Stoichiometric mismatch between phytoplankton and zooplankton consumers

Effects at contemporary, transgenerational, and evolutionary timescales

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Stoichiometric mismatch between phytoplankton and zooplankton consumers

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Stoichiometrisch onevenwicht tussen fyto- en zoöplankton: effecten binnen generaties, tussen generaties en op een evolutionaire tijdschaal

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

Introduction

All the organisms on earth are exposed to combinations of multiple factors in their environment, such as light, temperature, and nutrients. Changes in one of these factors may result in strong influences on organism performance. Particularly, living matters are made up of various unsubstitutable basic elements, such as carbon (C), nitrogen (N), phosphorus (P) and other elements, and have identical demands of materials for their growth and metabolism. These elements cannot be synthesized or interconverted by organism themselves but need to be extracted from the ambient environment. Therefore, whichever element that is in the shortest supply relative to demands of an organism will limit the growth of that organism (Liebig's minimum law, Liebig 1840).

In recent decades, biogeochemical cycles of essential elements such as carbon (Lorenz and Lal 2008; Regnier et al. 2013), nitrogen (Schlesinger 2009), and phosphorus (Yan et al. 2016) in natural systems have been strongly altered by anthropogenic activities. These changes may have far-reaching consequences for organisms, and scale up to communities, or even entire ecosystems.

Ecological stoichiometry (ES)

Historically, an early cornerstone in contributing the development of ecological stoichiometry is the work by Redfield (1963), who found that the atomic ratios of C, N, and P in marine seston were the same as the ratios of differences of dissolved nutrients in ambient waters (C_{106}:N_{16}:P_1, Redfield ratio). This congruence in nutrient ratios further indicated a balanced flow of C, N, and P in and out of biota. Another key contribution to ecological stoichiometry is the findings that the consumer – driven nutrient cycling was affected by both algal and zooplankton characteristics (Consumer-driven nutrient cycling, CNR, Elser et al. 1988). Thereafter, many studies have applied the mass balance and stoichiometric approach to the studies investigating producer – consumer interactions (Sterner 1990; Elser and Hassett 1994; Elser and Urabe 1999). Defined as "The balance of multiple chemical substances in ecological interactions and processes, or the study of these balances, also sometimes refers to the balance of energy and materials" (Sterner and Elser 2002), ecological stoichiometry uses a multiple-nutrient approach to link the biochemical composition of organisms to their growth and reproduction, and investigates how the altered supply of the amount and ratios of these nutrients may generate influences on organisms, and eventually impact ecosystem structure and functioning. In recent

decades, ecological stoichiometry has developed into a powerful tool for ecologists to assess the mechanisms and consequences of the altered nutrient biogeochemical cycles on biota.

One fundamental concept in ecological stoichiometry is elemental homeostasis (Sterner and Elser 2002; Hessen et al. 2013). Stoichiometric homeostasis reflects the ability of an organism to maintain a constant internal body stoichiometry in response to variable ambient resource stoichiometry (Sterner and Elser 2002). Generally, ecological stoichiometry posits that autotrophs are flexible in the elemental composition of their body whereas heterotrophs are much more constant (Anderson and Hessen 1991; Anderson et al. 2005; van de Waal 2010). Therefore, any mismatch in elemental ratios between autotrophs and the requirements of consumers ('stoichiometric mismatch') may result in strong performance reductions of consumers (Sterner and Elser 2002; Hessen et al. 2013).

Phosphorus (P) in aquatic systems

Although element N is considered as a very important element for the growth of organisms (Mengel et al. 2001), the main focus of work in ecological stoichiometry is nevertheless element P (Sterner and Elser 2002; Hessen et al. 2013). P is an element whose biogeochemical cycle is strongly altered by anthropogenic activities (Elser and Bennett 2011). P is an important component for various molecules with a variety of biological functions, such as RNA, DNA, and ATP (Elser 2012). For many metazoans, it is also essential as a component of exoskeletons or bones (Elser et al. 1996). Particularly, element P is an important component of ribosomal RNA (rRNA), which drives the synthesis of protein, and further influences the growth of organisms. Based on this three-part correlations, ecological stoichiometry posits that low C:P ratios in rapidly growing organisms reflect increased allocation to P-rich rRNA (Growth rate hypothesis, GRH; Elser et al. 2000; Sterner and Elser 2002; Elser et al. 2003).

The availability of P to freshwater biota is highly variable (Anderson and Hessen 1991; Hessen 1992). Availability depends on the limnological features of the freshwater system, the geology of the drainage system and anthropogenic input. Sewage spill-overs, intensive agriculture and aquaculture have caused strongly increased P-loads to freshwater systems in many areas of the world (Csathó et al. 2007). Conversely, successful restoration efforts also result in strong reductions in P-availability (van Donk et al. 2008). These human activities result in a bidirectional consequences in lake systems, either oligotrphication (Stockner et al. 2000; Elser et al. 2009) or eutrophication (Schindler 2008; Smith and Schindler et al. 2009). The relative

availability of P may strongly determine the molar C:P ratio in seston and as such affect the quality that the seston has as food for zooplankton. Nutrient oligotrophic to mesotrophic lakes (Hecky et al. 1993; Elser et al. 2001; van Donk et al. 2008) typically have seston C:P values > 400, whereas hypereutrophic lakes have seston C:P values < 80. A recent survey by Sterner et al. (2008) assembled data sets of seston C:P from a variety of waterbodies including small lakes and great lakes, and reported that up to 10% of these waterbodies had seston C:P values higher than 380, whereas 15% of them were lower than 100.

Mismatch of C:P ratios at producer - consumer interface

Most studies in ecological stoichiometry investigating the consequences and mechanisms of stoichiometric mismatch are focusing on the effects of P limitation on the producer – herbivore interface. An early evidence providing support for the idea that P alone is able to strongly reduce the growth of consumers came from the lab study of Rothhaupt (1995), who applied an elegant P-enrichment method and showed that the addition of inorganic P to P-limited algal food strongly stimulated the growth of the rotifer *Brachionus rubens*. This result was further confirmed by Urabe et al. (1997) for *Daphnia*. Elser et al. (2001) applied the P-enrichment method to lake seston and showed that consumers in the field were limited by P. Notably, in addition to the lowered P availability, the reduced performance of consumers confronting P limitation may also result from the effects of excess C content (Hessen et al. 2008). Those negative effects of P limitation may not only affect the performance of individual primary consumers, but may also travel up the food chain (Boersma et al. 2008; Rowland et al. 2015), and affect the diversity, composition and functioning of entire communities (Hall 2009; Hillebrand et al. 2009).

Associated with its effects on consumer growth, P limitation may also strongly affect the behavior and general physiology of herbivorous consumers. A well-studied behavior of consumers in response to P limitation is the increased ingestion rates, i.e. compensatory feeding (Suzuki-Ohno et al. 2012; Urabe et al. 2018), through which consumers try to elevate P acquisition from P limited food. In addition, other physiological processes are also shown to respond to P-limited food, such as P assimilation efficiency (DeMott et al. 1998; Urabe et al. 2018), P excretion rates (Frost et al. 2004, 2005), and respiration rates (Jensen and Hessen 2006; Hessen and Anderson 2008). The changes in these processes may further be related to the expression of specific genes which is affected by environmental P supply (Jeyasingh et al. 2007). In addition, given that nutrient requirements of an organism may vary between

subsequent life stages (Urabe and Sterner 2001; Villar-Argaiz et al. 2002; Bullejos et al. 2014), P limitation has also been shown to differentially affect key life history traits of consumers (Jensen et al. 2006; Felpeto and Hairston 2013).

In contrast to the well-studied consequences of P limitation, less is known about the effects of excess P in food on consumers. A number of studies have reported that food with very low C:P ratios also leads to reductions in consumer growth (Plath and Boersma 2001; Boersma and Elser 2006). Recent studies suggested that consumers within wide taxa may be living on a stoichiometric knife edge, where too high or too low food C:P will reduce their performance (Elser et al. 2006; Bullejos et al. 2014; Benstead et al. 2014; Laspoumaderes et al. 2015; Elser et al. 2016). The underlying mechanisms that account for the negative effects of low C:P food are currently unclear. One possibility is that a very low C:P ratio results in C limitation for consumers growth (Threshold elemental ratio, TER; Frost et al. 2006). However, more studies attribute the negative effects of low C:P food to the disadvantages associated with excess P (Boersma and Elser et al. 2006; Hessen et al. 2013), as a result of costs related to P storage (Persson et al. 2010) or its elimination (Anderson et al. 2005; Boersma and Elser 2006). In addition, excess P itself may also be toxic to animals (Karasov and Martinez del Rio 2007). Alternatively, Plath and Boersma (2001) observed that low food C:P ratios resulted in decreased food ingestion rate of *Daphnia*. They suggested that feeding rates of consumers were adjusted to their P demand, and as such indirectly resulted in C limitation and thus growth reductions of consumers.

Remaining knowledge gaps

Although the physiological and ecological consequences of C:P mismatch have been studied for decades, there are still several important questions that need to be addressed. Firstly, P limited food affects consumers in complex ways. In addition to the direct effects of stoichiometric mismatch, i.e. through an imbalanced C:P ratio, other indirect non-stoichiometric effects may also play important roles (Rothhaupt 1995). The relative importance of these two types of effects is still unclear. Secondly, although many studies in ecological stoichiometry have shown negative effects of stoichiometric mismatch between food and consumers, knowledge about how these effects vary with the degree and type of stoichiometric mismatch, and to what extent consumers are able to maintain their elemental homeostasis, is still limited. Thirdly, even less is known about how P limitation may affect consumers across generational time scales, i.e. through maternal effects (but see Frost et al. 2010) or by driving

rapid evolutionary responses (Declerck et al. 2015). Finally, organisms in nature are often confronted with multiple threats simultaneously or sequentially. P limitation in aquatic systems may be accompanied with other stressors, such as increased temperature (Hessen et al. 2005), salinization (van Dijk et al. 2015), chemical pollutants or the presence of predators. We currently have poor understanding about how such stressors may interact with P limitation, and how the effect of such interactions may also be mediated by consumer evolutionary history (**Figure 1.1**).

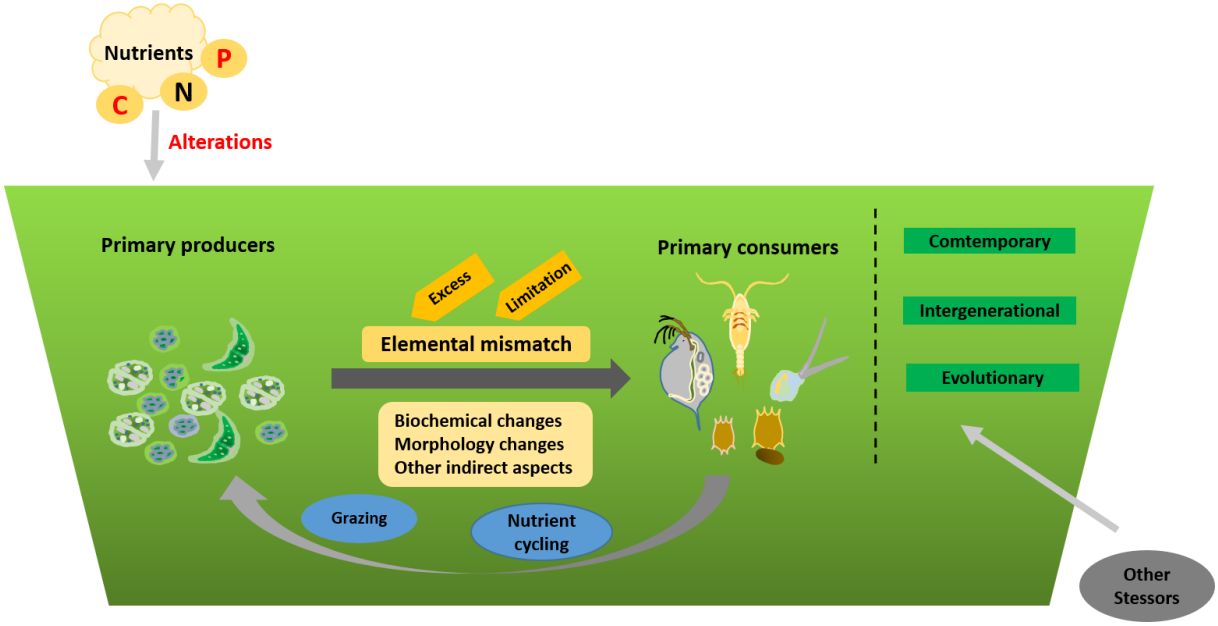

Figure 1.1 Schematic overview of how changes in nutrient supply rates in aquatic systems affect interactions between primary producers and primary consumers. Altered algal C:P ratios, for example, may affect consumers directly through stoichiometric mismatch and indirectly through other quality aspects of the food (e.g. biochemical composition; palatability). Responses of consumers to these changes may feedback to the growth of the producers via changed grazing rates and their impacts on the relative rates at which nutrients are recycled.

Stoichiometric mismatch at contemporary time scales

Relative importance of direct and indirect effects of P limitation

Many studies in the framework of ecological stoichiometry have repeatedly shown that resource P limitation strongly reduces the growth rates, reproduction, and survival of consumers (Sterner and Hessen 1994; Sterner and Schulz 1998; Bukovinszky et al. 2012). However, the underlying mechanisms that account for these negative effects are still in debate.

In addition to the lowered P availability (Sterner and Hessen 1994; DeMott 1998) and excess C supply (Darchambeau et al. 2003; Hessen et al. 2008), P limited food may also affect consumers through indirect non-stoichiometric effects. The first evidence supporting those indirect effects of P limited food comes from Rothhaupt (1995), who reported that the reduction in rotifer consumers cannot be fully explained by the elemental mismatch in algal food.

The indirect effects may result from the changes of non-stoichiometric traits in algae with a P limited growth history. For example, P-limitation has been shown to decrease the amount of highly unsaturated fatty acids (Müller-Navarra 1995; Weers and Gulati 1997; Spijkerman and Wacker 2011; Challagulla et al., 2015) in algal cells, which are important components for consumer growth and reproduction (Weers and Gulati 1997; Ravet and Brett 2006). Alternatively, algae may also respond to ambient P limitation by increasing their cell size and the thickness of their cell walls (van Donk and Hessen 1995; van Donk et al. 1997), which further affects their palatability and digestibility as food for consumers (Lürling and van Donk 1997; van Donk et al. 1997; DeMott 1998).

Currently, many stoichiometric models were developed with the aim of establishing a full understanding of the impacts of nutrient limitation of primary producers on consumers (Malzahn et al. 2010). Models typically ignore indirect non-stoichiometric effects which may result in biased outcomes. To address this, the first step is to estimate the relative strength of indirect compared to direct effects of P limitation on consumers.

Challenges to consumers along a stoichiometric knife edge

Recent studies have revealed that organisms among a wide taxa may be living on a 'stoichiometric knife edge', along which growth performance will be maximal at intermediate food C:P levels but strongly reduced with increasingly stoichiometric mismatch towards opposite sides of the food C:P gradient (i.e. towards high or low food C:P, Elser et al. 2006; Bullejos et al. 2014; Benstead et al. 2014; Laspoumaderes et al. 2015; Elser et al. 2016).

In contrast to the assumption of strict stoichiometric homeostasis in heterotrophs, there is accumulating evidence supporting that consumers may have some degree of flexibility in their somatic elemental composition (Small and Pringle 2010; Prater et al. 2017; Teurlincx et al. 2017). In ecological stoichiometry, the strength of stoichiometric homeostasis is typically quantified as the slope of the response of consumer body stoichiometry to changes in resource stoichiometry (1/H; where H is the homeostasis coefficient; Sterner and Elser 2002; Persson et

al. 2010; Hessen et al. 2013). However, homeostasis of a consumer is maintained by the combination of various physiological processes (Frost et al. 2005; He and Wang 2008), such as nutrient ingestion (Suzuki-Ohno et al. 2012; Urabe et al. 2018), assimilation (DeMott et al. 1998; Urabe et al. 2018), and excretion (Frost et al. 2004, 2005). Each of these physiological processes seems to have their limits and it is therefore unlikely that homeostatic strength is constant along a broad elemental gradient (Meunier et al. 2014). While growth reduction of consumers has been reported at both extremes of the stoichiometric knife edge, not much is known about how and to what extent organisms maintain their homeostasis along a stoichiometric knife edge.

Effects of stoichiometric mismatch across generations: maternal effects

The phenotype of one organism is not only determined by its contemporary environment and its genetic constitution but also by the environment that maternal generations have experienced, i.e. 'maternal effects' (Marshall and Uller 2007). In some scenarios, mothers may be able to anticipate future environments and prepare their offspring to be better able to cope with the environment they will encounter (adaptive maternal effects; Marshall and Uller 2007; Sheriff et al. 2013). Alternatively, negative effects of an unsuitable environment may be transferred to the next generation, reducing the performance of the offspring (transmissive maternal effects). The influence of maternal effects on consumers has been reported for many different taxa and various stressors (Kaneko et al. 2011; Warner and Lovern 2014; Moore et al. 2015; Stahlschmidt and Adamo 2015; Beyer et al. 2017). However, with few exceptions (Frost et al. 2010; He et al. 2016) the field of ecological stoichiometry lacks studies on maternal effects of stoichiometric mismatch, and we don't know if such effects reflect an adaptive strategy or are merely transmissive.

Even less is known about to what extent the direct and indirect effects of P limitation can affect consumers through maternal effects. Most likely, mothers adjust their offspring quality through changes in allocating the limiting nutrient to their eggs (Urabe and Sterner 2001; Frost et al. 2010). This has also been found for consumers under PUFAs limited conditions (Sperfeld and Wacker 2015). Given that the indirect effects of P limitation may potentially involve the effects of biochemical alterations in algal cells, it is therefore important to assess the relative importance of maternal effects of direct and indirect aspects of P limitation on consumer performance.

Effects of stoichiometric mismatch across evolutionary time scales

Microevolutionary trait changes in populations can be rapid and occur at similar time scales as ecological interactions (Hendry 2016; Govaert et al. 2019). As a result, ecological and evolutionary processes may interact with each other and evolutionary change has the capacity to change trajectories of food webs and ecosystems through time. For example, zooplankton consumers have been shown to rapidly adapt to stoichiometric imbalance with their food in the time span of less than a growing season (Declerck et al., 2015). This study showed that populations adapted to high C:P food were better able to suppress such food compared to populations adapted to P rich food and thus provides strong proof of concept for rapid adaptation to stoichiometric imbalance and its potential importance for eco-evolutionary feedbacks.

In natural aquatic systems, organisms are often confronted with multiple threats simultaneously or sequentially. Stoichiometric imbalance resulting from P-limitation may thus not be the only stressor that herbivores have to cope with (**Figure 1.1**). An important question is how stoichiometric mismatch may interact with other stressors, and how adaptation to P limitation may mediate the ability of organisms to deal with other stressors.

Salinization, for example, is increasingly considered as an important threat to freshwater systems (Kaushal et al. 2005; Cañedo-Argüelles et al. 2016). Increased salinity may be associated with the consequences of climate change, such as raised sea levels (Alley et al. 2005) and increased evaporation (Lenters et al. 2005), or result from anthropogenic activities, such as irrigation (Barica 1972), industrial pollution (Hamawand et al. 2013), and the use of road salts (Likens and Buso 2010; Dugan et al. 2017). High salinity is shown to be detrimental to freshwater organisms, mainly due to the increased ambient osmotic stress and the toxicity of salts ions (Munns and Tester 2008; Latta et al. 2012). Given that in freshwater systems nutrient limitation is sometimes also accompanied by increased salinization (van Dijk et al. 2015), it is therefore important to assess how local adaptation to P limitation affects the ability of organisms to cope with increased salinity and vice versa.

Experimental organism

For the work of this thesis, we used the monogonont rotifer species, *Brachionus calyciflorus*, as our experimental organisms. Together with cladocerans and copepods, rotifers play important roles in ecosystem functions of freshwater systems. They form an important link

between the primary producers and higher trophic levels of the classical food chain (i.e. phytoplankton, zooplankton, fish) in pelagic systems.

Due to very short generation times and their ability to reproduce asexually, *B. calyciflorus* have very high population growth rates, which allow them to respond quickly to changes in their environment. Furthermore, their populations consist of an assembly of clones. Similar to other monogonont rotifers, aphids and cladocerans, rotifers have a cyclic parthenogenetic reproduction mode. As shown in **Figure 1.2**, the life cycle starts with an amictic (i.e. asexual) female neonate that hatches from a resting egg and that will produce offspring that have the same genotype as their mother, i.e. they propagate clonally. However, under specific circumstances (e.g. overcrowding, strong changes in seasonal environmental conditions), amictic females will be induced to produce mictic (i.e. sexual) females. Mictic females will produce mictic eggs that will develop into haploid males when unfertilized or diploid resting eggs when fertilized by males. A neonate hatched from a resting egg represents a new genotype initiating a new phase of clonal propagation. Together with their fast population growth and small body size, these life history characteristics make monogonont rotifers an ideal model organism to investigate the effects of environmental change on their populations and to study rapid microevolutionary change through experimental evolution (Declerck and Papakostas 2016; Tarazona et al. 2017).

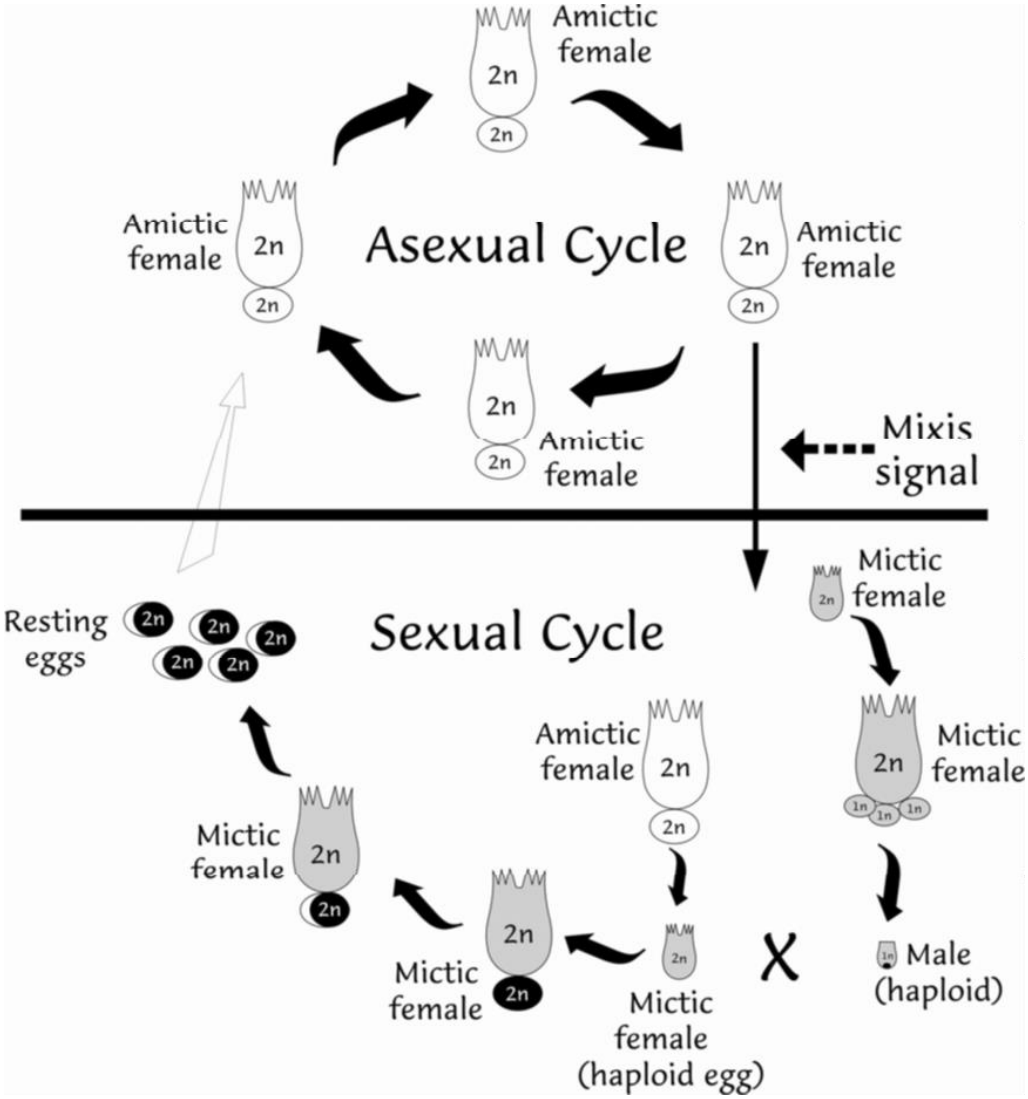

Figure 1.2 The sexual and asexual life cycles of rotifer *Brachionus calyciflorus*. Modified from Denekamp et al. (2009).

Thesis outline

The main aims of this thesis are

1) to assess the relative importance of direct and indirect effects of resource P limitation on consumer performance and life history strategy.
2) to explore the effects of a broad gradient of stoichiometric mismatch on consumer performance and homeostatic strength, and assess whether relaxation of stoichiometric homeostasis reflects an adaptive strategy or merely an inability to cope with stoichiometric imbalance.
3) to investigate the relative importance of direct and indirect maternal effects of P limitation in affecting consumer performance, and evaluate whether these effects are adaptive or transmissive.
4) to study how the response of consumers to food P limitation and increased salinity is mediated by their evolutionary history.

To address these questions, I apply a combination of research approaches, including growth rate experiments (both at population and individual levels), life history experiments, grazing experiments, and measurements of nutrient intake and loss rates.

In Chapter 2, I assess the relative impact of direct stoichiometric and indirect non-stoichiometric effects of resource P limitation on the performance and life history of rotifer consumers. Given that nutrient requirements of animals vary with ontogenetic phases, I expect that the relative importance of these two effects vary with different life history traits.

In Chapter 3, I investigate the growth and key physiological responses of rotifer consumers along a broad gradient of food C:P ratios. In addition, I study the homeostatic strength of rotifers along this gradient using a physiology-oriented approach. I hypothesize that both too high or too low food C:P ratios will lead to reductions in consumer growth, and that consumers will show different behavioral and physiological responses at the opposite sides of the gradient. Given that the diverse physiological processes through which animals maintain elemental homeostasis likely have their limits, I expect variable homeostatic strength along the gradient, especially at its more extreme ends.

In Chapter 4, I investigate the direct and indirect maternal effects of P limitation on key life history traits of rotifer consumers. Given that consumers have been shown to be able to adjust

the biochemical composition of their offspring, I expect that maternal effects caused by the indirect consequences of P limitation will also be important in affecting the performance and life history of rotifer offspring, although this will likely depend on the traits under consideration.

In Chapter 5, I first explore the interactive effects of food P limitation and increased salinity on the population growth of rotifer consumers. Then I assess how the adaptation to P limitation may affect the ability of rotifer consumers to cope with increased salinity, and vice versa. I also investigate how adaptive benefits to P limitation can be affected by increased salinity, and vice versa. Excess carbon in P-limited algal food may create a burden to consumers. In the meantime the physiological mechanisms that allow consumers to cope with osmotic stress and ion toxicity are energetically costly. As a result, whereas both high C:P food and high salinity may negatively affect the performance of rotifer consumers, they may dampen each other's effects when occurring simultaneously. Indeed, excess C in P limited food may provide the additional energy needed to regulate osmotic homeostasis. Furthermore, adaptation to P limitation may involve a more efficient elimination of excess C. If constitutive, this could result in a reduced ability to cope with salinity. I therefore hypothesize that adaptation to P limited food may reduce the ability of consumers to cope with increased salinity.

