvae of the phantom midge *Chaoborus* (Boersma *et al.* 1998; Reger *et al.* 2018). However, prey is likely to face co-occurring predators, and the response of *Daphnia* is proposed to depend on whether predators are functionally equivalent, functionally inverse, or functionally diverse (Herzog & Laforsch 2013).

Predators can induce several morphological defenses in *Daphnia*, including various forms of helmets (Bungartz & Branstrator 2003), lengthened spina (Dzialowski *et al.* 2003), neck teeth (Lüning 1992; Sperfeld *et al.* 2020), and thickened carapax (Rabus

et al. 2013). These responses are *Daphnia* species-specific and partially also predator-specific, i.e., depend on predator modality. Hence, when faced simultaneously with functionally inverse or diverse predators, specific morphological traits or combinations of these traits might be selected for.

In *Daphnia*, reproduction is characterized by an environmentally triggered shift between asexual and sexual reproduction (cyclical parthenogenesis). During most parts of the season, *Daphnia* reproduce parthenogenetically and *Daphnia* populations are thus mainly composed of clones of asexually produced individuals. These *Daphnia* clones can differ in their responses to abiotic (Barber et al. 1990; Palaima & Spitze 2004; Connelly et al. 2016), and biotic pressures (Hairston et al. 2001; Isanta-Navarro et al. 2021). Likewise, there is evidence that *Daphnia* clones differ quantitatively and qualitatively in their anti-predator responses (Boeing et al. 2006b; Reger et al. 2018; Tams et al. 2018). Hence, when facing multiple co-occurring and functionally inverse or diverse predators, *Daphnia* may either evolve toward a capacity to respond to all predators (“jack-of-all-trades” Palaima & Spitze 2004), using varying combinations of different defensive traits, or toward a specific response to the most prevalent predator.

The cladocerans *Bythotrephes longimanus* and *Leptodora kindtii* are important predators of zooplankton, including *Daphnia* spp. (Wojtal et al. 2004; Manca et al. 2008). They co-occur in many lakes in Central and Northern Europe (Hessen *et al.* 2011; Horváth et al. 2017) and more recently, since the invasion of *Bythotrephes*, also in North America (Cavaletto *et al.* 2010). Both are typical components of the zooplankton communities of the deep pre-Alpine lakes in Europe (Molinero et al. 2007; Manca et al. 2008; Straile 2015; Horváth et al. 2017). In these lakes both predators show similar spatio-temporal patterns, i.e., they occur at rather similar water depths and show similar seasonal dynamics with peak abundances around the same time of the year (Molinero et al. 2007; Seebens et al. 2012).

Bythotrephes and *Leptodora* belong to sister suborders within the order Diplostraca and are closely related (Richter et al. 2001). Both species are cruising predators of roughly similar size, suggesting that they select for a similar suite of defensive traits in their prey. However, both species differ in how they locate and catch their prey (Manca et al. 2008). *Bythotrephes* locates its prey either via mechanoreceptors or visually and then captures its prey using long feeding appendages (Schulz & Yurista 1998; Manca et al. 2008). In contrast, *Leptodora* requires direct contact with its prey to

initiate an attack (Branstrator 1998; Manca et al. 2008) and employs a feeding basket to catch its prey. The latter limits the maximum prey size for *Leptodora*. Hence, *Leptodora* feeding might be more size-limited compared to *Bythotrephes* feeding, and defences aimed at increasing body size might be more efficient against *Leptodora* predation. In contrast, *Daphnia* defences aimed at reducing visibility are expected to be successful only against the visual predator *Bythotrephes*. Taken together, this implies that both predators are functionally diverse (*sensu* Herzog & Laforsch 2013), but this has not been studied experimentally yet.

Here, we explored life history (age at first reproduction (AFR) and number of offspring) and morphological responses (head size, spina size, body width, and eye diameter, see Fig. 1) of eight clones of *D. galeata* to the presence of *Bythotrephes* and *Leptodora*. We analysed whether both predators are functionally diverse, that is whether *Daphnia* responded differently to the two predators and whether the expression of defences to both predators did entail demographic costs, i.e., reduced offspring production. Finally, we pro-posed that there is clonal variability in response to predators, as well as in the demographic costs of the responses.

2.3. Methods

Study Design

We tested the response of eight *Daphnia galeata* clones (A-H) to predator kairomones. The clones were hatched from ephippia, which were isolated from the upper 20 cm of a sediment core taken from Lake Constance. All clones were already cultured in the laboratory for several months prior to the experiment. The experiment consisted of three treatments, namely control (C), *Bythotrephes* (B), and *Leptodora* (L) with four replicates each containing six daphniids. Mortality of *Daphnia* (38 %) caused the loss of 4 experimental units (two in the L treatment (clones G and H), one in B (clone H) and one in the C treatment (clone C) resulting in a total of $(8 \times 3 \times 4) - 4 = 92$ experimental units. Each experimental unit was composed of a 200 mL beaker filled with 180 mL filtered ($< 0.2 \mu\text{m}$) lake water. All beakers contained a cylindrical plastic cage (4 cm diameter and 6 cm height) with a 140- μm nylon mesh that ensured separation between predators and *D. galeata* while guaranteeing the exchange of kairomones. While we cannot exclude the possibility that daphniids might also be able to see the predators using this design, we consider this highly unlikely. Furthermore, other experiments (Baludo et

al. personal communication.) have shown that *D. galeata* will also respond to kairomones of these two predators without the possible presence of visual cues. On the first day of the experiment, five individuals of *Bythotrephes* and *Leptodora* were placed in the plastic cages (B and L treatment), respectively; control (C) cages were left empty. Daphniids were fed daily with 1 mg C L⁻¹ of the green alga *Scenedesmus obliquus* (Culture Collection of Algae, University of Göttingen, Germany, SAG 276-3a), which was grown semi-continuously at 20 °C in 5-L batch cultures in Cyano medium (Jüttner et al. 1983) and harvest in the late-exponential growth phase (illumination at 120 μmol quanta m⁻² s⁻¹). The animals were transferred into new jars containing freshly prepared food and cages with freshly collected living predators every other day. Prior to each transfer, predators were isolated from Lake Constance zooplankton samples taken with net hauls towed behind a boat.

The experiment started with 1-day-old daphniids and was terminated when the daphniids reached age 10 days. At this age, daphniids of all clones and in all treatments had produced neonates in at least one replicate. For logistical reasons, the experiment was split into two blocks (hereafter: temporal blocks) that were run separately (4 weeks apart), with four clones in each block. Block I contained clones A, D, E, and F, and block II contained clones B, C, G, and H.

Life history and body size measurements

Neonates were observed in 91 out of 92 replicates (beakers) when the experiment was terminated (i.e., at day 10 of the experiment). Beakers were daily checked for neonates and the day neonates were first observed in a beaker was noted as AFR of daphniids in this beaker. For statistical analyses, AFR was assumed to be 11 days in the replicate without neonates, as embryos were already present in the brood chambers of the daphniids. To account for mortality during the experiment, reproductive output during the 10 days of the experiment was calculated as per capita total number of neonates. At the end of the experiment, daphniids were stored in 70% ethanol (Black & Dodson 1990) and subsequently examined using a Stemi 2000- C binocular equipped with a camera and an image analysing software. Body size, body width, head size, spina size, and eye diameter were measured as shown and described in Figure 1.

Data Analysis

Only *Daphnia* were used for morphological measurements, which allowed quantification of all morphological traits. This required the exclusion of some *Daphnia* that were damaged and with broken spina (i.e., for which spina size was less than the 95% prediction interval based on their body size). This reduced the data set from 359 to 337 daphniids; on average 3.5 daphniids per replicate were measured. Statistical analyses were run with trait averages within each replicate. Variation in body size, head size, spina size, body width, eye diameter, AFR, and per capita number of neonates was analysed using linear mixed-effect models (lmer function in lmerTest package) (Kuznetsova et al. 2017). Body size (except for models with body size and AFR as dependent variables) and treatment (*Bythotrephes*, Control, and *Leptodora*) were considered as fixed effects and clonal identity nested in temporal block as random effect. We used the step function in lmerTest to eliminate non-significant fixed and random effects from the full model, considering interaction between the fixed effects, as well as random intercepts (mean clonal effects) and random slopes (mean clonal responses to treatments). Likelihood ratio tests were used to test for the significance of fixed and random effects (ANOVA and RANOVA functions) (Kuznetsova et al. 2017). If significant treatment effects were observed, we used the emmeans package (Lenth 2020) to perform pairwise post hoc comparisons (Tukey test). In case models proposed by the step function resulted in singular fits, that is random effects explained zero or small variance, models were further simplified via removing temporal block as a random factor (Matuschek et al. 2017). In those cases, predictions of models without temporal block yielded identical predictions to models that included temporal block as a random factor. Residuals were checked for normality and heteroscedasticity. Relationships between trait expressions and offspring per *Daphnia* were analysed using clonal means and linear regression. All statistical analyses were performed in R v 4.02.0 (R Core Team 2020).

2.4. Result

Daphnia in the *Bythotrephes* treatment reproduced later than in the control ($t = 3.1$, $p < 0.001$) whereas *Daphnia* in the *Leptodora* treatment differed neither from the *Bythotrephes* treatment ($t = 2.2$, ns) nor from the control (Fig. 2a). Both predator treatments resulted in a larger *Daphnia* body size in comparison to the control (Tukey test, $p < 0.005$), but body sizes did not differ between predators (Tukey test, $p > 0.05$, Figure 2b). Body sizes were larger in block I than in block II ($p < 0.005$) and showed a

tendency for clonal variability nested in blocks ($p < 0.1$, Table 1). Across all treatments later AFR was associated with larger body size at the age of 10 days (fixed effect of age, $t = 2.2$, $p < 0.05$).

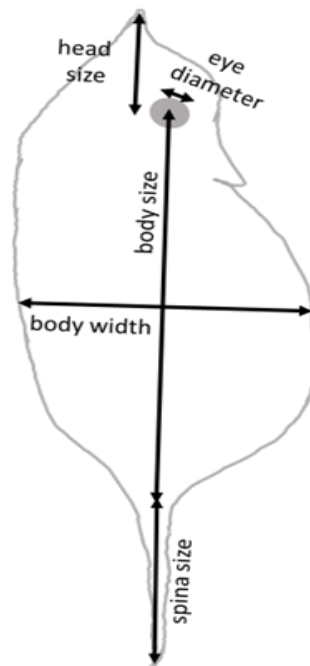


Figure 1. Body dimensions of *D. galeata*. Body size was measured from the base of the spina until the mid of the eye and described the body axis of the *Daphnia*. Spina size was measured from the top until the base of the of the spina. Body width was measured perpendicular to the body axis at the midpoint of body size. Head size was measured in parallel to the body axis from the top of the head (or top of the helmet) until the line perpendicular to the body axis crossing the midpoint of the eye. Eye diameter was measured at the largest dimension of the eye.

Trait specific analyses showed that all traits except head size were significantly influenced by body size (Fig. 3), although no significant treatment \times body size interaction was observed for any traits. Per capita number of offspring at the age of 10 days was lower in the *Bythotrephes* treatment as compared to the *Leptodora* ($t = -6.7$, $p < 0.0001$) and control ($t = -5.8$, $p < 0.0001$) treatments (Figs. 3,4). Head size was significantly larger in the *Bythotrephes* treatment than in the *Leptodora* treatment and in the control (Figs. 3,4). In contrast body width was related to body size (Fig. 3) but did not differ between treatments (Figs. 3,4). Spina size and eye diameter increased with increasing body size (Figs. 2, 3) and differed between treatments (Table 1). Spina size in both predator treatments was larger than in the control treatment (Tukey test, $p < 0.01$). Eye diameter in the *Bythotrephes* treatment was larger than in the control treatment (Tukey test, $p < 0.01$).

and tended to be larger in the *Bythotrephes* treatment than in the *Leptodora* treatment (Tukey test, $p < 0.1$).

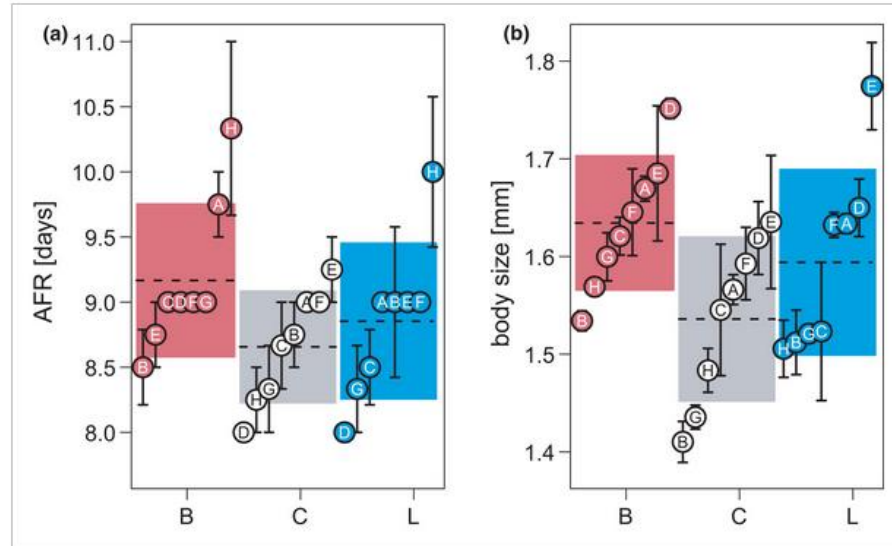


Figure 2. (a) Age at first reproduction (AFR) and (b) body size at the age of 10 days (means \pm 1 SE) of eight *Daphnia* clones grown in the *Bythotrephes* (B, red), control (C, grey), and *Leptodora* (L, blue) treatments. Clones F, A, E, and D were grown in block I and clones B, G, H, and C were grown in block II. Clones are arranged with increasing AFR or body size, respectively, within each treatment

Models for all traits revealed significant clonal variability (random intercepts) either nested in temporal block (body size, body width, and offspring per *Daphnia*) or un-nested (AFR, head size, spina size and eye diameter; Table 1). Random block effects contributed to the best model for body size, offspring per *Daphnia* and body width only (Table 1), but in the case of offspring per *Daphnia* the temporal block effect was not significant. Random slope effects (i.e., clone specific treatment effects), did not significantly contribute to models of any trait variable. Treatment responses of individual clones were most homogenous in respect to head size (Fig. 4a). For other traits, treatment effects were evident only after adjusting for body size differences between replicates (Fig. 4, middle panel), or after adjusting for body size and clonal differences (Fig. 4, lower panel). The larger head size of *Daphnia* in the *Bythotrephes* treatment (Fig. 4a) resulted from the formation of helmets in this treatment, whereas no helmets were formed in the two other treatments.

The mean number of offspring produced per clone across treatments was significantly negatively related to AFR ($t = -4.7$, $p < 0.0001$), head size ($t = -4.3$, $p < 0.0001$), and spina size ($t = -2.7$, $p < 0.05$), but not to eye diameter ($t = -1.1$, ns; Fig. 5). Furthermore, there was a significant negative relationship between neonates per *Daphnia* and head size within the *Bythotrephes* treatment ($t = -2.8$, $p < 0.05$, Fig. 5b) and a negative relationship between neonates per *Daphnia* spina size in the *Leptodora* treatment ($t = -2.5$, $p < 0.05$, Fig. 5c). Within the *Bythotrephes* treatment, AFR was not significantly related to neonates per *Daphnia* ($t = -1.96$, ns), whereas within the *Leptodora* treatment AFR was significantly related to neonates per *Daphnia* ($t = -2.6$, $p < 0.05$).

2.5. Discussion

Our experiments revealed that *D. galeata* is responsive to kairomones from both predators, *B. longimanus* and *L. kindtii*. However, the responses differed in the traits that were affected and the overall demographic costs of defences. *Daphnia galeata* clones developed larger body sizes and spines when exposed to both predators, whereas increased head sizes, due to helmet formation, and increased eye diameters were induced only by *Bythotrephes*. Demographic costs (i.e., a reduction in per capita number of offspring), were observed only in response to *Bythotrephes*, not in response to *Leptodora*. This implies that the two predators induce different responses in *Daphnia* and thus should be considered distinct predators when assessing inducible defences in zooplankton.

Kairomones from both predators induced a larger body size in *D. galeata*, which is in line with the typical response pattern of *Daphnia* to invertebrate predators (Diel et al. 2020). As all morphological traits, except head size, scaled positively with body size, predator treatments increased all body dimensions. However, body size-adjusted responses varied between predator treatments and between traits considered. Typical helmets and larger eyes were observed only in the *Bythotrephes* treatment, whereas longer spines were found in both predator treatments.

The most obvious difference in *D. galeata* antipredator response was the formation of a typical helmet, which was observed only in the *Bythotrephes* treatment. *Bythotrephes* as well as *Leptodora* have been shown previously to induce helmets or larger heads in other *Daphnia* species: *Bythotrephes* has been shown to induce helmets in *Daphnia mendotae* (Bungartz & Branstrator 2003) and *Leptodora* has been shown to induce larger heads in *D. mendotae* (Tanner & Branstrator 2006) and *D. cucullata*

(Laforsch & Tollrian 2004). However, no previous study has compared the responses to both predators using a single *Daphnia* species. The differences in helmet induction in *D. galeata* between *Leptodora* and *Bythotrephes* that were shown here can potentially be explained by *Leptodora* being able to prey upon a lower maximum prey size, which is determined by the size of its feeding basket. Hence, growing a helmet may be more effective against *Bythotrephes* predation than against *Leptodora* predation. We cannot exclude that *Leptodora* exposure would have resulted in a stronger head/helmet size response in sub-sequent *D. galeata* generations (transgenerational effects) that have not been explored here experimentally. However, morphological defence responses are typically rather quick (Weiss et al. 2015), and experiments with *D. mendotae* have shown that a larger helmet can be induced as early as in the first generation after kairomone exposure (Tanner & Branstrator 2006).

Kairomones of a large variety of predators, including fish (Spaak & Boersma 1997), *Chaoborus* (Lüning 1995; Sell 2000; Sperfeld et al. 2020), *Copepods* (Caramujo & Boavida 2000), and *Notonecta* (Kruppert et al. 2017), have been shown to induce larger spines in *Daphnia*. Likewise, *Leptodora* kairomones have been shown to induce larger spines in *D. cucullata* (Laforsch & Tollrian 2004), whereas spina enlargement in response to *Bythotrephes* kairomones had not been shown previously.

Visual predators may preferentially target individuals with large and dark pigmented eyes (Zaret & Kerfoot 1975). We proposed that *Bythotrephes*, but not *Leptodora* kairomones, would induce a reduction in eye diameter in *Daphnia*, because only the former is considered a visual predator (Jokela et al. 2013). In contrast to our expectation, *Bythotrephes* kairomones induced an increase in eye diameter in *D. galeata*. As larger eyes are costly to build (Brandon & Dudycha 2014) and increase the detectability to predators (Zaret & Kerfoot 1975), benefits of large eyes need to outweigh these costs for selection to occur. Potential benefits of larger eyes in zooplankton are not well understood. In *Daphnia*, larger eyes have been suggested to increase the ability to detect and thus escape fish predators (Beston et al. 2019). Likewise, *D. galeata* might use larger eyes to detect and avoid predation by *Bythotrephes*. Alternatively, larger eyes might benefit *Daphnia* in deeper water layers. *D. mendotae* has been shown to migrate to deeper water layers in the presence of *Bythotrephes* (Pangle & Peacor 2006). Whether the vertical migration behaviour of *D. galeata* in Lake Constance is influenced by *Bythotrephes* has not yet been studied.

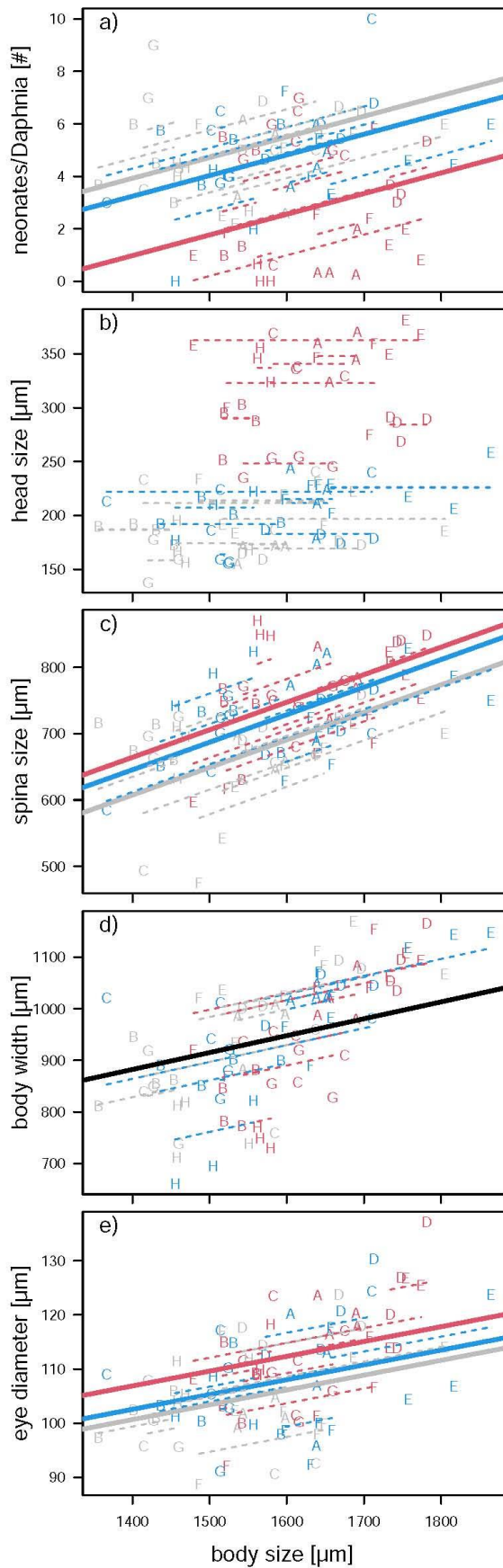


Figure 3. Relationships between body size and a) neonates per *Daphnia*, b) head size, c) spina size, d) body width, and e) eye diameter. Solid lines show fixed effects of treatments (control: grey, *Bythotrephes*: red, *Leptodora*: blue) and dashed lines show random intercepts for clones. As there was no relationship of head size with body size, only random intercepts are shown in b). As no significant treatment effects for body width were found (Table 1), only the body size relationship across all treatments is shown in d).

Theory predicts that induced antipredator responses should bear costs (Diel et al. 2020; Tollrian & Dodson 1999). However, these costs are difficult to quantify (Tollrian & Dodson 1999; Diel et al. 2020; but see Riessen & Sprules 1990; Riessen 2012). Moreover, possible demographic costs of morphological defences are difficult to separate from demographic costs of life history responses (Tollrian & Dodson 1999).

In our study, significant demographic costs (i.e., a reduced number of offspring), were only observed for the *Bythotrephes* treatment. This suggests that the combined morphological responses to *Leptodora* kairomones (i.e. larger body size and spina elongation), were less costly than the combined responses (larger body size, later AFR, elongated spina, helmet formation, and larger eyes) to *Bythotrephes* kairomones. As *Bythotrephes* kairomones induced various responses, it is difficult to quantify the contributions of each response to overall costs. However, as daphniids grew larger and developed larger spines in response to both predators, and did not differ significantly regarding their AFR responses, it seems likely that these three traits did not contribute a major part of the costs in the *Bythotrephes* treatment. Furthermore, the significant negative correlation of offspring numbers with head size, but not with eye diameter within the *Bythotrephes* treatment, suggests that helmet formation contributes strongly to overall costs in this treatment, although developing larger eyes has also been shown to be costly (Brandon & Dudycha 2014).

Although offspring production in the *Leptodora* treatment was not reduced compared to the control, there was a significant negative correlation between spina size and offspring production within the *Leptodora* treatment, suggesting that spina elongation is also costly. However, as these costs seem to be rather small, spina elongation costs might have been outweighed by other costs, such as those associated with helmet formation, in the *Bythotrephes* treatment. In response to predators, *Daphnia* will have to weigh costs of individual responses against their benefits in respect to mortality reduction. Hence, the absence of helmet induction in response to *Leptodora* kairomones might reflect an alternative fitness optimum in which higher mortality rates, due to the absence of helmet formation, are outweighed by enhanced birth rates.

Several studies have shown that there is clonal variability in antipredator defences of *Daphnia* (e.g., Boeing et al. 2006a; Carter et al. 2017; Dennis et al. 2011; Hammill et al. 2008; Reger et al. 2018; Sperfeld et al. 2020; Weiss et al. 2016). In our study, clonal differences were evident in head sizes, spina sizes, and in eye diameters (significant

random intercepts), but we did not detect clone-specific treatment responses (significant random slopes). Presumably, the number of replicates per clone (three or four) was not sufficient to identify treatment specific responses. However, the observed negative correlation between head sizes and neonate production indicates that individual clones differed regarding their position in the defence costs trade-off. Changing abundances of *Bythotrephes* versus *Leptodora* might hence result in changing selection regimes and consequently changes in the fitness of individual clones.

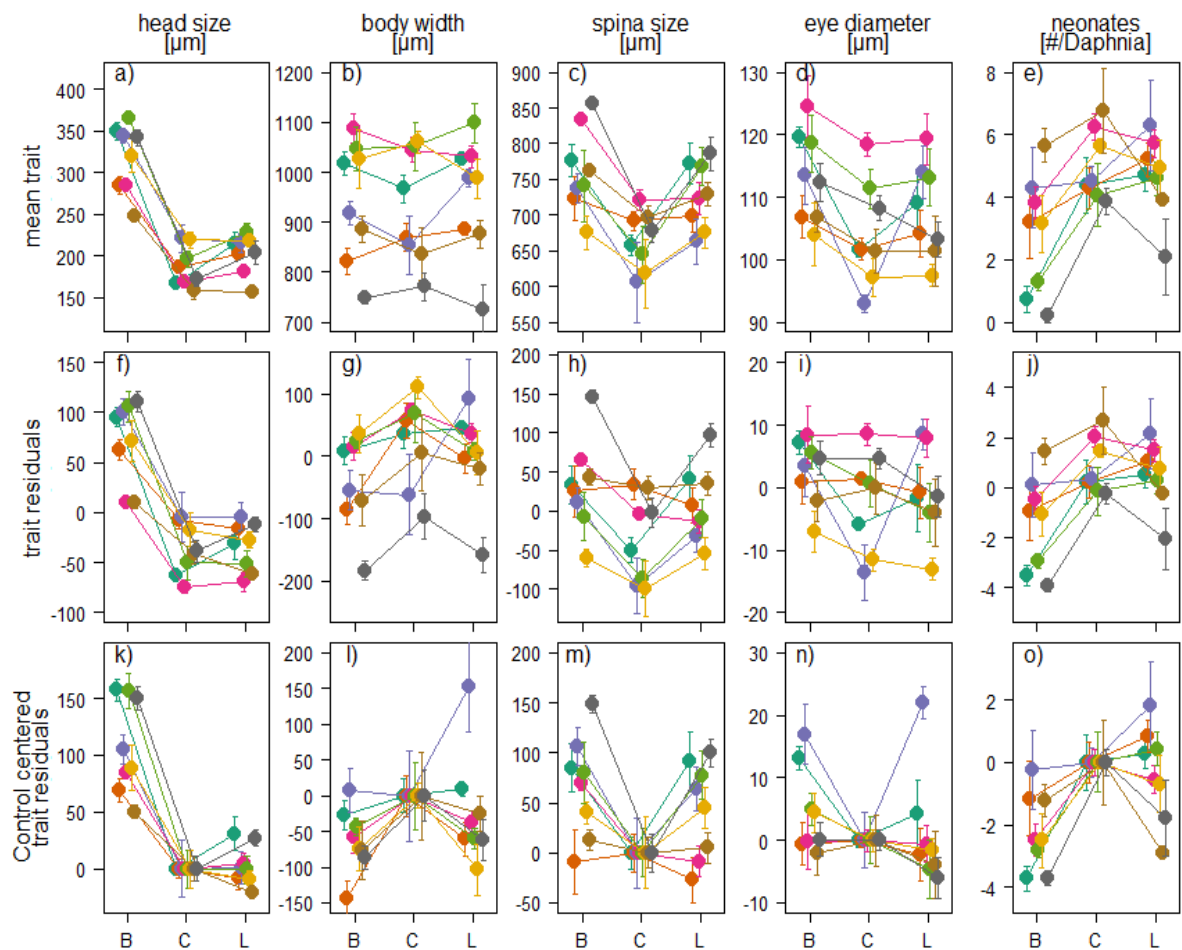


Figure 4. *Bythotrephes* (B) and *Leptodora* (L) treatment differences in comparison to the control treatment (mean \pm SE) of individual clones for helmet size, body width, spina size, eye diameter and neonates per *Daphnia* shown as mean values for each trait (upper panel, a – e), as mean values for traits corrected for body sizes (middle panel, f – j), and traits corrected for body size and centered around the control values of each clone (lower panel, k – o).

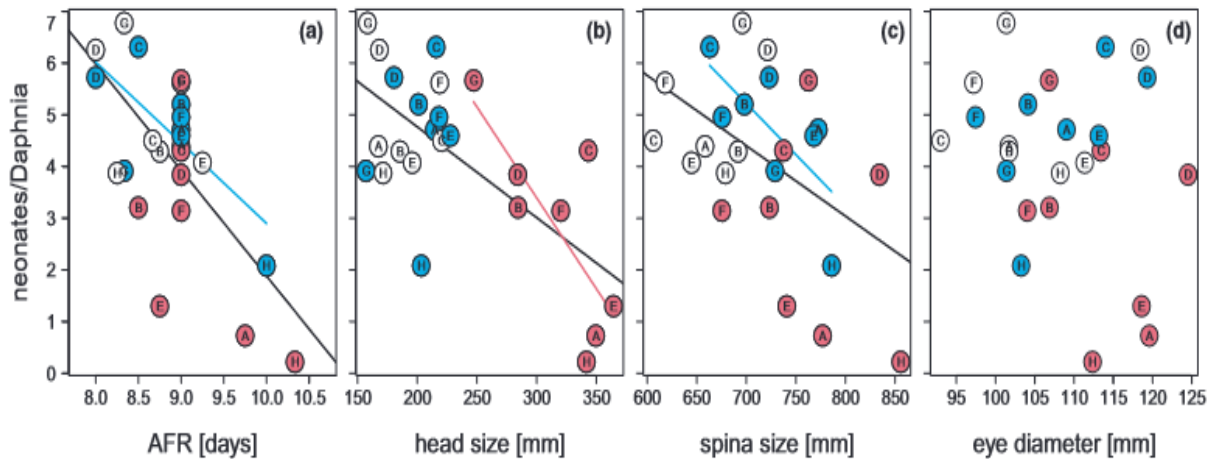


Figure 5. Relationship between clonal means of a) AFR, b) head size, c) spina size, d) eye diameter and clonal means of neonates per *Daphnia* in the control (white circles), *Leptodora* (blue circles), and *Bythotrephes* (red circles) treatment. Letters within circles indicate clone notations (A-H). Black lines indicate regression lines across all treatments, blue and red lines show the regression lines for the *Leptodora* and *Bythotrephes* treatments, respectively. Only regression lines significant at $p < 0.05$ are shown.

While we showed that *Bythotrephes* and *Leptodora* kairomones induced morphological changes in *D. galeata* clones, an important next step will be to study whether these changes indeed reduce predation by one or both predators. The mode of action of inducible defences and how they protect against certain predators is often unclear. For example, a longer spina may increase the escape ability because of a faster swimming speed (Mort 1986) or may decrease the catchability due to a suboptimal attack distance (Laforsch & Tollrian 2004). Investigating the role of helmets and elongated spines in the defence against *Leptodora* versus *Bythotrephes* predation is a difficult task, because these two predators are difficult to handle in the laboratory.

In conclusion, we showed that the antipredator responses of *D. galeata* differ between two closely related invertebrate predators, *Bythotrephes* and *Leptodora*. Hence, these two predators should be regarded separately in studies assessing the impact of invertebrate predation on natural zooplankton communities. Functional diversity might also partially explain the strong impact of *Bythotrephes* invasion on zooplankton communities in North American lakes (Foster & Gary 2009; Lehman & Cáceres 1993). The successful establishment of *Bythotrephes* in these lakes probably added a new invertebrate predator type to zooplankton communities and did not simply replace a functionally similar predator (i.e., *Leptodora*). Moreover, our results revealed predator-

specific defence demographic cost trade-offs, suggesting that the presence of these invertebrate predators can drive different evolutionary processes in natural zooplankton communities. Multi-trait, multi-clone studies are needed to disentangle the phenotypic responses of prey species to co-occurring predators.

Table 1. Statistics of fixed effects (type II ANOVA with Satterthwaite's method) and random effects (Likelihood ratio tests) in models relating age at first reproduction (AFR), body size, per capita number of offspring, helmet size, spina size, body width and eye diameter to the fixed effects of body size and treatment (T), and the random intercept effects of clone nested in temporal block (1|clone:block), temporal block (1|block), and clone (1|clone)

	Fixed effects		Random effects		
	Body size	T	1 clone:block	1 block	1 clone
AFR	-----	$F_{2,84} = 5.1$ **			$LRT_1 = 9.6$ **
body size	-----	$F_{2,81.4} = 14.65$ ***	$LRT_1 = 3.74$.	$LRT_1 = 7.89$ **	
Number of offspring	$F_{1,73.6} = 13.8$ ***	$F_{2,81} = 26.4$ ***	$LRT_1 = 20.9$ ***	$LRT_1 = 0.26$ ns	
helmet size		$F_{2,81} = 292$ ***			$LRT_1 = 44.6$ ***
body width	$F_{1,85.5} = 16.7$ ***		$LRT_1 = 17.35$ ***	$LRT_1 = 5.4$ *	
spina size	$F_{1,87} = 37.2$ ***	$F_{2,81} = 9.1$ ***			$LRT_1 = 34.3$ ***
eye diameter	$F_{1,83.7} = 7.2$ **	$F_{1,82.4} = 4.9$ **			$LRT_1 = 22.0$ ***

Note: Only fixed and random effects contributing to the best non-singular model as identified by backward elimination of factors are shown. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$, . $p < 0.1$.

Antipredator responses of three *Daphnia* species within the *D. longispina* species complex to two invertebrate predators

Marjohn Yucada Baludo, Pelita Octorina, Andrew Beckermann, & Dietmar Straile

3.1. Abstract

Prey communities in natural environments face a diverse array of predators with distinct hunting techniques. However, most studies have focused only on the interactions between a single prey species and one or more predators and typically only one of many induced defense traits, which limits our understanding of the broader effects of predators on prey communities. In this study, we conducted a common garden experiment using five clones each of three *Daphnia* species (*D. cucullata*, *D. galeata*, and *D. longispina*) from the *D. longispina* species complex to investigate the plasticity of predator-induced defenses in response to two predators in a community ecology setting. Five clones from each species were subjected to predator kairomones from two closely related invertebrate predators that are common in several European lakes, *Bythotrephes longimanus* or *Leptodora kindtii* for a duration of ten days, and the morphological traits of body size, head size, spina size, and the presence of spinules on the ventral and dorsal carapace margins were measured. We show that among the species within this species complex there are different antipredator reactions to the invertebrate predators. The induced responses exhibited were species, trait, and predator-specific. Notably, *D. galeata* and *D. cucullata* developed distinctive helmets as defensive mechanisms, while microdefenses were induced in *D. galeata* and *D. longispina*, but not in *D. cucullata*. This demonstrates that the expression of micro- and macrodefenses across species was unrelated, highlighting the possible independent evolution of microstructures as defensive modules in *Daphnia*'s anti-predator strategies. This study is the first to document both micro- and macrodefensive phenotypic plasticity in three co-occurring *Daphnia* species within the *D. longispina* species complex. The differences in inducible defenses may have a substantial impact

on how these three species cohabit with *Bythotrephes* and *Leptodora*.

Keywords: anti-predator strategies, *Daphnia*, *Daphnia longispina* species complex, *Bythotrephes*, *Leptodora*, morphological defenses

3.2. Introduction

Predation is a crucial evolutionary force that shapes ecological communities and drives the development of antipredator defenses in many prey species (Tollrian and Harvell 1999). The evolution of antipredator defenses is determined by several factors. One of these factors is the presence of a reliable cue that indicates the proximity of a threat and activates a defense response in the prey. Additionally, the effectiveness of the prey's defenses against the predator, and the balance between the costs and benefits of developing defenses play crucial roles in this evolutionary process (Tollrian and Harvell 1999). Prey employ various inducible defenses against their predators, such as morphological changes (Laforsch and Tollrian 2004; Octorina et al. 2022; Sperfeld et al. 2020), behavioral modifications (Stich and Lampert 1981), and life-history traits (Kruppert et al. 2017; Stibor 1992) to enhance their fitness and reduce predation risk, which are essential for their survival in the environment (Riessen 2012).

A wide range of organisms, including, for example, protozoans, vascular plants, rotifers, arthropods, and vertebrates have been observed to possess inducible antipredator defenses (Tollrian and Harvell 1999). In natural environments, prey encounter various predators, each employing distinct hunting and capturing techniques (Laforsch and Tollrian 2009).

Prey can gather and evaluate information about the risk of predation e.g., through chemical cues emitted by predators or their conspecifics (Turner 2008; Weiss et al. 2012). They can also differentiate between predator species, as seen in *Daphnia pulex* and a freshwater snail *Radix balthica* exhibiting predator-specific traits when facing multiple predators (Beckerman et al. 2010; Lakowitz et al. 2008; Miner et al. 2005). Nevertheless, most studies examining predator-prey interactions have been limited to investigating interactions between one prey and one predator species (Weiss et al. 2015; Tams et al. 2018; Sperfeld et al. 2020) and single induced traits. Even while

there has been some research conducted on prey with multiple predators and traits (Laforsch and Tollrian 2004; Herzog et al. 2016; Diel et al. 2021), there are still surprisingly few studies that concentrate on coexisting prey alongside their complete predator assemblage. This limited scope hinders a comprehensive understanding of the breadth of predator-induced responses in the prey species that comprise natural ecological communities (Laforsch and Tollrian 2004; Miner et al. 2005).

The zooplankton genus, *Daphnia* stands out as a well-studied example of inducible defenses. *Daphnia* exhibits inducible defenses against both vertebrate predators and invertebrate predators (Diel et al. 2020; Tollrian and Harvell 1999). Most individual studies on the responses of *Daphnia* species have examined their defenses against *Chaoborus* (Beckerman et al. 2010; Carter et al. 2017; Hammill et al. 2008; Laforsch and Tollrian 2004; Lind et al. 2015; Reger et al. 2018; Sperfeld et al. 2020; Wolinska et al. 2007), *Notonecta* (Diel et al. 2021; Herzog et al. 2016; Ritschar et al. 2020; Weiss et al. 2015), *Triops* (Diel et al. 2021; Herzog et al. 2016; Petrussek et al. 2009; Rabus et al. 2013; Ritschar et al. 2020), and fish (Adamczuk 2009; Beckerman et al. 2010; Carter et al. 2017; Lind et al. 2015; Reger et al. 2018; Winder et al. 2004; Wojtal-Frankiewicz et al. 2010).