In Chapter 6, I summarize the results presented in this thesis, and discuss them in a broader context of ecological stoichiometry and rapid evolution. Furthermore, I also provide suggestions for further research.

Chapter 2

Direct and indirect effects of resource P-limitation differentially impact population growth, life history and body elemental composition of a zooplankton consumer

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Abstract

One of the central tenets of ecological stoichiometry is that consumer growth rate is strongly determined by food phosphorus (P) content. In planktonic organisms population growth rates of zooplankton have repeatedly been shown to be reduced when fed with P-limited algal food sources. However, P-limitation may also affect other quality-related aspects of algae, such as biochemical composition or palatability. We studied the population growth, detailed life history and body elemental composition of the herbivorous rotifer, *Brachionus calyciflorus*, in response to three different food quality treatments: algae cultured in high phosphorus conditions (average algal molar C:P ≈112,'HP'), algae cultured in low P conditions (molar C:P ≈ 631, 'LP') and low-P cultured C:P ≈ 631, 'LP') and low-P cultured algae spiked with P just before feeding (molar C:P ≈113, 'LP+P'). LP+P algae thus combined high P content with a history of growth under P-limited conditions. Total P content and the C:P ratio of rotifers in the LP+P treatment equaled those of rotifers in the HP treatment. Rotifer population growth rates were higher in HP than in LP and intermediate in the LP+P treatment. Similarly, many life history traits observed for animals in the LP+P treatment, such as somatic growth rate, age at maturity, and egg production rate were also intermediate to those observed in the LP and HP treatments. However, there were important deviations from this pattern: size at first reproduction and egg mortality in the LP+P treatment equaled the HP treatment, whereas size and development time of the first eggs equaled those of the LP treatment. Our results indicate that elemental limitation cannot fully explain reduced performance of consumers fed with P-limited algae and strongly suggest that indirect, non-stoichiometric effects of P-limitation, e.g. via changes in biochemical composition or morphology of the algae also play a major role. Furthermore, our study highlights that such indirect effects have a differential impact on major fitness components and may as such also determine the population dynamics and demographic structure of consumer populations.

Introduction

As a major component of the macromolecules DNA, RNA and ATP, phosphorus (P) is an essential element for the growth and reproduction of organisms. Due to this dependence, the availability of P may strongly limit the productivity of primary producers and higher trophic levels (Hessen 1992; DeMott and Gulati 1999; McCarthy et al. 2006). Human activities increasingly alter the amounts and ratios of biogenic elements (e.g. carbon, nitrogen and phosphorus) in natural systems and cause many freshwater systems to become P-limited (Stockner et al. 2000, Elser et al. 2009). A better mechanistic understanding of how P-limitation impacts the organisms in these ecosystems is therefore urgently needed.

Laboratory studies have shown strong reductions in the growth and reproduction of primary consumers when fed even high amounts of P-limited food (Sterner and Hessen 1994, Sterner and Schulz 1998). Such reduced performance has stimulated considerable debate about the underlying mechanisms. One potentially important cause of reduced consumer performance is pure mineral limitation: when the food resource has a very low P-content, the supply to a consumer may be too low even when food intake of the latter is at its maximum (Sterner and Hessen 1994; DeMott 1998). Furthermore, stoichiometric mismatches between the nutrient content of producers and consumers may also incur costs for the consumer, such as those associated with the disposal of excess C and other elements (Darchambeau et al. 2003). However, in addition to such direct effects, P-limitation may also affect the quality of producers indirectly. P-limitation in algae, for example, has been shown to decrease the amount of highly unsaturated fatty acids (Müller-Navarra 1995; Weers and Gulati 1997a; Spijkerman and Wacker 2011; Challagulla et al. 2015) which are important components for consumer growth and reproduction (Weers and Gulati 1997b; Ravet and Brett 2006). P-limitation has also been shown to result in changes of algal cell size and cell wall morphology (van Donk and Hessen 1995; van Donk et al. 1997). van Donk et al. (1997) and Lürling and van Donk (1997) explained reduced performance of *Daphnia* grown on P-limited algae by the lower digestibility of their thickened cell walls. DeMott (1998) demonstrated that the performance of *Daphnia* may be limited by energy even when fed high C:P algal food because of the low digestibility of P-deficient algae. These studies thus all indicate that food P-limitation may negatively affect consumers in direct as well as indirect, non-stoichiometric ways.

Ecological stoichiometry (Sterner and Elser 2002, Hessen et al. 2013) has so far been the predominant framework contributing to a better understanding of the impact of nutrient

limitation and stoichiometric mismatch on primary and secondary productivity (Malzahn et al. 2010), grazer top down control and nutrient cycling (Sistla and Schimel 2012), the strength of trophic cascades (Hall 2009) and trophic transfer efficiency (Rowland et al. 2015). Potentially, stoichiometric models still underestimate the full impact of nutrient limitation because indirect effects are typically not taken into account. The general lack of consideration of such indirect effects probably results from our poor understanding of the causal mechanisms underlying such effects, from the scarcity of information on their relative importance and from the difficulties inherent to incorporating these effects in mathematical models.

P-supplementation tests may provide us with a powerful experimental tool to address the relative importance of indirect, non-stoichiometric effects, even when knowledge about the causes is lacking. The approach makes use of the fact that P-limited algae are able to quickly absorb inorganic P from their environment (Lehman and Sandgren 1982) and hinges on the assumption that the process of P-uptake is much faster than responses in other traits, such as abundance, biochemical composition or morphological features (Boersma 2000; Elser et al. 2001). The relative importance of direct stoichiometric and indirect non-stoichiometric effects can be estimated through a comparison of the performance of consumers fed equal biomasses of P-replete (HP), P-limited (LP), and P-supplemented LP algae (LP+P). Equal performance of consumers in the LP+P as in the HP treatment indicates that direct P-limitation is the only cause of reduced performance in the LP treatment (Figure 2.1, Scenario I). Conversely, low consumer performance in the LP treatment can completely be attributed to indirect effects of P-limitation if P-supplementation results in no improved consumer performance compared to the LP treatment (Figure 2.1, Scenario III). If performance of consumers in the LP+P treatment is intermediate to the LP and HP treatments, then the relative importance of direct and indirect mechanisms can be inferred from the position of the LP+P treatment compared to LP and HP (Figure 2.1, Scenario II). A key requirement is that algae in the LP+P treatment acquire a C:P ratio equal to the HP algae.

Only few studies have used such experimental approach to evaluate the relative importance of direct and indirect effects of P-limitation on consumers. Rothhaupt (1995) found that although supplementation of P-limited algae enhanced the exponential population growth rate of the rotifer *B. rubens* it still remained considerably below that in P-rich algae and he suggested biochemical limitation as the mechanism underlying the observed indirect effect. DeMott (1998) found strong improvements of somatic growth to P-supplementation of P-limited algae

in multiple *Daphnia* species; although growth of most species almost approximated the levels observed with P-rich algae, they still remained somewhat lower in most cases. Boersma (2000) and Becker and Boersma (2003) cross-combined P-treatments (LP, HP and LP+P) with fatty acid supplementation treatments and concluded that biochemical limitation by fatty acids only becomes important when phosphorus is present in ample supply, and suggested that other factors were still at work since the joint effects of P and highly unsaturated fatty acids could not fully explain the higher growth rate observed in HP algae. Ravet and Brett (2006) demonstrated a stronger negative impact of indirect than direct P-limitation effects on *Daphnia* somatic growth and reproduction.

Nutritional requirements of a consumer organism differ between its life stages. This has been shown for stoichiometric (Urabe and Sterner 2001; Villar-Argaiz and Sterner 2002; Færøvig and Hessen 2003) as well as for biochemical requirements (Martin-Creuzburg and Von Elert 2004; Boëchat and Adrian 2006; Wacker and Martin-Creuzburg 2007). So far, P supplementation studies have mainly assessed the response of consumers to food quality treatments by considering general performance criteria, such as somatic growth (Boersma 2000; Elser et al. 2001) or population growth (Rothhaupt 1995). As a result, it remains unclear how the relative impacts of direct and indirect food quality effects vary among life history traits or major fitness components. Such information is, nevertheless, key to a better understanding of the consequences of nutrient limitation on the dynamics and demographic structure of consumer populations.

An implicit assumption of the P-supplementation method is that the accessibility of P to consumers should be equal in both LP+P and HP treatments. This may not necessarily be so. For example, a reduced digestibility of algae associated with P-limitation (van Donk et al. 1997) may result in a reduced availability of P to the consumers. Furthermore, when supplied to P-starved algal cells, anorganic phosphates may initially be stored under the form of polyphosphates in attendance of further metabolization (Eixler et al. 2006). If consumers are less able to take up and assimilate P from polyphosphates than from other P-containing biomolecules (e.g. DNA, RNA, ATP, phospholipids) then polyphosphate storage in LP+P algae could result in a reduced growth of consumers compared to those fed with HP food. To our knowledge, none of the P-supplementation studies so far have considered the possibility that a reduced accessibility of P in LP+P algae to consumers may unduly emphasize the importance of indirect effects.

With this study, using a P-supplementation approach we aimed at studying the relative importance of direct and indirect effects of P-limitation on population growth performance and a variety of life history traits, using the rotifer *B. calyciflorus* as consumer model. In an effort to evaluate whether differences exist in accessibility of P to consumers between LP+P and HP algae, we simultaneously studied the effect of food quality treatments on consumer elemental content and composition. Our results show that, whereas P-supplementation of P-limited algae enhanced P-content of algae as well as of rotifers to levels equal to those of P-replete conditions, population growth, somatic growth as well as individual fitness remained lower, indicating an important impact of non-stoichiometric, indirect effects. These effects seemed to have a differential impact on fitness components as life history traits responded in various ways to the supplementation treatment.

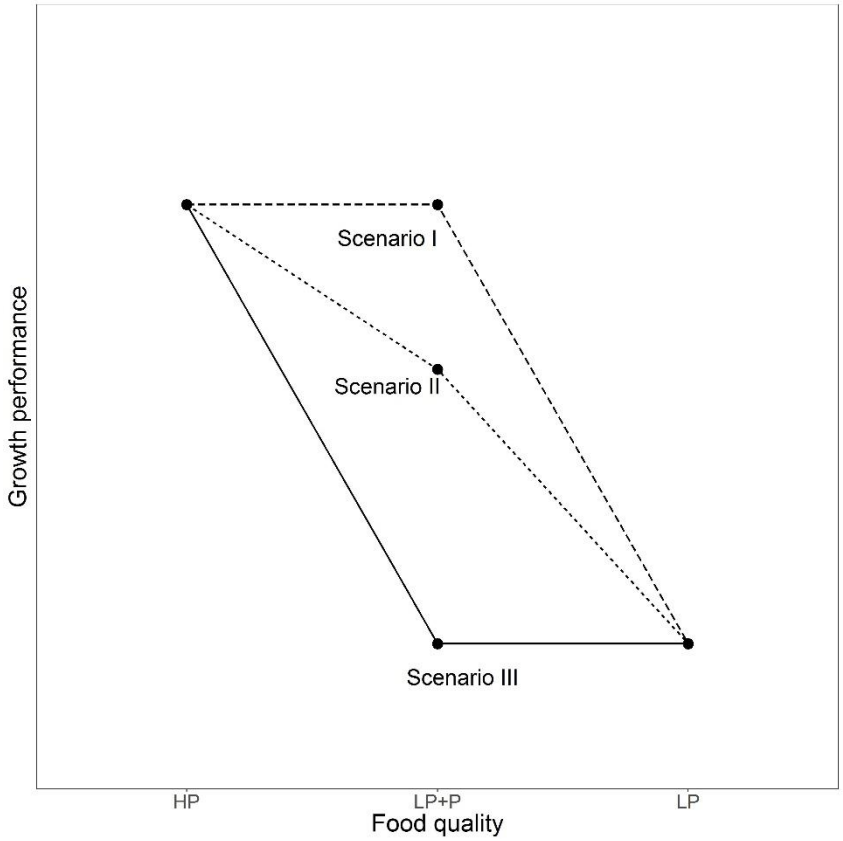

Figure 2.1 Three potential scenarios of how the performance of consumers may respond to the food quality treatments in a P-supplementation experiment. Scenario I depicts a case where the growth reduction of consumers fed P-depleted food is uniquely caused by direct, stoichiometric effects of P-limitation. Conversely, in Scenario III, this reduction in growth performance is entirely due to non-stoichiometric indirect effects of P-limitation. In scenario II both direct and indirect effects are of large importance. HP: P-saturated food; LP: P-deficient food; LP+P: P-deficient food enriched with a P-supplement.

Methods

Rotifer and Algae Cultures

Three clones of the rotifer *B. calyciflorus* were obtained from the resting egg banks of two Dutch lakes (D12 and D61 52°01'31.2"N, 4°11'16.8"E; E1 52°38'41.9''N, 4°43'81.7''E). *B. calyciflorus* consists of a species complex containing at least four putative species (Papakostas et al 2016). Based on ITS1-sequences clones D12 and D61 belong to the evolutionary unit 'C' and E1 to 'D' as denoted by Papakostas et al. (2016). Stock cultures were maintained at room temperature under continuous light conditions and fed daily with the nutrient replete green alga *Chlamydomonas reinhardtii* (1000 µmol C L^{-1}). Every three days the rotifers were transferred to new containers with fresh medium.

All experiments were based on a comparison between three different food quality treatments: (1) algae cultured in high phosphorus conditions (molar C:P = 112±2.6 SE, further referred to as 'HP'), (2) algae cultured in low P conditions (molar C:P = 631±14.9 SE, 'LP') and (3) algae cultured in low-P media which was then spiked with inorganic phosphate prior to feeding to the rotifers (molar C:P = 113±2.7 SE, 'LP+P'LP+P algae thus combined high P content with a history of growth under P-limited conditions.

C. reinhardtii was cultured in 10 continuous 2L-chemostats at 23±1 ˚C using modified WC (Woods Hole Chu-10) medium (Guillard and Lorenzen, 1972) at a dilution rate of 0.33/day. Five replicate chemostats with HP algae were cultured in media with 65 μmol L^{-1} P under ≈40 μmol quanta m^{-2} s^{-1} of continuous light. Five replicate chemostats with LP algae were cultured in media with 15 μmol L^{-1} P under ≈120 μmol quanta m^{-2} s^{-1} of continuous light. All chemostats were at steady state for at least one month prior to the experiments.

The algae for the HP and LP treatments were harvested daily from the chemostats, centrifuged (2500 rpm for 10 minutes) and resuspended in nutrient free WC medium. To create the LP+P treatment, inorganic phosphate (K_2HPO_4, 0.05 mol L^{-1}) was added to centrifuged and resuspended LP algae 90 minutes before being fed to experimental rotifer cultures. The amount of added P was based on the algal C content estimated from cell counts (Multisizer^{tm} 3 Coulter Counter, Beckman Coulter). For all three treatments, algae were kept in the dark for 90 minutes between their harvest and the feeding of the rotifers.

Population level growth rate experiment

Population growth rate in each food quality treatment (HP, LP and LP+P) was estimated for all clones at ad libitum food concentrations. Each clone by food treatment had five resource replicates (45 experimental units, i.e. 3 clones × 3 food quality treatments × 5 chemostat replicates). Experimental units were initiated by randomly selecting ten juvenile rotifers from a stock culture and transferring them into a 16 mL well filled with 8 mL of WC medium containing 1000 μmol C L^{-1} algae. Over the course of 22 days, wells were checked every 24 hours and the number of females counted. After counting, each unit was reinitiated by transferring ten haphazardly selected individuals to a new plate with fresh medium. Only juveniles, females without eggs or females with parthenogenetic eggs were transferred, males

or females with sexual eggs were not transferred. Plates were incubated at 23 ˚C under continuous darkness.

Life table experiments

Using a life table experiment, we studied the effect of the three food quality treatments on rotifer life history. The design of the life table experiment consisted of a total of 225 experimental units, i.e. 3 resource qualities × 5 food chemostat replicates × 15 individuals. For reasons of feasibility and because all clones showed similar response patterns to the food quality treatments in the growth rate experiment, we only used one clone, D12.

To initiate the experiment, we used cultures as described for the growth rate experiment as a starting point. For each experimental unit in the life table design we isolated at least ten females with parthenogenetic eggs from these cultures and transferred them to a new well with the corresponding food treatment. These wells were checked hourly for newly hatched neonates over the course of 8 hours. Once observed, a neonate was individually transferred into a 3mL well with 1 ml of algal suspension (1000 µmol C L^{-1}) of the same food quality and incubated at 23±1 ˚C in the dark at random locations in an incubator.

After the initial eight hours of their incubation, animals in all experimental units were checked every two hours until the conclusion of the experiment. At each time point we recorded the number of eggs, the number of neonates produced during the latest interval (which were then removed), and survival. If an individual produced male eggs they were no longer monitored. In the HP and LP+P treatments individuals were monitored until the production of a fourth neonate. As development was much slower in the LP treatment these individuals were instead monitored for the first 62 hours.

To obtain estimates on adult body and egg size at first reproduction, we conducted an additional but shortened version of a life table experiment. This experiment had the same design as the full life history experiment, except that only 5 individuals were used per resource replicate (75 experimental units, i.e. 3 resource qualities × 5 food chemostat replicates × 5 individuals). Neonates were collected in the same manner as in the life history experiment and checked hourly after eight hours. Gravid individuals were preserved in 4% formalin 2 hours after the production of their first egg. Body and egg volume were measured manually under a microscope.

Algae and Rotifer Stoichiometry

Molar C:P ratios of phytoplankton in the food quality treatments were measured at day 1, 6, 11, 16 and 21 of the growth rate experiment. For the life table experiment, the algal C:P ratios were measured just before and after the experiment. Rotifer density was too low in the growth rate experiment to collect enough animals for elemental analysis. For this reason, we scaled up culture conditions of the growth rate experiment to 200 mL batch cultures. The design of this experiment consisted of 30 units, i.e. 2 clones (D12 and D61) × 3 food quality treatments × 5 food replicates. Flasks with 1000 μmol C L^{-1} of algae were initially seeded with rotifers at a density of 15 individuals mL^{-1}. Every other day rotifer density was estimated and a volume representing 3000 rotifers was transferred to a new flask, this volume was then reduced to 20 mL and 180mL of fresh media was then added to the vessel. This method allowed rotifers to be cultured in a state of constant exponential growth with ad libitum food, similar to the cultures in the growth rate experiment. Prior to elemental analysis rotifer individuals with one egg were isolated in nutrient free WC medium for one hour to allow emptying of the guts. C and N contents were determined using a FLASH 2000 organic element analyzer (Interscience B.V., Breda, The Netherlands), while P content was determined by a QuAAtro segmented flow autoanalyzer (Beun de Ronde, Abcoude, The Netherlands). Each of these analyses was based on a sample of 150 individuals. During this experiment we also measured molar C:P ratios of phytoplankton in the food quality treatments at two occasions.

Data analysis

Exponential population growth rate was repeatedly calculated for each unit of the population level experiment as $R = \frac{\ln Nt - \ln N0}{t}$, where N_0 and N_t represents the population size at the start and end of each 24-hour period. Growth rate for each unit was calculated as the mean growth rate for the last 16 days of the experiment (i.e. the period during which growth rates had stabilized).

Life table data was used to calculate mortality rate of focal individuals and of eggs, age at first egg production, egg development time, and egg production rate. Egg production rate was calculated as the total number of eggs produced per hour during a time interval encompassing at least two egg production events per individual. Finally, for each replicate we calculated the instantaneous population growth rate *r* using the Euler-Lotka equation $1 = \sum lx * mx * e^{(-r*x)}$

(Stearns, 1992), where l_x represents the fraction of individuals surviving from birth to age class *x*, and m_x is the fraction of offspring in age class *x*.

Body volume at first reproduction was calculated as $Vb = \pi * Lb * (Wb/2)^2$, where L_b and W_b are body length and width at first reproduction, respectively. The volume of parthenogenetic eggs was calculated with the geometric formula for an ellipsoid: $Ve = \left(\frac{4}{3}\right) * \pi * (Le/2) * (We/2)^2$, where L_e and W_e represent egg length and egg width. Somatic growth was estimated as the difference between the body volume of an individual at first reproduction and egg volume of the first egg for the same individual divided by the amount of time to mature from a juvenile to first egg production.

In all experiments, phytoplankton chemostats represented the true level of replication. For population growth rate, intrinsic rate of population increase *r*, phytoplankton and rotifer C:P we obtained one value for each independent replicate. Therefore, we analyzed the effect of food quality on *r* and phytoplankton C:P with one-way ANOVA whereas we evaluated the effect of food quality and its interaction with 'clone' on population growth rate and rotifer C:P with a two-way ANOVA. Whereas clone should in fact represent a random factor we still specified it as a fixed factor because it only comprises three levels. In contrast, for all other life history variables we collected data from multiple individuals per chemostat replicate. We accounted for the intrinsic dependency of these data using general linear mixed models. In these models, food chemostat replicates were specified as random factor and food quality as fixed factor. For all life history variables the significance of food quality was evaluated with a likelihood ratio test comparing the full model with the corresponding intercept model. All ANOVA and linear mixed models were studied in more detail with Tukey *post hoc* comparisons to assess the significance of differences among factor levels. All statistical analyses were performed in R software environment 3.3.1 (R Core Team 2016). Mixed effects analyses were performed with the lme4-package (Bates et al. 2015) in R (R Core Team 2016).

Results

Growth rate experiment

Food quality had a strong effect on rotifer population growth rates (Figure 2.2A). A two-way ANOVA detected a significant interaction between food quality and clone identity for mean population growth rate (Table 2.1): growth rate differences among clones were clearly

expressed in the HP and LP+P treatments, however such differences proved relatively small in the LP treatment (Figure 2.2B). Yet, all clones showed a very similar response pattern to the food quality treatments: the HP treatment had the highest mean population growth rate, while the LP+P treatment was intermediate to the HP and LP treatments (Table 2.3).

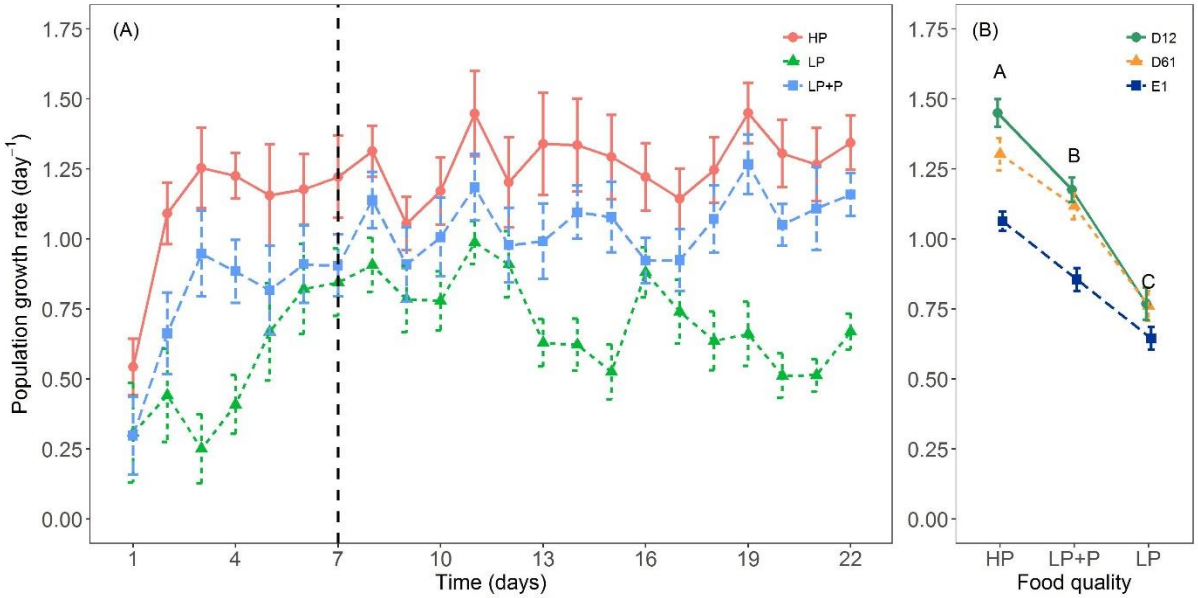

Figure 2.2 Response of rotifer population growth rates to the three food quality treatments in the growth rate experiment. (A) Mean growth rate of the different food treatments for each day over the course of the experiment and (B) mean population growth rate (Day 7- 22) of the three clone lines. Circles represent clone D12, triangles clone D61 and squares clone E1. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison across clones. Symbols and error bars represent the mean ± 2 standard error, respectively.

Table 2.1 Summary of ANOVA results for population growth rate and algal and rotifer C:P ratios. Note that an additional factor 'clone' was incorporated in the analyses of population growth rate and rotifer C:P. SS: Sum of squares; MS: mean square; df: degrees of freedom.

	SS	MS	df	F value	p
Population-level growth rate experiment					
Population growth rate					
Food	2.27	1.14	2	478.4	<0.001
Clone	0.62	0.31	2	144.1	<0.001
Food * Clone	0.14	0.04	4	11.9	<0.001
Life table experiments					
Intrinsic growth rate *r*					
Food	0.04	0.02	2	21.9	<0.001
Algae and rotifer stoichiometry					
Algal C:P ratio					
Food	7.23×10^5	3.61×10^5	2	119.8	<0.001
Rotifer C:P ratio					
Food	3.37×10^4	1.69×10^4	2	179.6	<0.001
Clone	80.0	80.0	1	0.9	0.365
Food * Clone	1609	804	2	8.6	0.002

Life table experiments

The intrinsic rate of population increase *r* was significantly different between all treatment combinations (Figure 3A, Table 1). *r* was highest in the HP, lowest in the LP and intermediate in the LP+P treatment (*post hoc* test: HP-LP, $p < 0.001$, HP-LP+P, $p=0.021$, LP+P-LP, $p=0.012$). *r*-values were positive in the HP and LP+P treatments but negative in the LP treatment.

The mortality rate of experimental individuals was 8.0% in the LP, 1.4% in the LP+P and 0% in the HP treatment. Larger differences were observed in egg mortality where 23.1% of rotifer eggs died before hatching in the LP treatment, in contrast to the HP and LP+P treatments where no eggs died.

The age at first egg production was lowest in the HP and highest in the LP treatment (Figure 3B, Table 2.2, $\chi^2(2)=148.07$, $p<0.001$). Although values for this variable were higher in the LP+P treatment than in the HP treatment, they approached more those of the HP than of the LP treatment (Figure 2.3B; Table 2.3). A similar pattern was found for the ages at which

subsequent eggs were produced. The development time of first egg was similar in the LP and LP+P treatments and longer than in the HP treatment (Figure 2.3C; $\chi^2(2)=24.384$, $p<0.001$; Table 2.2, 2.3). The development time of subsequent eggs differed significantly among all treatments (Figure 2.3C). Egg production rate was highest in the HP and lowest in the LP ($\chi^2(2)=338.67$, $p<0.001$; Table 2.2). Egg production rate in the LP+P treatment was intermediate but approached more that of the HP treatment (Figure 2.3D; Table 2.3).

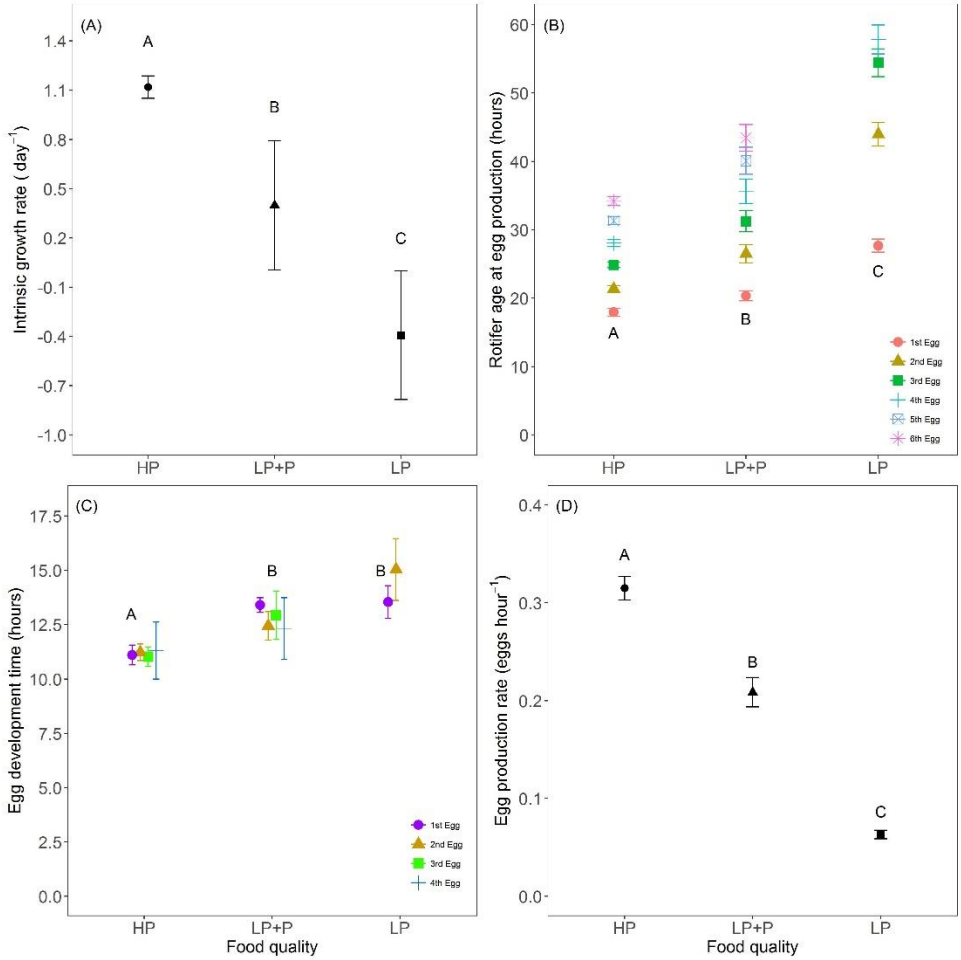

Figure 2.3 Life history traits in response to food quality treatments. (A) Intrinsic population growth rate (B) age at egg production, (C) egg development time, and (D) egg production rate. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison. Letters in (B) and (C) only represent analysis results for the first eggs produced. Symbols and error bars represent the mean ± 2 standard error, respectively.

Body size at first egg production in the HP did not differ significantly from the LP+P treatment (Figure 2.4A). However in both treatments body size was significantly larger than in the LP treatment ($\chi^2(2)= 12.983$, $p<0.002$; Table 2.2, 2.3). In contrast, the size of first egg was not significantly different between the LP+P and LP treatments (Figure 2.4B; Table 2.3), but in both treatments it was significantly larger than in the HP treatment ($\chi^2(2)=12.931$, $p<0.002$; Table 2.2). Somatic growth rate differed among all three treatments (Figure 2.4C; $\chi^2(2)= 51.508$, $p<0.001$;). Somatic growth rate was highest in the HP treatment and intermediate in the LP+P treatment (Table 2.3).

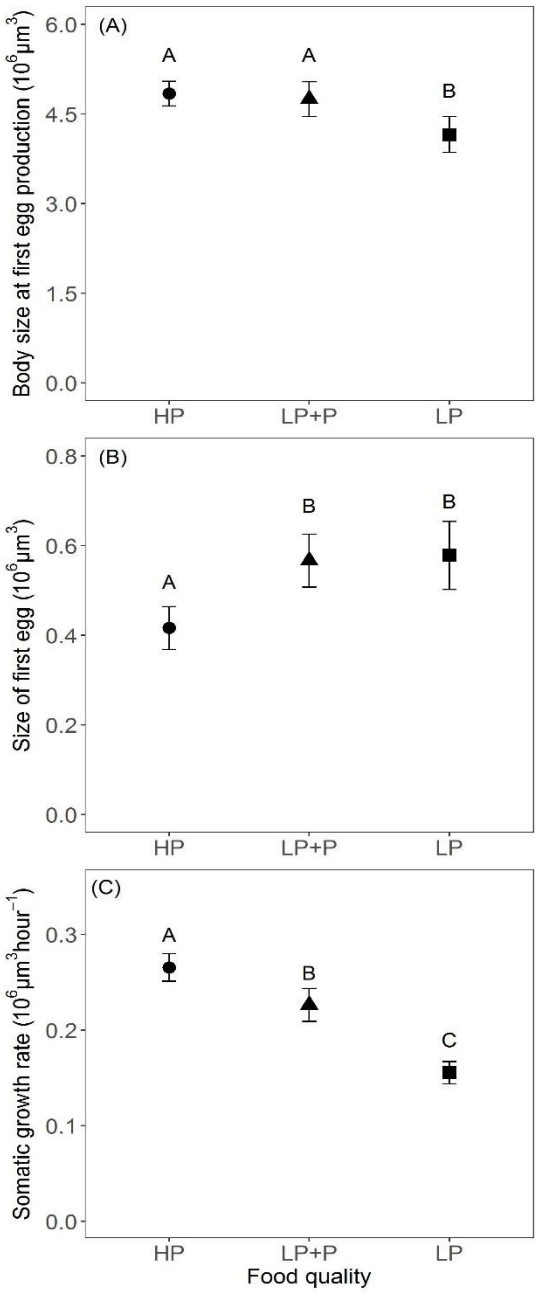

Figure 2.4 Size-related traits in response to food quality treatments. (A) Estimated body size at first egg production, (B) estimated size of first egg and (C) estimated somatic growth rate. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison. Symbols and error bars represent the mean ± 2 standard error, respectively.

Table 2.2 Summary of mixed model analyses for life table results. Food quality was specified as fixed effect in the models. SS: sum of squares; MS: mean square; df: degrees of freedom. P-values were obtained through application of the ratio likelihood test and are reported in the text of the Results section.

Fixed Effect	SS	MS	df	F value
Life table experiment				
Age at first egg				
Food quality	2372	1186	2	166.7
Development time of first				
Food quality	90.2	45.1	2	14.2
Egg production rate				
Food quality	1.68	0.84	2	604.5
Body size at first egg				
Food quality	3.40	1.70	2	7.8
Size of first egg				
Food quality	0.18	0.09	2	7.8
Somatic growth rate				
Food quality	0.07	0.03	2	59.6

Algal and Rotifer Stoichiometry

Throughout the experiment the C:P ratio of the LP algae was much higher than in the other two treatments (Figure 2.5A, Table 1). No significant difference in the C:P ratio was observed between the HP and LP+P treatment.

A significant interaction between food quality treatment and clone was observed for rotifer body C:P ratio as well as body P and C content (Figure 2.5B,C,D; Table 1). However, both clones showed a very similar response to food quality and the majority of the variation was explained by the food quality treatment (Table 1). The body C:P of rotifers from the LP treatment was significantly higher than of rotifers from the HP and LP+P treatments. No significant difference in the C:P ratio was observed between the HP and LP+P treatment. These patterns were driven by variation in total body P (Table 2.3). Animals in the LP treatment contained less C than animals in the HP and LP+P treatments (Table 2.3). Nevertheless, their C:P values were higher due to a proportionally very low P content (Figure 2.5C,D; Table 2.3).

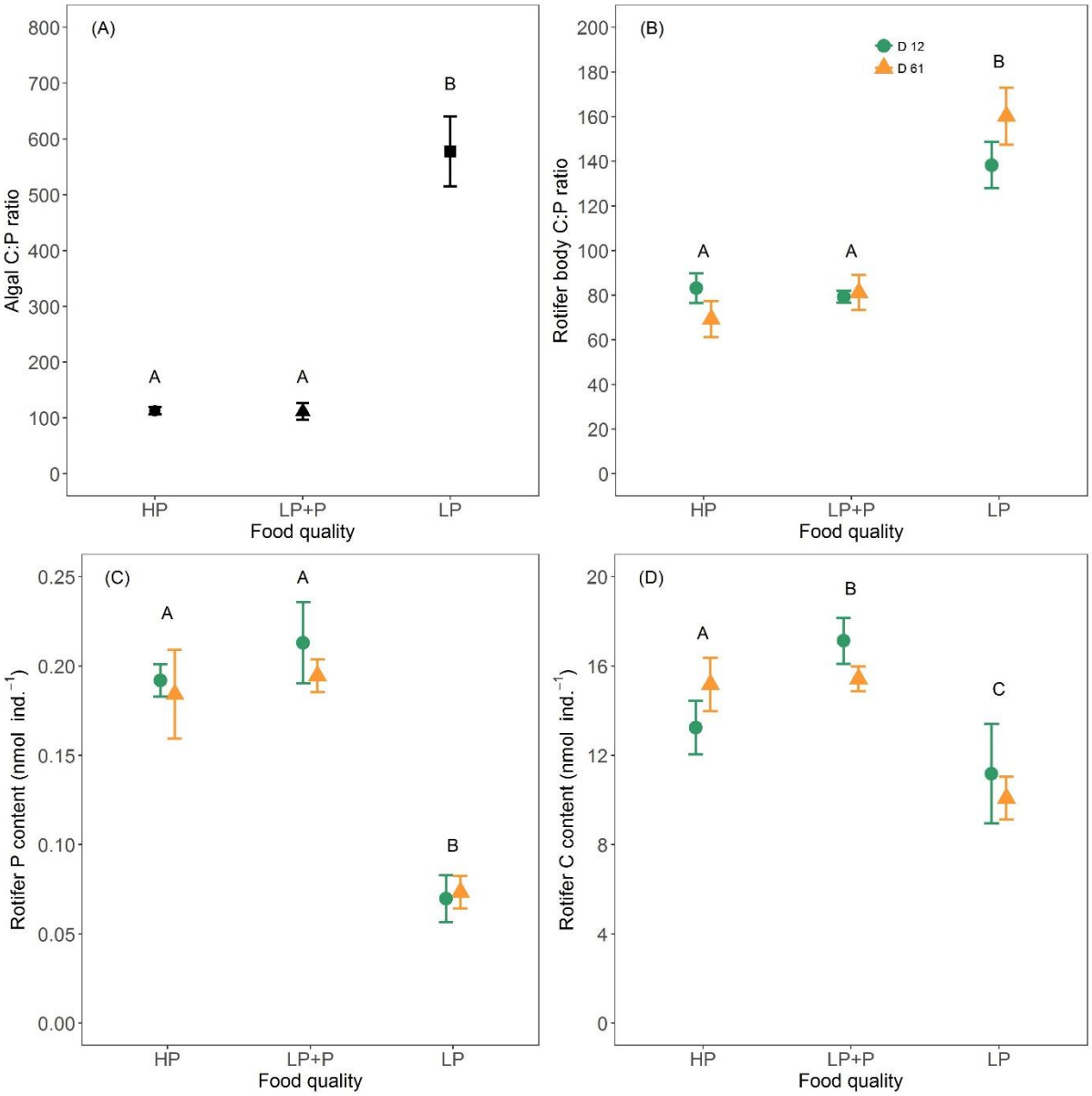

Figure 2.5 Stoichiometric ratios of algal food and rotifers. (A) Averages over time of the molar C:P ratios of the three food quality treatments, (B) body C:P ratios of rotifers raised on the three food quality treatments, (C) body P content of rotifers raised on the three food treatments, and (D) body C content of rotifers raised on the three food treatments. Green circles represent clone D12, and yellow triangles clone D61. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison across clones. Symbols and error bars represent the mean ± 2 standard error, respectively.

Table 2.3 Overview table with estimates of the relative impact of direct and indirect effects of P limitation on the investigated traits of *B. calyciflorus*. The relative impact of direct effects was calculated as $(\mu_{LP}-\mu_{LP+P})/\mu_{HP}.100$, whereas the relative impact of indirect effects was calculated as $(\mu_{LP+P}-\mu_{HP})/\mu_{HP}.100$, where μ refers to the mean value across replicates of a food quality treatment. A negative number indicates reductions in trait values by P-limitation. P-values were obtained through Tukey *posthoc* comparisons.

Traits	Effect Source	Relative Differences	p
Population growth rate			
(LP+P)-HP	Indirect	-17.5%	<0.001
LP-(LP+P)	Direct	-25.0%	<0.001
Somatic growth rate			
(LP+P)-HP	Indirect	-18.6%	0.001
LP-(LP+P)	Direct	-27.4%	<0.001
Age at first egg production			
(LP+P)-HP	Indirect	19.1%	<0.001
LP-(LP+P)	Direct	45.0%	<0.001
Development time of first egg			
(LP+P)-HP	Indirect	20.7%	<0.001
LP-(LP+P)	Direct	1.2%	0.940
Egg production rate			
(LP+P)-HP	Indirect	-33.8%	<0.001
LP-(LP+P)	Direct	-46.1%	<0.001
Egg mortality			
(LP+P)-HP	Indirect	0.0%	1
LP-(LP+P)	Direct	-23.1%	0.007
Body size at first egg production			
(LP+P)-HP	Indirect	-1.9%	0.889
LP-(LP+P)	Direct	-12.3%	0.007
Size of first egg			
(LP+P)-HP	Indirect	36.2%	0.005
LP-(LP+P)	Direct	2.9%	0.956
Rotifer C:P ratio			
(LP+P)-HP	Indirect	5.3%	0.67
LP-(LP+P)	Direct	90.6%	<0.001
Rotifer C content			
(LP+P)-HP	Indirect	14.6%	0.01
LP-(LP+P)	Direct	-39.8%	<0.001
Rotifer P content			
(LP+P)-HP	Indirect	8.3%	0.14
LP-(LP+P)	Direct	-70.3	<0.001

Discussion

In line with previous work (Rothhaupt 1995; DeMott 1998; Boersma 2000; Becker and Boersma 2003), our P-supplementation study shows that P-limitation of primary producers negatively affects zooplankton consumers not only directly through a reduced availability of P, but also indirectly via non-stoichiometric, qualitative effects. Indeed, general performance measures of rotifers, such as somatic and population growth rates proved to be affected almost as strongly by indirect as by direct effects (Table 2.3). Novel to our study is that we were able to evaluate the relative importance of these direct and indirect effects on multiple life history traits simultaneously. Intriguingly, the response of these traits proved to differ very strongly. Some traits such as size and age at first reproduction and egg mortality were largely affected by the direct effects of P-shortage, whereas other traits (e.g. egg size and first egg development time) seemed only affected by indirect effects of P-limitation. The P content and C:P ratio of rotifers fed P-supplemented LP algae (LP+P) was equally high as in rotifers fed HP algae. This indicates that the observed reduction of rotifer performance in the LP+P compared to the HP treatment cannot be explained by a lower accessibility of P in LP+P food.

Animals provided with P-limited algae had lower somatic growth rate, older age of maturity, lower egg production rate, longer egg development time and higher egg mortality compared to animals grown with P-rich algae. These responses are largely in line with other studies reporting the effects of P-limitation on zooplankton life history, although most of such work has been done on *Daphnia* (Færøvig and Hessen 2003; Urabe and Sterner 2001; Lukas et al. 2013). To our knowledge, there are only two studies reporting on the impact of P-limitation on rotifer life history. When feeding *B. calyciflorus* P-limited algae, Jensen et al. (2004) observed a lower somatic growth rate, an older age at first egg production and a shorter reproductive period compared to animals fed P-replete algae although egg mortality and total life span remained unaffected. Conversely, in a study of the rotifer *Keratella cochlearis* Ramos-Rodríguez and Conde-Porcuna (2003) observed a lower offspring production, a higher age at maturity, and a lower life span in animals fed with P-replete compared to P-limited *Cryptomonas* algae. However, in this experiment the C:P of the nutrient sufficient *Cryptomonas* was higher than that of the P-limited *Cryptomonas.*

In our study, the enhancement of growth performance following supplementation of P-limited algae with inorganic P supports the idea that consumer productivity is strongly impacted by the quantitative lack of P and the associated stoichiometric imbalance. However, our results also

indicate that such direct effects of P-limitation cannot fully explain the decreased performance of rotifers under P-limited food conditions. The C:P ratio of algae in the LP+P treatment was equal to that of the HP algae. Similarly, the body P content and the C:P ratio of adult rotifers fed LP+P food was similar to that of animals fed with HP food, and both were substantially different from rotifers in the LP treatment. We therefore conclude that it is unlikely that morphological changes induced by a history
of P-limitation or that the form of P-storage in LP+P algae has reduced accessibility of P to the consumers. Nevertheless, population growth rate remained considerably lower than in rotifers fed HP algae. This result suggests that P-limitation induced non-stoichiometric qualitative changes in phytoplankton which negatively affected its suitability as food for zooplankton.

Our results are in line with a number of other P-supplementation studies (Rothhaupt 1995; DeMott 1998; Boersma 2000; Becker and Boersma 2003) which suggested important indirect effects of food P-limitation on zooplankton consumer performance. Furthermore, through our life table data, we are able to assess the relative importance of direct stoichiometric and indirect non-stoichiometric effects of algal P-limitation on multiple fitness components, simultaneously. Most life history traits seemed to respond to P-addition, but still bore a clear signature of indirect effects of P-limitation. Similar to the population growth rates measured in the population-level culture experiment, somatic growth rate and intrinsic rate of population increase reached values in the LP+P treatment that were intermediate to that in the LP and HP treatments. Similarly, egg production rate and age at first egg production in the LP+P treatment were also intermediate to LP and HP although they appeared to be more strongly influenced by P addition because their values approached more those of the HP than the LP treatment.

However, other traits deviated strongly from such pattern. Both size at first reproduction and egg mortality in the LP+P treatment equaled that of the HP treatment suggesting these traits are exclusively impacted by the direct effects of P-limitation. Conversely, size and development time of the first egg showed no response to P-addition and appeared to be entirely controlled by indirect effects of P-limitation. Our results therefore clearly demonstrate a differential sensitivity of different fitness components to indirect and direct effects of P-limitation in the food resource. Likely this is reflective of the fact that both stoichiometric (Urabe and Sterner 2001; Villar-Argaiz and Sterner 2002; Færøvig and Hessen 2003, Becker and Boersma 2003) and biochemical requirements (Martin-Creuzburg and Von Elert 2004; Wacker and Martin-Creuzburg 2007) vary among the different predominant physiological

processes that characterize ontogenetic stages of the consumers. For example, fast somatic growth of juvenile stages is known to be highly dependent on the availability of P (cf. 'growth rate hypothesis', Elser et al. 2003). In contrast, egg development may be more dependent on the availability of specific biochemical substances. For example, *Daphnia* eggs have been shown to contain disproportional amounts of fatty acids compared to somatic tissue (Wacker and Martin-Creuzburg 2007), especially polyunsaturated fatty acids (PUFA's) such as eicosapentaenoic acid (EPA). Wacker and Martin-Creuzburg (2007) demonstrated that poor biochemical quality of food reduced the amount of these essential fatty acids in *Daphnia* eggs, and suggested an important role of biochemical compounds for egg development. Possibly, the slower development rate of eggs in the LP+P and LP treatments may have been the result of lower biochemical quality. We can only speculate about the mechanisms that may underlie our observation of larger eggs in the LP and LP+P treatments compared to the HP treatment. Larger eggs often reflect increased allocation of carbon resources of the mother to its progeny (Gliwicz and Guisande 1992; Kirk 1997). It is possible that mother animals in the LP treatment discarded excess C into their eggs (Urabe and Sterner 2001). Rotifers of clone D12 contained more C in the LP+P treatment than in the HP treatment, despite equal C-availability and C:P ratio of these food treatments. Possibly, the larger egg size observed in the LP+P treatment also reflected a C allocation strategy of adults towards their eggs similar as in the LP treatment.

Morphological changes in phytoplankton have also been suggested to be the cause of reduced consumer performance under conditions of P-limitation. Algae have been reported to respond to nutrient limitation with an increase in cell size (van Donk and Hessen 1995) and increased thickness of their cell wall (Van Donk and Hessen 1993; Van Donk et al. 1997). In filter feeders like *Daphnia*, these morphological changes improve viable gut passage and explain reduced clearance and population growth rates of these grazers when fed P-limited algae (Lürling and van Donk 1997; Van Donk et al. 1997). However, although cell size increased in response to P-limitation in our experiment they remained well within the limits of the food particle size range ingestible for *B. calyciflorus* (Rothhaupt 1990). Additionally, in contrast to *Daphnia*, rotifers crush ingested food with a specialized stomach (mastax; Gilbert and Starkweather 1977), hence, it is doubtful that cell wall thickening would allow gut passage of intact cells. Rothhaupt (1995) observed no reduction in grazing rates of *B. rubens* on P-limited algae, whereas P-limitation has also been found to result in increased clearance rates (Suzuki-Ohno et al. 2012). Finally, in our experiment, rotifer body C and P content did not decrease in the

LP+P compared to the HP treatment, suggesting no reduction in C and P ingestion and assimilation efficiencies.

Our study highlights that the performance of consumers provided with a phosphorus limited resource is not exclusively affected by the quantitative reduction of available P and the corresponding stoichiometric mismatch with their elemental requirements. Consumer performance was also impacted by the qualitative deterioration of the food as a result of the resource growth environment that acted independently of elemental content or stoichiometric ratios of the final food resource. In our study, such indirect qualitative effects proved to contribute strongly to the observed reductions in consumer population growth under P-limited conditions. Importantly, the magnitude of the impact of these indirect effects seemed to differ between different key fitness components of consumers. Given the strong link between life history and population demography, this suggests that such effects may also have an important impact on the structure and dynamics of consumer populations. Furthermore, the relatively large impact of the indirect effects of P-limitation in our results highlight their potential importance in determining the strength of producer-consumer bottom-up control and the efficiency of energy transfer between trophic levels. A better knowledge of the consequences of non-stoichiometric food quality effects of P-limitation on consumer populations may therefore be crucial for a better understanding of the true nature of P-limitation effects in natural communities.

Acknowledgements

We wish to thank Dennis Waasdorp, Els Faassen, and Dedmer van de Waal for their advice regarding the set-up of the phytoplankton chemostats. We also thank Nico Helmsing and Erik Reichman for carrying out nutrient analyses as well as Caelen Miller and Laurens Verhage for their help in the lab.

Appendix S1

Figure S1.1 Photograph of the phytoplankton chemostat system under two different nutrient conditions.

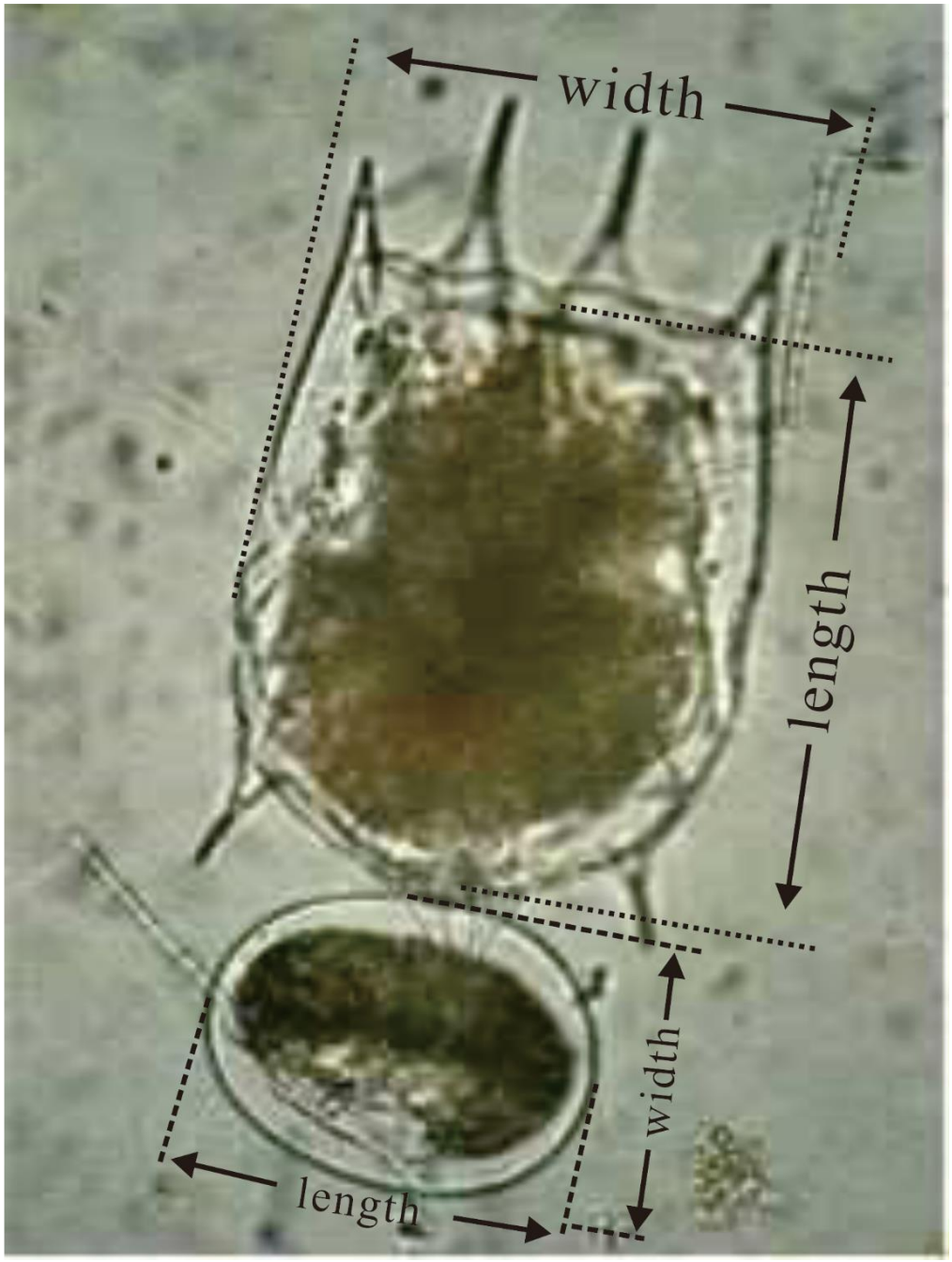

Figure S 1.2 Photograph for the measurement of rotifer body and egg size.

Chapter 3

Herbivore consumers face different challenges along opposite sides of the stoichiometric knife-edge

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Abstract

Anthropogenic activities have reshaped the relative supply rates of essential elements to organisms. Recent studies suggested that consumer performance is strongly reduced by food that is either very high or very low in relative phosphorus content. However, the generality of such 'stoichiometric knife-edge' and its underlying mechanisms are poorly understood. We studied the response of a planktonic rotifer to a tenfold food carbon:phosphorus (C:P) gradient and confirmed the existence of the stoichiometric knife-edge. Interestingly, we observed a complete homeostatic breakdown associated with strong growth reductions at high food C:P. In contrast, at low food C:P animals maintained homeostasis despite pronounced performance reductions. Our results suggest that the mechanisms underlying adverse effects of stoichiometric imbalance are determined by both the identity of elements that are limiting and those that are present in excess. Negative effects of excess P reveal an additional way of how eutrophication may negatively affect consumers.