Despite this huge amount of research done during the last decades (Diel et al. 2020), new types of inducible defenses, particularly morphological defenses, in *Daphnia* continue to be discovered, including the alteration of tiny microstructures (Diel et al. 2021; Ritschar et al. 2020). According to Diel et al. (2021), changes in an organism's microstructure might either be developmentally connected to more obvious changes in its features, or represent independent defensive structures to fine-tune protection against a predator. The first reasoning would suggest that changes at the microstructural level are strongly related to the more significant induced changes that take place during development. For instance, Laforsch and Tollrian (2004) noted that *D. cucullata* displays a strengthened carapace and extended helmet in response to various invertebrate predators, a positive correlation between the macro (helmet) and micro (carapace structure) induced defense. However, in accordance with the "concept of modality" of predators, specialized defenses have generally surpassed general ones throughout evolution. In order to improve the overall defense strategy, subtle changes may thus be important. For example, spinules, micro spike structures along the carapace, might additionally play a role in refining or fine-tuning the induced

phenotype to provide optimal protection against a particular predator with distinctive methods of capture or manipulation (Diel et al. 2021).

In many European lakes, *Daphnia* of the *Daphnia longispina* complex, *D. longispina*, *D. galeata*, and *D. cucullata* co-occur and dominate the grazing pressure on phytoplankton. In many of these lakes, these *Daphnia* species are exposed to one or two cladoceran predators, *Leptodora kindtii* and *Bythotrephes longimanus*. These two predators differ in predation modes: *Leptodora* is a tactile predator and *Bythotrephes* is a visual predator (Octorina et al. 2022). *Bythotrephes* relies on mechanoreceptors or its large medial compound eye to detect its prey. It then uses its long feeding appendages to grasp the prey and subsequently shred it (Manca et al. 2008). In contrast, *Leptodora* employs a strike tactic for capturing prey and requires direct contact with the prey before initiating an attack (Browman et al. 1989; Manca et al. 2008) and uses a "trap basket" for capturing prey (Branstrator 2005). Unlike *Leptodora*, *Bythotrephes* are likely capable of successfully feeding on larger prey items because they are not constrained by a feeding basket (Manca et al. 2008). Previous studies have shown that *L. kindtii* induces significantly longer helmets and tail spines on *D. cucullata* (Laforsch & Tollrian 2004), while *B. longimanus* induces a typical helmet on *D. galeata* (Octorina et al. 2022), but the responses of *D. longispina* to both predators have not been explored.

Here, we investigate the predator-induced defenses in a community ecology context, evaluating responses among three species within the *D. longispina* species complex which co-occur with the two invertebrate predators in Lake Constance. We examine plasticity in head, respectively helmet, body, and spina size, as well as in proposed microstructures, the extension of the ventral and dorsal spinule areas, and the length of the ventral spinule (Diel et al. 2021) in response to the two predators.

Both *D. galeata* and *D. longispina* are morphologically similar and adult individuals of these species typically range in body size from 1.2 to 2.5 mm. As *D. cucullata* is smaller (Ogorelec et al. 2022; Stich et al. 2005) compared to the other two species, we expect that small *D. cucullata* might be more vulnerable to invertebrate predation. Besides size, the species also differ in anti-predator behavior. *D. longispina* performs diel vertical migration (DVM) (Geller 1986; Stich and Lampert 1981), while *D. cucullata* and *D. galeata* stay in the upper water strata (0-20 m) (Geller 1986, Ogorelec et al. 2022; Stich and Lampert 1981). As the two invertebrate predators also

inhabit the upper water layers (Stich 1989), *D. galeata* and *D. cucullata* (not performing DVM), but not *D. longispina* (performing DVM) are exposed to the two predators throughout a 24 hour day.

Given this background and the potential for covariation between macro and micro induced defense traits, we formulated and tested four hypotheses: 1) based on the differences in predation behavior between the two predator species, we anticipate distinct responses in the daphnids; 2) we expect variations in the expression of defensive traits among the *Daphnia* species, with the most vulnerable *D. cucullata* showing the strongest responses, while the least vulnerable *D. longispina* displaying only weak responses to predator kairomones; 3) given the ‘connectedness sensu Diel et al. (2021)’ between small-scale defensive qualities and the large-scale defensive traits against invertebrate predators, we expect that all three species will display microdefenses in response to both predators; 4) finally, we expect that the expression of microdefenses would be directly related to the expression of macrodefenses, emphasizing the complexity of the defensive strategies.

3.3. Materials and Methods

Origin of Daphnia clones

Daphnia clones were collected in Upper Lake Constance, located in Central Europe and bordering Germany, Switzerland, and Austria, which is the main basin of the large peri-alpine Lake Constance (Güde and Straile 2016). The number of *Daphnia* species has changed there during the last century. While until the mid-20th century only *D. longispina* was found in the lake, *D. galeata* was able to invade the lake with eutrophication in the 1950s (Straile 2015). More recently, *D. cucullata* established large densities (Ogorelec et al. 2022) and currently all three species co-occur in the lake. Both predators, *B. longimanus* and *L. kindtii*, have been regularly found in the lake since more than one century (Straile and Geller 1998). Clones of *Daphnia galeata* were hatched from ephippia, which were collected from the upper 20 cm of a sediment core taken from the lake. In contrast, *Daphnia longispina* and *Daphnia cucullata* clones were collected in Lake Constance using a plankton net (mesh size 140 µm) drawn from 40-0 m. The lineages established from these individuals of *D. cucullata* and *D. longispina* are thus isolates, and it is not 100% certain that they represent

different clones. However, given the large clonal diversity of *Daphnia* spp. in Lake Constance (Beninde 2021), and our low numbers of isolates obtained from 100's sampled, we consider it unlikely that two randomly picked individuals from several hundred individuals in one sample share their clonal identity. Consequently, in this paper, we referred to them as clones, acknowledging the potential that two isolates may share the same clonal identity. The three species were cultured in the laboratory for several months before the experiment. The third clutch from the third generation of maternal lines was collected as study organisms. In order to reduce the influence of extraneous variables, all stock cultures of clones were kept under the same conditions (e.g., feeding volume, feeding schedules, temperature, light). Maternal cultures were kept in 1 L glass jars with 800 mL lake water in 20 °C (range: 19 – 21 °C) with a 16:8 L:D photoperiod cycle. These cultures were fed with 2 mg C/L green alga *Tetradesmus obliquus* (Culture Collection of Algae, University of Göttingen, Germany, SAG 276-3a) three times per week and were transferred to a new medium until they reached the third generation.

Lake water was prepared for both *Daphnia* culturing and the experiment by undergoing a process involving filtration through a 0.2 µm mesh sieve. Subsequently, the filtered water was aerated and allowed to age for 24 hours to prevent expression of morphological defenses in *Daphnia* cultures.

Experimental setup and measurements of morphological traits

Since our focus was on studying species differences rather than clonal variations within species, we conducted replications at the species level, with five clones each, rather than at the clonal level. The five clones of each *Daphnia* species, *D. cucullata*, *D. galeata*, and *D. longispina*, were placed in three different treatments: *Bythotrephes* (B), Control (C), and *Leptodora* (L). For each clone and treatment, we added three to four 1-day old *Daphnia* individuals to 200-mL glass jars containing 150 mL lake water. The number of experimental animals at the start of the experiment was 3 species x 5 clones (5 jars) x 3 treatments x 3-4 daphnids per jar, in total of 45 jars and 164 individuals. Unfortunately, 63 daphnids died during the experiment resulting in 101 daphnids used for morphological measurements. Mortality was mostly caused by the transfer of daphniids into new jars every second day and was highest for fragile *D. cucullata* (65 % of all mortality). This caused the loss of two clones of *D. cucullata* in the *Leptodora* (L) treatment. The analysis of spina length variability was

conducted using only daphnids with intact spines ($n = 96$). This exclusion involved the removal of two clones of *D. cucullata* in the *Bythotrephes* (B) treatment due to broken spina. Hence, the number of independent experimental units was 43 (3 species x 5 clones x 3 treatments – 2) for analyses of body and head sizes and microstructures, and 41 for the analysis of spina size.

Five predators were added to each jar in the predator treatments, which were collected from the lake using a plankton net towed behind a boat. These predators were placed in a cylindrical plastic cage (4 cm diameter and 6 cm height) with a 140- μ m nylon mesh (Octorina et al. 2022) and were not fed with *Daphnia*. In the control treatment, the plastic cage was left empty. Every second day, daphnids were transferred to new jars filled with lake water with *T. obliquus* and freshly collected predators. Throughout the experiment, daphnids were fed with 1 mg C/L of the green alga *T. obliquus* for the first seven days and 2 mg C/L for the remaining three days to ensure also that large and matured daphnids have unlimited food. The experiment was conducted at a constant temperature of 20 °C (range: 19 – 21 °C) and a 16:8 L: D photoperiod cycle.

At an age of 10 days, the daphnids were preserved in 70% ethanol following shock treatment in 95% ethanol (Black and Dodson, 2003). We chose age 10 for all three species because this age is sufficient to guarantee that all individuals were mature in all treatments. Subsequently, morphological defenses of *Daphnia* individuals were measured in R version 4.2.2 (R Core Team 2021) with a self-written script using photographs of the daphnids taken with a Bresser MikroCamII 12 MPu mounted to a Stemi 2000-C stereomicroscope (Carl Zeiss Werk, Göttingen, Germany). For each *Daphnia*, we measured body size, head size, spina size, and the spinules on the ventral and dorsal carapace margins (Fig. 1). Body size was determined from the base of the spina to the mid-eye, while spina size was measured from the end of the tail spine to the base of the spina. Head size was measured in parallel to the body axis from the top of the head or top of the helmet until the line perpendicular to the body axis crossing the midpoint of the eye. All *Daphnia* individuals were examined for the presence of spinules in the dorsal and ventral carapace margins, and for the presence of neckteeth. The ventral spinules bearing area (ventral SBA) was measured from the first ventral spinule to the base of the tail spine, while the dorsal spinules bearing area (dorsal SBA) was measured from the first dorsal spinule to the base of the tail spine. Additionally,

the length of the ventral spinules was measured from the base of the spinule to its tip, with five spinules per animal measured (Diel et al. 2021). Unfortunately, one *D. longispina* and eight *D. galeata* pictures were not suitable to measure the microstructures. This reduced the number of observations, but not the number of independent experimental units in our dataset regarding microdefenses.

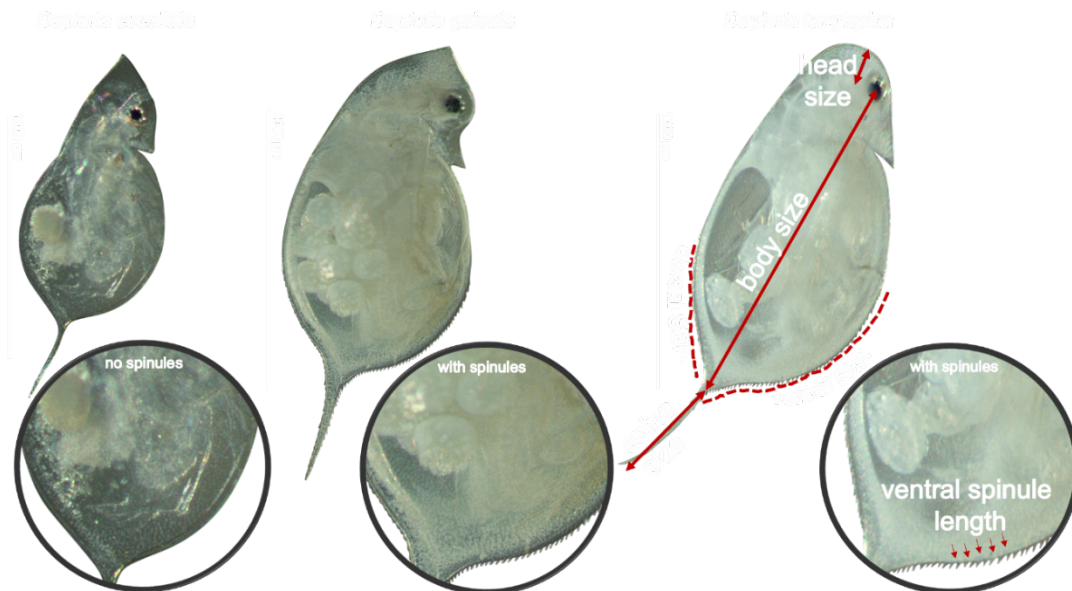


Figure 1. Measurements of various morphological traits of *Daphnia* species and the presence/absence of dorsal and ventral spinules.

Test of hypotheses 1 & 2: predator-specific responses and different level of defensive trait expressions in Daphnia

Variations in body size, head size, spina size, dorsal SBA, ventral SBA, and ventral spinule length were then analyzed using linear mixed-effect models (Kuznetsova et al. 2017) with body size, treatment, and species as fixed effects and clonal identity as a random effect. This accounts for non-independence of the daphnids from each clone which were kept in one jar. Body size was centered in the model to eliminate co-variation between body size and *Daphnia* species identity. Hence, centered body size in the models predicts within species trait variability, whereas between species trait variability is predicted by species identity. We used likelihood ratio tests (ANOVA and RANOVA functions) (Kuznetsova et al. 2017) to evaluate the significance of fixed and random effects. If significant treatment effects were found, we conducted pairwise post hoc comparisons using the Tukey method (Lenth et al. 2023).

Test of hypothesis 3: microdefenses in all three species

To test the predictions regarding the occurrence of microdefenses in all three species, we calculated the mean trait residual for dorsal SBA, ventral SBA, and ventral spinule length, and compared these values among the *Daphnia* species. The linear mixed-effect model for dorsal SBA, ventral SBA, and ventral spinule length was then utilized to analyze treatment and species effects, followed by post-hoc comparisons.

Test of hypothesis 4: covariation of macrodefenses and microdefenses

Finally, to examine covariation between macrodefenses and microdefenses, we first calculated the residuals of regressions between each trait and body size separately for each species. Next, we calculated the mean residual values for each trait and clone and determined the correlation for each combination of pairwise traits and across *Daphnia* species. This allowed us to assess the presence of trade-offs versus positive covariation between the different traits. For multiple comparisons, we corrected the *p*-values using a Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995).

Statistical analyses were conducted using R version 4.2.2 (R Core Team 2021). All models were constructed using lme4 (Bates et al. 2015) and analyzed using lmerTest (Kuznetsova et al. 2017). Post hoc testing, where appropriate, was carried out using the emmeans package (Lenth et al. 2023). Figures were generated using the ggplot2 package (Wickham 2016) and the pairs R function.

3.4. Results

Hypotheses 1 & 2: *Predator-specific responses and the differences in the defensive trait expressions in Daphnia*

After a growth period of 10 days, it was evident that the body size of *D. cucullata* was significantly smaller, mostly below 1000 μm , compared to the other two species, which generally reached body sizes between 1500 and 2000 μm (Figs. 2, 3a). The presence of predators led to an increase in the body size of *D. galeata* (B – C: $t_{84.7} = 2.769$, $p = 0.0187$; C – L: $t_{84.2} = -3.355$, $p = 0.0034$) (Figs. 2, 3a), while the effects of predator kairomones on the body size of the other two species were not as clear (Table 1a, significant treatment x species interaction).

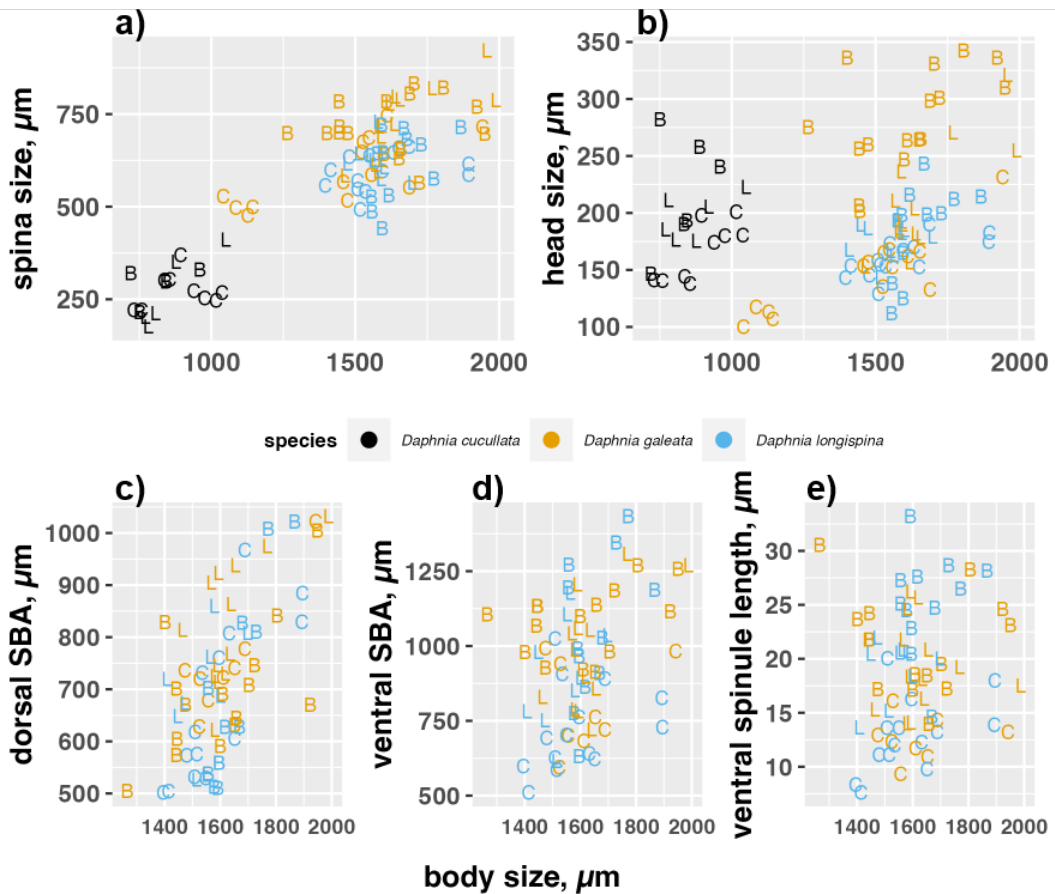


Figure 2. Relationship between body size and spina size, head size, dorsal SBA, ventral SBA, and ventral spinule length for the three *Daphnia* species. Each treatment condition is shown with distinct letters (*Bythotrephes* (B), Control (C), and *Leptodora* (L)).

We did not observe any neckteeth for any *Daphnia*. All other traits were influenced by predator kairomones in a species-specific manner, and except for the ventral spinule length, all trait lengths were related to body length (Figs. 2, 3). This relationship held even when body lengths were centered for each species (Table 1). Regarding body size, head size, and spina size, significant clonal variability was found, indicated by significant random intercepts (Table 1).

Table 1. Summary of the linear mixed-effects model on the morphological trait responses of *Daphnia* community against two co-occurring invertebrate predators.