Introduction

In recent decades, anthropogenic activities have strongly altered the relative supply rates of key elements, such as carbon (C), nitrogen (N), and phosphorus (P) to organisms (Smith and Schindler et al. 2009; Elser et al. 2009). The elemental composition of autotrophs is known to be different and more flexible than that of their consumers (Sterner and Elser 2002; Persson et al. 2010; Van De Waal et al. 2010). Mismatches of elemental ratios between producers and consumers may have negative effects on consumer performance (Elser et al. 2001; Hessen et al. 2013; Wagner et al. 2013), trophic transfer efficiency (Grover 2003; Rowland et al. 2015) and the strength of top-down control (Hall 2009; Declerck et al. 2015). To understand how altered elemental supply rates affect food web structure and ecosystem functioning, it is important to know when stoichiometric mismatch occurs, how it affects consumers and how these respond accordingly.

Generally, the growth of organisms is thought to be constrained by the nutrient that is relatively in the shortest supply (Liebig's law of minimum; Liebig 1840; Hessen et al. 2013). Accordingly, a well-developed concept in ecological stoichiometry (ES) is the threshold elemental ratio (TER; Urabe and Watanabe 1992; Frost and Elser 2002; Anderson and Hessen 2005; Frost et al. 2006), which represents the ratio of two elements at which the identity of the limiting element shifts from one to the other element. TER predicts a unimodal response of consumer performance to a gradient of food elemental ratios, with optimal growth close to the requirements of the organisms (TER) and a reduction in both directions toward the extremes of the gradient (Anderson and Hessen 2005; Frost et al. 2006; Khattak et al. 2018). This prediction has been supported for a wide range of taxa (coined as the "stoichiometric knife-edge"; Elser et al. 2006; Bullejos et al. 2014; Benstead et al. 2014; Laspoumaderes et al. 2015; Elser et al. 2016). However, most of these studies attribute the performance reduction of consumers fed low C:P food more to disadvantages associated with excess P than to C limitation (Boersma and Elser 2006; Hessen et al. 2013). For example, storage (Persson et al. 2010) or the elimination of excess P (Anderson et al. 2005; Boersma and Elser 2006; Elser et al. 2006) are thought to be highly costly, and to be associated with a growth penalty in heterotrophs. P may itself also be toxic to animals, e.g. because of its harmful effects on symporter functions of gut epithelial cells (Karasov and Martinez del Rio 2007). Alternatively, Plath and Boersma (2001) suggested that a low food C:P ratio may indirectly result in C limitation of *Daphnia* because they adjust their feeding rates in function of their P intake.

Another key concept in ES is elemental homeostasis, which reflects the ability of an organism to keep its somatic elemental composition constant in the face of varying elemental supply ratios (Elser and Urabe 1999; Sterner and Elser 2002). In contrast to autotrophs, animal consumers have long been considered as being very homeostatic (Andersen and Hessen 1991; Sterner and Elser 2002; Anderson et al. 2005). However, evidence for considerable plasticity in consumer body stoichiometry has recently accumulated for a wide range of organisms (Small and Pringle 2010; Prater et al. 2017; Teurlincx et al. 2017), raising the notion that organisms may take different positions along a continuum between strict regulators and strict conformers (Persson et al. 2010; Meunier et al. 2014). Most work on consumer homeostasis assumes that the somatic elemental composition of consumers responds proportionally to changes in the elemental composition of its food source and that the strength of this response is constant across the whole stoichiometric food quality range (Sterner and Elser 2002; Persson et al. 2010; Hessen et al. 2013). However, Meunier et al. (2014) introduced a more physiology-oriented view on the concept and questioned the linearity of this response. Indeed, homeostasis is maintained by the simultaneous action of multiple regulating processes (Frost et al. 2005; He and Wang 2008; Hessen et al. 2013), such as feeding (Suzuki-Ohno et al. 2012; Urabe et al. 2018), assimilation (DeMott et al. 1998; Urabe et al. 2018), allocation (Urabe and Sterner 2001; Frost et al. 2010), excretion (Frost et al. 2004, 2005) and respiration (Jensen et al. 2006; Hessen and Anderson 2008). Variation in the efficiency of these processes along a stoichiometric food gradient is very likely and is expected to result in a variable homeostatic strength across such gradient. Meunier et al. (2014) also suggested that conformers and regulators should differ in the shape of their stoichiometric response curves. Lack of suitable datasets has so far prevented further confirmation of these expectations.

Variation in degree of homeostasis likely reflects different adaptive strategies of organisms, however, benefits and disadvantages of homeostasis are still poorly understood (Persson et al. 2010; Hessen et al. 2013; Leal et al. 2017). Organisms require various biomolecules (e.g. proteins, nucleic acids, lipids) in specific ratios (Elser et al. 1996). At optimal growth, somatic elemental composition reflects the ideal ratios of these biomolecules and their elemental make-up (Sterner and Elser 2002; Franklin et al. 2011; Manzoni et al. 2017). By maintaining elemental homeostasis, regulators strive towards the maintenance of a specific somatic elemental composition to secure optimal functioning. The disadvantage of this strategy is that as food elemental composition deviates from this optimum, growth will be limited by the element in shortest supply whereas excess elements need to be eliminated. Such strategy will

thus inevitably result in a reduced resource exploitation efficiency (Anderson et al. 2005; Frost et al. 2005; Manzoni et al. 2017). In contrast, conformers have the ability to track the elemental composition of their food. Such relaxation of homeostasis may potentially be achieved without relevant performance reductions if excess elements can be stored without costs (Persson et al. 2010; Hood and Sterner 2010) or if the utilization efficiency of the limiting element can be increased (Jeyasingh et al. 2009; Prater et al. 2017). Alternatively, however, stoichiometric plasticity may also reflect an inability to cope with stoichiometric imbalance. For example, the capacity of regulators to maintain homeostasis has likely its limits. Once the degree of stoichiometric mismatch trespasses these limits we expect abrupt deviations in somatic elemental composition to coincide with strong reductions in performance.

A question that so far has largely remained unaddressed is to what extent responses of organisms to a broad food stoichiometry gradient are consistent along the opposite sides of their growth optimum. Studies on the stoichiometric knife-edge (Boersma and Elser 2006; Elser et al. 2006; Elser et al. 2016) implicitly suggest that organisms face different challenges when being confronted to opposing directions of stoichiometric mismatch, i.e. costs associated with P-limitation and excess C at high C:P versus costs associated to excess P at low C:P conditions. Currently, we don't know much yet about how organisms respond to these different challenges and whether the strategies to cope with them also involve different levels of homeostatic strength. Such knowledge is nevertheless a prerequisite if we want to be better able to understand and predict the direct consequences of stoichiometric mismatch for the role of consumers in ecosystem functions such as nutrient cycling (Elser and Urabe 1999; Atkinson et al. 2017) and the trophic transfer of energy and materials (Boersma et al. 2008; Rowland et al. 2015), not only when nutrients are limiting but also under conditions of severe anthropogenic eutrophication.

The experimental exposure of organisms to broad gradients of food elemental composition allows studying the association of organism performance with variation in their somatic elemental composition. This may not only help the interpretation of deviations from homeostasis but also cast a light on the costs and benefits from contrasting strategies with which organisms face stoichiometric mismatch. For this study we subjected a metazoan planktonic consumer, i.e. the monogonont rotifer *Brachionus calyciflorus* to a tenfold gradient of food C:P ratios and measured the response of two measures of performance (somatic and population growth rate), somatic elemental composition and two key regulating physiological functions (i.e. food uptake and P-loss rates). First, we wanted to study the consumer performance response

to food elemental content along a broad C:P range, and test for the existence of a stoichiometric knife-edge. Second, given that organisms may face different challenges along opposite directions of a stoichiometric food gradient we assessed variation in the degree and modes of stoichiometric regulation in response to stoichiometric imbalance along both sides of the growth optimum (i.e. towards very high and very low resource C:P). Third, by relating consumer performance with the degree of stoichiometric plasticity along each of these directions we evaluate to what extent homeostatic regulation implies reduced performance, and whether an apparent relaxation of such regulation reflects a capacity to deal with stoichiometric mismatch or, alternatively, an inability to maintain elemental homeostasis. Finally, we discuss the potential implications of these results for real world systems.

Methods and materials

Rotifer and algae cultures

The *B. calyciflorus* clone used in our study was obtained from the dormant egg bank of a Dutch lake (52°5'26.50"N; 4°20'18.40"E). To exclude the influence of indirect effects of P-limitation on the nutritional quality of algae (e.g. Zhou et al. 2018), we created a stoichiometric food quality gradient by enriching P-limited chemostat grown green algae (*Chlamydomonas reinhardtii*) with different concentrations of P shortly before feeding to the rotifers (see Appendix S1). In this way, we created nine food quality treatments representing a ten-fold food C:P gradient ranging from 53 to 587. This gradient was chosen to realize a strong stoichiometric mismatch between producers and consumers via both high and low food C:P values, while still being representative for realistic field conditions (Hessen 2006; Sterner et al. 2008).

Population growth rate

The response of rotifer exponential population growth rate to the respective food quality treatments was studied in flasks filled with a 200 mL algal food suspension at *ad libitum* concentrations (1000 µmol C L^{-1}), 24 ˚C and continuous darkness. We kept the cultures in an exponential growth by daily restarting populations with a subsample of approximately 4000 individuals in a fresh food suspension (see Appendix S1). Exponential population growth rate was repeatedly calculated as $(\ln N_t - \ln N_0)/t$ on daily basis, where N_0 and N_t represent the population size at the start and end of each 24-h period. Population growth rate for each treatment was calculated as the mean of 12 consecutive 24-hour periods.

Somatic growth rate

For somatic growth rate we collected a cohort of 100 newborns (age < 2 hours) from the populations of the population growth rate experiment, incubated them during 18 hours (t) in the respective food quality treatments and measured their total C (M_t). We also measured the carbon content of groups of 100 neonates born in the respective food quality treatments (M_0; see Appendix S1). Mass-specific somatic growth rate was calculated as $(\ln M_t - \ln M_0)/t$.

Food ingestion, P intake and P loss rate

For the measurement of food ingestion, P intake and P loss rates, we used animals from the population growth rate experiment to ensure that animals were physiologically adapted to different food qualities. All rates were measured on the same sets of animals. For the ingestion rate experiment, we allowed groups of 200 rotifers to feed for four hours on algae suspensions of the different food quality treatments (see Appendix S1). Ingestion rates were calculated as $(C_t\text{-}C_0)\ v/(nt)$ (Peters 1984), where C_t and C_0 represent the initial and final algal concentration in each vial, v is the volume of food suspension in each vial (8 ml), n is the rotifer number and t is the time period of incubation, respectively. Algal concentrations were calculated by estimating algal biovolume using a coulter counter (Multisizer-tm 3 Coulter Counter, Beckman Coulter). These biovolumes were subsequently converted into molar concentrations of C using a previously established biovolume-carbon regression equation. P intake rate was estimated by dividing the C ingestion rate by the molar C:P ratio of the respective food quality treatment.

For the measurement of P loss rates, we transferred the rotifers from the grazing experiments into 1.5 mL volumes of nutrient free WC medium (Guillard and Lorenzen 1972). After an incubation of four hours in the dark on a rotating plankton wheel (30 r.p.m), the medium from each unit was filtered through a 30 µm mesh to remove rotifers and transferred into a 1.5 ml glass vial. After being autoclaved for 30 minutes under 121 ˚C, the samples were stored for the later measurement of dissolved P (i.e. ortho-phosphates). P loss rates were estimated as $(Pv)/(nt)$, where P refers to sample P content, v and t represent sample volume and incubation time, n is the rotifer number in each treatment.

Stoichiometric measurements

During the experiment we measured algal C, N, P content of each food quality treatment at three occasions. For the measurement of rotifer elemental content we used animals from the

population growth rate experiment. From each culture, we incubated two samples of 150 females each carrying one parthenogenetic egg in nutrient free WC medium during 30 minutes to allow evacuation of the guts. C and N content of algae and rotifer samples were determined using a FLASH 2000 organic element analyzer (Interscience B.V., Breda, Netherlands). For P, the algal and rotifer samples were first incinerated at 550℃ for 45 minutes and autoclaved in 2.5% potassium persulfate ($K_2S_2O_8$) solutions at 121℃ for 30 minutes. These samples and the samples from the P loss-rate experiment were measured using a QuAAtro segmented flow autoanalyzer (Beun de Ronde, Abcoude, Netherlands). All stoichiometric ratios are expressed as molar ratios.

Data analysis

The responses of rotifer growth rates, somatic elemental ratios, food ingestion rates, and P intake and loss rates to diet C:P were statistically evaluated by contrasting three alternative models, i.e. a non-quadratic linear model, a quadratic linear model, and a piecewise regression model. Models were ranked based on the Akaike Information Criterion (AIC). Generally, the model with the lowest AIC value was used for interpretation. However, if alternative models proved equally good (i.e. with ΔAIC < approximately 2; Burnham and Anderson 2004), they were chosen to maximize comparability across analyses. Where relevant, quadratic and piece-wise regression models were used to calculate the location of the optima of unimodal responses. Elemental ratios, P-intake and P-loss rates were $\log_2$-transformed prior to analysis. All the statistical analyses were performed in R (R Core Team, 2016). The piecewise regression was conducted using the package 'segmented', and the Davies test provided by this package was applied to determine the significance of differences between slopes (Muggeo 2008).

Results

The addition of P to P-limited algae strongly increased their P content and reduced their C:P ratios (ranging from 53-587), but did not change their N content and C:N ratios (Appendix S3.2 Figures 3.1 and 3.2a-b).

Rotifer population growth rate showed a unimodal response to diet C:P ratio with a maximum at intermediate diet C:P values and strong reductions towards the extremes of the resource gradient (Fig. 3.1a; Appendix S3.2 Table 3.1, 3.2). Using the quadratic regression model, the population growth rate maximum was estimated at a diet C:P ratio of 171. Mass specific

somatic growth rate showed a very similar response (Fig. 3.1b; see Appendix S3.2 Table 3.1) with a maximum at an estimated diet C:P ratio of 165.

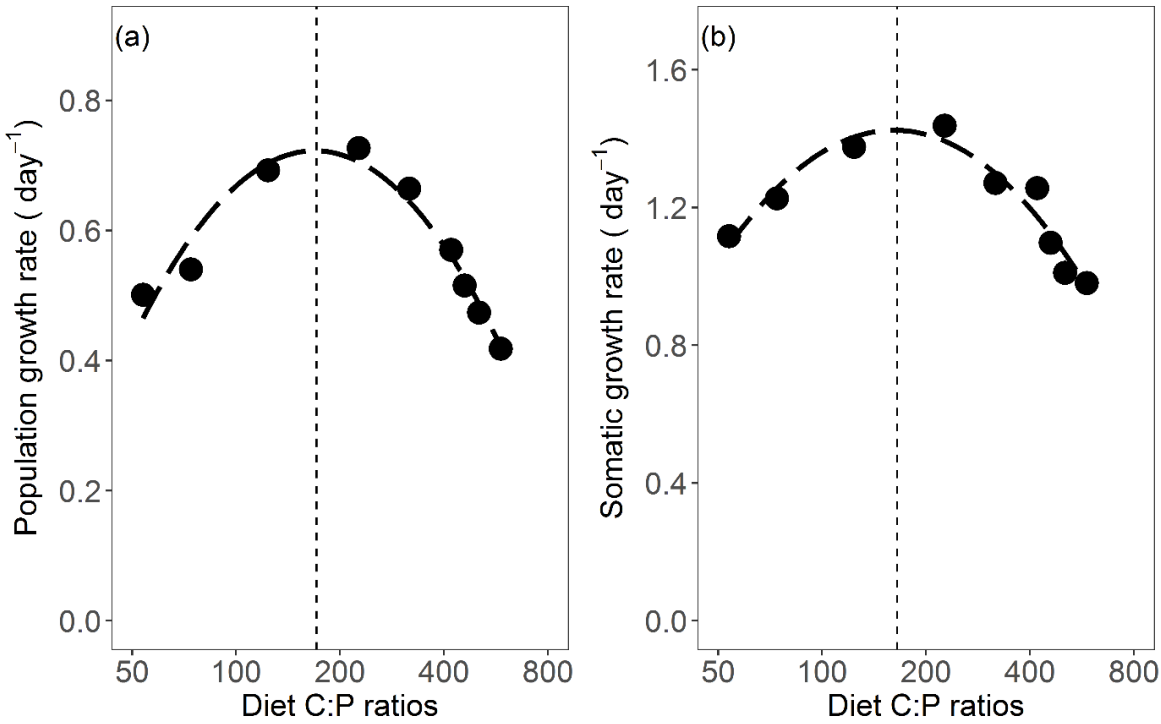

Figure 3.1 (a-b). Response of rotifer population growth rate (a), and mass-specific somatic growth rate (b) to the experimental food C:P gradient. The dashed lines represent the expected values according to the quadratic regression models; the vertical dotted lines represent the food C:P ratio corresponding to the maximal growth rate values as calculated by the quadratic regression models. Symbols in (a) represent means of values from 12 subsequent 24-hour time intervals, and symbols in (b) are the means of three technical replicates. Note the log-scale of the X-axes.

The relationship between rotifer C:P and food C:P was best described by a two segments piecewise regression model and its breakpoint was situated at a food C:P of 391 (Figure 3.2a, Appendix S3.2 Table 3.2). Above the breakpoint, the slope approximated 1.02, showing that rotifer C:P followed changes in food C:P ratio proportionally. In contrast, the slope of the regression equation below the breakpoint was much lower (0.125) but still differed significantly from zero (Figure 3.2a; see Appendix S3.2 table 3.2). Although the C:P response above the breakpoint coincided with an increased rotifer C content it was mainly driven by a reduction in rotifer P content (Figure 3.2b-c; Appendix S3.2, S3.2 Table 3.4). Rotifer C:N ratios increased slightly along the C:P gradient mainly due to an increased rotifer C content (Appendix S3.2 Figure 3.3).

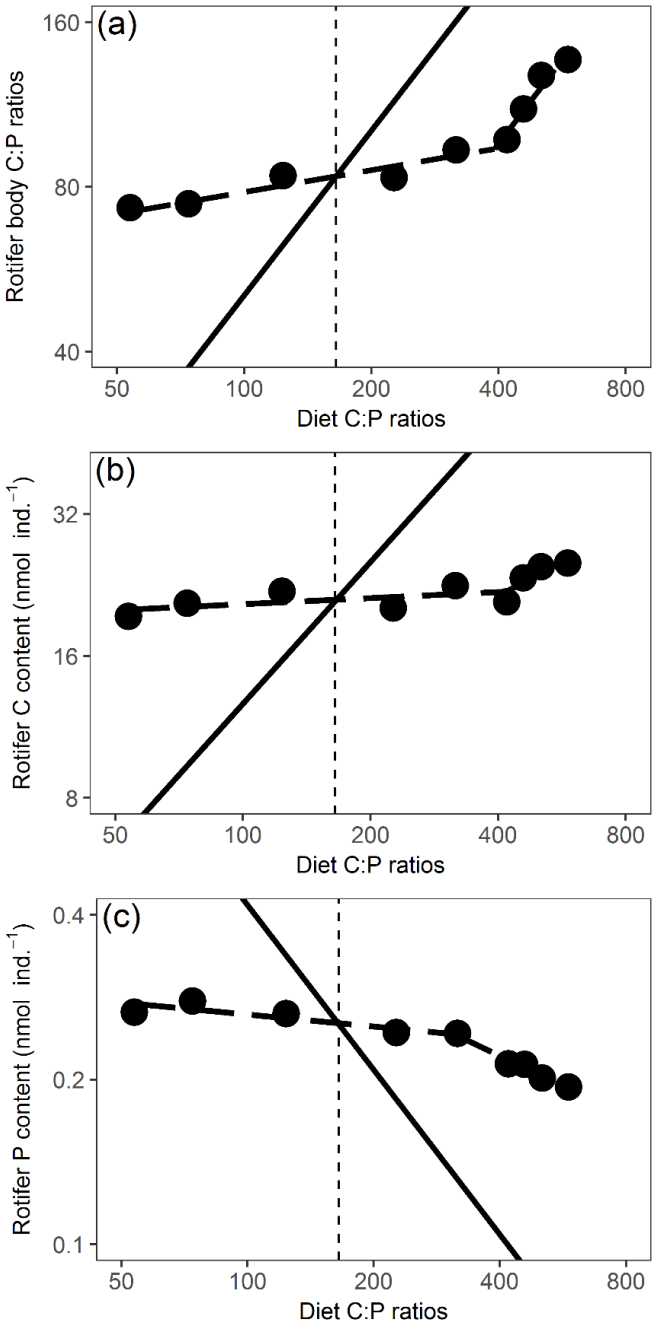

Figure 3.2 (a-c). Response of rotifer stoichiometry to the experimental food C:P gradient. (a) Rotifer C:P ratio, (b) rotifer C content, and (c) rotifer P content. The vertical dotted lines represent the optimal food C:P at which rotifer somatic growth was estimated to be maximal. In each figure, the solid line represents a 1:1 proportional change between diet C:P and the respective variables. Symbols are the means of three repeated measurements in time. Note the log-scale of the axes.

According to piecewise regression (Appendix S3.2 Table 3.2), maximum population growth rate coincided with a rotifer somatic C:P ratio of 80 (Figure 3.3a). Similarly, somatic growth rate reached a maximum at a diet C:P ratio of 77 (Figure 3b; Appendix S3.2 Table3.2).

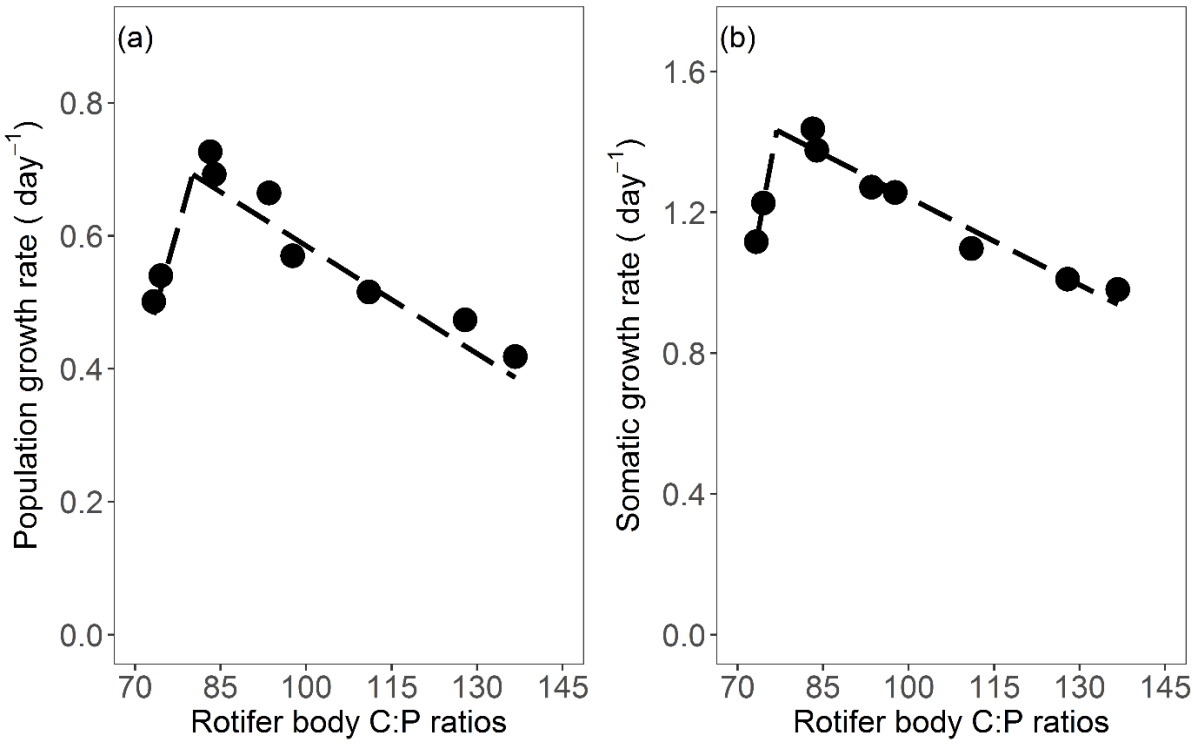

Figure 3.3 (a-b). Association between rotifer population growth rate (a) and rotifer somatic growth rate (b) with rotifer C:P ratio. Symbols represent means of population growth rates across 12 subsequent 24-hour time intervals.

Both food ingestion and corresponding P intake rates were strongly affected by food quality (Figure 3.4a-b; Appendix S3.2 Table 3.1, 3.2, 3.3). Below the food C:P growth optimum, food ingestion rates decreased with decreasing food C:P ratios (Figure 3.4a) while P intake rates increased but less than predicted by the 1:1 line (Figure 3.4b). Above the growth optimum, ingestion rates increased with increasing food C:P ratios but leveled off above food C:P ratios of 400 (Figure 3.4a). Despite increased food ingestion rates, estimated P intake rates decreased strongly with increasing food C:P but slower than predicted by the 1:1 line (Figure 3.4b). P loss rates were largely proportional to P intake rates (Fig. 3.5), with low loss rates under high C:P food and high loss rates when animals were fed low C:P food. The response of P loss rate to increasing P intake rate nevertheless tended to saturate at the lowest levels of food C:P (Figure 3.5).

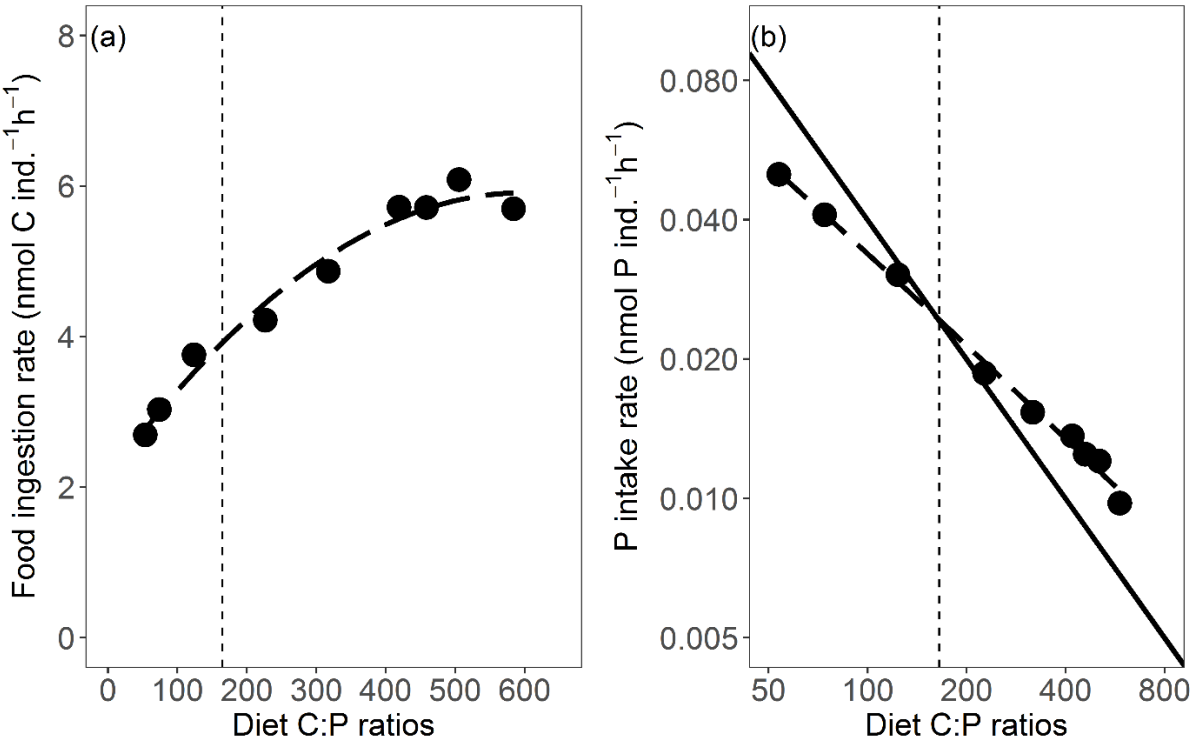

Figure 3.4 (a-b). Response of rotifer food ingestion (a) and P intake (b) rates to a gradient in food C:P ratios. The vertical dashed lines represent the optimal food C:P at which rotifers showed the highest growth rates. The solid line in Figure 3.4b represents a 1:1 proportional change between diet C:P ratio and P intake rate. Symbols are the mean values across three repeated measurements in time. Note the log-scale of the X and Y axes in Figure 3.4b.

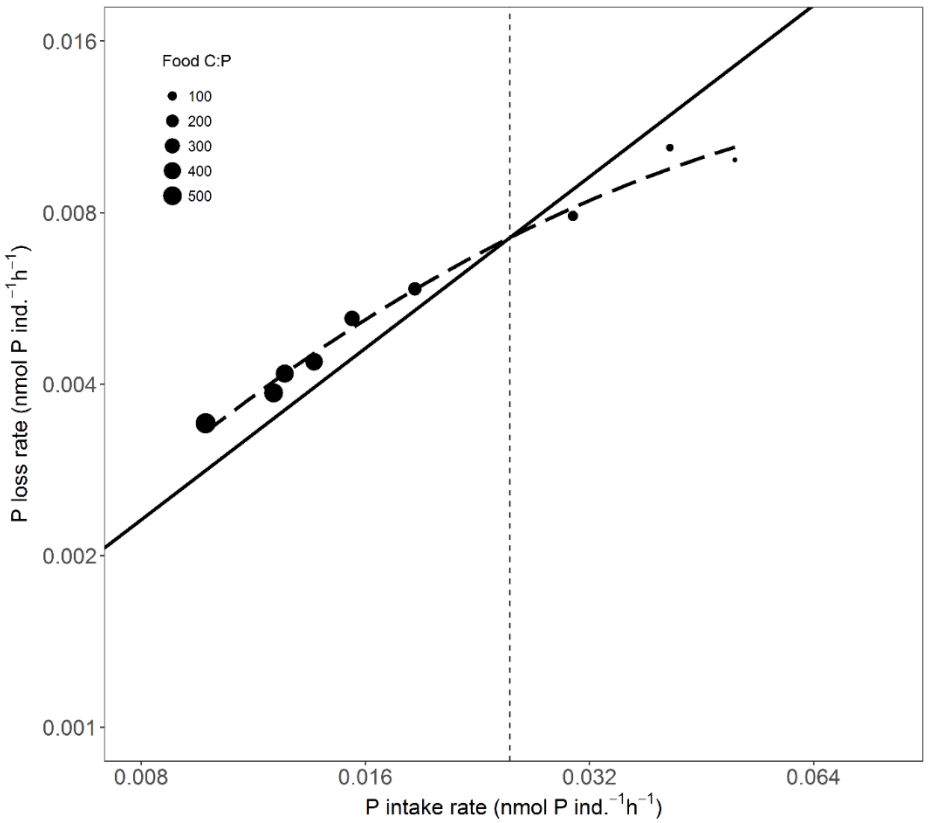

Figure 3.5 Association between rotifer P loss rates and P intake rates. The vertical dashed line represents the optimal food C:P at which rotifers showed the highest somatic growth rates. The solid line represents a 1:1 proportional change between the two variables. Symbols represent averages of P loss rates of three repeated measurements in time. Symbol size indicates food C:P ratio. Note the log-scale of the axes.

Discussion

Our study clearly demonstrates the existence of a stoichiometric knife-edge for somatic as well as population growth rates. Both variables showed a unimodal response along the food C:P gradient, with optimal growth at intermediate food C:P ratios and reduced growth rates towards the extremes of the food quality gradient. The results are well in line with studies reporting a stoichiometric knife-edge for a variety of organism groups (Bullejos et al. 2014; Benstead et al. 2014; Laspoumaders et al. 2015; Elser et al. 2016). In our study, the food C:P ratios at which somatic and population growth were found to be optimal were strikingly similar (i.e. approximately 170) although the slopes of the responses tended to be steeper for population than for somatic growth rate. Population growth rate integrates life history and demographic processes and may therefore be a more sensitive indicator of the adverse effects of stoichiometric mismatch than somatic growth rate. Indeed, stoichiometric mismatch is known to strongly affect life history traits such as survival, fecundity and development time (Jensen

and Verschoor 2004; Felpeto and Hairston 2013; Zhou et al. 2018). The more pronounced negative response of population growth rate to stoichiometric mismatch likely reflects these additional effects.

In contrast to what is widely assumed for consumers in general, the elemental composition of rotifers showed a non-linear response to the food stoichiometry gradient. Within a food C:P range between 53 and 391, rotifers proved to be strong regulators. Rotifer C:P increased with food C:P but the extent of this increase was very limited, suggesting strong homeostasis (Persson et al. 2010). In contrast, when food C:P trespassed the value of 391, homeostasis broke down entirely as rotifer C:P increased proportionally with food C:P. These results demonstrate an important heterogeneity in the degree to which an organism is able to adhere to homeostasis. The evaluation of the homeostatic strength of a consumer is often based on a single value (regulation coefficient, H; Sterner and Elser 2002) assuming a constant response strength of consumer to producer elemental composition across the whole stoichiometric food quality spectrum. However, care should be taken with the use of this metric, especially when organisms are being compared that have grown under different ranges of food C:P (see also Persson et al. 2010; Meunier et al. 2014).

The simultaneous study of consumer growth performance, body elemental composition and key physiological responses along a ten-fold food C:P gradient provides insights in a number of fundamental questions, such as which are the challenges organisms are confronted with along opposite sides of the food C:P optimum, and how and to what extent organisms are able to respond to these challenges. The approach also casts a light on whether deviations from elemental homeostasis reflect adaptive physiological responses or, alternatively, an inability to cope with stoichiometric mismatch. In an almost threefold food C:P range surrounding the growth optimum (120-320), maintenance of a relatively strong degree of homeostasis resulted in no considerable performance reductions. These results indicate that rotifers strive towards maintaining an optimal body elemental composition to safeguard optimal growth and that they are also well able to do so when facing food with quite pronounced but still moderate deviations of their food C:P optimum. In contrast, at lower food C:P values (< 75) we observed a strong reduction in performance despite a relatively strong maintenance of elemental homeostasis. This performance reduction likely reflects large costs associated with maintaining homeostasis. Contrary to the Threshold Elemental Ratio idea, costs associated to the avoidance and removal of P were likely more important than effects of C limitation, given that we worked at food satiating conditions, and that carbon is a very abundant element even in food with a very low

C:P. Indeed, animals responded to lowering food C:P by reducing feeding and thus C- and P-intake rates (Fig. 4) and by increasing P loss rates (Fig. 5; Frost et al. 2004, 2005; He and Wang 2007). Both strategies, however, come at costs and have their limitations. Reduced food uptake rates may secondarily result in a reduced acquisition of energy (Plath and Boersma 2001; Suzuki-Ohno et al. 2012) and other resources. Excretion of excess P has also been suggested to be energetically costly (Anderson et al. 2005; Boersma et al. 2006; Elser et al. 2006) and the relative decline of P-loss at the lowest levels of food C:P are also suggestive of physiological limitations to the rates at which P may be excreted. Finally, although rotifers responded with a mere 14% relative increase of somatic P to an approximately twofold increase of P relative to C in their diet, this increase in somatic P may nevertheless have negatively impacted the performance of the rotifers through toxic effects (Karasov and Martinez del Rio 2007) or costs associated with its storage (Persson et al. 2010).

When food C:P exceeded 391, a complete breakdown of homeostasis (Fig. 2) coincided with a strong reduction in somatic and population growth rates (Fig. 3). This shows a strongly reduced ability of rotifers to cope with such high degree of stoichiometric mismatch and also demonstrates very negative performance effects of deviations from an optimal body stoichiometry. Rotifers thus do not seem flexible conformers but regulators that have limited capacities to maintain homeostasis when confronted with high C:P food. Above the optimum, rotifers responded to an increasing food C:P with increased ('compensatory') feeding, a well-documented response of consumers to compensate for the reduced intake of essential nutrients when facing nutrient deficient food (Fink and von Elert 2006; Suzuki-Ohno et al. 2012;). However, above a food C:P of 400 feeding rates leveled off, possibly due to a trade-off between food-ingestion and P-assimilation rates (DeMott et al. 2010) and the need to avoid excess C intake (Hessen and Anderson 2008). Rotifers also responded to increased food C:P with a strong reduction of P loss rates, although they seemed unable to achieve a reduction of P-loss stronger than proportional to what they acquired through feeding. The dramatic increase in rotifer C:P above the food C:P optimum tended to be more driven by a reduced P than an increased C content suggesting that homeostatic breakdown was mainly caused by the inability to cope with lack of P than excess carbon, although the latter also likely played a role.

Our results support the conclusions of Meunier et al. (2014) of a non-linearity in the response of consumer body stoichiometry to food elemental composition. Application of the framework of Meunier et al. (2014) to our results nevertheless remained difficult. According to Meunier et al. (2014), breakdown of homeostasis in regulators occurs at both sides of the food optimum

when food C:P values surpass specific thresholds. Although we indeed observed such pattern at high food C:P , no breakdown of homeostasis was observed at the low food C:P end. This is most likely because the lowest C:P value of our experimental food quality range was still too high and we cannot exclude the possibility that a breakdown would still be observed at lower food C:P levels. It is nevertheless remarkable that rotifers were better able to maintain homeostasis when facing low compared to high C:P extremes. Indeed, in the range above the optimum, homeostatic breakdown was observed close after the food C:P ratio doubled compared to the optimal food C:P. In contrast, in the range below the optimal food C:P, homeostatic breakdown was not observed when the ratio of P to C in the food tripled. This asymmetry in response is likely a reflection of the fact that organisms are facing fundamentally different challenges along these opposite directions of the resource quality gradient. When faced with increasing C:P above the optimum, animals suffer from the simultaneous combination of P-limitation and excess C in their food. In contrast, given that we worked at food satiating conditions, animals exposed to low C:P food only had to face excess P but no limited availability of C. The combined effect of excess C and limitation of P may therefore explain the faster breakdown of homeostasis in the higher than in the lower food C:P ranges.

Relevance to natural systems

The strong negative effects of excess P on the performance of consumers may cast a new light on the consequences of eutrophication (Elser et al. 2016). In the latest decades, many water bodies worldwide have undergone strong eutrophication due to anthropogenic N and P inputs. These nutrient inputs and associated changes in light regimes (light-nutrient hypothesis; Sterner et al. 1997; Elser et al. 2003) have strongly lowered C:nutrient ratios of primary producers in many of these systems. The low C:P-levels applied in our study correspond well with the lower range of values reported for lake seston in surveys (Hessen 2006; Sterner et al. 2008). Indirect negative effects of eutrophication on primary zooplankton consumers, e.g. via the promotion of toxic or inedible cyanobacterial blooms (Carpenter 2008; Schindler et al. 2008) or the enhancement of planktivorous fish stocks (Sereda et al. 2008) are quite well understood. Remarkably, much less attention has been given to the potential detrimental direct effects of stoichiometric mismatch caused by high levels of nutrient supply (Boersma et al. 2006; Elser et al. 2016). The results of our study support the idea that the high P content in food seston itself may be an underappreciated factor that may not only contribute to the reduction of primary consumer performance in eutrophied systems (Elser et al. 2016) but that may also affect ecosystem functions, e.g. by altering mass specific rates of grazing and P excretion.

As consumers of primary producers, recyclers of nutrients and food source of higher trophic levels, zooplankton take a key position in the pelagic food web. An important question is to what extent the observed heterogeneity in homeostatic strength impacts these functions along broad gradients of relative nutrient supply rates. Several models in ecological stoichiometry describe consumer-producer and nutrient cycling dynamics assuming strict consumer homeostasis (Sterner 1990; Loladze et al., 2000), whereas more recent models (Mulder and Bowden 2007; Anderson et al. 2013) suggest that a relaxation of homeostasis may change model outcomes. Another prominent question is to what extent homeostatic breakdown of primary consumers may result in a stoichiometric mismatch with higher trophic levels. P-limitation of primary consumers has indeed been shown to travel up the food chain (Malzahn et al. 2007; Boersma et al. 2008), and subsequently affect energy transfer efficiencies among higher trophic levels (Grover 2003; Rowland et al. 2015). Recent studies have also shown that consumer prey with excess P may negatively affect the performance of their predators (Benstead et al. 2014; Laspoumaderes et al. 2015), and one may hypothesize that such bottom-up cascades may also be driven by very high P supply under hypereutrophic conditions. However, given the limited response of rotifer P-content and C:P ratios to P-rich food, our results provide limited support for such mechanism.

Although our results were obtained in well-defined laboratory conditions they may form a poor representation of the complexity of real field conditions. Our results focus on the response of a single consumer to elemental mismatch with one producer. In natural systems, however, consumer communities consist of multiple species that may differ from each other in their responses to elemental mismatch (Currier and Elser 2017). Furthermore, consumers are exposed to a mixture of food source types that may vary in elemental composition and on which they may feed selectively. In addition, consumer populations may also be negatively affected by indirect, non-stoichiometric effects of elemental imbalance in their environment (Rothhaupt 1995; Zhou et al. 2018). For example, algae grown in a P-limited environment have been shown to have a reduced digestibility (van Donk et al. 1997) or changed biochemical composition (Spijkerman and Wacker, 2011; Challagulla et al., 2015). We are not aware of studies that have demonstrated such indirect effects for P–rich environments, but the possibility that such indirect effects further aggravate the effects of excess P cannot be a *priori* be excluded. Therefore, although our results provide strong proof of concept for a number of important ideas that so far have received limited attention in ecological stoichiometry, there is the need for more studies to address their relevance to the complex situation of natural systems. With that respect, some

attempts have recently been undertaken, e.g. regarding the strength of the knife-edge effect in multiple *Daphnia* species using lake seston (Currier and Elser 2017) or elemental plasticity at species and community level in outdoor mesocosms (Teurlincx et al. 2017).

Acknowledgements

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Appendix S 3.1

Algae cultures and food preparation

We used the green algae *C. reinhardtii* as food source for the rotifers. The algae were cultured in continuous 2L-chemostats using modified WC medium (Guillard and Lorenzen, 1972) at room temperature of 25 ˚C and a dilution rate of 0.33 day^{-1}. The WC medium contained 15 µmol L^{-1} P. Phytoplankton chemostats received ≈120 µmol quanta m^{-2} s^{-1} of continuous light. Algal biomass and stoichiometric composition were in a steady state at least for three months prior to the experiment (see Zhou et al. 2018).

To create food with different C:P ratios, we first harvested algae directly from the chemostat, and then manually added the inorganic phosphate (K_2HPO_4, 0.05 mol L^{-1}) 90 minutes before feeding them to experimental rotifers. The algae were then kept in a shaking incubator under darkness to ensure the adequate absorption of P and to avoid photosynthesis and growth of the algae (Zhou et al. 2018). The concentrations of algal food were calculated based on the equation: $Y = 21.456\ X$ (R^2=0.9899), where Y is the carbon concentration of algal food (µmol L^{-1}) whereas X represents the biovolume (10^6 μm^3) as measured from the coulter counter. This equation was also used for calculating the carbon concentrations in the food ingestion rate experiment.

Estimation of population growth rates

Before the experiment, we acclimatized nine rotifer populations to each of the food quality treatments for 10 days by initiating cultures of each food quality treatment in small scales (20 ml flasks) and expanding the cultures of each population. At the end of this period we transferred 4000 individuals into 200 mL flasks with a 1000 µmol C L^{-1} food suspension of the corresponding food quality treatments. To achieve continuous exponential growth we daily restarted these populations in a fresh food suspension with a subsample of approximately 800 individuals. At the end of each 24-hour period, we estimated total population size in each of the cultures by counting the number of rotifers in two independent 5 mL subsamples. Based on these counts a culture volume equivalent to 4000 rotifers was rinsed on a 60 µm mesh and used to restart the respective populations with fresh medium. All the flasks were incubated in a shaking incubator at 24 ˚C under continuous darkness. We repeated this culturing process during the whole experimental period (i.e. 10 days of acclimatization followed by 12 days of experiment).

Estimation of somatic growth rates

The somatic growth rate experiment aimed to study the response of somatic growth rate to the experimental C:P gradient. For each food quality treatment we kept a rotifer population in exponential growth (see 'population growth rate'). The experiment was started by transferring at least 400 mothers with parthenogenetic eggs form each culture into a fresh food suspension (1000 µmol C L-1) of the respective quality. After 2 hours, we incubated 100 newborns from these cultures in a glass vial with 5 ml of the corresponding food suspension, covered these vials with aluminium foil, and placed them on a rotating plankton-wheel (30 rotations per minute) to keep the algal food in suspension. After 18 hours, each cohort was prepared for the measurement of total C content (Mt). For each food quality treatment we also measured total carbon content for a group of 100 newborns with a maximum age of 2 hours (M0). Mass-specific somatic growth rate was calculated as (lnMt – lnM0)/t, with t = 18 hours, and subsequently converted to somatic growth rate at time interval of 24 hours (day-1).

Estimation of food ingestion rates

To start the grazing experiment, we isolated three replicate groups of 200 rotifer individuals from each of the nine food quality treatments in the population growth rate experiment. To avoid variation due to rotifer age, sex or size, we selected exclusively adult females carrying one parthenogenetic egg. These groups of animals were incubated in 10 ml glass vials filled with 8 ml of food suspension (1000 µmol C L^{-1}) of the respective food quality treatments and allowed to feed for four hours. During the incubation, the glass vials were wrapped in aluminium foil to prevent growth of algae and placed on a rotating plankton wheel to keep algal food in suspension (30 rotations $minute^{-1}$). Immediately after the incubation period, all rotifers of each vial were retrieved and used to start the subsequent P loss experiment (see P loss experiment).

Appendix S 3.2:

Appendix S 3.2 Table 3.1 Results of quadratic linear regression models. Models in bold are those selected for interpretation based on AIC and consistency (see Methods).

Variables		AIC	df	F	p	R^2	Adjusted R^2	Equation
Dependent variables	**Explanatory variables**							
Population growth rate	**Diet C:P ratios[I]**	**-33.92**	**$DF_{2,6}$**	**50.53**	**<0.001**	**0.944**	**0.925**	**$y=-4.439+1.395X - 0.094X^2$**
Somatic growth rate	**Diet C:P ratios[I]**	**-23.6**	**$DF_{2,6}$**	**34.58**	**<0.001**	**0.920**	**0.894**	**$Y=-5.716+1.947X - 0.132X^2$**
Food ingestion rate	**Diet C:P ratios**	**2.7**	**$DF_{2,6}$**	**130**	**<0.001**	**0.977**	**0.970**	**$Y=2.125+0.0126X-0.0000105X^2$**
P intake rate[I]	**Diet C:P ratios[I]**	**-186.73**	**$DF_{2,24}$**	**51.98**	**<0.001**	**0.813**	**0.797**	**$Y=0.241-0.0469X+0.0024X^2$**
P loss rate[I]	**P intake rate[I]**	**-16.2**	**$F_{2,6}$**	**233.6**	**<0.001**	**0.987**	**0.983**	**$Y=-7.33-0.740X-0.131X^2$**
Rotifer C:P[I]	Diet C:P ratios[I]	-10.2	$F_{2,6}$	33.27	<0.001	0.917	0.890	$Y=10.40-1.312X+0.103X^2$
Population growth rate	Rotifer C:P ratios	-16.1	$F_{2,6}$	4.36	=0.067	0.593	0.457	$Y=-0.513+0.024X -0.00013X^2$
Somatic growth rate	Rotifer C:P ratios	-10.4	$F_{2,6}$	5.69	=0.041	0.655	0.540	$Y=0.072+0.027X-0.00016X^2$
Rotifer C:N ratios[I]	Diet C:P ratios[I]	-23.2	$F_{2,6}$	26.24	=0.001	0.897	0.863	$Y=4.32-0.591X + 0.046X^2$

[I] represents the values that have been Log2-transferred during the analysis.

Appendix S3.2 Table 3.2 Results of piecewise regression models. Models in bold are those selected for interpretation based on AIC and consistency (see Methods).

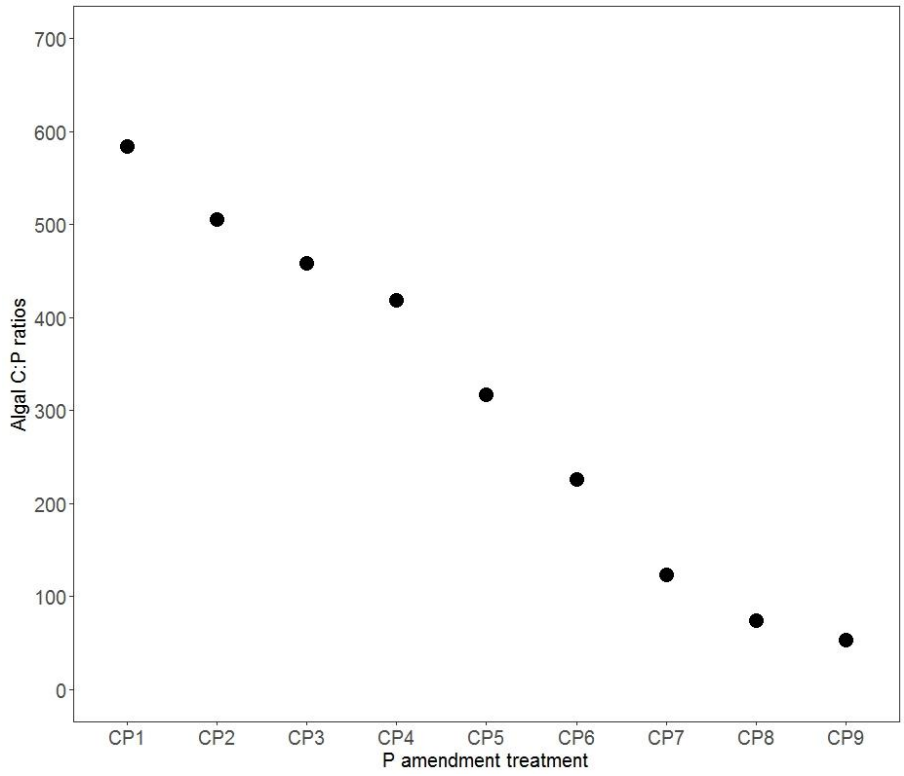
Variables		AIC	Equation 1	Equation 2	Breakpoint	Slope1	95% CI	Slope2	95% CI	p_{Davies}
Dependent variables	**Explanatory variables**									
Population growth rate	Diet C:P ratios[Ł]	-36.45	Y=-0.4317+0.16X	Y=3.4-0.232X	200.9	0.16*	(0.09,0.23)	-0.39*	(-0.29,-0.18)	<0.001
Somatic growth rate	Diet C:P ratios[Ł]	-22.5	Y=-0.113+0.215X	Y=4.098-0.338X	196.2	0.215*	(0.055,0.374)	-0.338*	(-0.461,-0.215)	0.004
Food ingestion rate	Diet C:P ratios	1.5	Y=2.54+0.007X	Y=8.47-0.005X	493.7	0.007*	(0.006,0.009)	-0.005	(-0.014,0.004)	0.023
P intake rate[Ł]	Diet C:P ratios[Ł]	-184.8	Y=0.14-0.016X	Y=0.064-0.006X	187.4	-0.016*	(-0.03,-0.006)	-0.006	(-0.014,0.002)	0.212
P loss rate[Ł]	P intake rate[Ł]	NA	NA	NA	NA	NA	NA	NA	NA	NA
Rotifer C:P[Ł]	**Diet C:P ratios[Ł]**	**-20.04**	**Y=5.4665+0.1255*X**	**Y=-2.266+1.0235X**	**390.7**	**0.125***	**(0.05,0.20)**	**1.023***	**(0.58,1.47)**	**0.008**
Population growth rate	**Rotifer C:P ratios**	**-31.6**	**y = 1.79-0.031X**	**y=3.657-0.037X**	**80.1**	**0.031**	**(-0.06,0.13)**	**-0.037***	**(-0.007,-0.004)**	**0.004**
Somatic growth rate	**Rotifer C:P ratios**	**-27.2**	**Y=-5.355+0.0883X**	**Y=2.067-0.00825X**	**76.9**	**0.0883**	**(-0.031,0.208)**	**-0.00825***	**(-0.01,-0.006)**	**0.006**
Rotifer C:N ratios[Ł]	**Diet C:P ratios[Ł]**	**-24.0**	**Y=2.482-0.008X**	**Y=0.86+0.207X**	**186.1**	**-0.008**	**(-0. 15,0.14)**	**0.207***	**(0.094,0.320)**	**0.1**

*slope differing significantly from 0; p_{Davies}: significance of difference between the two slopes; Ł Log2-transformed prior to statistical analysis. NA: no breakpoint detected.

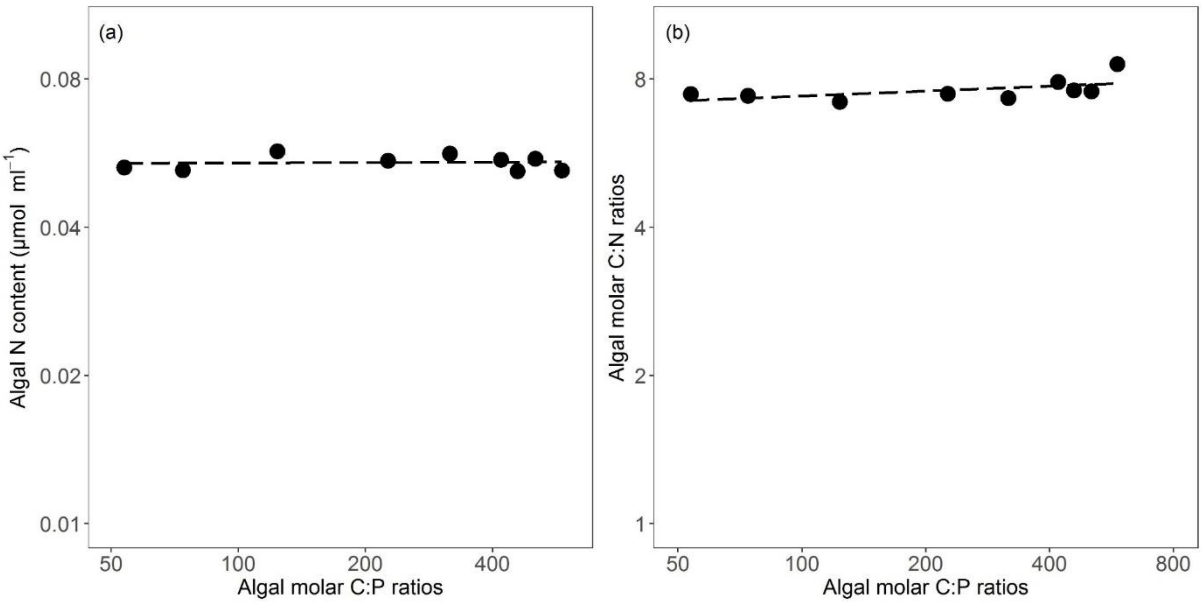
Appendix S3.2 Table 3.3 Results of linear regression models. None of these models were selected for further interpretation given their low performance compared to quadratic or piecewise regression models.