Traits	Fixed Effects							Random Effect
	Body size	Tr	species	Body size: Tr	Body size: species	Tr: species	Body size: Tr: species	1 clone
a. Body size		F _{2,86.0} = = 0.901 ns	F _{2,13.0} = 91.1 ***			F _{4,85.8} = 3.33 *		LRT ₁ = 4.91 *
b. Head size	F _{1,81.9} = 18.6 ***	F _{2,75.9} = 50.6 ***	F _{2,10.9} = 5.81 *	F _{2,77.751} = 2.47 ns	F _{2,83.711} = 0.134 ns	F _{4,75.5} = 16.3 ***	F _{4,75.6} = 1.59 ns	LRT ₁ = 13.7 ***
c. Spina size	F _{1,65.6} = 15.9 ***	F _{2,74.7} = 2.52 ns	F _{2,12.6} = 82.2 ***	F _{2,78.443} = 1.21 ns	F _{2,78.744} = 1.07 ns	F _{4,74.2} = 2.65 *	F _{4,75.0} = 2.90 *	LRT ₁ = 16.1 ***
d. Dorsal SBA	F _{1,53.4} = 55.8 ***	F _{2,55.3} = 7.17 **	F _{1,6.64} = 8.76 *	F _{2,53.986} = 2.13 ns	F _{1,53.403} = 2.45 ns	F _{2,55.3} = 0.246 ns	F _{2,53.9} = 5.04 **	LRT ₁ = 2.33 ns
e. Ventral SBA	F _{1,54.8} = 6.37 *	F _{2,56.1} = 18.2 ***	F _{1,8.47} = 1.28 ns	F _{2,55.095} = 1.72 ns	F _{1,54.850} = 0.81 ns	F _{2,56.1} = 0.347 ns	F _{2,55.1} = 0.830 ns	LRT ₁ = 3.43 ns
f. Ventral spinule length	F _{1,49.6} = 0.522 ns	F _{2,56.9} = 38.6 ***	F _{1,8.66} = 1.93 ns	F _{2,56.057} = 0.17 ns	F _{1,49.653} = 1.89 ns	F _{2,56.9} = 1.21 ns	F _{2,56.1} = 0.102 ns	LRT ₁ = 1.05 ns

Note: The analysis included fixed effects such as centered body size, head size, spine size, dorsal spinule bearing area (SBA), ventral SBA, and ventral spinule length using Type III Analysis of Variance with Satterthwaite's method to examine the significance of these fixed effects. Clonal identity was treated as a random effect, and Likelihood ratio tests were employed to evaluate its impact on the model. "Tr" refers to the three treatments: *Bythotrephes*, Control, and *Leptodora*. The model employed an equal count of observations (n = 101) for body size and head size, whereas the count differed for spina size (n = 96) because data points were excluded for daphnids with broken

spines in the *Bythotrephes* treatment. Additionally, the observation count varied for the microdefenses model ($n = 71$) due to the absence of these defenses in *D. cucullata*. Significance levels are given as: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

The kairomones of both predators increased head sizes due to the development of a helmet in *D. cucullata* (B – C: $t_{77.8} = 4.222$, $p = 0.0002$; C - L: $t_{78.5} = -2.808$, $p = 0.0171$) and *D. galeata* (B – C: $t_{72.6} = 13.204$, $p < 0.0001$; C - L: $t_{72.1} = -3.224$, $p = 0.0053$). However, this effect was not observed in *D. longispina* (Figs. 2, 3b, Table 1b, significant species x treatment interaction). Notably, the increase in *D. galeata* head sizes was greater in the *Bythotrephes* treatment compared to the *Leptodora* treatment ($t_{71.5} = 9.637$, $p < 0.0001$), but no significant difference was detected in *D. cucullata* between the two predator treatments ($t_{76.2} = 1.455$, $p = 0.3181$).

The influence of kairomones on spina length depended on body size and *Daphnia* species (significant three-way interaction). This was mainly due to the response of *D. galeata* in the *Leptodora* treatment, where the spina length - body size slope was steeper than in the *Bythotrephes* and control daphnids, resulting in large spines only for large daphnids (Fig. 2a). The spina length - body size slopes were comparable between the control and *Bythotrephes* treatments for *D. galeata* and larger spines were observed in the *Bythotrephes* treatment compared to the control treatment at similar body sizes ($t_{68.5} = 3.939$, $p = 0.0006$).

Hypothesis 3: *Microdefenses in all three species*

We found qualitative differences between the three species regarding microdefenses: *D. cucullata* did not develop microstructures in the presence of predators in contrast to the other two species. Consequently, we used only the data for *D. galeata* and *D. longispina* in the mixed models for microstructures. The effect of kairomones on dorsal SBA depended on body size and *Daphnia* species (significant three-way interaction). This threefold interaction was mostly caused by the *Bythotrephes* treatment response, where dorsal SBA for *D. longispina* increased more steeply with body size than SBA of *D. galeata* (Fig. 2c). Ventral SBA and ventral spinulae lengths did not differ between the two species, but were larger in both kairomones treatments compared to the control (Figs. 3e: *D. galeata* (B – C: $t_{53.7} = 4.118$, $p = 0.0004$) and *D. longispina* (B – C: $t_{57.8} = 4.541$, $p = 0.0001$; C – L: $t_{53.9} = -$

3.023, $p = 0.0105$), Fig. 3f: *D. galeata* (B – C: $t_{54.1} = 5.261$, $p < 0.0001$; C – L: $t_{52.5} = -3.957$, $p = 0.0007$) and *D. longispina* (B – C: $t_{58.6} = 7.198$, $p < 0.0001$; C – L: $t_{55.0} = -3.217$, $p = 0.0061$).

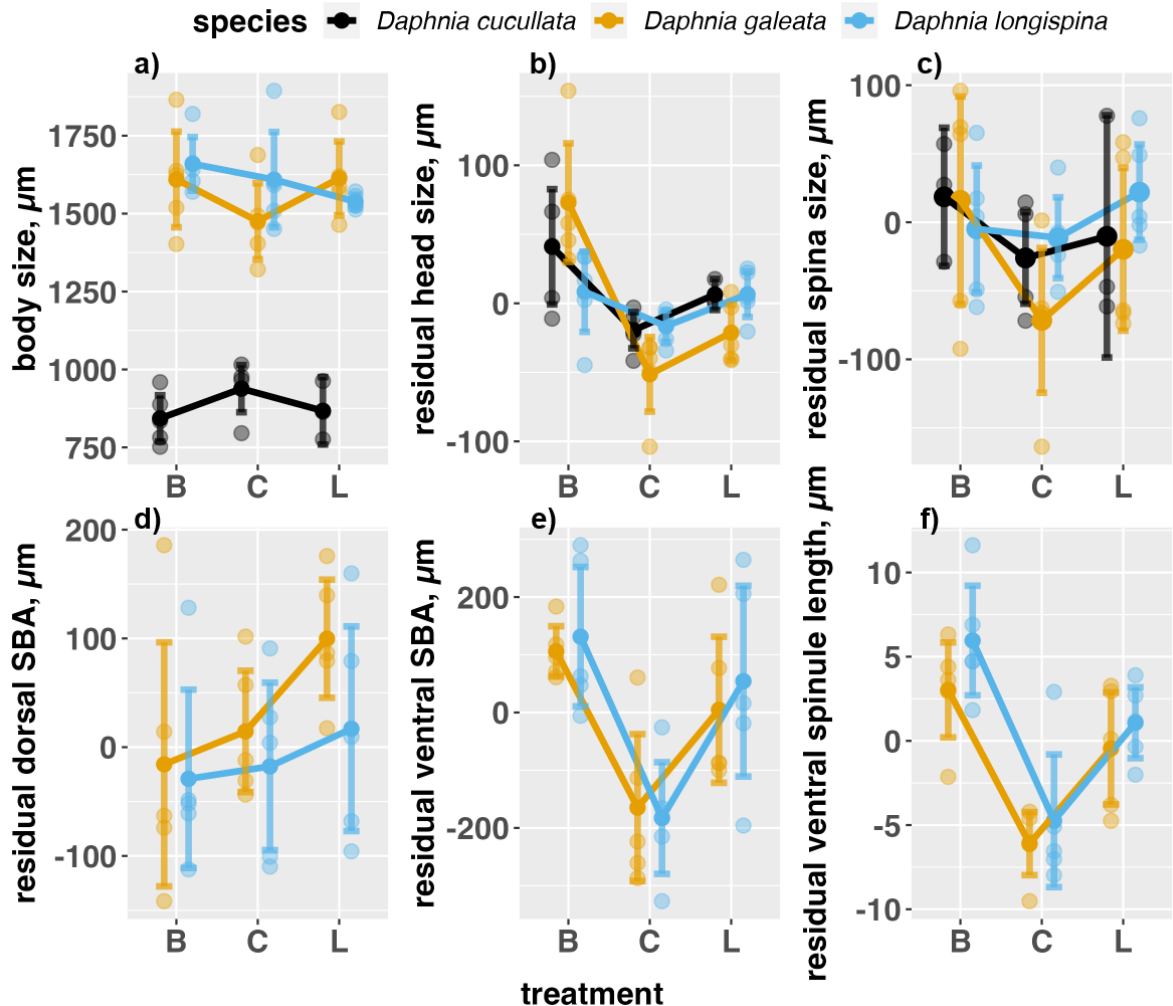


Figure 3. Responses (means \pm 2 standard errors) of the three *Daphnia* species to three treatments (B: *Bythotrephes*, C: Control, L: *Leptodora*) for the traits body size, residual head size, residual spina size, residual dorsal SBA, residual ventral SBA, and residual ventral spinule length. Small dots represent the clonal means for the different treatment-species combinations.

Hypothesis 4: Covariation of macrodefenses and microdefenses

Morphological traits adjusted for body size (Fig. 4) co-varied across species and treatments in 4 out of 10 trait combinations. Across all species and treatments, *Daphnia* with species and body size specific large head/helmet were found to have also a species and size specific large spina ($r = 0.47$, $p < 0.05$). Likewise, residual head size and residual ventral SBA ($r = 0.49$, $p < 0.05$), residual head size and residual

ventral spinule length ($r = 0.52$, $p < 0.05$), and residual ventral SBA and residual ventral spinule length ($r = 0.69$, $p < 0.0001$) were significantly and positively related after correction for multiple testing. No co-variation of trait expressions was observed between residual dorsal SBA and any other trait (Fig. 4). Correlations conducted within species suggest that *D. galeata* had positive significant covariation between residual head size and spina size ($r = 0.62$, $p < 0.05$), between residual head size and residual ventral spinule length ($r = 0.70$, $p < 0.05$), between residual head size and residual ventral SBA ($r = 0.70$, $p < 0.05$), between residual spina size and residual ventral spinule length ($r = 0.58$, $p < 0.05$) and between residual ventral SBA and residual ventral spinule length ($r = 0.71$, $p < 0.05$). No significant co-variation was found between traits of *D. longispina* and between residual head size and residual spina size of *D. cucullata*.

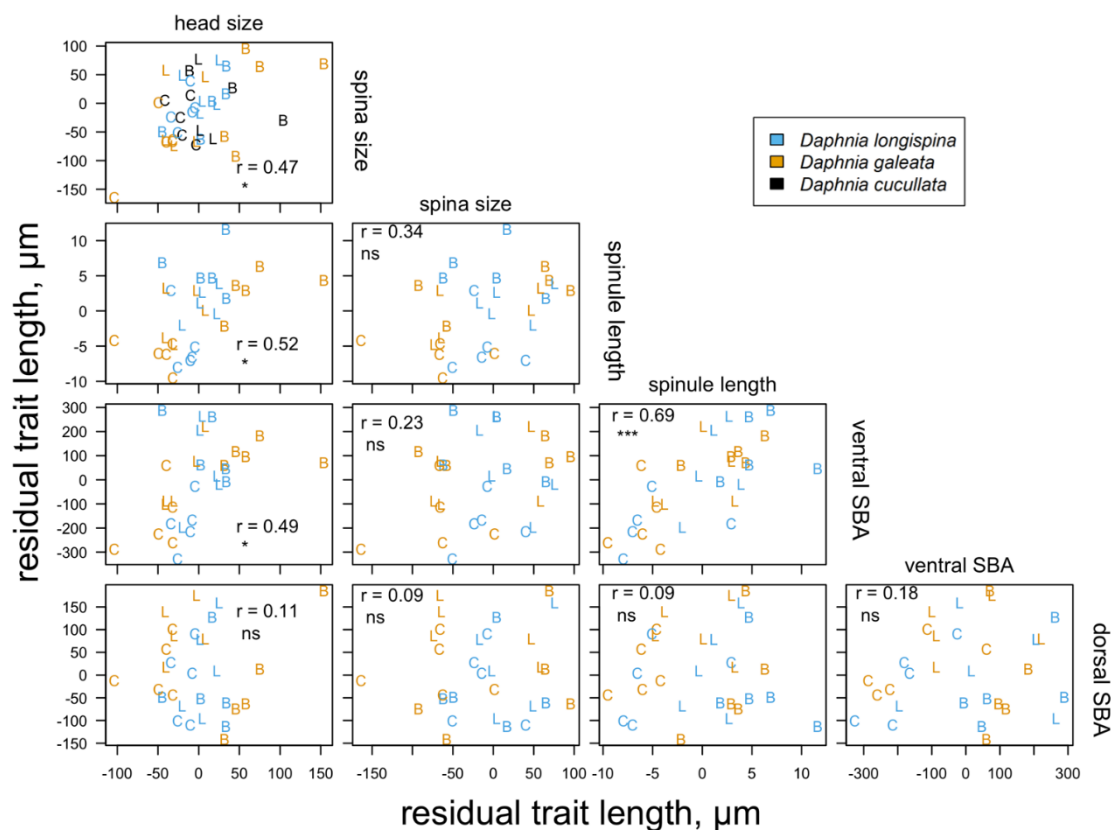


Figure 4. Relationships among various residual mean traits. The colored letters in the figure correspond to three treatments, (*Bythotrephes* (B), Control (C), and *Leptodora* (L)). The r values in each box in the figure displays the correlation coefficient for the respective pair of residual traits, and asterisks indicate significant co-variation between the respective traits (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Ross, 2017)).

3.5. Discussion

The invertebrate predators *Bythotrephes longimanus* and *Leptodora kindtii* are able to induce morphological defenses within all three species of the *D. longispina* complex. We found that the morphological responses of *D. longispina* species complex were trait -, *Daphnia* – species, and predator – species specific. However, our results only partially support our expectations. *D. longispina* was indeed less responsive to the two predators as compared to the other two species but only regarding macrodefenses. Microdefenses were induced in *D. longispina* and *D. galeata*, but not in *D. cucullata*. Hence, microdefenses were expressed independently of macrodefenses across the three *Daphnia* species.

We did neither observe the induction of an expanded head form (helmet) nor spina elongation for *D. longispina*. The absence of helmet induction corresponds to the typical appearance of *D. longispina* in Lake Constance (Güde and Straile 2016). This suggests that diel vertical migration of this species in Lake Constance seems to provide sufficient protection against the predation pressure of both invertebrate predators against the visual predator *Bythotrephes*, but also against the tactile predator *Leptodora*. The lack of strong morphological responses here suggests a trade-off may exist between behavioral and morphological defenses and suggests different strategies to deal with predation risk can evolve among species in this complex. The supposed behavioral protection seems to remove potential fitness benefits of elongated heads and spines, causing absence of helmets not only *in situ*, but also in our laboratory environment, in which daphnids were exposed to kairomones and light during daytime. Hence, helmet induction seems to be not a part of the defensive repertoire of *D. longispina*, and is not only switched off *in situ* by DVM induced reduction of light availability. This is in contrast to life history anti-predator strategies of *D. magna* in response to fish kairomones, which expression depends on light intensity (Effertz and von Elert 2014).

Absence of *D. longispina* morphological defenses was also observed in Norwegian deep lakes (Sperfeld et al. 2020). However, *D. longispina* clones from small and shallow water bodies in Norway developed longer spines and neck teeth in response to *Chaoborus* kairomones (Sperfeld et al. 2020). Interestingly, some *D. longispina* with neck teeth have also been observed occasionally in Lake Constance (Güde and Straile 2016) despite the absence of *Chaoborus* in this deep lake. As no neck teeth were observed in our experimental animals this might suggest that either a

different predator induces neck teeth induction in Lake Constance *D. longispina*, or that neck teeth expression is highly clone specific. In the latter case, neck teeth were not part of the defensive repertoire of the five clones used in our study. This reasoning is consistent with the rarity of neck teeth observations in Lake Constance.

The strong response of *D. galeata* observed in this study confirms our recent study showing that *Bythotrephes* and *Leptodora* will induce helmets and longer spines in this species (Octorina et al. 2022). In the same study, using eight clones with replicates at the clonal level, the age at first reproduction was observed to be the latest and demographic costs, characterized by a reduced number of offspring, were found to be the highest in the presence of *Bythotrephes* treatment. Helmet formation of *D. galeata* is also typically observed in Lake Constance during the summer season (Güde and Straile 2016). Likewise, helmet formation of *D. galeata-mendotae* has been experimentally induced by other invertebrate predators such as *Chaoborus* and *Notonecta* (Dodson 1988). However, *D. galeata* clones from various lakes, including Lake Constance, did not produce a helmet in response to fish kairomones (Tams et al. 2018). Longer spines in response to copepod kairomones have been observed for a *D. longispina* x *D. galeata* hybrid clone (Caramujo and Boavida 2000), and for *D. galeata* and *D. galeata* hybrids in response to fish kairomones, however, the latter only at high food concentrations (Spaak and Boersma 1997).

Induction of *D. cucullata* helmet formation in response to invertebrate predators is widespread and has been experimentally demonstrated in response to *Chaoborus*, copepod, and *Leptodora* kairomones (Laforsch and Tollrian 2004). Likewise – and in contrast to our results – spina elongation was demonstrated in response to *Chaoborus* and *Leptodora* kairomones (Laforsch and Tollrian 2004). Unfortunately, we lost two *D. cucullata* clones within the *Leptodora* treatment, and thus had a lower statistical power to detect effects of *Leptodora* kairomones for this species. We can therefore not exclude the possibility that also Lake Constance *D. cucullata* might elongate their spines in response to *Leptodora* and *Bythotrephes*.

Our study is the first to show the induction of three microdefenses in *D. longispina* and *D. galeata*, and the absence of these defenses in *D. cucullata*. Such induction of microdefenses has been reported previously in several *Daphnia* species, e.g., *D. barbata*, *D. similis*, *D. magna*, and *D. longicephala* (Herzog and Laforsch 2013, Ritschar et al. 2020) in response to the invertebrate predators *Triops cancriformis* and *Notonecta maculata* suggesting that these defenses are widespread

among *Daphnia*. However, no increase of dorsal and ventral SBAs, nor of spinule lengths of *D. magna* have been found in response to kairomones of a fish species, *Leucaspius delineatus*, suggesting that microstructure induction might also be a predator-specific adaptation (Diel et al. 2021). Similarly, in *D. cucullata*, we found no dorsal or ventral spinules in any of the treatments, including the control and the two predator treatments. This suggest that also spinules are not expressed in all *Daphnia* species. However, we detected that spinules were present in neonates of *D. cucullata* in all treatments (data not shown), suggesting that the expression of microdefenses likely depends on developmental stage. Such stage-specificity has been shown for neck teeth development of *D. pulex* in response to *Chaoborus* kairomones (Tollrian 1995). However, the strongest evidence for microstructures as independent modules of the defense strategy and not merely by-products of other defensive structures is the presence and length induction of microspines in *D. longispina* despite the absence of morphological macrodefenses.

The multitude of *Daphnia* induced defenses have been suggested to be uncoupled (Boersma et al. 1998) following a modular concept, which favors the evolution of defenses tailored towards individual predator species (Herzog and Laforsch 2013). We found that body size adjusted traits in the *Daphnia longispina* complex covaried positively with other body-size adjusted traits both between and within *Daphnia* species. For example, adjusted head, respectively helmet sizes, were significantly related to all other traits besides dorsal SBA. In contrast, the expression of adjusted dorsal SBA was not related to any other morphological trait. This might suggest that specific combination of traits might be especially favorable in some predator environments and might act synergistically. In contrast, other traits might provide only additive benefit when exposed to predators, and thus show no covariation with other traits.