Variables		AIC	df	F	R^2	Adjusted R^2	p	Equation
Dependent	**Explanatory**							
Population growth	Diet C:P ratios[I]	-10.5	$DF_{(1,7)}$	0.377	0.05	-0.08	=0.56	Y=0.71-0.019X
Somatic growth rate	Diet C:P ratios[I]	-4.0	$DF_{(1,7)}$	0.984	0.12	-0.002	=0.35	Y=1.54-0.043X
Food ingestion rate	Diet C:P ratios	1.5	$DF_{(1,7)}$	91.98	0.93	0.92	<0.001	Y=2.74+0.006X
P intake rate[I]	Diet C:P ratios[I]	-	$DF_{(1,25)}$	92.91	0.79	0.78	<0.001	Y=0.11-0.011X
P loss rate[I]	P intake rate[I]	-12.5	$DF_{(1,7)}$	287.3	0.98	0.97	<0.001	Y=-3.49+0.697X
Rotifer C:P[I]	Diet C:P ratios[I]	-4.49	$DF_{(1,7)}$	28.91	0.81	0.78	=0.001	Y=4.78+0.23X
Population growth	Rotifer C:P ratios	-14.0	$DF_{(1,7)}$	3.90	0.36	0.27	=0.089	y =0.84-0.003X
Somatic growth rate	Rotifer C:P ratios	-9.1	$DF_{(1,7)}$	7.15	0.51	0.43	=0.032	Y= 1.67-0.005X
Rotifer C:N ratios[I]	Diet C:P ratios[I]	-18.3	$DF_{(1,7)}$	24.68	0.779	0.748	=0.0016	Y=1.80+0.098X

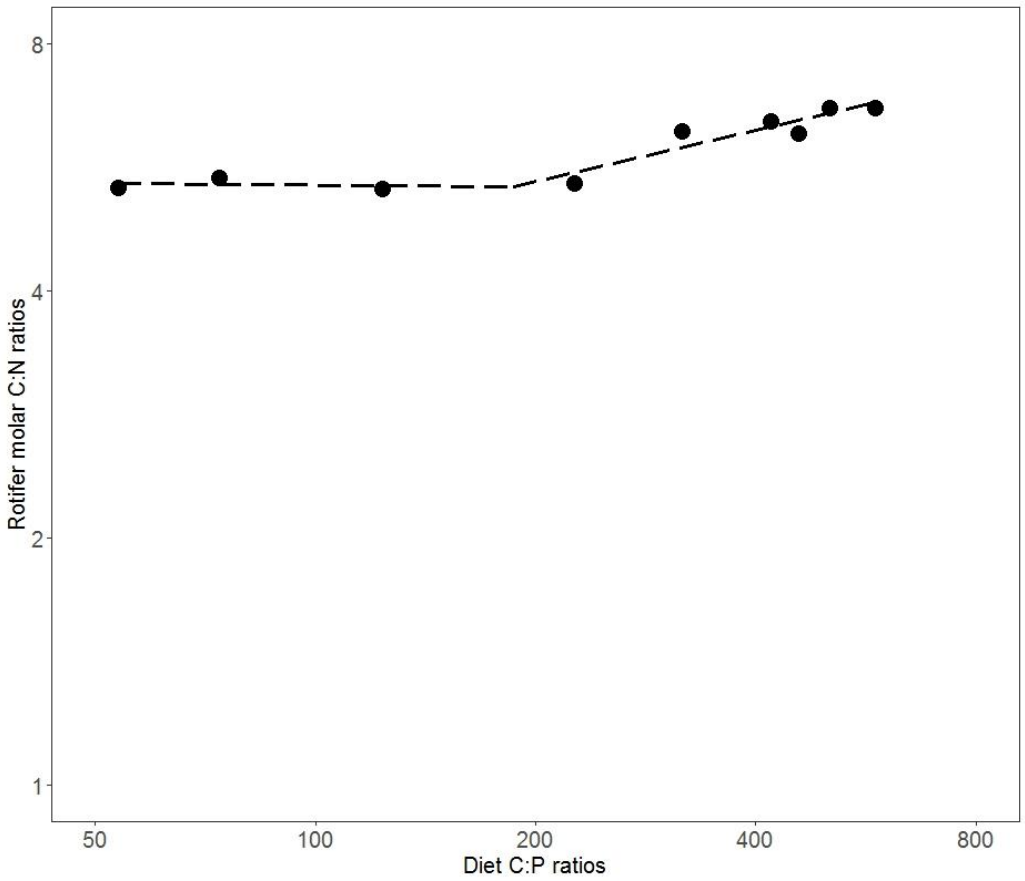
[I] represents the values that have been Log2-transferred during the analysis.



Appendix S3.2 Figure 3.1 Algal molar C:P ratios of each food quality treatment. Symbols are the mean values of three repeated measurements in time.



Appendix S 3.2 Figure 3.2 (a-b) Algal N content (a) and C:N ratios (b) of each food quality treatment. Linear regression models showed a slope of 0.0024 for algal N content which was not significantly different from 0 (p=0.88), and a slope of 0.032 for algal C:N ratios in response to algal C:P ratios (p=0.128). Symbols are the mean values across three repeated measurements in time. Note the log-scale of the axes.



Appendix S 3.2 Figure 3.3 Rotifer C:N ratios in each of the food quality treatments. The segmented regression shows a break point located at an algal C:P ratio of 186. The slope of the regression equaled -0.01 below and 0.21 above this breakpoint. These slopes did not differ significantly (Davies-test). Symbols represent the mean values across three repeated measurements in time. Note the log-scale of the axes.

Results for Rotifer C and P analysis

At the breakpoint food C:P of 391, rotifer C:P equaled 93.5. At the highest food C:P level (584), rotifer C:P equaled 137. Above the breakpoint, rotifer C:P thus responded with a 46% increase to a 50% augmentation of the C:P of its food. Such dramatic increase of rotifer C:P appears to have resulted from the joint effects of increased total somatic C content and reduced total somatic P content. Indeed, for rotifer C content, a two segments piecewise regression model suggested a breakpoint at a food C:P of 418. The slope of the regression above this breakpoint was steeper than the slope of the regression below this breakpoint (0.485 and 0.042, respectively; Figure 2b), although no significant difference between these two slopes was detected ($p=0.259$; Appendix S2 Table 4). For rotifer P content, a breakpoint occurred at a food C:P of 312. With increasing food C:P the rate at which rotifer P content decreased was larger above than below this breakpoint ($p = 0.019$; see Appendix S2 Table4).

To assess the relative contribution of changes in somatic C and P content to variation in rotifer C:P ratio, we calculated rotifer C:P ratios keeping one element (either C or P) constant while allowing the other one to vary. At the highest food C:P level assuming an invariable rotifer P, the observed increase in C content would result in a rotifer C:P of 107, corresponding to a 19% increase. With an unchanged rotifer C content, the decrease in P content would result in a rotifer C:P of 134, corresponding to a 43% increase. These results suggest that the breakdown of homeostasis at high food C:P ratios was mainly driven by reductions of body P content, although augmentations in C content also had a considerable contribution.

Appendix S 3.2 Table 3.4 Results of piecewise regression models for rotifer C and P contents

Variables			AIC	Equation 1	Equation 2	Breakpoint	Slope1	95% CI	Slope2	95% CI	p_{Davies}
Dependent variables		**Explanatory variables**									
Roitfer content	C	Diet C:P ratios	-16.5	Y=0.042X+4.087	Y=0.485X+0.231	417.6	0.042	(-0.05,0.13)	0.485	(-0. 06,1.02)	0.259
Rotifer content	P	Diet C:P ratios	-27.9	Y=-0.071X-1.457	Y=-0.369X+1.01	311.9	-0.071*	(-0.14,-0.01)	-0.369*	(-0.52,-0.22)	0.019

*slope differing significantly from 0; p value: significance of difference between the two slopes ($p<0.05$ represent a significant difference); data was log2-transferred before analysis.

Chapter 4

Maternal effects in zooplankton consumers are not only mediated by direct but also by indirect effects of phosphorus limitation

Libin Zhou and Steven A. J. Declerck

Oikos **(Minor revision)**

Abstract

Nutrient limitation of primary producers has repeatedly been shown to negatively affect consumers, directly through stoichiometric mismatch and indirectly via alterations in the producer's biochemical quality or palatability. In this study, we assessed whether direct and indirect impacts of phosphorus-limitation on a planktonic consumer are transferred to the next generation via maternal effects and whether these effects reflect an anticipatory adaptive strategy. For this, we subjected cultures of the algivorous monogonont rotifer *Brachionus calyciflorus* to three food quality treatments, i.e. P-limited (LP), P-replete (HP) and P-enriched LP algae (i.e. algae with an LP-growth history but with molar C:P ratios equal to those of HP-algae). After two generations, we subjected offspring of these cultures to each of the three food quality treatments and monitored life history traits. In addition, we tested starvation resistance. Our results showed very strong negative maternal effects of low P food on offspring performance. These negative effects prevailed irrespective of contemporary diets, suggesting transmissive and selfish maternal effects rather than anticipatory adaptive effects. The relative strength of direct and indirect maternal P-limitation effects varied among different traits. Adult body size was predominantly determined by direct effects of P-shortage in maternal as well as contemporary food (LP < LP+P and LP+P = HP). In contrast, whereas egg size was negatively affected by direct effects of P-limitation in the maternal diet, a contemporary diet of LP and LP+P algae resulted in larger eggs than HP algae. Animals born from such larger eggs showed no higher growth rates, but they were more resistant to starvation, likely as the result of higher maternal allocation of energy rich molecules to the eggs. The present study shows that maternal food conditions represent an important factor that should be taken into account in studies of stoichiometric mismatch between producers and consumers.

Introduction

The phenotype of an organism is the product of the interaction between its genotype and its environment. In addition, organisms may also be profoundly affected by the environment that has been experienced by previous generations (Marshall and Uller 2007, Yanagi and Tuda 2010, Pajk et al. 2012, Kuijper and Hoyle 2015, Harney et al. 2017). As such, maternal generations are able to affect the quality of their offspring via non-genetic transgenerational mechanisms ('maternal effects', Mousseau and Fox 1998, Marshall and Uller 2007). When confronted with a new stressor or threat, organisms may increase their fitness by anticipating the environment of the future generation and by enhancing the success of their offspring in this environment ('anticipatory maternal effects', Marshalland Uller 2007, Sheriff et al. 2013). For example, upon reception of cues by predators, prey may induce the expression of traits in their offspring that reduce their vulnerability to predation (Yin et al. 2015, Yule and Burns 2017), while deteriorating feeding conditions may result in changed allocation patterns of energy resources among eggs (Yanagi and Tuda 2010, Kaneko et al. 2011, Stahlschmidt and Adamo 2015, Harney et al. 2017). In contrast, organisms may also respond to worsening environmental conditions by increasing their own fitness at the expense of their offspring ('selfish maternal effects', Marshall and Uller 2007), e.g. by allocating fewer resources to offspring under worsening feeding conditions (Frost et al. 2010, He et al. 2016). Alternatively, some maternal effects represent the legacy of maternal growing conditions but lack adaptive value ('transmissive maternal effects', Marshall and Uller 2007). For example, exposure of the mother generation to food limitation, toxins or parasites may translate in a reduced performance of the offspring (Guinnee et al. 2007, Beyer et al. 2017).

A good understanding of phenotypic variation in consumers requires knowledge of their response to environmental change and how the effects of these responses propagate across generations (West-Eberhard 2003, Kuijper and Hoyle 2015). One important factor that may strongly affect the phenotypes of animals is resource quality. Resource quality is often determined by the relative ratios of essential biogenic elements, such as nitrogen (N), phosphorus (P) and carbon (C) (Hessen et al. 2013). Many studies have demonstrated that a mismatch between the elemental requirements of consumers and their prey (i.e. 'stoichiometric mismatch') results in altered phenotypes and reduced performance of consumers (Elser et al. 2001, Frost et al. 2002, Declerck et al. 2015). However, the role of maternal effects in

determining the phenotypic response to stoichiometric mismatch is still poorly understood. It is also unclear to what extent such maternal effects represent an adaptive strategy.

In addition to the negative effects of a stoichiometric mismatch, imbalanced nutrient supply rates may also influence the performance of consumers by causing non-stoichiometric alterations in their food (Rothhaupt 1995, Ravet and Brett 2006, Zhou et al. 2018). P-limitation of primary producers often result in changes in their biochemical composition (Müller-Navarra, 1995, Weers and Gulati, 1997, Spijkerman and Wacker, 2011, Challagulla et al., 2015) and morphology (van Donk and Hessen 1995, van Donk et al. 1997), which may affect consumer performance independently of their P-content. Although never studied, it is likely that such indirect effects may also be transferred to next generations through maternal effects. Variation in the biochemical composition of maternal diets has been shown to affect the performance of subsequent generations (Sperfeld and Wacker 2015), likely as the result of altered allocation of biochemical components to eggs (Sperfeld and Wacker 2009, Sperfeld and Wacker 2012). Changes in the biochemical quality of food as result of P-limitation could be transferred to the next generation in a similar way and it remains an open question to what extent such indirect effects would be transmissive or adaptive.

Zhou et al. (2018) showed that individuals of the zooplanktonic rotifer *B. calyciflorus* produced larger eggs when fed P-limited algae (LP) compared to animals that were fed with a diet of P-replete algae (HP). Given that egg size tends to be associated with higher offspring quality in many taxa (Dias and Marshall 2010, Krist et al. 2011, Segers and Taborsky 2011, Moore et al. 2015), such increased egg size may reflect an anticipatory adaptive response. Using a P enrichment method, Zhou et al. (2018) were able to disentangle direct from indirect effects of P-deficient food by creating a food quality treatment (LP+P) involving algae with an LP growth history but having molar C:P ratios equal to that of the HP treatment. They showed that animals underperformed in LP+P compared to HP diets, possibly as the result of indirect effects of P-limitation (e.g. disadvantageous alterations in algal biochemical composition or morphology). Notably, the eggs of animals grown in LP+P were also larger than in HP and equaled the size of eggs produced in LP. This leads to the question whether the egg size response in the LP+P treatment has any adaptive value or, conversely, reflects a legacy of the indirect effects of P-limitation.

The objective of our study was to assess whether both direct and indirect impacts of P-limitation on the rotifer *B. calyciflorus* are transferred to the next generation via maternal

effects and to evaluate to what extent these effects reflect anticipatory adaptive strategies or merely transmissive effects. For this, we raised animals under LP, LP+P and HP conditions for multiple generations, confronted their offspring to either of the three food quality conditions according to a multifactorial design and monitored a number of key life history traits. We considered maternal effects to be anticipatory if offspring with an ancestral history of P-limitation (LP or LP+P) perform better under these respective conditions than offspring born from mothers grown under P-replete conditions. In addition, we tested if the increased egg size associated with exposure of the maternal generation to P-limitation or its indirect effects is associated with an increased starvation resistance of the offspring.

Methods

Rotifer and algae cultures

We cultured the green alga *Chlamydomonas reinhardtii* as food for the rotifers in ten 2L-chemostats with modified WC medium (Guillard and Lorenzen, 1972) at 23±1 ˚C and a dilution rate of 0.33 $day^{-.1}$. Five of the chemostats received phosphorus rich medium ('HP', 65 µmol L^{-1} K_2HPO_4) and 40 µmol quanta m^{-2} s^{-1} of light and produced algae with a low molar C:P ratio (C:P=120), whereas the other five chemostats were provided with a phosphorus poor medium ('LP', 15 µmol L^{-1} K_2HPO_4) and 120 µmol quanta m^{-2} s^{-1}, resulting in algal cultures with a high C:P ratio (C:P=600).

We used one *B. calyciflorus* clone obtained from the resting egg bank of a Dutch lake (52°5'26.50"N, 4°20'18.40"E). *B. calyciflorus* is actually known to consist of a species complex, of which four species have recently been (re-)described based on phylogenetic and morphological analyses (Papakostas et al. 2016, Michaloudi et al. 2018). Based on its ITS1-sequence information, the clone used for this study was identified as *B. calyciflorus* (Michaloudi et al., 2018). Rotifer stock cultures were maintained at room temperature under continuous light conditions, and fed with HP food.

Creation of food quality treatments for the experiment

The rotifers in the experiment were fed every 24 hours at a fixed time of the day. Algae were harvested from the LP and HP chemostats, centrifuged (2500 rpm for 10 minutes) and resuspended in nutrient free WC medium. Ninety minutes before feeding, we created an LP+P food quality treatment by adding inorganic phosphate (K_2HPO_4) to a suspension of LP algae. The amount of added P was based on the algal C content estimated from cell counts

(Multisizertm 3 Coulter Counter, Beckman Coulter). The algae of all three treatments were kept in the dark for 90 minutes between the moments of harvesting and feeding to the rotifers.

Maternal effects experiment

The aim of this experiment was to study how the effects of food quality on the performance and life history traits in a focal rotifer generation ("contemporary food quality") is mediated by the food quality conditions experienced by the maternal generations ("ancestral food quality"). The design of this experiment is illustrated in Figure 1. Starting from great-grandmaternal adults, we independently raised multiple clonally reproducing lines of single individuals under each of the three food quality types (i.e. HP, LP+P and LP) for two generations (the grandmaternal and maternal generations). We then randomly redistributed the offspring (further referred to as 'focal' generation) of the maternal generation across these same food quality treatments. Each food quality treatment was independently replicated by five chemostats. In the focal generation, each replicate in each cross-factorial food quality combination was represented by 4 animals from clonal cultures that had been independently raised since the grand-maternal generation. This thus resulted in an experiment with 180 experimental units (i.e., 3 ancestral food quality treatments × 3 contemporary food quality treatments x 5 chemostat replicates × 4 individuals).

To start the experiment, great-grandmothers with parthenogenetic eggs from stock cultures were randomly collected and used to start 180 individual cultures, i.e. 12 cultures per food quality replicate. The individuals were assigned to individual wells of tissue culture plates filled (volume: 1 ml) with a suspension of the respective food quality treatment (1000 μmol C L^{-1}). Plates were incubated at 23±1 ˚C in the dark at a random location in the incubator. Different food qualities cause differences in generation times (Zhou et al. 2018). To maximally synchronize the moment of birth of the focal generations in the three food quality treatments, we initiated the great-grandmaternal generation of the LP and LP+P cultures, respectively, 42 and 27 hours earlier than of the HP cultures. Every day the cultures were checked twice for the presence of neonates under a stereomicroscope. Females were discarded once their first parthenogenetically produced offspring was born, and the neonate was further cultured as the next generation. Following the production of their first egg, females of the mother generation were immediately transferred to a food suspension of the destined food quality treatment (see experimental design in Figure 4.1) to ensure that experimental animals of the focal generation were born in the target food quality treatment. These gravid females were checked on an hourly

basis to assess the time of birth of the neonate. From an age of 8 hours on, animals from the focal generation were checked every two hours and the age at first egg production was recorded. Two hours after the observation of the first egg the individuals were preserved in 4% formaldehyde for later microscopic measurement of body and egg size.

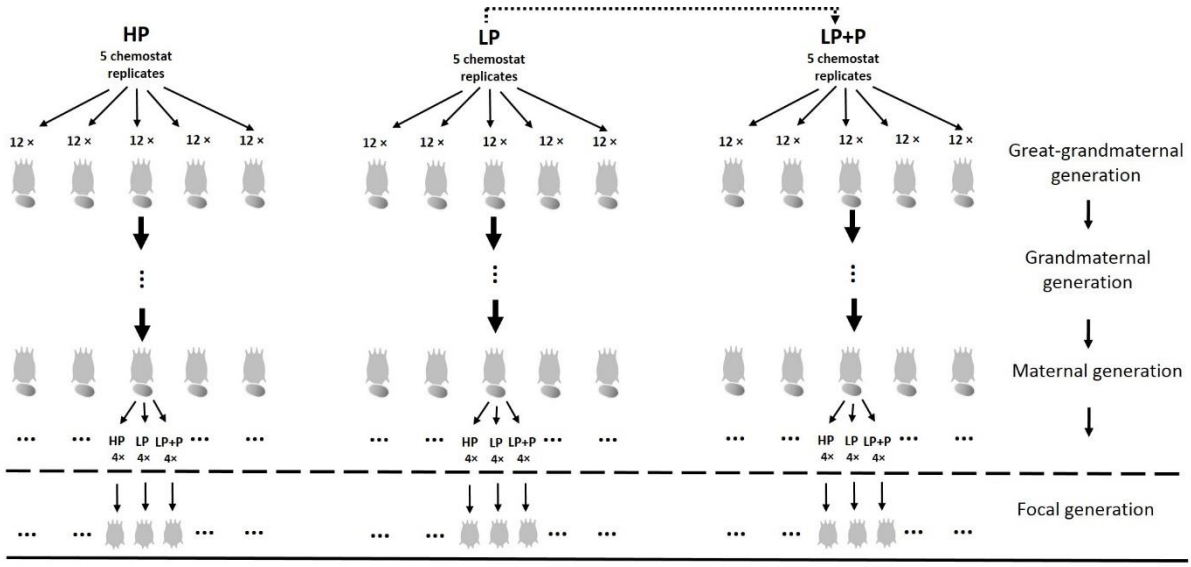


Figure 1. Design of the experiment. We created 3 food quality treatments (HP, LP+P, and LP) each derived from 5 replicate chemostats. We used randomly selected individuals from stock cultures to start 12 clonal lines per food quality replicate. Clonally producing lines were independently raised for two generations, i.e. the grandmaternal and maternal generations. Once individuals of the maternal generation produced their first parthenogenetic egg, they were haphazardly assigned to one of the three food qualities (i.e. four individuals per replicate of each multifactorial combination). These mothers were checked hourly for neonates (i.e. the 'focal generation'). When born, these neonates were individually raised and monitored to record life history traits.

Starvation experiment

This experiment was conducted to evaluate the effect of ancestral food quality treatments on starvation resistance of newborns. Starvation resistance was measured as the age at which animals died in the absence of food. Similar as with the maternal effects experiment, clonally reproducing culture lines of single individuals were established from stock cultures and independently grown under the three food quality treatments (i.e. LP, HP and LP+P) for two generations. The experiment consisted of 45 experimental units, i.e. 3 food quality treatments × 5 chemostat replicates × 3 lines. Upon production of their first egg, females from the maternal generation were transferred into nutrient-free WC medium and checked every hour. The hour of birth was recorded and the newly hatched neonates were transferred to an individual well

with 1 ml nutrient-free WC medium. The neonates were monitored every 2 hours until their death. Due to lab closing hours, we were unable to record death in 18% of the experimental units.

Algae stoichiometry

During the experiment, the molar C:P ratio of the phytoplankton in each food quality treatment was measured at two occasions. Algal samples were obtained by filtering culture medium on glass filters (GF/F) following by drying at 60 ℃ for at least 24 hours. C and N contents of algae were determined using a FLASH 2000 organic element analyzer (Interscience B.V., Breda, Netherlands). For P content, algal samples were incinerated at 550 ℃ for 45 minutes and then autoclaved in 2.5% potassium persulfate ($K_2S_2O_8$) at 121 ℃ . These samples were subsequently determined using a QuAAtro segmented flow autoanalyzer (Beun de Ronde, Abcoude, Netherlands).

Data analysis

Body volume was calculated as $Vb = \pi * Lb * (Wb/2)^2$, where L_b and W_b are rotifer body length and width, respectively. Egg volume was calculated with the geometric formula for an ellipsoid: $Ve = \left(\frac{4}{3}\right) * \pi * (Le/2) * (We/2)^2$, where Le and We represent egg length and egg width (Zhou et al. 2018). Somatic growth rate was estimated as g = (lnVb – lnVe)/t, where t is the age at which the first egg was produced (Zhou et al. 2018). By estimating the initial egg size of the focal generation by the size of the eggs they produced themselves we assumed that egg size of maternal and focal generations remained the same within food quality treatments.

To evaluate the effects of maternal and contemporary diets and their interactions on life history traits, we applied general linear mixed effects models specifying contemporary and maternal diet treatments as fixed factors and chemostat replicates for contemporary and maternal diets as random factors. The significance of maternal and contemporary diets, as well as their interaction were evaluated with a following ANOVA analysis. Size at first egg production and egg size were log_2-transformed prior to analysis. The effect of maternal diet on offspring starvation resistance was studied by taking maternal food quality as fixed factor and food chemostat replicate as random factor. Tukey *post hoc* comparisons were applied to evaluate the significance of differences among factor levels. All statistical analyses were performed in

R software environment 3.4.2 (R Core Team 2017). Mixed effects analyses were performed with the lme4 package in R (Bates et al. 2015).

Results

Throughout the experiment, the algal molar C:P ratios of the HP and LP+P food quality treatments were very similar (HP: mean: 131, sd: 22, n=5; LP+P: mean: 99, sd: 13, n=5) and much lower than of the LP treatment (mean: 582, sd: 59, n=5; see also Appendix S 4.1).

Mortality of focal animals during the life table experiment was low as only 4 out of 180 individuals died before maturation. Size at first egg production of the focal generation was determined by both maternal and contemporary diets, but there was no interaction between these factors (Figure 4.2a, Table 4.1). The contribution of the maternal diet to the variation in size at first egg production was considerably higher than the contribution of the contemporary diet (Table 4.1). Under any of the contemporary diets, HP and LP+P maternal diets resulted in a similar size at first egg production and both were significantly larger than animals born to mothers cultured in LP food (Figure 4.2a, Tukey post hoc comparisons: HP vs. LP maternal diets: $p<0.001$, LP+P vs. LP maternal diets: $p=0.001$). Similarly, for focal animals with a same maternal diet history, there was no difference in size at first egg production between HP and LP+P contemporary diets, but both were larger than in the LP contemporary diet (Figure 4.2a, Tukey post hoc comparisons: HP vs. LP contemporary diets: $p=0.005$, LP+P vs. LP contemporary diets: $p=0.008$).

The size of the first eggs was also affected by both maternal and contemporary diets while no significant interaction was found (Figure 4.2b, Table 4.1). Under a given contemporary diet, the size of the first eggs of focal animals born to HP and LP+P mothers were similar, and both were larger than those produced by offspring born to LP mothers (Figure 4.2b, Tukey post hoc comparisons: HP vs. LP maternal diets: $p=0.020$, LP+P vs. LP maternal diets: $p=0.006$). However, for animals with a same maternal diet, LP and LP+P food resulted in the production of larger eggs than HP food (Tukey post hoc comparisons: HP vs. LP+P contemporary diets: $p<0.027$, HP vs. LP contemporary diets: $p=0.005$), whereas no significant difference was found between LP+P and LP treatments.

Age at first egg production of rotifers was determined by both maternal and contemporary diets and a relatively weak but significant interaction between these two factors (Figure 4.2c, Table 4.1). Animals born to mothers with an LP history always needed a significantly longer time to

produce their first eggs than those born to mothers with an HP history (Figure 4.2c, Tukey post hoc comparisons: HP vs. LP maternal diets: $p<0.01$ in all levels of contemporary diets). Age at first reproduction of animals with a maternal LP+P diet was similar to those with an HP maternal diet and lower than in the LP maternal diet (Tukey post hoc comparisons: LP+P vs. LP maternal diets: $p<0.001$) except in an LP contemporary diet, where age at first reproduction of animals with a maternal LP+P diet was intermediate to the other maternal diets (Figure 4.2c, Tukey post hoc comparisons: LP+P vs. LP maternal diet: $p<0.001$, HP vs. LP+P maternal diet: $p=0.009$). Contemporary diets also strongly affected the age at first reproduction, given that animals in LP food needed a longer time to produce their first eggs than those in HP and LP+P diets (Figure 4.2c, Tukey post hoc comparisons: HP vs. LP contemporary diets: $p<0.001$, LP+P vs. LP contemporary diets: $p<0.001$).

Both maternal and contemporary diets significantly affected somatic growth rate (Figure 4.2d, Table 1). Maternal diets contributed considerably more to variation in somatic growth rate than the contemporary diet (Table 4.1). Under a given contemporary diet, animals in the HP treatment tended to have a higher somatic growth rate than those in the LP+P treatment, and both were higher than in the LP treatment (Figure 4.2d, Tukey post hoc comparisons: HP vs. LP+P maternal diets: $p=0.005$, HP vs. LP maternal diets: $p<0.001$, LP+P vs. LP maternal diets: $p=0.005$). The diet of the focal generation also strongly affected the somatic growth rate of animals. Animals in HP diets had a similar growth as those with an LP+P diet, whereas both were higher than animals in the LP treatment (Tukey post hoc comparisons: HP vs. LP contemporary diets: $p=0.002$, LP+P vs. LP contemporary diets: $p=0.004$).

Table 4.1 Summary of mixed model analysis results of the maternal effect experiment. Maternal and offspring diets and their interaction were specified as fixed factors in the models. SS: sum of squares, MS: mean square, DF: degrees of freedom.

Variables	SS	MS	DF	F value	p
Size at first egg production					
Maternal diets	1.34	0.67	2	22.6	<0.001
Contemporary diets	0.69	0.34	2	11.7	0.004
Maternal diets × Contemporary diets	0.09	0.02	4	0.8	0.516
Size of first eggs					
Maternal diets	0.45	0.22	2	9.9	0.006
Contemporary diets	0.40	0.20	2	8.8	0.006
Maternal diets × Contemporary diets	0.07	0.02	4	0.8	0.545
Age at first egg production					
Maternal diets	93.2	46.6	2	29.8	<0.001
Contemporary diets	282.2	141.1	2	90.1	<0.001
Maternal diets × Contemporary diets	30.6	7.7	4	4.9	0.001
Estimated somatic growth rate					
Maternal diets	5.2	2.6	2	67.0	<0.001
Contemporary diets	1.2	0.6	2	16.1	0.001
Maternal diets × Contemporary diets	0.1	0.04	4	0.9	0.163

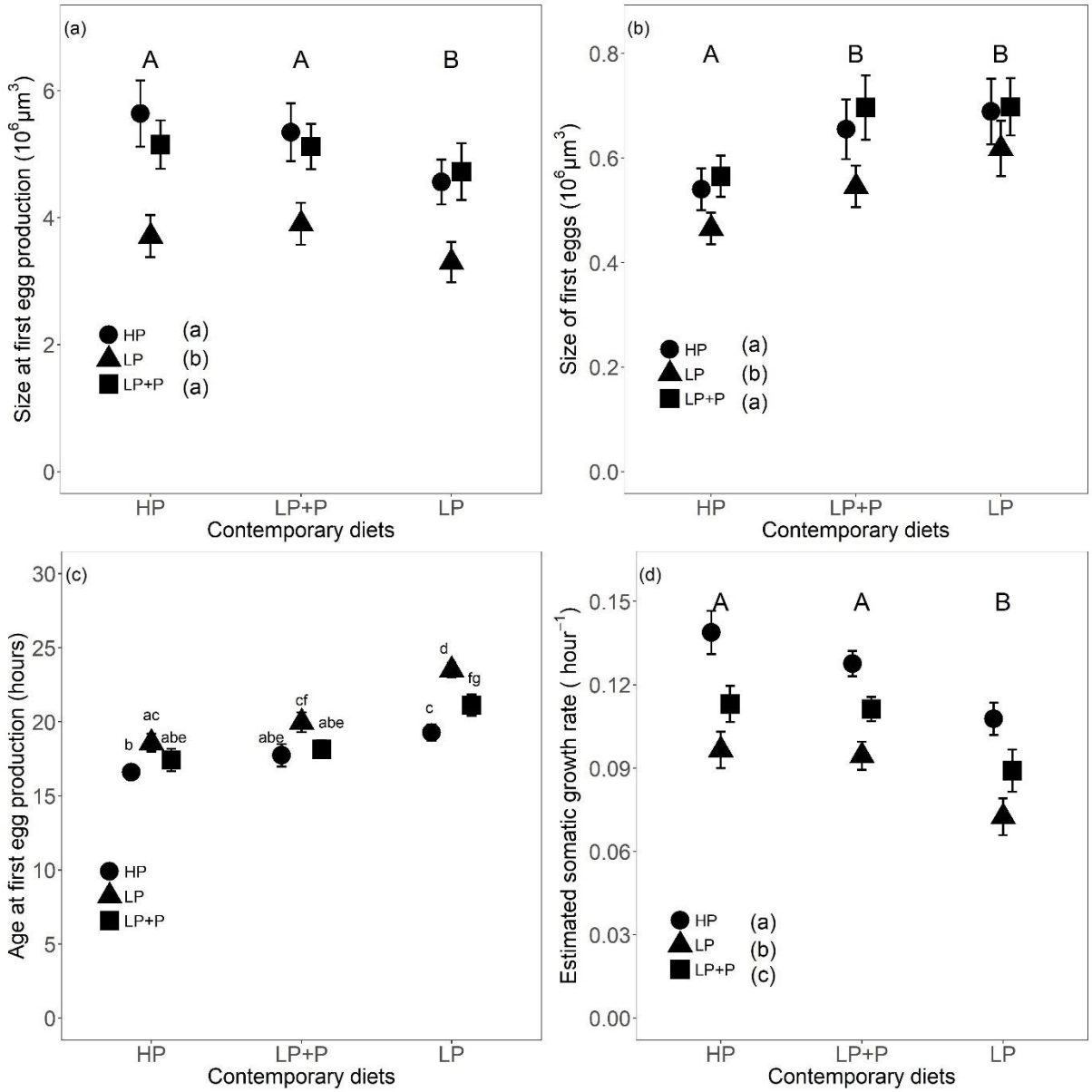


Figure 4.2 Response of rotifer life history traits in the focal generation to contemporary and maternal food quality treatments. a: Size at first egg production, b: Size of first eggs, c: Age at first egg production, d: Somatic growth rate. HP: P-rich diet, LP: P-poor diet, LP+P: LP diet enriched with inorganic P. The circles, triangles, and squares represent offspring for which the two preceding maternal generations were cultured in HP, LP, and LP+P food, respectively. Different uppercase letters indicate a significant difference among contemporary diet treatments according to Tukey post hoc comparisons. Different lowercase letters indicate significant differences among maternal diet treatments, except for age at first egg production (c) for which a significant two-way interaction was found and different lower case letters indicate differences among any multifactorial combinations. Symbols and error bars represent the mean ± 2 x standard error.

Neonate starvation resistance proved to be significantly affected by maternal diet. After being inoculated in a food free environment, neonates hatched from eggs produced by LP and LP+P mothers lived on average 63 and 59 hours, respectively. In contrast, neonates from HP mothers

only lived 51 hours (Figure 4.3, Tukey post hoc comparisons: HP vs. LP+P: p=0.05, HP vs. LP: p=0.002, LP vs. LP+P: p=0.51).

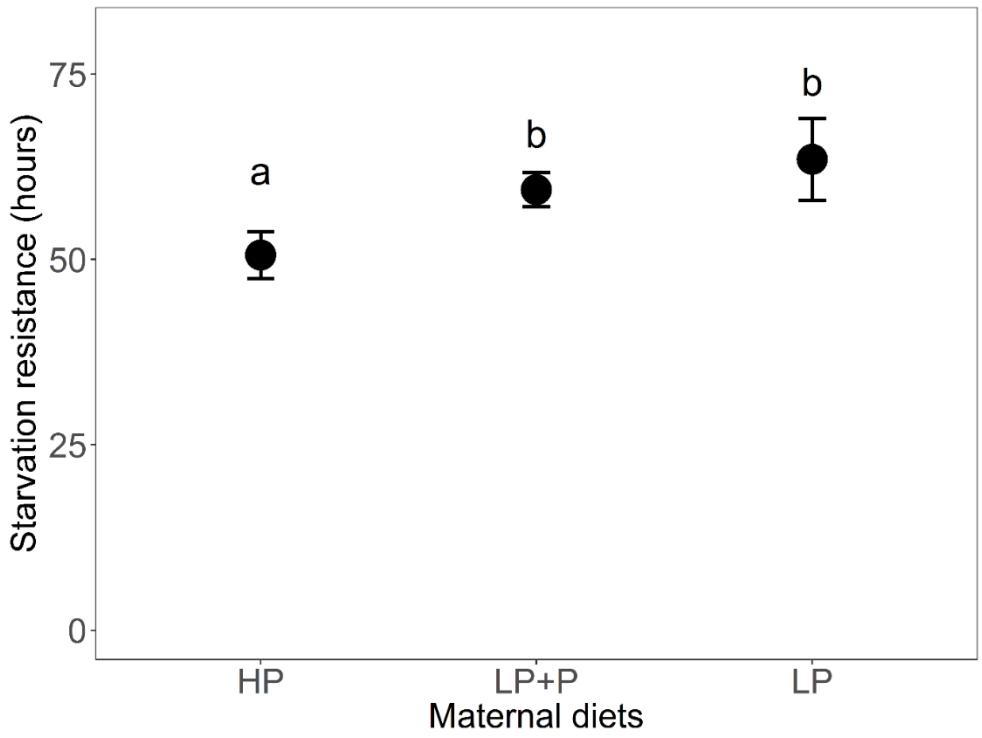


Figure 4.3 Starvation resistance of neonates in function of maternal diet. HP: P-rich maternal diet, LP: P-poor maternal diet, LP+P: LP maternal diet enriched with inorganic P. Different letters indicate significance differences according to Tukey post hoc comparison. Symbols and error bars represent the mean ± 2 × standard error.

Discussion

Our study demonstrates strong effects of maternal food quality on key life history traits of rotifer consumers. Consistent with our previous study (Zhou et al. 2018), rotifers that had been cultured in LP food across multiple generations produced larger eggs than those cultured in HP food. However, we found no evidence for an anticipatory maternal effect. In contrast to the idea that larger egg size should result in a higher offspring quality, offspring born to P-limited mothers had a reduced fitness (e.g. smaller size at first egg production, lower somatic growth rates, and a higher age at first reproduction) compared to offspring born to mothers reared with P-sufficient food. This pattern was found irrespective of the diet in which the focal generation was grown. More specifically, when fed LP food, animals with an ancestral history of P-limitation had no advantage to animals of which maternal generations had grown under P-sufficient conditions. The fact that LP raised mother animals produced larger eggs and neonates with higher starvation resistance than HP raised mother animals suggests that they increased

allocation of carbon under the form of energy rich biomolecules (e.g. lipids) to their offspring. Unless food scarcity typically follows periods of algal P limitation in the habitat where the population of the investigated genotype evolved, the increased C-allocation is more likely a coincidental positive side-effect (Urabe and Sterner 2001) rather than an anticipatory adaptive response.

The strong negative maternal effects of P-limited food likely result from the transmission of poor growing conditions to the offspring (i.e. transmissive maternal effects), possibly in combination with a selfish maternal strategy where the mother generation increases its own fitness at the expense of that of the next generation (i.e. selfish maternal effect, Cunningham and Russell 2000, Kudo 2006), e.g. by allocating less P or valuable biochemical resources or by shunting excess C towards the eggs. Somatic growth rates were more strongly determined by maternal than contemporary diets. Animals with a maternal history of P limitation underperformed strongly compared to those with an HP history. The magnitude in growth difference between both categories proved to be larger than those resulting from differences in contemporary food quality. These results show that animals have difficulties to recover from the negative effects of a P-deficient maternal diet even if their contemporary diet contains sufficient P. It is reasonable to question if such pattern can be well explained by transmissive effects alone given that it fits better to what would be expected from strong selfish maternal effects. Similar cases with relatively strong maternal effects have been reported for consumers exposed to food with imbalanced biochemical composition (Sperfeld and Wacker 2015), and toxic cyanobacteria (Beyer et al. 2017).

Only a few studies have provided evidence for negative transmissive maternal effects of stoichiometric mismatch. In *Daphnia*, P-limited mothers allocate less P to their eggs than P-sufficient mothers animals (Boersma and Kreutzer 2002, Frost et al. 2010), which seems to result in reduced offspring performance (Frost et al. 2010). Similarly, He et al. (2016) demonstrated that the copepod *Pseudodiaptomus annandalei* reduced its investment of N and the fatty acid DHA into its offspring when feeding on N limited food, which resulted in a longer naupliar development time and smaller body length at copepodite stage I compared to mothers fed with an N-rich diet. Interestingly, in contrast to our results, poor offspring performance due to nutrient limitation was systematically associated with reductions in egg size in these studies.

P-limitation of algal resources may affect the performance of consumers not only via direct effects of P-shortage but also through indirect effects via other food quality aspects (Rothhaupt

1995; Zhou et al. 2018). Unique to our study is that its design allows decomposing maternal effects of P-limitation in these two components. As such it has the potential to reveal hidden anticipatory maternal responses to indirect effects of P-limitation that are masked by non-adaptive transmitted effects of direct P-limitation or vice versa. If maternal generations allow their offspring to cope better with negative indirect effects of P-limitation, offspring with an LP+P ancestral feeding history should outperform offspring with an HP ancestral feeding history under LP+P feeding conditions. Alternatively, if maternal generations prepare their offspring only in coping with negative direct effects of P-limitation, offspring with an LP ancestral feeding history should outperform offspring with an LP+P ancestral feeding history under LP feeding conditions. However, our results provide no evidence for such anticipatory effects. Under LP+P feeding conditions, somatic growth rate was lower in animals with a LP+P than an HP ancestral feeding history while age at first reproduction was higher. Similarly, we found no evidence for a higher performance between animals with an LP compared to an LP+P ancestral feeding history under LP feeding conditions.

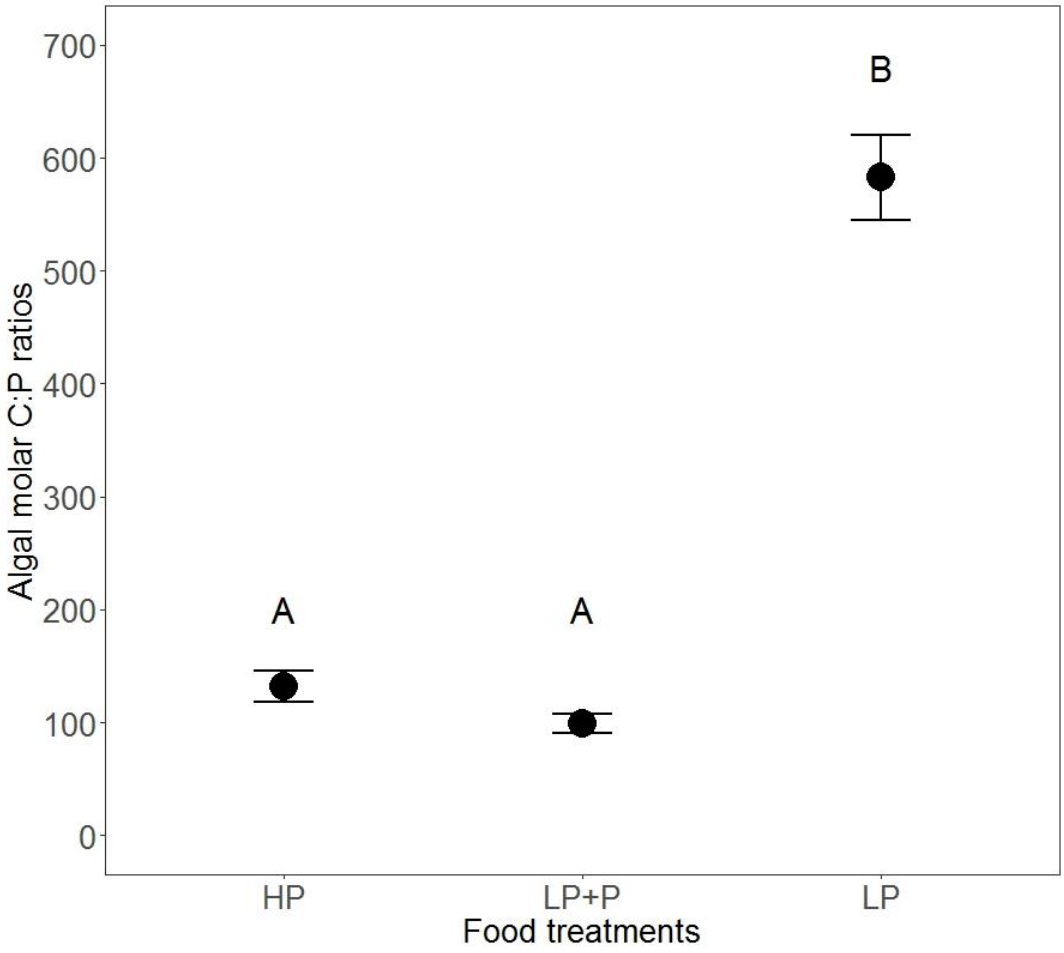
Our data also show that the relative strength of direct and indirect maternal effects of P limitation vary strongly among different life history traits. Maternal diets had large effects on the size at first egg production of the focal generations, which seemed entirely determined by the P content of the algae in the maternal diets and not by the culture history of these algae. In contrast, the indirect effect of P limitation was more pronounced in somatic growth rate and age at first egg production, for which values of offspring from LP+P mothers tended to be intermediate to those from HP and LP mothers. The most remarkable response, however, was found for the size of the eggs produced by the focal animals where effects of maternal diets were mainly generated by direct effects in contrast to the effects of contemporary diets which were largely determined by indirect mechanisms of food P limitation. This latter observation clearly indicates that the mechanisms through which maternal diets affect egg size of the focal animals differ from those of the contemporary diets. Generally, in a given environment, the initial size of animals is known to be positively correlated to the body size of their mothers (Boersma 1997, Neuheimer et al. 2015, Rollinson et al. 2016). Animals that were continuously cultured with HP or LP+P food across generations were consistently larger than animals cultured with LP food. In line with the idea of a positive body size-egg size relationship, we found a very similar pattern of size differences between eggs of different maternal feeding histories within each of the contemporary food quality treatments. However, this pattern was broken by the response pattern of egg size to contemporary food quality.

Our results were obtained from the study of one single clone. This limits our ability to generalize our findings. However, given that we worked with a haphazardly selected clone, it likely represents other genotypes of the same and possibly also other zooplankton species as well. In any case, our results can be considered as proof of concept for the idea that the direct and indirect consequences of P limitation may strongly impact zooplankton consumers through maternal effects. However, our results also indicate that the relative impact of these direct and indirect effects may largely be trait-dependent.

Conclusion

Our study demonstrates that phosphorus limitation of the maternal generation may strongly reduce the performance of zooplankton consumers through transmissive maternal effects, possibly in combination with selfish maternal effects. Depending on the traits under consideration, such maternal effects were not limited to the direct effects of P-limitation but were also caused by indirect effects of such limitation. We found no evidence for anticipatory maternal effects. Although P limitation resulted in the production of larger eggs and a higher starvation resistance, it did not contribute to a higher ability to cope with P-limitation. The relative strength of the maternal effects is remarkable, given that the growth rate of the focal generation was found to be more determined by the ancestral than by the contemporary diet. First, it demonstrates the need for taking into account maternal effects in the design of experimental studies by acclimatizing animals to experimental food quality treatments during at least one, but preferentially two or more maternal generations. Second, stoichiometric studies in which experimental manipulations of stoichiometric mismatch are created by artificially enriching P-limited algae to various degrees, may result in an underappreciation of the effects of such mismatch because they exclude indirect effects of P-limitation (see also Zhou et al. 2018). Third, strong maternal effects may also be important for the interpretation of consumer-producer dynamics under natural conditions, as they may decouple effects of P-limitation on consumer growth (Frost et al. 2010) and associated ecosystem functions, such as grazing and nutrient cycling (Suzuki-Ohno et al. 2010, Urabe and Yamaguchi 2018; Frost et al. 2004, He and Wang 2008). The latter is expected to be mainly relevant in aquatic systems where the time scale of recurrent changes in stoichiometric seston quality approximates the generation time of consumers.

Appendix S 4.1



Appendix S4 Figure 4.1. The molar C:P ratios of algae from different food treatments. No significant difference was found for molar C:P between HP and LP+P food, whereas both of which were significantly lower than that in LP food (One-way ANVOA, HP-LP: $P<0.001$, LP+P-LP: $P<0.001$). Symbols and error bars represent the mean ± 2 standard error, respectively.

Chapter 5

Microevolutionary adaptation to stoichiometric mismatch reduces cross tolerance of consumers to salinization

Libin Zhou, Shuaiying Zhao, Kimberely D. Lemmen, and Steven A. J. Declerck

To be sumbitted

Abstract

Interactive effects of multiple stressors on organisms are becoming the core to study how environmental changes affect consumers in nature. Recently, increasing evidence has also shown that evolution is able to happen at similar time scales as ecological interactions, and therefore should also be explicitly incorporated in ecological studies. Till now, how the evolutionary history and various environmental stressors can interactively affect organisms is still unclear. Here, we studied how the selection history may interactively affect a herbivourous rotifer, *Brachionus calyciflorus*, with two different common stressors in nature, i.e. P limitation and increased salinity. We took advantage of two categories of rotifer populations, i.e. P limitation adapted and saline adapted populations, which were obtained from two previous lab evolution experiments. We subjected these rotifer populations, as well as the ancestral (reference) populations to four combined stressor treatments, i.e. high P food with low salinity (HP-LS), high P food with high salinity (HP-HS), low P food with low salinity (LP-LS), and low P food with high salinity (LP-HS), respectively, and measured their population growth rates. Our results showed the under LP food, the increased salinity had no impact on reference populations. Interestingly, adaptation to P limitation reduced the ability of animals to cope with increased salinity, but not vice versa. The adaptive benefits to either P limitation were consistent across treatments, while the magnitude of advantages varied depending on the presence of an additional stressor. Our study suggests that evolutionary history may add another dimension of factor that interactively affect organism with various environmental stressors.

Introduction

Organisms in nature are simultaneously facing with multiple environmental threats exerted by natural processes and anthropogenic activities (). The interactions among different stressors on organisms are therefore becoming the key to better understand the consequence of various environmental changes (Sala et al. 2000; Gunderson et al. 2016; Jackson et al. 2016). However, a general prediction of the interactive effects of multiple stressors on organisms is difficult to obtain (Gunderson et al. 2016), because the consequence of joint effects may vary with the type of stressors (Karim and Mimura 2008; Berry et al. 2011). Generally, the effects of different stressors on organisms may highly depend on whether the effect of one single stressor interact with that of another stressor (Schäfer and Piggott 2018; Velasco et al. 2018). When different stressors independently affect organisms with no interaction, the consequence of multiple stressors on organisms equals to the sum of effects from each single stressor (additive; Crain et al. 2008; Jackson et al. 2016). Alternatively, the effects of one stressor on organisms may be affected by the presence of another (Sinclair et al. 2013; Uyhelji et al. 2016). The outcomes of such interactions can be either synergistic, i.e. greater than the sum of individual effects (Crain et al. 2008; Jackson et al. 2016), or antagonistic, i.e. less than the sum of individual effects (Jackson et al. 2016; Velasco et al. 2018). Particularly, when organisms share a common mechanism through which they are able to cope with different stressors, the exposure to one stressor can then protect them against another, i.e. cross tolerance (Todgham et al. 2005; Sinclair et al. 2013; Pallarés et al. 2017; Hintz et al. 2018; Velasco et al. 2018).

In contrast to the well-studied physiological responses, the knowledge of how adaptation to one stressor can mediate the responses of animals to a different stressor is poorly studied. One scenario is that the adaptation to one specific stressor may induce reciprocal mechanisms of organisms, e.g. basal expression of genes that generally protect themselves (Dhar et al. 2012), which enable them to better cope with another stressor, i.e. evolutionary cross protection. A nice example of evolutionary cross protection comes from the study of Hintz et al. (2018), which demonstrated that adaptation to one specific type of salt, i.e. NaCl, is able to increase *Daphnia* tolerance to other type of salts, i.e. $CaCl_2$ and $MgCl_2$. Other evidence supporting the evolutionary cross protection arises in the studies of micro-organisms, reporting that adaptation to salts enables organisms to better cope with oxidative stress (e.g. Bergholz et al. 2012; Dhar et al. 2012). However, evolutionary cross protection is by no means universal among stressors. It could also be possible that adaptation to one environmental stressor may come at the cost of fitness reduction when confront a different stressor (Hereford 2009; Jansen et al. 2011; Kelly

et al. 2016). This suggests a possible fitness trade-off of organisms being exposed to different environments.

Even less is known about how evolutionary history would mediate the responses of organisms to multiple stressors. An evolutionary cross protection enables organism to benefit from the adaptation to one stressor when simultaneously facing multiple stressors (e.g. Dhar et al. 2012). However, if adaptation to one stressor is not able to protect organisms against the other, the outcomes of joint effects may depend on the degrees of both adaptive benefits and the fitness trade-off under different stressors. For example, organisms may still benefit from adaptation to one specific stressor if this adaptive process does not affect their ability to cope with others. However, when the adaptation process strongly hampers organismal ability to cope with another stressor, the adaptive advantages may start diminishing. In this scenario, adaptation to one stressor may be even detrimental to organisms under simultaneous exposure to multiple stressors.

Currently, nutrient limitation (Stockner et al. 2000; Elser et al. 2009) and increased salinization (Kaushal et al. 2005; Canedo-Arguelles et al. 2013; Kefford et al. 2016) in freshwater systems are attracting increasing attentions of scientists. Previous studies have repeatedly showed that both phosphorus (P) limitation (Elser et al. 2001; Hessen et al. 2013; Zhou et al. 2018) and increased salinization (Arnér and Koivisto 1993; Lee et al. 2017) strongly affect the growth and reproduction of freshwater organisms and subsequently influence ecological interactions. The negative effects of P limitation are mainly attributed to deficient P (Rothhaupt 1995; Urabe at al. 1997) and excess C in food (Hessen et al. 2008). Whereas animals suffering from increased salinity are mainly due to the ion toxicity and increased ambient osmotic stress (Munns and Tester 2008; Latta et al. 2012). Given that the physiological regulation in response to increased ambient salinity is highly energy required (Pennak 1985; Kefford et al. 2016), we may expect that when expose animals to dual stressors, the excess C in P limited food provides extra energy source for animals to better cope with increased salinity to some degree. Currently, the joint effects of P limitation and increased salinity are nevertheless poorly understood (Velasco et al. 2018).