3.6. Future Research

Within the world of plasticity research, one of the ideas that receives too little attention is how among trait relationships (trade-offs or positive covariation) vary across environments and among species (Reger et al. 2018; Stearns 1989). This idea that trade-offs or positive covariation patterns are themselves plastic is important because if the traits involved relate strongly to fitness, as predator defense traits do, then context dependent variation in these relationships will be important to coexistence

under variable environments.

While we cannot analyse our data to explore bivariate reaction norms across environments as proposed by Stearns (1989), we do have enough data to propose a compelling hypothesis that relationships between traits are predator-environment dependent and that this dependency may vary among co-occurring prey species that share two common predators.

For example, in Figure 5, we can see that patterns of GxE vary among species (the variation and sign of the relationship for each species change in different ways across the treatments). Variability in particular seems to manifest in different ways among species for each predator. For example, head size is more variable under *Bythotrephes* whereas spina size is more variable under *Leptodora*. One might argue that only with *Bythotrephes* as a predator, we see more variability in both residual head and spina sizes, possibly indicating a positive relationship for two species (*D. galeata* and *D. longispina*).

Of course these data, with only 5 genotypes, are too sparse to draw firm conclusions via statistics, but our core analyses highlight substantial genetic variation and across species variation suggesting that increasing the number of genotypes assayed can help reveal the context dependency of trait relationships. Such an effort could start to shed light on how trade-offs and positive relationships among predator defense traits are tied to coexistence among multiple prey species sharing two predators.

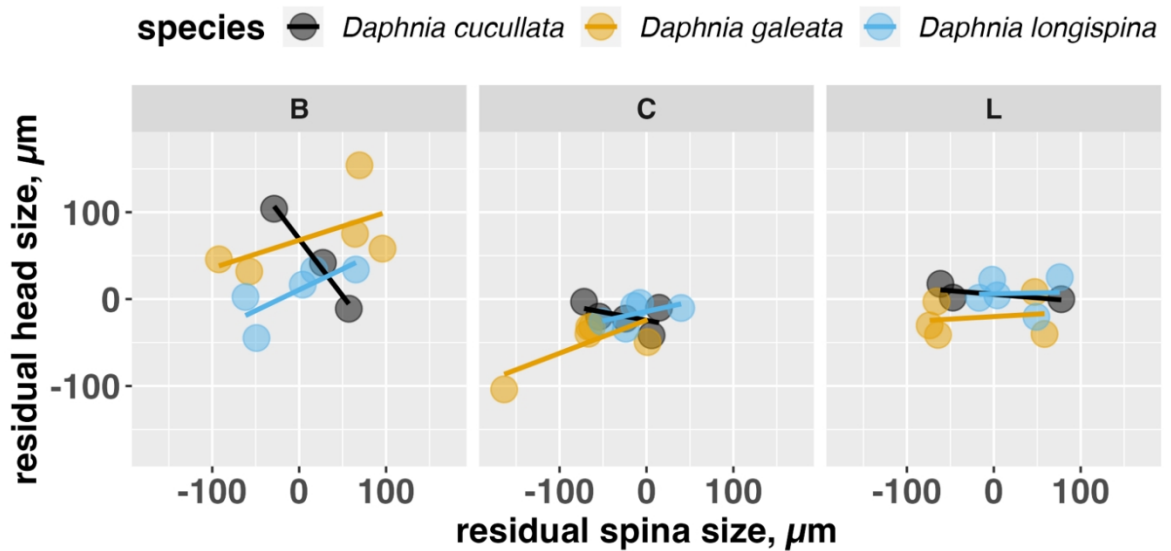


Figure 5. Relationship between residual head size and residual spina size among clonal means of each species in Lake Constance separately shown for the three predator treatments (*Bythotrephes* (B), Control (C), and *Leptodora* (L)). The lines represent the linear regressions of each species' residual head size as a function of its residual spina size.

This is the first study which explores induced defenses of *Daphnia* in a community context involving multiple *Daphnia* prey species, and multiple predators – all occurring in one lake ecosystem. Overall, our work demonstrates the presence of dorsal and ventral SBA microdefenses, highlighting the complexity of defensive mechanisms and their multifaceted contributions to survival strategies. Our study further reveals a captivating contrast between two *Daphnia* species. *D. cucullata* has a big helmet but no microdefenses. In contrast, *D. longispina* deploys microdefenses rather than macro-morphological ones, which is consistent with modularity theory, but challenges the view that microdefenses are mere developmental by-products of other induced morphological changes. The patterns in our data among three prey species and two predator species reveal a multifaceted set of strategies linking macro-morphological characteristics and smaller-scale defenses, which is further modified by the potential role of DVM behavioral responses in *D. longispina*. Clearly, further investigation is warranted to unveil the strategies defined across multiple types of induced traits among multiple species facing multiple predators.

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Morphological defence of *Daphnia galeata* during oligotrophication

4.1. Abstract

Trophic state changes have the potential to alter planktonic community dynamics, including prey and predator interactions. Prey organisms exhibit defensive responses in the presence of predators via morphological changes, which can vary depending on predator type and the age of the prey. These changes can occur through plasticity or microevolution. Here, we investigated the influence of trophic state changes, specifically oligotrophication, on the morphological changes of *Daphnia galeata*. We examined antipredator morphologies of 30 *Daphnia galeata* clones derived from resting eggs produced under different trophic conditions in Lake Constance induced by kairomones from two invertebrate predators, *Bythotrephes longimanus* and *Leptodora kindtii*. Our results identified clonal differences in morphological traits in juvenile and adult daphniids, particularly in spine length and head length. Microevolutionary changes in morphology were found already in the control treatment, whereas the effects of predator kairomones did not change between *Daphnia* clones of different trophic state. Hence, we show that oligotrophication shifts antipredator morphologies of *Daphnia*, but we found no evidence for the evolution of phenotypic plasticity. This study suggests a coupling of bottom-up and top-down control of *Daphnia* at the microevolutionary scale: large changes in food availability may result in microevolutionary adaptation in antipredator morphologies.

Keywords: morphological defence, *Daphnia galeata*, phenotypic plasticity, microevolution, oligotrophication.

4.2. Introduction

Oligotrophication has been shown to modify planktonic communities (Manca et al. 2000; Hsieh et al. 2011; Verbeek et al. 2018) and can alter planktonic prey-predator relationship (Manca & Ruggiu 1998; Krol et al. 2019). Food web interactions and predator-prey relationships are crucially influenced by the defences of prey species. Such defences are usually costly to express which resulted in to evolution of defences, which are expressed only during the presence of predator, that is, inducible defences. Inducible

defences are especially well studied in *Daphnia* and are induced by chemical compounds released by predators, so-called kairomones (Weiss et al. 2018; Ayelo et al. 2021). As reviewed by Diel et al. (2020) numerous studies have demonstrated that kairomones can cause the development of a variety of defences, for example, morphological, life history, and behavioural defenses which are known to increase *Daphnia* survival in the presence of predators.

Daphnia responses to kairomones may depend on the predator type or species (Kleiven et al. 1996; Ritschar et al. 2020; Octorina et al. 2022), on the *Daphnia* species (**chapter III**) and on the size, respectively instar of *Daphnia* (Hanazato 1991; Nagano & Yoshida 2020). Furthermore, food availability may also influence the expression of inducible defences. However, there are contrasting results whether low food availability will result in decreased (Hanazato 1991; Chang & Hanazato 2003) or increased (Parejko & Dodson 1990; Pauwels et al. 2010b) expression of defences. Oligotrophication of lakes thus may influence anti-predator responses of *Daphnia*. *Daphnia* produces resting eggs encapsulated in so-called ephippia, which can be sampled from lake sediments. *Daphnia* is known to hatch from ephippia possibly up to hundreds of years old (Fritsch et al. 2014). The research approach of establishing *Daphnia* clonal lines from ephippia and performing common garden experiments is called “resurrection ecology” (Hairston et al. 1999; Kerfoot & Weider 2004; Burge et al. 2018). Resurrection ecology allows studying microevolutionary responses to environmental changes such as changes parasite pressure (Decaestecker et al. 2007), global warming (Geerts et al. 2015) and eutrophication and oligotrophication (Hairston et al. 1999; Isanta-Navarro et al. 2021). However, whether there is microevolutionary change in *Daphnia* morphological antipredator responses during oligotrophication has not been yet studied.

Here we study microevolution of morphological antipredator responses to two invertebrate predators of *Daphnia galeata* in Lake Constance during the oligotrophication period of the lake using an resurrection ecology approach. The trophic state of Lake Constance has shifted during the last century from oligotrophic conditions (until the 1950-1960s), to eutrophic conditions (1960s to 1990s) and back to oligotrophic conditions (since the 2000s) (Milan et al. 2022). We hatch *Daphnia* from ephippia sampled from sediment layers dating back to the 1970s, and compare their morphologies under control conditions with those induced by the two invertebrate predators *Bythotrephes longimanus* and *Leptodora kindtii*. We analyse whether there is evidence

for the evolution of antipredator morphologies of *Daphnia galeata* during this period, and whether there is evidence for the evolution of phenotypic plasticity, i.e., whether the strength of induced anti-predator responses changes with oligotrophication.

4.3. Methods

Experiment animals

We established *D. galeata* clones from resting eggs collected from a sediment core collected in the Friedrichshafen Bay (47°37'N, 9°29'E, Wessels et al.1999) of Upper Lake Constance. The 50 cm long sediment core was sliced at 1 cm intervals. The samples then were washed under running water through a 250 µm mesh size sieve. The ephippia were isolated using a Stemi 2000-C binocular and immediately transferred to 24-well plates filled with age filtered lake water. Each well contained 1 ephippia and 0.05 ml-1 food suspension green algae *Acutodesmus obliquus* was added to assure food availability when the neonates hatched from the resting eggs. The well-plates were checked daily for hatched neonates. After hatching, one day old neonates were transferred to a new medium. These new *Daphnia* clones were cultivated in filtered aged lake water and kept at 20°C room, fed by green algae *Acutodesmus obliquus*.

Above four generation synchronized age mother were raised and their synchronized second clutch (neonates born within 12 hours) were used as experimental animals. Hatching of resting eggs, *Daphnia* culturing and the experiment were done in 20°C room temperature with artificial daylight (16:8 L:D). *Daphnids* were fed during both experiments with freshly prepared food the green alga *Acutodesmus obliquus* (Culture Collection of Algae, University of Göttingen, Germany, SAG 276-3a), which was grown semi-continuously at 20 °C in 2-L batch cultures in Cyano medium (Jüttner et al. 1983) and harvest in the late-exponential growth phase (illumination at 120 µmol quanta m⁻² s⁻¹).

Kairomone experiment

The morphological of body traits were investigated in cultured *Daphnia* exposed with kairomone predator. 12 Days old *Daphnids* (assumed has already reached the age at first reproduction) were the experiment animals and expected to have the second clutch during kairomone exposure. From the 50 cm sediment core we abled to hatch resting eggs down to a maximum of 18 cm sediment depth. This depth layer corresponds to approximately 1970 based on counting of sediment laminae and the presence of distinct

marker beds resulting from large flood events in specific years (Milan et al. 2022) . We chose 30 clones *D. galeata* whereas 15 clones were collected from sediment core below 10 cm and 15 clones collected from depths between 11-18 cm. 10 cm corresponds to approximately the mid-1990s, e.g. when total phosphorus concentration in the lake dropped below 25 $\mu\text{g TP/L}$ and *Daphnia galeata* in the lake strongly declined in abundance (Straile, 2015 and unpublished data). In the following we call the clones isolated from a layer depth > 10 cm “eutrophic” clones and clones from a layer depth < 10 cm ”oligotrophic” clones.

We used live predators *Leptodora kindtii* and *Bythotrephes longimanus* as kairomone source, which were caught from Lake Constance with net hauls towed behind a boat. The experiment consisted of three treatments, namely control (C), *Bythotrephes* (B), and *Leptodora* (L). Each experimental unit was composed of a 200 mL beaker filled with 180 mL filtered ($< 0.2 \mu\text{m}$) aged lake water containing five *Daphnids*. All beakers contained a cylindrical plastic cage (4 cm diameter and 6 cm height) with a 140- μm nylon mesh that ensured separation between predators and *D. galeata* while guaranteeing the exchange of kairomones. Five individuals of *Bythotrephes* and *Leptodora* were placed in the plastic cages (B and L treatment), respectively; control (C) cages were left empty. Plastic cages including predators were replaced every second day.

Daphniids were fed every second day with freshly prepared food 1 mg C L⁻¹ of the green alga *Acutodesmus obliquus*. The animals were transferred into new jars after 7 days and fed thereafter with 2 mg C L⁻¹ to account for the increased population sizes in each jar. The experiment was terminated after 15 days and daphniids were stored in 70 % ethanol (Black & Dodson 1990). Further they were subsequently examined using a stemi 2000-C binocular equipped with a camera and an image analyzing software. Body length, body width), head length, head width, spina size, and eye size (length, diameter, and area), and rostrum size were measured as shown in Figure 1.

Data analysis

Only those *Daphnia* were used for morphological measurements which allowed quantification of all morphological traits. This required the exclusion of some *Daphnia* which were damaged and which spina was broken, i.e., for which spina size was lower than the 95 % prediction interval based on their body size (Octorina et al. 2022). Data analyses was run separately for large (adult) and small (juvenile) individuals assuming

that adults were larger than 1100 μm as around 1100 μm body size there was a distinct gap in body size distribution in the data set (see Fig. 2).

Variation in morphological traits was analyzed using linear mixed-effect models (lmer function in lmerTest package) (Kuznetsova et al. 2017). Morphological traits was set as dependent variables while body length (except body length model), treatment and sediment depth served as fixed effect and clonal identity (CI) as random effects. We run the analysis with step function to eliminate non-significant fixed and random effects from the full model. Likelihood ratio tests (LRT) were used to test for the significance of fixed and random effects (anova and ranova functions) and further pairwise post hoc comparisons (Tukey test) as described in Octorina et al. (2022). All statistical analyses were performed in R v 4.02.0 (R Core Team 2020).

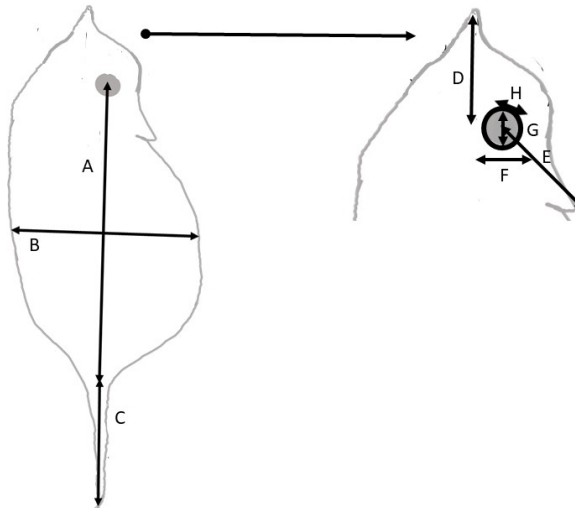


Figure 1. Body dimensions of *D. galeata*. Body size was measured for (A) body length, (B) body width, (C) spine length, (D) Head length, (E) rostrum, (F) eye diameter, (G) eye length, (H) eye area.

4.4. Results

After the growth period of 15 days daphniids have growth to a mean population size of 39.2 ± 16.2 (standard deviation) daphniids per jar. Population sizes did not differ between treatments ($F_{2,90} = 1.64$, ns) nor did show any relationship with sediment depth ($F_{1,90} = 1.24$, ns). Likewise the proportion of daphniids smaller than 1200 μm ($52.3\% \pm 19.1\%$) did not differ between treatments ($F_{2,90} = 0.03$, ns) nor was related to sampling depth ($F_{1,90} = 0.01$, ns).

Body lengths of juvenile and adult *Daphnia* were strongly correlated with all of their body dimensions (Fig. 2). Adult *Daphnia* substantially altered all morphological traits in reaction to kairomones, with the exception of eye length (Table 1). However, the effect of kairomone was only shown in interactions with body length for certain adult body characteristics, such as body width, spine length, and head length. Spine length, head width, head length, and eye diameters of juveniles were also altered by kairomones. Moreover, the interaction between kairomone and body length altered the length of the juveniles' heads and rostrums.

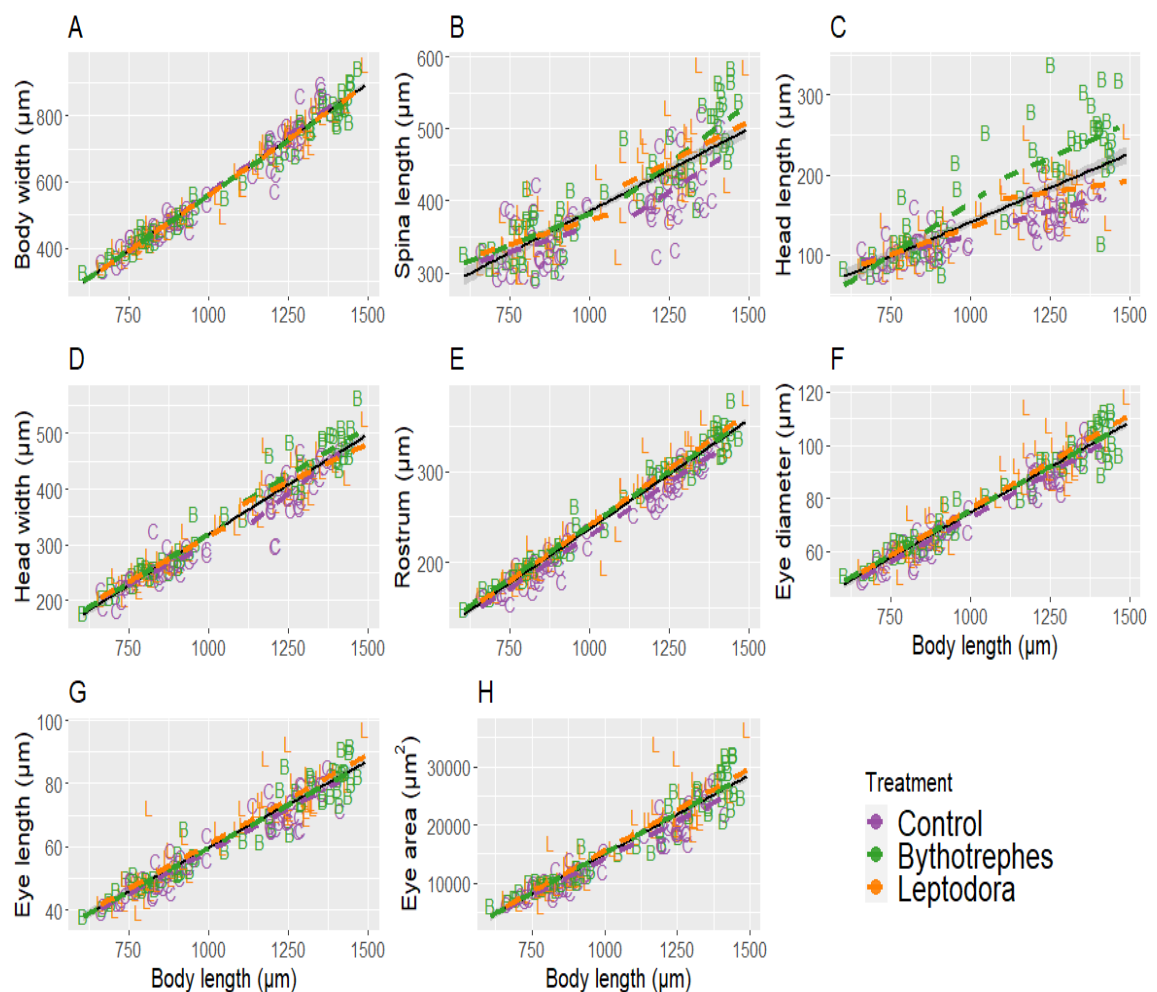


Figure 2. The relationship between body traits and body length of adult and juvenile *Daphnia galeata* in response to kairomones B (*Bythotrephes longimanus*), L (*Leptodorakindtii*), and non-kairomone / Control (C).

Post hoc pairwise tests revealed that compared to the *Leptodora* kairomone, the *Bythotrephes* kairomone exerted a greater influence on adult body length and head traits, particularly head length and width ($p < 0.05$). There was no significant difference in adult body length and head width between the *Leptodora* and control groups. Yet, the *Leptodora* kairomone promoted an increase in the adult eye's diameter and area compared to the control. Regarding juvenile body attributes, both kairomones induced increase in spine size, rostrum size, and eye traits ($p < 0.05$), but did not influence the length of the body (Fig. 3), and there was no difference in size between those induced traits.

Mean body size increased with sediment core depth (which further we'll just call "depth") for juvenile *Daphnia*, but not for adult *Daphnia* (Tables 1,2, Figs. 3,4). It is important to note that we measured the body lengths after a population growth period of 15 days. Hence, differences in mean body length between clones likely reflect differences in growth or reproduction between the clones of different depths, and not specific traits such as size of neonates or size of first reproduction. In the models, body length is thus always included as a predictor to address only body dimension differences adjusted for body length.

Depth had a strong effect on body size dimensions for both adult and juvenile *Daphnia* (Tables 1, 2). Adult body length was not influenced by depth, whereas with decreasing depth (increasingly oligotrophic conditions) *Daphnia* clone adults showed larger residual spines, but smaller residual head length, head width, and eye lengths (Table 1). Juvenile body length strongly increased with decreasing depth, while residual spina size increased, and residual head length (Fig. 4) decreased with decreasing depth. Furthermore, for several traits there was a significant body size: depth (BS:D) interaction suggesting that the depth response differed between individuals of different length. There was no significant interaction between depth and treatment for either adult or juvenile body features. However, for juvenile *Daphnia* the interaction between body length: treatment: depth (BS:TR:D) was significant for head length and rostrum length (Table 2). For all body traits, significant clonal differences (random slopes) were detected using ranova for the corresponding mixed models.

Table 1. ANOVA results for linear mixed-effects regression model of adult body traits with body length as covariates, and the fixed effects treatment, depth, and their interaction. Treatment:depth interactions are not shown in the table as they were not significant for any trait. Statistical significance is denoted by asterisks (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, NS: $p > 0.1$).

Adult body traits	Body length	Treatment	Depth	BS:TR	BS:D
Body length		$F_{2,24.82} = 13.367^{***}$	NS	NS	NS
Body width	$F_{1,1084.56} = 664.24^{***}$	$F_{2,923.01} = 2.36$	$F_{1,734.69} = 2.98$	$F_{2,993.64} = 3.77^*$	$F_{1,1085.34} = 4.75^*$
Spina length	$F_{1,1074.92} = 57.437^{***}$	$F_{2,874.99} = 1.22$	$F_{1,556.27} = 7.41^{**}$	$F_{2,924.20} = 3.35^*$	$F_{1,1076.18} = 4.49^*$
Head length	$F_{1,1069.81} = 89.47^{***}$	$F_{2,797.12} = 0.32$	$F_{1,733.19} = 7.92^{**}$	$F_{2,1004.77} = 3.25^*$	$F_{1,1072.17} = 8.62^{**}$
Head width	$F_{1,1075.01} = 416.32^{***}$	$F_{2,28.81} = 7.55^{**}$	$F_{1,716.93} = 13.63^{***}$	NS	$F_{1,1079.96} = 17.52^{***}$
Rostrum length	$F_{1,1007.3} = 1667.58^{***}$	$F_{2,24.4} = 19.07^{***}$	NS	NS	NS
Eye diameter	$F_{1,1088.29} = 834.31^{***}$	$F_{2,32.95} = 4.40^{**}$	NS	NS	NS
Eye length	$F_{1,1045.21} = 71.09^{***}$	NS	$F_{1,785.74} = 3.14^*$	NS	$F_{1,1069.26} = 4.03^*$
Eye area	$F_{1,1088.30} = 964.02^{**}$	$F_{2,33.66} = 4.28^*$	NS	NS	NS

4.5. Discussion

We found strong evidence for microevolutionary change of body dimensions of *Daphnia galeata* during oligotrophication. Likewise, we show the induction of antipredator-defences by predator kairomones. However, we found no strong evidence for the evolution of phenotypic plasticity, that is, the strength of induced anti-predator defences did not change with oligotrophication.

The body traits of both size groups - regardless of their origin (eutrophic versus oligotrophic clones) - increased in size in response to *Bythotrephes* and *Leptodora* kairomones. This response is commonly observed in *Daphnia* when confronting with invertebrate predators (Diel et al. 2020). The observed responses in the juvenile group suggest that the ability to detect the present of kairomones is established early in life. This finding aligns with the research by Weiss et al. (2016), which also demonstrated that kairomone sensitivity develops already during embryogenesis.

Table 2. ANOVA results for linear mixed-effects regression model of juvenile body traits with body length as covariates, and the fixed effects treatment, depth, and their interaction. Treatment:depth interactions are not shown in the table as they were not significant for any trait. Statistical significance is denoted by asterisks (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, NS: $p > 0.1$).

Juvenile body traits	BS	Treatment	Depth	BS:TR	BS:D	BS:TR:D
Body Length		NS	$F_{1,26,6}=6.05^{***}$	NS	NS	NS
Body Width	$F_{1,1904,1}=1425.80^{***}$	NS	$F_{1,398,6}=4.09$	NS	$F_{1,1951,3}=9.15^{**}$	NS
Spina Length	$F_{1,1932,6}=42.90^{***}$	$F_{2,28,59}=4.10^*$	$F_{1,133,5}=14.16^{***}$	NS	$F_{1,1950,4}=9.42^{**}$	NS
Head Length	$F_{1,1958,26}=203.93^{***}$	NS	NS	NS	$F_{1,1955,95}=6.0594^*$	$F_{2,1920,95}=6.49^{**}$
Head Width	$F_{1,1963,31}=6166.68^{***}$	$F_{2,565,26}=4.50^*$	NS	$F_{2,1839,36}=6.14^{**}$	NS	NS
Rostrum length	$F_{1,1910,2}=844.44^{***}$	NS	NS	NS	NS	$F_{2,1695,48}=3.34^*$
Eye Diameter	$F_{1,1947,08}=528.34^{***}$	$F_{2,25,66}=6.13^{**}$	NS	NS	$F_{1,1959,18}=7.76^{**}$	NS
Eye Length	$F_{1,1908,99}=431.22^{***}$	NS	NS	NS	$F_{1,1932,81}=5.38^*$	NS
Eye Area	$F_{1,1940,69}=444.15^{***}$	NS	$F_{1,235,43}=3.14^*$	NS	$F_{1,1950,62}=9.33^{**}$	NS

In Chapter II, we analysed the antipredator response of *D. galeata* using one day old *Daphnia* of 8 different clones from a highly controlled life history experiment (Octorina et al. 2022) . Here we use *Daphnia* from unknown age of 30 clones produced in a population growth experiment. On one side the larger number of clones should result in a higher power to detect differences between predator treatments, on the other side, population growth within the individual jars resulted in different densities, which consequently might have resulted in food availability differences between jars. This might have increased overall variability thereby lowering statistical power.

This chapter yielded results similar to Octorina et al. (2022) for various traits. For example, *Bythotrephes kairiromones* yielded strongest anti-predator responses regarding *Daphnia* head sizes, spina sizes and eye diameters. However, in contrast to Octorina et

al. (2022) we found also larger residual head sizes in the *Leptodora* treatment (Fig. 5) presumably due to increased power in this experiment. However, development of a typical helmet was also in this experiment restricted to the *Bythotrephes* treatment.

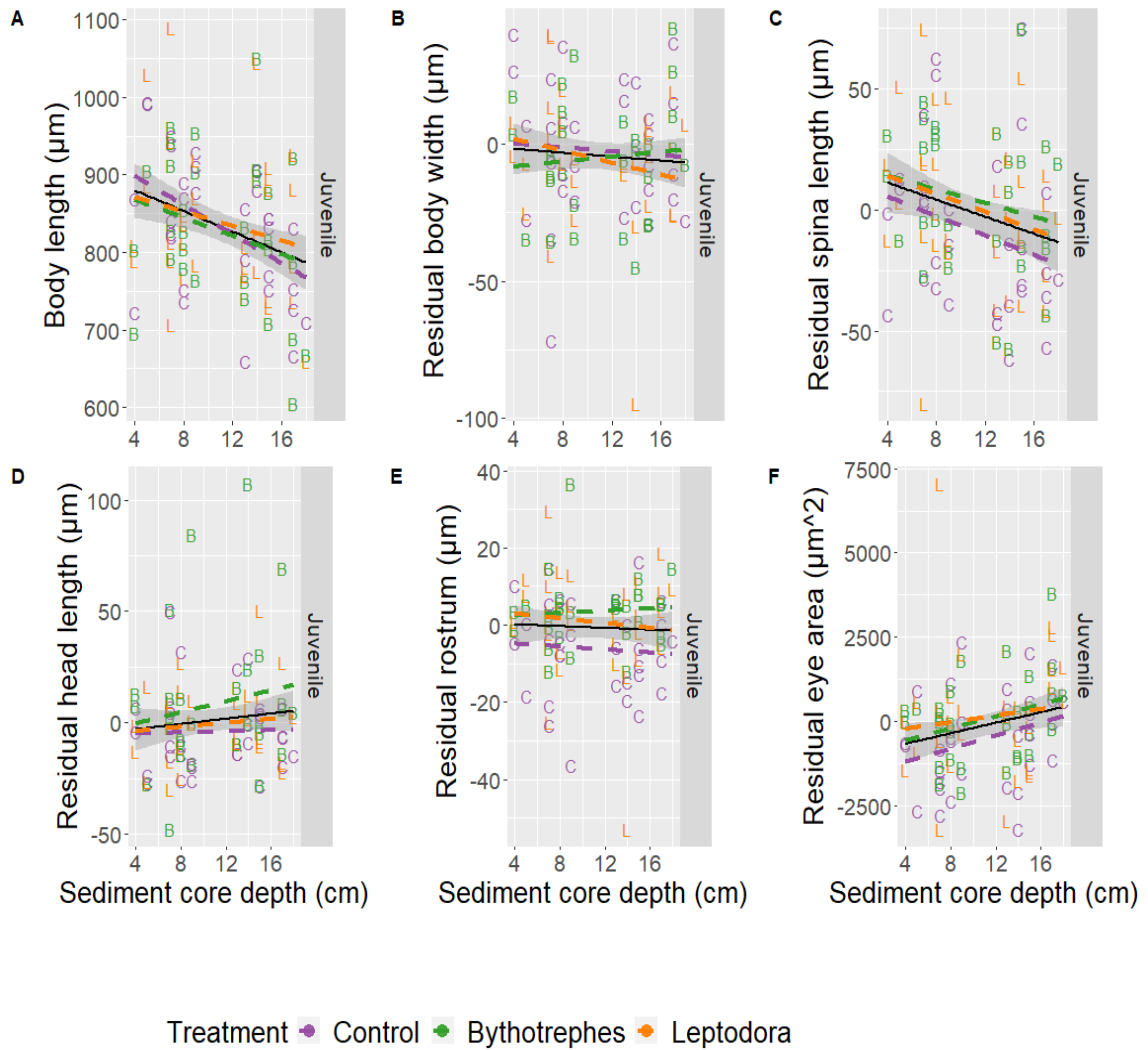


Figure 3. The relationship between body size and residual body traits versus sediment core depth for juvenile *Daphnia galeata*. Only traits significantly related to depth are shown.

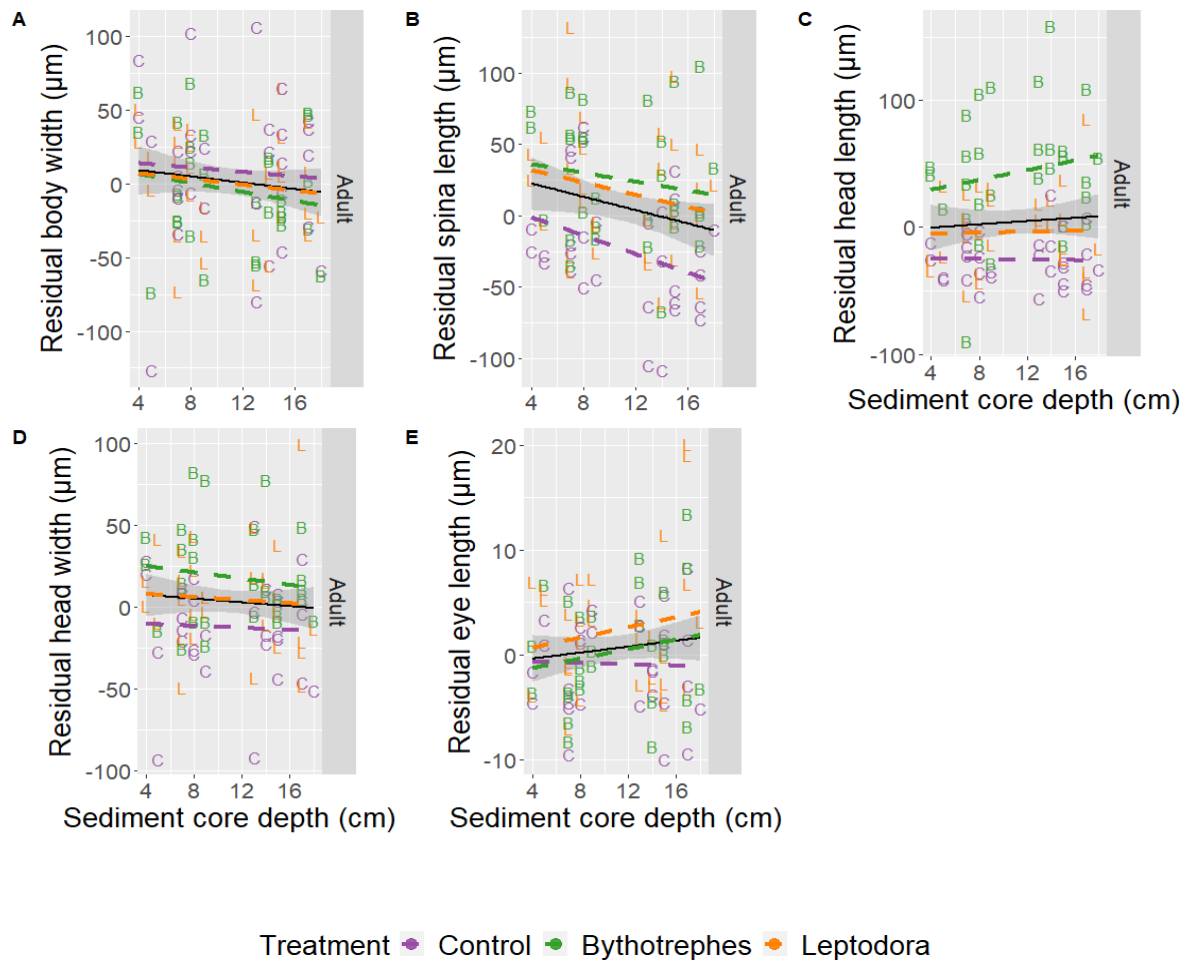


Figure 4. The relationship between residual body traits versus sediment core depth for adult *Daphnia galeata*. Only traits significantly related to depth are shown.

Oligotrophic clones did develop larger spines, but smaller eyes and head sizes compared to eutrophic clones. With oligotrophication, algal biomass declined (Jochimsen et al. 2013) suggesting reduced food availability for *Daphnia*. This might result in favouring *Daphnia* clones investing less in costly defences. On the other side, reduced food availability will result in lower clutch sizes and consequently lower birth rates. Persisting in the system with lower birth rates might only be possible if daphniids are able to reduce their mortality rates. This reasoning would predict higher investment in antipredator defences for oligotrophic clones. While no previous study examined microevolutionary changes in anti-predator defences caused by oligotrophication, some studies examined phenotypic plasticity of anti-predator defences at different food availability. Hanazato (1991) observed that *Daphnia ambigua* produced smaller helmets with lower food concentrations in response to *Chaoborus* kairomones. Likewise, Spaak

and Boersma (1997) found that spina length induced by fish in the *Daphnia galeata* complex was shorter with low food as compared to high food levels. In contrast, fish predation induced stronger anti-predator life history responses in *Daphnia magna* under food stress as compared to a high food environment (Pauwels et al. 2010a) supporting the hypothesis that *Daphnia* may re-allocate resources from growth to antipredator defences under low-food conditions.

Our results suggests that the microevolutionary change in anti-predator responses to reduced food availability might differ between the traits examined: While daphniids developed larger spines with oligotrophication, head sizes and eye lengths decreased. Octorina et al. (2022) suggested that establishment of long heads and helmets might be more costly than spina elongation. Likewise, the large compound eyes of *Daphnia* are expensive to build and the size of *Daphnia* eyes depends on food availability (Walsh & Gillis 2021). This suggests that with oligotrophication *Daphnia* increased the size of a less costly defense (spina), but decreased the size of more costly defenses (heads/helmets and eyes). These changes can already be seen in non-induced animals, that is in the control treatment. We did not detect significant treatment depth (TR:D) interactions, i.e., no evidence for the evolution of phenotypic plasticity. Only for the juvenile daphnids, a significant interaction between body size, treatment, and depth (BS:TR:D) was observed for head and rostrum lengths.

In conclusion, this chapter demonstrates the microevolution of body traits of *D. galeata* during roughly four decades of trophic changes, from the 1970s towards the 2010s. While *Daphnia galeata* clones throughout this period were sensitive to predator kairomones, there was no evidence for the evolution of phenotypic plasticity. Rather, changes in body traits were found to occur already in the control treatment. Hence, oligotrophication of Lake Constance did not only result in the microevolution of traits involved in bottom-up control (e.g. see chapter 6 and Hairston *et al.* 1999, Isanta-Navarro *et al.* 2021), but also in traits presumably mitigating top-down control by invertebrate predators.

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Morphological and life history responses of *Daphnia galeata* to food availability during oligotrophication

5.1. Abstract

Long term trophic state changes influence pelagic food web relationship via regulating food availability for consumers. *Daphnia* has been shown to exhibit phenotypically plastic responses to food differences in food quantity including changes in reproductive allocation strategies affecting e.g. age and timing of reproduction as well as morphological changes in filter comb dimension. However, microevolutionary responses to food limitation have not been studied yet. Here we use a resurrection ecology approach to study microevolutionary change of *Daphnia galeata* to oligotrophication of Lake Constance. 30 *Daphnia* clones retrieved from various sediment depths deposited during eutrophic and oligotrophic conditions, were exposed in a common garden experiment to high and low food concentrations. While *Daphnia* showed plastic responses to food concentrations in filter-comb dimensions and life history patterns, microevolutionary change was only observed for life history patterns. At low food concentrations oligotrophic clones evolved later timing of first and second reproduction suggesting evolution of plasticity in response to food quantity.

Keywords: food level, plasticity, microevolution, morphological changes, life history traits.

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5.2. Introduction

The trophic status of lakes is an important driver of the composition of aquatic food webs via regulating primary productivity and food availability (Barbiero et al. 2012). Long-term changes in trophic status are likely to change food availability for pelagic consumers, which consequently need to adapt either phenotypically or evolutionary to changing conditions. Adaptation might occur via changes in morphology, life history, and/or behaviour, resulting in changes in the characteristics of aquatic organisms such as

reproduction, population density (Leoni et al. 2014), and body size (DeMott et al. 2001) of populations.

Daphnia's responses to changing food availability have been widely studied (Stuchlík 1991; Lampert & Brendelberger 1996; Spaak & Boersma 1997; Repka et al. 1999). *Daphnia* are filter feeders whose filter comb morphology influence its food uptake. Under conditions of low food concentration, *Daphnia* has been shown to increase the area of its filter combs, adjust mesh size, and modify seta length and thickness in order to increase food uptake (Gophen & Geller 1984; Repka et al. 1999; Bednarska 2006; Wejnerowski et al. 2017). In addition to morphological changes, *Daphnia* might also phenotypically adjust its life history strategy (Goos & Jeyasingh 2020). For example, low food concentrations may result in the reduction of energy allocated into reproduction in favour of growth and maintenance (Glazier & Calow 1992). In addition, life history traits such as age and size at maturity might be influenced by food concentrations (McCauley et al. 1990).

Trait adjustment can occur via phenotypic plasticity, which allows organisms to respond to immediate challenges without involving genetic changes (Cavalheri et al. 2019). Phenotypic responses are not passed on to future generations. However, changes in trophic status may also result in microevolutionary changes. For example, changes in trophic status often result in changes in phytoplankton community composition including changes in the concentrations of toxic cyanobacteria. *Daphnia* has been shown to adapt to changing concentrations of toxic cyanobacteria in their diet (Hairston et al. 1999, Isanto-Navarro et al. 2021).

Microevolutionary changes of *Daphnia* in lake ecosystems can be studied using the resurrection ecology approach (Kerfoot et al. 1999; Pauwels et al. 2014; Stoks et al. 2016) in which resting eggs of *Daphnia* are retrieved from the sediment and used for the establishment of clonal lineages. This approach has been successfully used to study adaptation of *Daphnia* to changing food quality (Hairston et al. 1999, Isanto-Navarro 2021, Moody et al. 2021), but it is not clear whether *Daphnia* will also adapt to changing food quantity.

Here we study the microevolutionary response of *Daphnia* in Lake Constance to changing food quantity using a resurrection ecology approach. We compare the life history and filter comb morphology of 30 *Daphnia* clones grown at high and low food concentrations to test the hypothesis that *Daphnia* will evolve life history strategies as

well as filter comb morphologies which increase its fitness under oligotrophic conditions with low food concentrations.

5.3. Methods

Experiment animals

The resting eggs were collected from the Upper Lake Constance sediment core. The 50 cm long sediment core was cut into 1 cm sections, and the samples were washed under running water through a 250 μm mesh size sieve. The remaining ephippia, each containing two resting eggs, were isolated using a Stemi 2000-C binocular and transferred to 24-well plates filled with age-filtered lake water. Green algae *Acutodesmus obliquus* food suspension was added to each well (0.05 ml⁻¹) to ensure food availability for the offsprings upon hatching. The resting eggs were then incubated at 20°C under artificial daylight (16:8 L:D) until the eggs hatched. One-day-old offsprings were transferred to new medium and defined as one clone. Unhatched resting eggs were also transferred to new medium to await hatching of other offsprings.

Hatched *Daphnia* clones were cultivated in filtered aged lake water and kept at 20°C under artificial daylight (16:8 L:D). Offsprings from mothers with synchronized ages (above four generations) were used as experimental animals, specifically their second clutch at 12 hours of age. All culturing and experimental processes were conducted at 20°C under artificial daylight (16:8 L:D). During the experiments, *Daphniids* were fed with freshly prepared food, the green alga *Acutodesmus obliquus* (Culture Collection of Algae, University of Göttingen, Germany, SAG 276-3a). The algae were grown semi-continuously at 20°C in 2-L batch cultures using Cyano medium (Jüttner et al. 1983) and harvested during the late-exponential growth phase with illumination at 120 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$.

Experimental design and experimental procedure

30 clones of *Daphnia* hatched from ephippia collected from sediment depth between x and 18 cm were used in the experiment, with 15 clones from sediments depths below 10 cm and 15 clones from sediment depths above 10 cm (“oligotrophic” versus “eutrophic” clones). *Daphniids* were cultured individually either in 0.2 mg C L⁻¹ *Acutodesmus obliquus* for the low food (LF) treatment or 2 mgC L⁻¹ for the high food (HF) treatment. In total 388 *daphniids* survived either until their first or second

reproduction. Depending on production of neonates during preparation of experimental animals 12.9 ± 5.9 (SD) daphniids per clone were used in the experiment. For roughly 50 % of the daphniids ($n = 184$), the experiment was stopped after their first reproduction. This was necessary as *Daphnia* handling to measure body sizes results in high mortality for *Daphnia galeata*. For the remaining daphniids, the experiment was continued until 2nd reproduction. Age at first and second reproduction were recorded for all daphniids. Neonates and daphniids from all jars were preserved in 70% ethanol for subsequent body size measurements and filter preparation of adult daphniids and counting of neonates.

For analyses of filter morphologies, we dissected the thoracic limb of the third pair from the mothers, and each sample was further processed under a Stemi 2000-C binocular to analyze filter area and setae length. We used a light microscope to analyze filter setae number and SEM to analyze setulae diameter and mesh size. SEM measurements could only be done for daphniids from the LF treatment as we interested in the ontogenetic variation within similar food condition (low food level).

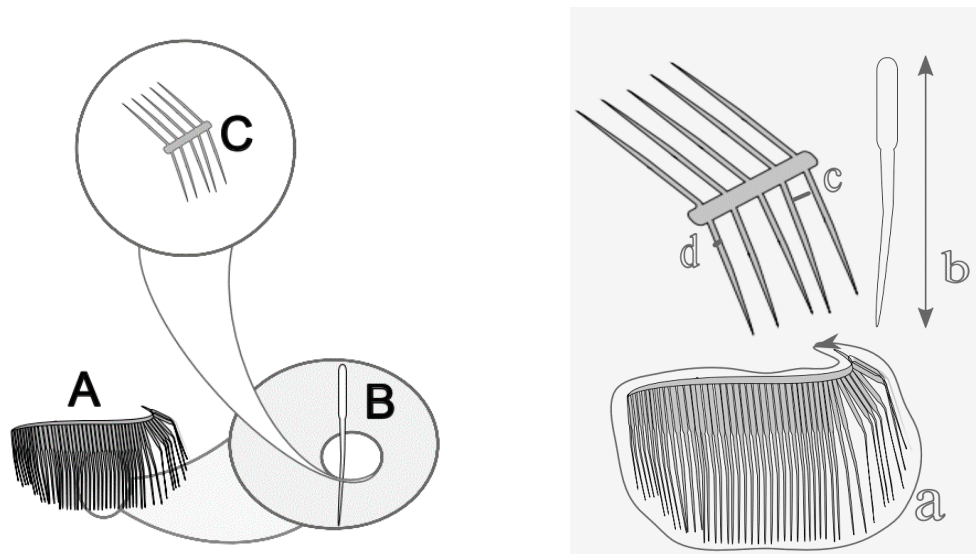


Figure 1. Filter combs dimensions of *D. galeata*. We measured dimensions of the whole filter comb (A), setae (B) and setulae (C) such as filter area (a), setae length (b) mesh size (c) and setulae diameter (d). Body size of *Daphnia* was measured as shown in Octorina et al. (2022).

Data analysis

Variation in morphological and life history traits was analyzed using linear mixed-effect models (lmer function in lmerTest package) (Kuznetsova et al. 2017). Morphological traits was set as dependent variables while body length (except body length model), treatment and sediment core depth served as fixed effect and clonal identity (CI) as random effects. We run life history analysis only for food level experiment, whereas age at first reproduction (AFR) and second reproduction (ASR) and their offspring number (clutch number) were the life history traits.

We run the analysis with step function to eliminate non-significant fixed and random effects from the full model. Likelihood ratio tests (LRT) were used to test for the significance of fixed and random effects (anova and ranova functions) and further pairwise post hoc comparisons (Tukey test) as described in (Octorina et al. 2022) All statistical analyses were performed in R v 4.02.0 (Rcore 2022).

5.4. Results

Food concentration had a significant impact on the body length, clutch size and age of *Daphnia galeata* at their first and second reproduction (Table 1, Fig. 2). Sediment core depth was not related to body sizes at first and second reproduction, whereas age at first and second reproduction, as well as clutch size at 2nd reproduction was influenced by sediment depth – however only after accounting for the effects of body lengths and food treatment in three-fold interactions (Fig. 3). Age at 1st and 2nd reproduction increased with decreasing sediment depth especially at high food concentrations. Likewise clutch size at 2nd reproduction decreased with sediment core depth. For all life history traits, there was a significant random slope, i.e. clones responded differently to the food treatment (Table 2).

Table 1. Anova results of body lengths and life history traits with the fixed effects body length, treatment, depth, and their interactions. Statistical significance is denoted by asterisks (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, NS: $p > 0.1$).

Traits	Body Length (BL)	Treatment (T)	Depth (D)	BL: T	BL:D	T:D	BL: T:D
Body length at AFR		$F_{1,22.7} = 104^{***}$	NS			NS*	
Body length at ASR		$F_{1,182.4} = 185.2^{***}$	NS			NS*	
AFR	NS	$F_{1,153.82} = 6.28^*$	$F_{1,162.00} = 4.68^*$	$F_{1,153.03} = 6.15^*$	$F_{1,154.06} = 4.46^*$	$F_{1,150.40} = 5.01^*$	$F_{1,148.87} = 5.69^*$
ASR	$F_{1,15} = 7.24^{**}$	$F_{1,13} = 6.10^*$	$F_{1,17} = 7.36^*$	$F_{1,13} = 3.40$	$F_{1,16} =$	$F_{1,13} = 4.89^*$	$F_{1,13} = 5.03^*$
FR-clutch size	$F_{1,18} = 51.63^{***}$	$F_{1,17} = 29.19^{**}$	NS	$F_{1,17} = 34.68^{***}$	NS	NS	NS
SR-clutch size	$F_{1,18} = 4.54^*$	$F_{1,19} = 6.58^*$	NS	$F_{1,19} = 8.04^{**}$	NS	$F_{1,19} = 4.28^*$	$F_{1,19} = 4.04^*$

Table 2. Likelihood ratio test (LRT) results for body length, filter comb, and life history traits with 'Tr | clone' random effects.

Traits	LRT (Tr Clone)
Body length	20.035***
Filter area	5.359*
Setae length	NS
Setae number	43.1***
Mesh size	NS
AFR	12.088**
ASR	8.731*
FR clutch size	6.438*
SR clutch size	8.905*

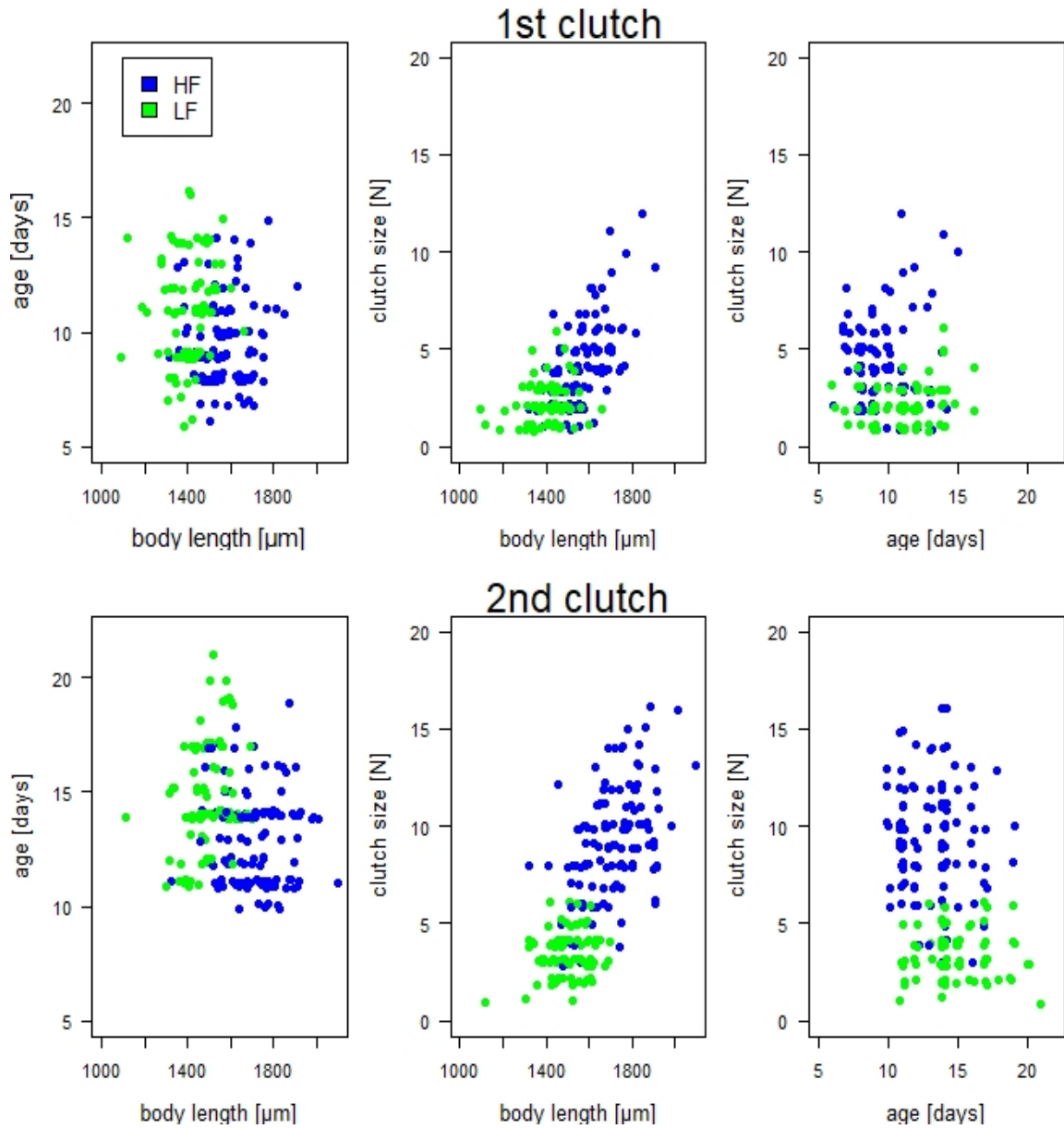


Figure 2. Relationships between body lengths, ages at reproduction and clutch sizes at first reproduction (upper panel) and 2nd reproduction (lower panel) in the two food treatments. Age at reproduction and clutch sizes are shown jittered to avoid complete overlap of data points.

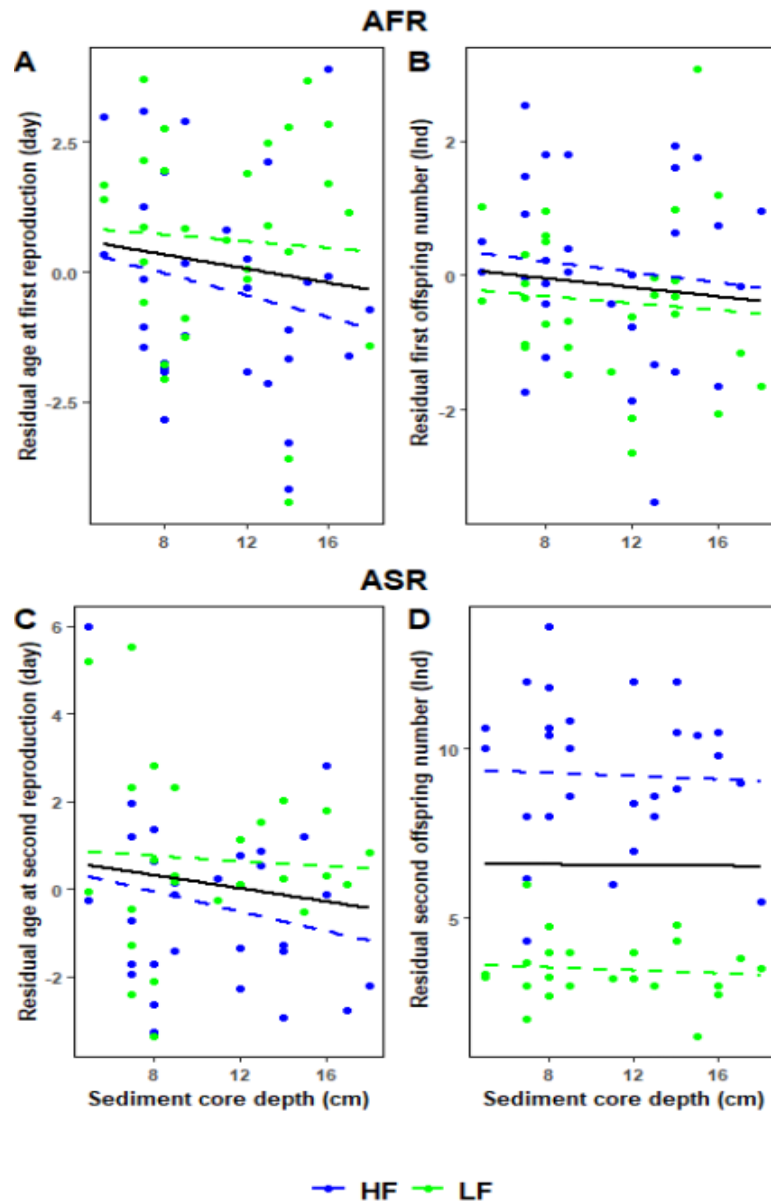


Figure 3. Relationships between sediment core depth, residual ages at reproduction and residual offspring number (clutch sizes) at first reproduction/AFR (upper panel) and 2nd reproduction/ASR (lower panel) in the two food treatments.

All filter comb traits with the exception of setae number and setulae diameter were significantly related to *Daphnia* body size (Table 3, Fig. 4). Food concentration significantly affected filter screen area and setae length but not the setae number and seta diameter (Table 3). We found no significant relationship of filter dimensions to sediment core depth (Table 3). Clones showed significant differences to food concentrations (significant random slopes) for all traits except of mesh size and setae length.

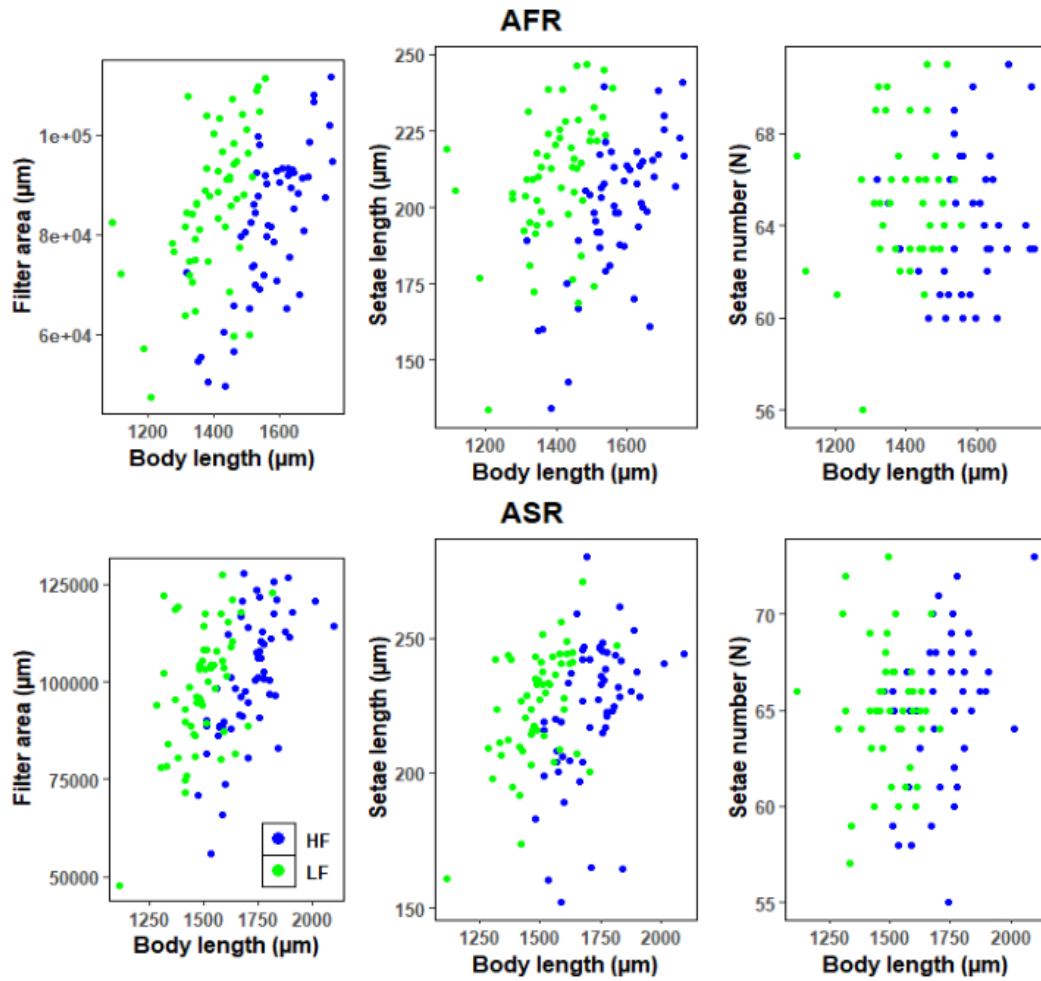


Figure 4. Relationships between body lengths, filter area, setae length and setae number at first reproduction/AFR (upper panel) and 2nd reproduction/ASR (lower panel) in the two food treatments, high level (HF) in blue and low level (LF) in green.

Table 3. Anova results of body and filter comb traits with body length as covariates, and the fixed effects treatment, depth, and their interaction. Note that mesh sizes and setulae diameters were analysed only for the low food treatment. Statistical significance is denoted by asterisks (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, NS: $p > 0.1$).

Traits	Body Length (BL)	Treatment (T)	Depth (D)	T:D	BL:D
Filter area	$F_{1,11}=66.60^{***}$	$F_{3,96}=26.41^{***}$	NS	NS	NS
Setae length	$F_1=41.31^{***}$	$F_3=19.57^{***}$	NS	NS	NS
Setae number	NS	NS	NS	NS	NS
Setulae diameter	NS		NS		NS
Mesh size	$F_1=21.99^{***}$		NS		NS

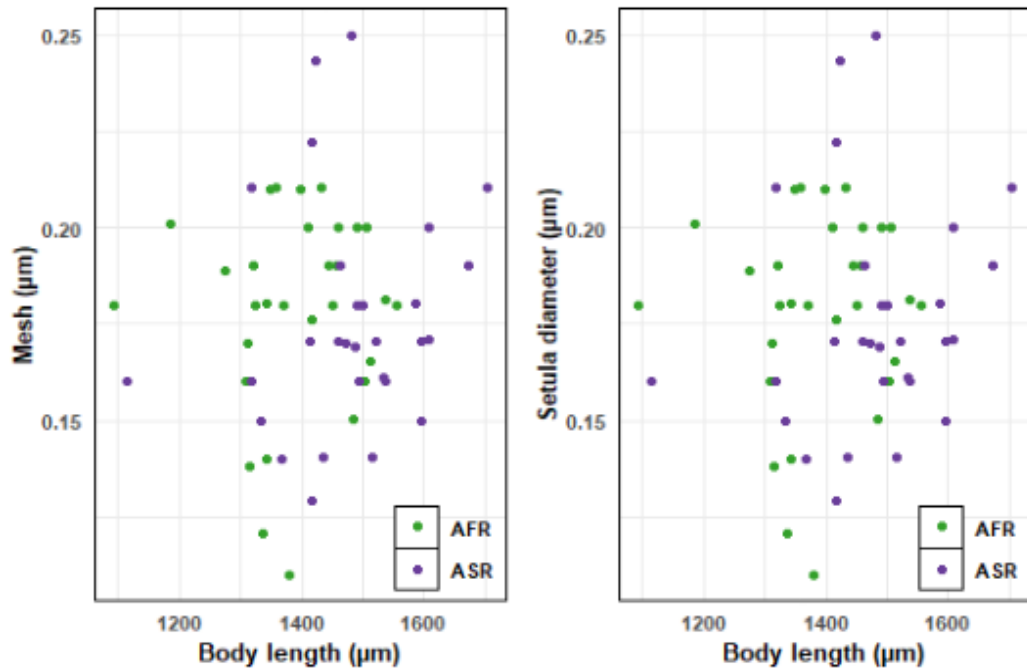


Figure 4. Relationships between body lengths, mesh size and setula at first reproduction/AFR and 2nd reproduction/ASR in the low food treatments.

5.5. Discussion

Using a resurrection ecology approach we show that the *Daphnia galeata* life history response to food limitation changed during oligotrophication of Lake Constance. *Daphnia galeata* evolved different reproductive schedules (age at 1st and 2nd reproduction) at two food concentrations suggesting evolution of phenotypic plasticity. In contrast, we found no evidence for evolutionary changes of body length and filter comb traits of *Daphnia* during the oligotrophication period.

Daphnia's filter comb play a vital role in efficient food gathering and energy intake. The size of this filtering apparatus scales with body size where larger *Daphnia* have larger combs (Egloff & Palmer 1971; Brendelberger & Geller 1985; Pop 1991). However, filter comb dimensions are also shaped by food availability beyond growth effects. The observed responses of filter comb traits to food availability support previous results showing that *Daphnia* exposed to low food levels have larger filtering areas compared to those exposed to high food levels (Gophen & Geller 1984; Pop 1991; Repka 1999). Likewise, the length of *Daphnia*'s setae and mesh sizes was influenced by food conditions: food limited conditions resulted in longer setae, indicating an adaptive response to improve feeding efficiency in low food environments. However, we did not

observe significant differences in setae number between different food levels or ontogenetic stage. This corresponds to the suggestion that setae number of *Daphnia* remains constant throughout an individual's life and cannot be adjusted to different food conditions during ontogenetic development (Wejnerowski et al. 2017). In contrast to strong treatment effects on filter dimensions, we found no evidence for microevolutionary change of filter dimensions during oligotrophication.

Similarly, to filter comb dimension also life history traits were strongly influenced by food concentrations and support previous results showing that at low food concentration *Daphnia* does grow more slowly, mature and reproduce later, and produce smaller clutches (Goos & Jeyasingh 2020; Klumpen et al. 2021). The allocation of energy into somatic growth versus reproduction follows a fine-tuned strategy to increase fitness under different food concentrations. We provide evidence that with oligotrophication there is microevolutionary change in these resource allocation patterns resulting in late timing of reproductive events for *Daphnia* from the oligotrophic time period. Adaptation to different trophic status was also found when comparing *Daphnia pulicaria* clones from hypereutrophic versus eutrophic lakes. The former showed increased fecundity and ontogenetic growth rates when raised on phosphorus fertilized seston (Moody et al. 2021). Likewise, previous studies have shown *Daphnia galeata* evolve to better cope with cyanobacteria in their diet (Hairston et al. 1999), but lost this capacity again with oligotrophication (Isanta-Navarro et al. 2021). In these studies, *Daphnia* was shown to microevolutionary respond to different aspect of food quality, whereas we show that there is a also microevolutionary response to food quantity.

In contrast to reproductive timing we found no evidence for microevolutionary changes in body lengths at first and second reproduction. This might suggest that daphniids evolved different reproductive timings to maintain body lengths at reproduction constant. However, more detailed analysis of our data including modelling of reproductive strategies at different food conditions seem to necessary for a full understanding of the observed patterns, and whether these changes indeed increase fitness in oligotrophic conditions.

In conclusion, our study provides evidence for the evolution of phenotypic plasticity of *Daphnia* in response to varying food quantity. *Daphnia* evolved later reproductive timings at low food concentrations. In contrast, we found no evidence for

the evolutionary change in filter comb dimension. This study adds further aspects of *Daphnia* microevolutionary change in response to trophic change of lake ecosystems.

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This final chapter discusses various aspects of *Daphnia* ecology and evolution in deep Lake Constance. First, I will discuss the role of *Daphnia* spp. in pelagic food webs. Subsequently, I focus on morphological changes induced by invertebrate predators including their life-history costs. Finally, I will discuss the effects of oligotrophication on microevolutionary changes of *D. galeata* morphological and life history traits.

Large *Daphnia longispina* as keystone species

In Lake Constance three *Daphnia* species from the *Daphnia longispina* species complex coexist: *D. longispina* (native), *D. galeata* (invaded during eutrophication in the 1950s) and *D. cucullata* (invaded in the 2010s presumably as a result of increased predation pressure due to the invasion of sticklebacks in Lake Constance). Daphniids are widely known as keystone herbivores that link different trophic levels resulting in cascading effects to lower trophic levels. Our field mesocosm experiment (**chapter I**) allowed us to study which species from the *Daphnia longispina* species complex acts as a keystone species, as by definition a keystone species should be characterised by highest centrality and strongest impact (Jiang & Zhang 2015; Cagua et al. 2019; Fan et al. 2023).

We found that large bodied *D. longispina* experienced the strongest predation pressure from fish among all zooplankton taxa. Likewise, this species also more strongly controlled phytoplankton abundance compared to other zooplankton taxa and *Daphnia* species. As fish predation is size specific (Jemelian & Schindler 2023) large bodied *D. longispina* most strongly declined in mesocosms with fish. This presumably released other zooplankton species – including small *D. cucullata* - from competition by *D. longispina*. As the third *Daphnia* species in Lake Constance, *D. galeata* strongly declined with oligotrophication in the lake, its response to predation was not analysed in this mesocosm experiment. The decrease of *D. longispina* abundance reduced overall zooplankton grazing and thereby top-down control of phytoplankton. Hence, cascading effects on phytoplankton biovolumes were observed in the fish mesocosms.

***Daphnia* morphological responses to invertebrate predator kairomones**

Observations on morphological changes of *Daphnia* due to induction by invertebrate predator kairomones are provided in **chapters II, III** and **IV**. Regarding predator kairomone effects on *D. galeata* all three chapters found similar effects with some differences presumably originating from differences in statistical power between

studies as in **chapter III** 8 clones, in **chapter IV** 5 clones and in **chapter V** 30 clones of *D. galeata* were used. In **chapter III** we analysed whether *D. galeata* anti-predator responses differ between the closely related cladoceran predators *Bythotrephes longimanus* and *Leptodora kindtii*, which co-occur in Lake Constance with *D. galeata*. *D. galeata* developed larger body size under the influence of both predator kairomones as typically observed for invertebrate predator kairomones (Diel et al. 2020). In addition we observed longer spines, larger head size with helmet formation and larger eye size in response to *Bythotrephes* kairomones. In contrast, no helmets and no increased eye sizes were induced as a response to *Leptodora* kairomones. Also lower costs could be detected for the defenses induced by *Leptodora* kairomones. It would be highly interesting to study whether – despite the differences in costs – antipredator responses provide similar benefits against both predators. In this case, *D. galeata* might be able to express defenses against *Leptodora* at lower energy and food availability as compared to *Bythotrephes*. An interesting subsequent study would be to analyse the induction of antipredator defenses at different food concentrations for *Daphnia*.

The adaptive benefit of larger eyes in the presence of *Bythotrephes* is not clear. Especially so, as *Bythotrephes* is a visual predator (Pangle & Peacor 2006) and might detect daphniids with larger eyes more easily. However, larger eyes might also allow *Daphnia* to detect *Bythotrephes* earlier, thus minimizing contact with the predator. Such a reasoning would be consistent with the recent finding that *Daphnia* in lakes with high fish predation pressure evolved larger eyes compared to *Daphnia* in lakes with low fish predation pressure (Muirhead & Sprules 2003; Pangle & Peacor 2006; Beston et al. 2019). The results in **chapter II** confirmed that *D. galeata* morphological defenses are predator specific also when considering two different cladoceran predators *Bythotrephes* and *Leptodora*. Consequently, the presence of these invertebrate predators may trigger different evolutionary trajectories in natural zooplankton communities due to predator-specific and clone specific defense-cost trade-offs.

Chapter III shows that responses to predator kairomones of *Daphnia* species within the *Daphnia longispina* species complex differ between the *Daphnia* species. This supports previous studies showing that different *Daphnia* species may employ different antipredator strategies (Kiehnau & Weider 2022). Our results indicate similar responses of *D. galeata* and *D. cucullata* which both developed a helmet in response to predator kairomones. Regarding *D. galeata* this confirms our findings from **chapter II**. In contrast,

D. longispina did not show any head extension, presumably because *D. longispina* performs diel vertical migration in Lake Constance which might protect this species also from visually hunting *Bythotrephes*. Consequently, a costly elongation of its head might be not adaptive for this species. Likewise, we did not detect formation of neck teeth in *D. longispina*, which has been observed in *D. longispina* from other lakes presumably as a response to *Chaoborus* predation (Sperfeld et al. 2020). However, we also observed development of microdefenses in *D. longispina* as a response to predator kairomones. This suggests that also species performing diel vertical migration invest in morphological defenses to finetune its antipredator strategies. In addition, it shows that microdefenses are induced independently of the usually studied macrodefenses such as tail spines and helmets.

Microevolutionary responses of *D. galeata* to oligotrophifications

Changes in lake nutrient levels over long periods of time such as eutrophication or oligotrophication will modify plankton communities and food web dynamics (Krol et al. 2019; Marina Manca et al. 2000; M. Manca & Ruggiu 1998; Rose et al. 2021). Such processes will directly or indirectly via food web interactions result in changes in genetic structure and species traits via microevolution (Brede et al. 2009; Isanta-Navarro et al. 2021). **In Chapters IV and V**, I analysed how oligotrophication has affected daphniid microevolutionary responses to both invertebrate predation pressures and food availability. By hatching *Daphnia* eggs stored in the lake's sediment layers from different time periods, I compared clones that are presumably adapted to periods characterized by high versus low nutrient concentrations. This allowed me to trace microevolutionary changes within this single species across generations experiencing declining nutrient concentrations.

Chapter IV analysed differences between *Daphnia* clones from varying sediment depths, representing past time periods with higher vs lower nutrients. *Daphnia* spina lengths showed decreasing trends with increasing core depth for both juvenile and adult daphniids. In contrast head lengths and eye sizes were larger for eutrophic clones, however, this differences was more pronounced in juvenile daphniids compared to adult ones. This suggests that the reduction of nutrient concentrations indirectly resulted in microevolutionary changes in defensive traits against invertebrate predators. Our results are thus in line with studies analyzing phenotypic plasticity of defenses at different food concentrations, which found both, increasing investment in defenses (Pauwels et al.

2010), but also decreasing investment in defenses (Hanazato 1991) with lower food concentrations. *Daphnia galeata* from Lake Constance seems thus to have evolved larger investment of less-costly defenses (spines), but smaller investment in more-costly defenses (heads, eyes). Microevolution of defenses thus might differ between individual defenses due to differences in costs associated with each defense. Increased investment in less-costly defenses might help *Daphnia* to reduce predator-induced mortality also during periods of limited energy availability. However, we did not detect any evidence for the evolution of plasticity in respect to anti-predator morphologies as microevolutionary changes were observed already in the control treatments and there was no microevolution regarding the strength of induced responses. Hence, despite the costs of defenses induced by *Bythotrephes* kairomones were found to be larger than those induced by *Leptodora* kairomones (**chapter II**), also *D. galeata* clones from the oligotrophic time period, did invest in similar defenses as more “eutrophic” clones. It would be interesting to repeat this experiment with lower food concentrations for *Daphnia* to see if lower food availability would allow to detect plasticity evolution of *D. galeata* antipredator defenses. In addition to anti-predator defenses, we found also microevolution of life history strategies in response to oligotrophication (**chapter V**): more oligotrophic clones reproduced later at low food concentrations compared to more eutrophic clones. Hence, in response to different food concentration we detected evolution of plasticity of daphniids reproductive allocation strategies. Hence, the traits investigated in chapters V and VI seem to differ strongly in respect to their evolvability with oligotrophication. It will be important to develop a better understanding why some traits showed rather large evolutionary change, whereas others did not or hardly change. Likewise, the consequences of the observed changes for food-web interactions and the persistence of *D. galeata* in the Lake Constance ecosystem are unknown. The complex and multiple microevolutionary responses of *D. galeata* during oligotrophication of Lake Constances, however, suggest that understanding the role of evolutionary change for *Daphnia* in Lake Constance will require studying the combined effects of multiple traits and stressors. My thesis is a first step in this direction.

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Chapter I: Large *Daphniids* are keystone species that link fish predation and phytoplankton in a trophic cascade

Ž.O.: Conceptualization (equal); data curation (equal), investigation (equal); methodology (equal); formal analysis (equal); writing – review and editing (lead); final approval for publication (equal). **C.W.**: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); formal analysis (equal); writing – review and editing (lead); final approval for publication (equal). **A.J.K.**: Conceptualization (equal), investigation (equal); methodology (equal); formal analysis (equal); data curation (equal), writing – review and editing (equal); final approval for publication (equal). **P.O.**: data curation (equal), investigation (equal); methodology (equal); writing – review and editing (equal); final approval for publication (equal). **J.I.N.**: Conceptualization (equal); investigation (equal); methodology (equal); formal analysis (equal); writing – review and editing (equal); final approval for publication (equal).

Chapter II: Morphological defenses and defense–cost trade-offs in *Daphnia* in response to two co-occurring invertebrate predators

P.O.: formal analysis (equal); writing – original draft preparation (lead); writing – review and editing (equal); final approval for publication (equal). **A.B.**: data curation (equal); investigation (equal); methodology (equal); writing – review and editing (equal); final approval for publication (equal). **D.M.C.**: writing – review and editing (equal); final approval for publication (equal). **D.S.**: Conceptualization (equal), funding acquisition (lead); project administration (lead); supervision (lead); methodology (equal); formal analysis (equal); writing – review and editing (equal).

Chapter III: Antipredator responses of three *Daphnia* species within the *D. longispina* species complex to two invertebrate predators

M.Y.B.: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); formal analysis (equal); writing – original draft preparation (lead); writing – review and editing (equal); final approval for publication (equal). **P.O.**: data curation (equal); investigation (equal); methodology (equal); writing – review and editing

(equal); final approval for publication (equal). **A.B:** formal analysis (equal); writing – review and editing (equal); final approval for publication (equal). **D.S:** Conceptualization (equal), funding acquisition (lead); project administration (lead); supervision (lead); methodology (equal); formal analysis (equal); writing – review and editing (equal); final approval for publication (equal).

Chapter IV: Morphological defense of *Daphnia galeata* during oligotrophication

P.O: Conceptualization (equal); data curation (equal), investigation (equal); methodology (equal); formal analysis (equal); writing – original draft preparation (lead); writing – review and editing (equal). **D.S:** Conceptualization (equal), funding acquisition (lead); project administration (lead); supervision (lead); methodology (equal); formal analysis (equal); writing – review and editing (equal).

Chapter V: Morphological and life history responses of *Daphnia galeata* to food availability during oligotrophication

P.O: Conceptualization (equal); data curation (equal), investigation (equal); methodology (equal); formal analysis (equal); writing – original draft preparation (lead); writing – review and editing (equal). **D.S:** Conceptualization (equal), funding acquisition (lead); project administration (lead); supervision (lead); methodology (equal); formal analysis (equal); writing – review and editing.