Recently, few studies reported that fast-grow herbivores are able to rapidly adapt to either saline (Coldsnow et al. 2016; Zhao et al. in prep.) or P limited environments (Declerck et al. 2015). Animals that have adapted in a stressed environment were shown to have higher fitness than those have not been selected under the same stressed condition. Adaptation to salinity likely

involves complex mechanisms, mainly osmoregulation related components, such as membrane structure and regulation, and ATPase activity (Kefford et al. 2016; Coldsnow et al. 2017). The mechanisms that consumers adapt to P limitation were still rarely studied, probably involved the increase in P use (Frisch et al. 2014) and C elimination efficiencies (Hessen et al. 2008), or physiological and behavior adjustments (Suzuki-Ohno *et al.* 2012). However, whether adaptation to one of these two stressors would affect the ability of animals to cope with the other, and whether the interactive effects of these two stressors on consumers would be affected by their evolutionary history are currently unclear.

In the present study, by subjecting a cyclically parthenogenetic rotifer species, *Brachionus calyciflorus*, we aimed to 1) assess how adaptation to one stressor, i.e. P limitation, would affect the ability of rotifer consumers to cope with another stressor, i.e. increased salinity, and vice versa; 2) how the adaptive benefits would be affected by the presence of dual environmental stressors. In the present study, we took the advantage of two evolution experiments that performed by Lemmen et al. (in prep.) and Zhao et al. (in prep,), from which they obtained the LP adapted and saline adapted rotifer populations, respectively. Our former results showed that both LP food adapted (Lemmen et al. in prep.) and saline adapted (Zhao et al. in prep.) populations had higher growth performance than the naïve populations under the environment where they were selected from. Here, we subjected those two categories of adapted rotifer populations, as well as the naïve populations (reference populations), to a following-up common garden experiment, and estimated their population growth rates in response to LP food, saline food, and LP combined with saline food, respectively. Given that P limited food may provide extra energy source for rotifers to cope with increased salinity in the environment, we hypothesized that the interactive effects of these two stressors are antagonistic. Given that adaptation to P limitation may involve altered efficiencies in excess C elimination, and this may decrease the C allocation of animals to regulate osmotic homeostasis under high saline conditions, we further hypothesized that adaptation to P limitation may decrease the ability of animals to cope with increased salinity.

Methods

Rotifer and Algae cultures

Green algae *Chlamydomonas reinhardtii* was taken as food in our study. We cultured *C. reinhardtii* in 2L-chemostats using modified WC medium (Guillard and Lorenzen, 1972) under

23±1 ˚C at a dilution rate of 0.33 day^{-1}. Six replicate chemostats with three high phosphorus cultured algae ('HP', algal C:P =120) and three with low phosphorus cultured algae ('LP', algal C:P=600) were cultured under the P-supply and light regimes of 65 µmol L^{-1} (K_2HPO_4) under 40 µmol quanta m^{-2} s^{-1} continuous light and 15 µmol L^{-1} (K_2HPO_4) under 120 µmol quanta m^{-2} s^{-1} continuous light, respectively. All chemostats were in steady states at least three months before the experiment. HP and LP algal food were directly harvested from the chemostats with a following dilution process using nutrient-free WC medium. Algae density from each chemostat was determined using Coulter counter (Beckman Coulter Multisizer 3) and diluted to a target concentrations 1000 µmol C L^{-1}. Saline food was created by adding fixed amount of NaCl solutions to each food treatment.

All the clones of rotifer *B. calyciflorus* were obtained from the resting egg banks in our lab. These clones were used as the original clones for the following evolution experiments. Stock cultures were maintained at room temperature under continuous light conditions and fed with MP food at daily basis. Every three days the rotifers were transferred to new containers filled with fresh food medium.

Saline evolution experiment

This lab evolution experiment was conducted to obtain the saline adapted clones by exposing 44 randomly selected original rotifer clones to either saline treatment at ad libitum food concentrations (0.5×10^6 cells/ml). At the start of the experiment, we set a relatively low salinity (1 g/L) to avoid a high salinity shock to original clones. We then gradually increased salinity throughout the whole experiment. Three replicate populations were created, with each population was initiated by randomly transferring 20 individuals from each of the 44 clones to 250 ml flasks that filled with HP food suspension at the concentration of 1000 µmol C L^{-1}. These populations were maintained in an incubator at 23±1 ˚C under continuous light. This culturing methods allowed animals to grow at an exponential rate. Once the population density was getting high, we then expanded each population by transferring the animals to 500 ml flasks, and these animals were continuously cultured in flasks to produce resting eggs.

During the experimental period, swimming rotifers of each population were strained with a 15 µm sieve and immediately transferred to new flask every two days. The sediment left from each flask was collected and examined for resting eggs. These resting eggs were transferred to corresponding new flasks and all the animals in new flasks were continuously cultured when

the number was less than 50. Once the number of resting was above 50, these resting eggs were collected and independently hatched, and meanwhile the cultures of the population was terminated. For these newly hatched genotypes, we individually transferred them to 24 well plates with 1 ml food suspension, and set up 50 new clones for each population.

New populations for each population was restarted by again transferring 20 individuals from each of the 50 clones to new flasks filled with algal food suspension. These new populations were maintained at the same manner as mentioned above to produce resting eggs. We repeated this process 4 times during our evolution experiment, and every time we increased the salinity at an interval of 0.5 g/L for the food suspension. Therefore, this experiment was ended by collecting resting eggs under the salinity of 3 g/L for saline treatment. The saline adapted clones were set up by hatching the resting eggs collected from the last round of the experiment and individually maintained in 24 well plates.

Phosphorus limitation evolution experiment

This evolution experiment was conducted to obtain the low P adapted populations. Thirty of the 44 reference clones that used in the first experiment were randomly selected. We created 7 populations for this experiment. To initiate each population, two individuals standardized as with one parthenogenetic egg from each clone (60 individual in total) were randomly selected and transferred to 100 mL sterile flask and provided 48mL of the designated LP food suspension at the concentration of 1550 μmol L^{-1} C. Throughout the experiment these populations were cultured in the dark at a constant temperature of 24±1°C.

During the course of 36 days, we haphazardly selected 60 individuals from each population and transferred them to new culturing flask with new food suspension to restart the population. Genotypes that had the highest growth were most likely to be transferred, and this resulted in a selection for fast growth. All viable resting eggs were recorded and also transferred, this allowed newly produced genotypes to be established in the populations. Additionally, this method also ensured that the daily refreshed food resources were always stay at ad libitum, preventing exploitative competition between genotypes.

Common garden experiment

This common garden experiment was conducted to test: 1) how adaptation to salinity can affect the ability of rotifer B. calyciflorus to cope with P limitation, 2) 1) how adaptation to salinity

can affect the ability of rotifer B. calyciflorus to cope with P limitation, and 3) how adaptation to either salinity or P limitation determines the performance when the two stressors occur simultaneously. We assessed the population growth rate response of the salt adapted populations from Zhao (in prep) and the LP adapted populations from Lemmen (in prep) to a multifactorial combination of salt and LP conditions. In addition, to be able to directly compare the performance of populations with different selection histories, three randomly selected reference populations from Zhao and Lemmen experiments were subjected to the same multifactorial combinations. In each multifactorial combination, each population was replicated three times and each of those replicates were fed with algae from one of the replicate chemostats. In summary, the experimental design consisted of a total of 108 unites, with 2 food quality treatments (HP and LP food) × 2 salinity treatments (high saline, HS, and low-saline, LS treatments) × 3 selection histories (reference, saline adapted, and LP adapted populations) × 3 populations × 3 replicates. Initially, we created a salt concentration of 3 g/L for HS treatments, which was the same in the condition where the saline adapted populations selected from. However, the combined effects of a 3g/L salts and LP food sharply decreased the growth and quickly lead to the extinction of populations with no saline adapted background. To guarantee the survival of animals, we therefore decreased the salinity level of LP-HS treatment to a NaCl concentration of 1.5 g/L.

The common garden experiment was performed in 6 well plates and the growth rates of populations in each unit were examined at a daily basis (see also Zhou et al. 2018). We initiate this experiment by transferring 10 randomly selected individuals from the stock cultures to the plates, with each well filled with 8 ml algal food at the concentration of 1000 μmol C L^{-1}. The plates were incubated at 23±1 ˚C under continuous darkness. Every 24 hours, wells were checked under a stereomicroscope, the number of females were counted and 10 individuals from each unit were haphazardly selected and transferred to a new well filled with fresh food suspension. We repeated this process until population sizes stabilized and then continued for an additional three days.

Algal stoichiometry

Molar C:P ratios of phytoplankton were measured during the experiment. Algal samples were obtained by filtering culture medium on glass filters (GF/F) following by drying at 60 ℃ for at least 24 hours. Algal C and N contents were determined using a FLASH 2000 organic analyzer

(Interscience B.V., Breda, The Netherlands), while P content was determined by a QuAAtro segmented flow auto-analyzer (Beun de Ronde, Abcoude, The Netherlands).

Data analysis

Exponential population growth rate was repeatedly calculated for each experimental unit as $(\ln N_t - \ln N_0)/t$, where N_0 and N_t represent the population size at the start and end of each 24-hour period, and where t is the time interval between the two counts. In the calculation, only the last three days of the experiment were considered. The adaptive benefits to one environmental stressor were estimated by calculating the growth differences between adapted and the reference populations (ΔR), i.e. $\Delta R = R_{selected} - R_{reference}$. Where $R_{selected}$ represents the growth rate of adapted population under a given environment and $R_{reference}$ is the average growth of original clones in the same condition.

The salinity difference between HP-HS and LP-HS treatments precluded consideration of a three-way interaction. Instead, we evaluated within each of the LP and HP food levels how population selection history interacts with salinity. . To assess how the growth rates of reference populations was affected by increased salinity, student's t test was applied in HP and LP treatment, respectively. To assess how adaptation to one specific stressor may affect the ability of rotifer consumers to cope with another, student's t test was used to detect the growth difference between the adapted populations and the reference populations under a given environment. To assess how adaptive benefits may be affected by contemporary environment, We applied two-way ANOVA separately for HP and LP food treatments to detect how ΔR may change in response to different selection histories and food salinity. All ANOVAs were further studied in details to assess the significances of differences among factors with Tukey post hoc comparisons. All statistical analyses were performed in R software environment 3.4.1 (R Core Team 2017).

Results

Increased salinity strongly decreased the growth rates of original populations in HP food (Student's t test, $p<0.001$) whereas it did not change the growth of original populations in LP treatments (Figure 5.1).

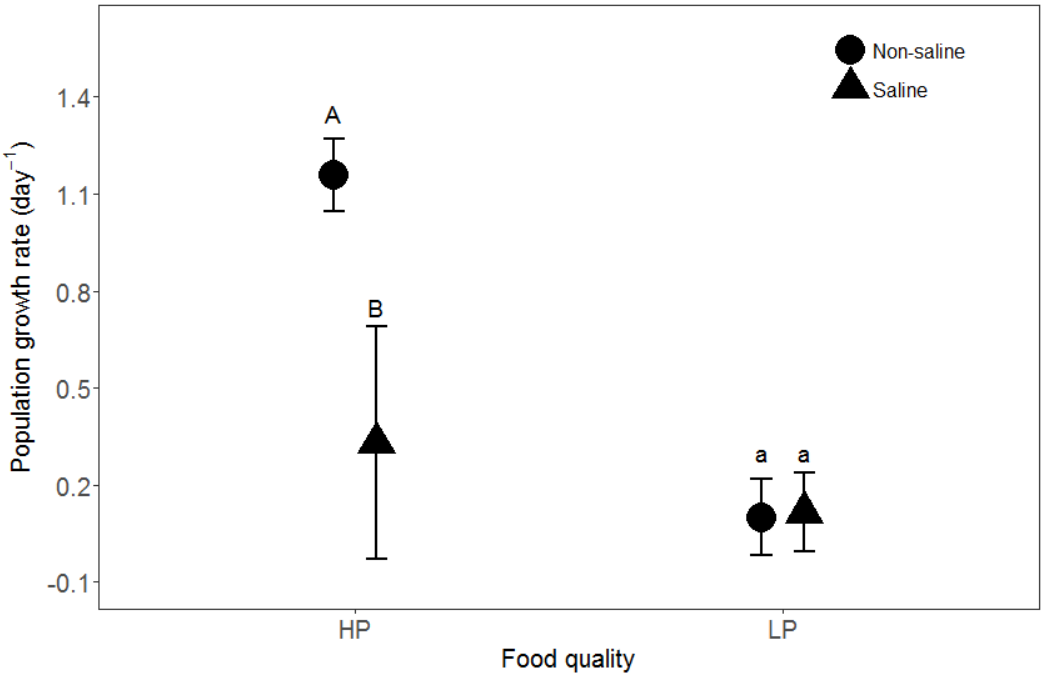


Figure 5.1 Population growth rate of reference populations. The circles and triangles represent the population growth rates s under LS and HS conditions, respectively. LS: low salinity, HS: high salinity; HP: high P food; LP: low P food. Symbols and error bars represent mean values ± 2 times the standard error. Note that salinity treatments in the LP and HP treatments differed (LP: 1.5 mg/L; HP: 3mg/L) and can therefore not directly be compared (see Methods).

When fed HP food, no significant difference of growth rates was found among the populations with different selection histories in LS conditions (Figure 5.2A). Whereas in HP-HS treatment, saline adapted populations had higher growth rates than the reference populations (Student's t test: $p<0.001$) whereas the LP adapted populations had lower than the reference populations (Figure 5.2A, Student's t test: $p=0.002$). ΔR was mainly determined by rotifer selection history (two-way ANOVA; $p=0.021$) and the interaction between selection history and salinity (two-way ANOVA; $p=0.002$). The addition of salt strongly increased the adaptive benefits of saline adapted populations ($\Delta R_{S\text{-}O}$; Tukey post hoc: $p=0.015$). Although statistical results showed that increased salinity did not significantly decrease adaptive benefits of LP adapted populations ($\Delta R_{LP\text{-}O}$), the average value of $\Delta R_{LP\text{-}O}$ in HS condition were 3.9 times lower than that in LS treatment (Figure 5.2A).

Under LP-LS treatment, LP adapted populations had the highest growth whereas saline adapted animals had similar growth to reference populations (Figure 5.2B, Student's t test: LP adapted vs. reference $p<0.001$). However, in LP-HS treatment, both categories of adapted animals had

significantly higher growth rates than the reference populations (Figure 5.2B, Student's t test: saline adapted vs. reference $p<0.001$, LP adapted vs. reference $p=0.04$). In LP food, ΔR were mainly attributed to the interactions between food salinity and selection history (two way ANOVA; $p<0.001$). Addition of salt significantly decreased ΔR_{LP-O} (post hoc test; $p=0.041$) but strongly increased ΔRS-O (post hoc test; $p=0.012$).

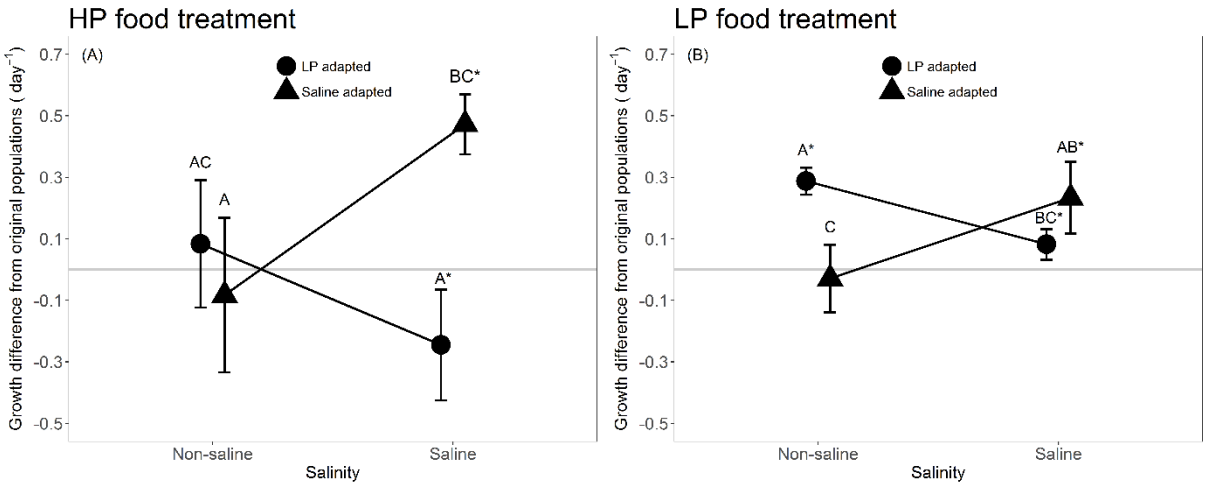


Figure 5.2 Growth rate differences with non-evolved reference populations (ΔR) for populations with different selection histories in response to the salinity treatments under both HP (A) and LP food conditions (B). The circles and triangles represent ΔR-values for LP and HS adapted populations, respectively. The horizontal grey line represents absence of population growth rate differences between adapted and reference populations (ΔR=0). Stars (*) represent significance growth rate differences between adapted and references populations. Differences in uppercase letters indicate significant differences between saline and non-saline conditions both in HP and LP food accordingly Tukey post hoc comparisons. Symbols and error bars represent mean values ± 2 times the standard error.

Discussion

In contrast to HP food conditions, the combination of a high salinity (3g NaCl/L) with LP food resulted in the extirpation of the experimental populations. To allow continuation of these populations, we had to reduce the salinity level in this specific treatment (to 1.5g/L), which precluded us from studying the interactive effects of food quality and salinity on rotifer population growth performance. In line with observations in other studies (Coldsnow et al. 2017; Zhao et al. in prep.), a salinity of 3g/L strongly decreased growth of genotypes of the ancestral, non-adapted population under HP conditions. In contrast, the addition of a lower concentration of salts (1.5 g/L) showed no negative effects under LP conditions. The lack of a negative response to this level of salinity may simply reflect the fact that such level of salinity was too low to cause any negative impacts on the populations. Alternatively, these results may provide

support for the idea that high food C:P ratios associated with P limited algal growth may mitigate the negative effects of moderate levels of salinity. Indeed, the physiological processes that enable freshwater organisms to maintain osmotic homeostasis and neutralize the toxic effects of ions in a salinized environment are highly energy demanding (Kelly et al. 2015; Hintz et al. 2018) and may be more easily covered by the high energy content of C-rich P-limited food (Hessen et al. 2008). There are three lines of strong evidence rejecting the first explanation. First, for four *B. calyciflorus* genotypes subjected to a salinity of 2g/L, Zhao et al (in prep.) found 21-45% reductions in population growth rate compared to populations grown under non-saline conditions. Second, at a salinity of 1.5 g/L under LP-conditions, LP-adapted populations showed a significant growth reduction compared to the respective non-saline treatment. Third, in the same treatment, the saline-adapted population performed better than clones of the ancestral populations, suggesting an adaptive benefit of adaptation to salinity although the salt concentration was low. The fact that no such differences between these populations and their ancestral clones were found in the non-saline treatments, indicates that this level of salinity must have effectively impacted the performance of populations. Taking all this evidence together, we conclude that the lack of effect of a moderate salinity in the LP-food treatment suggests that antagonistic effects between a diet of P limited algal food and increased salinity enable rotifer consumers to cope with the challenges that would be imposed by each of these stressors in isolation, i.e. excess C in the case of LP food and increased energy expenditure in the case of increased salinity.

The fact that we had to work at a lowered salinity level in the LP-HS treatment hampers the study of how microevolutionary adaptation may mediate the interactive effects of salinity and P-limited food. Our data nevertheless allow us to evaluate how adaptation to LP food mediates the ability to cope with increased salinity under the two different food quality contexts separately. We found that adaptation to resource P limitation comes at the cost of the ability to cope with increased salinity. In P sufficient food, high salinity resulted in a stronger decrease of population growth rates for LP adapted populations than for their ancestral genotypes. Currently, the mechanisms through which zooplankton herbivores adapt to P limited food is still poorly understood (Declerck et al. 2015). Irrespective of possible changes in P use efficiency (Frisch et al. 2014), adaptation to P limited food may also involve altered feeding rates (Suzuki-Ohno et al. 2012; Urabe et al, 2018), or the reduced cost for the elimination of excess C (Darchanmbeau et al. 2003; Hessen et al. 2008). Although those mechanisms seem to enable consumers to better cope with excess C in LP limited food, they may constitutively

reduce the energy allocation in maintaining osmotic homeostasis. When confront high salinity, such changes in energy budget may further inhibit the ability of adapted animals to cope with increased ambient osmotic stress.

However, saline adapted populations had similar growth rates to that of reference populations in LP-LS conditions, indicating that adaptation to salinity did not affect the ability of consumers to cope with food P limitation. Adaptation to high salinity may involves increased ATPase activity, which allows animals to better regulate osmotic stress (Coldsnow et al. 2017; Hintz et al. 2018). Given that P limited food also involves relatively excess C content, such mechanisms nevertheless are independent of that for animals to cope with excess C. Similar independent adaptive response was also reported by Dhar et al. (2012), who found that adaptation to salinity also did not enable yeast to better cope with oxidative stress. However, instead of inhibiting the ability of organisms to cope with another stressors, some previous studies nevertheless reported that adaptation to NaCl enables organisms to better cope with other stressors, i.e. evolutionary cross protection. For example, recent studies showed that adaptation to NaCl is able to increase the tolerance of *Daphnia* to other type of salts, such as $CaCl_2$ (Hintz et al. 2018; Zhao et al. in prep.) and $MgCl_2$ (Hintz et al. 2018). More evidence supporting evolutionary cross protection comes from studies for microbiome. Bergholz et al. (2012) reported that the adaptation of *Listeria monocytogene* to salt stress led to increased tolerance to hydrogen peroxide, and this is potentially attributed to the increased transcript levels of catalase.

Benefits of being adapted to a specific stressor were found to be consistent across treatment levels of the other stressor, although the magnitude of the advantage varied depending on the presence of a second stressor. When fed LP food, LP adapted populations had consistently higher population growth rates than ancestral populations, but this advantage was lower under saline then under non-saline conditions. Similarly, under saline conditions, salinity adapted populations had an advantage compared to their ancestral genotypes, and this was true under both LP and HP conditions. In the latter case, however, we could not evaluate how the relative benefit depended on the second stressor because the lower salt concentration in the LP food treatment may have reduced the benefit of being salt adapted. In addition, LP food itself may also have interacted antagonistically with the salt in reducing the relative benefit of being salt-adapted.

Adaptation to either salinity or P limitation did not seem to involve a constitutive cost for populations. When cultured under benign condition, i.e. HP-LS treatment, no significant growth difference was found between the adapted and reference populations. Similarly, Latta et al. (2012) also showed no fitness cost of for salt-adapted *Daphnia* genotypes when comparing to a salt-nontolerant genotype under a low saline condition. These results nevertheless differed from the findings of Hintz et al. (2018), who found evolved tolerance of *Daphnia* comes at the cost of slower population growth in the absence of salt. Animals that adapted to other stressors, such as warming (Kelly et al. 2015), were also reported to show an evolutionary cost. Possibly, the induction of evolutionary costs may highly depend on the contemporary environments, and we may still observe a fitness trade-off of the adapted populations along other environmental axes.

It should be noted that the evolved populations used in our experiment originated from two different experimental evolution studies that took place in different moments and their culture conditions during selection also differed. The saline evolution experiment was performed by circularly collecting resting eggs and restarting populations, whereas the LP selection was maintained under constant conditions and the genotypes with the fastest growth were selected. One may think that the adaptive benefits of adapted populations showed in our study may be an artifact resulting from the specific set-ups for the two evolution experiments. However, this possibility could be excluded based on the observation that in HP-LS condition, neither of the adapted populations showed significant benefits comparing to the reference populations. Clear adaptive benefits were observed when the contemporary environment involves the stressor where the adapted populations were selected from. These results suggested that the growth differences between both adapted populations and the reference populations were most likely due to the adaptation to the corresponding environmental stressor.

The present study provides a pioneer research to investigate how the evolutionary response of populations to selection by one type of stressor may affect their ability to cope with another stressor. It nevertheless leaves the underlying mechanisms open. For example, knowledge of the physiological mechanisms in planktonic consumers that underlie the maintenance of elemental homeostasis in the face of stoichiometric imbalance caused by P limitation is still poorly understood (Declerck et al. 2015; Lemmen et al. in prep; but see Jeyasingh et al. 2009; Sherman et al. 2017), which further constrains the improvement of our knowledge about the pathways through which adapted animals modulate their ability to deal with another simultaneously occurring stressors. It is therefore essential to explore the molecular

mechanisms that show how cross tolerance is realized and account for the trade-off of animal abilities to cope with different stressors. In addition, we showed that adaption to LP food inhibits the ability of rotifer consumers to cope with high salinity, but not vice versa. These results suggest that whether the adaptation to one specific stressor can affect the ability of organisms to cope with another stressor may also highly depend on the type of stressors under consideration. This also elicits the necessity to investigate how evolution can mediate the performance of organisms in the face of other combinations of stressors in future studies, such as combinations among varied salinity, temperature, and food quantity.

Natural environments are variable in time and space. The interactions of multiple stressors on organisms are able to strongly affect the community composition in natural habitats by altering competition among different species (Knillmann et al. 2013), and therefore affect ecosystem functions (Rogell et al. 2009; Dinh Van et al. 2013). Given that the ecological and evolution processes are able to happen at the same time scale (Hendry 2016; Govaert et al. 2019), we suggest that rapid adaptation should be incorporated more explicitly in studies that address the implications of interactive effects among multiple environmental stressors in natural systems. In addition, the decreased adaptive benefits of LP adapted populations from LP-LS to LP-HS treatments in our study may also have important implications for study that explore the feedbacks between ecology and evolution, i.e. eco-evolutionary dynamics. It has been shown that adaption of herbivorous consumers to P limited food was able to affect their food biomass through alterations in population structure and grazing pressure (Declerck et al. 2015). However, our results indicate that the consequences of evolutionary feedbacks can vary with the type of stressors in the contemporary environment, at least under the presence of increased salinity.

Chapter 6

General discussion

Anthropogenic activities and climate change are increasingly introducing multiple threats to natural ecosystems worldwide (Ripple et al. 2017). Major alterations in the biogeochemical cycles of essential elements are increasingly receiving attention of ecologists. Depending on the situation, due to the redistribution of these elements ecosystems may be become polluted or limited by nutrients (Elser and Bennett 2011). This may have generated far-reaching consequences for biota. Ecological stoichiometry provides a powerful tool to investigate the ecological causes and consequences of altered nutrient supply ratios (Sterner and Elser 2002). Many studies in the framework of ecological stoichiometry have been devoted to investigate the impacts of P limitation on phytoplankton and zooplankton in freshwater systems (Elser et al. 2001; Hessen et al. 2013). However, many knowledge gaps still exist.

The aim of this thesis was to obtain a better understanding of the impacts of stoichiometric imbalanced food on zooplankton consumers. We investigated the impacts of C:P imbalanced food on the performance of rotifer consumers while considering effects at different time scales. To address these questions, I combined multiple experimental approaches, such as population-level culture experiments, life history experiments, and grazing and nutrient loss rate experiments. In this chapter, I briefly discuss the major findings and discuss these results in a broader context in the framework of ecological stoichiometry and rapid evolution. In addition, I will provide some suggestions for future research.

Effects of stoichiometric imbalance on consumers at contemporary time scales

When fed with P limited food, the direct effects of stoichiometric mismatch, i.e. lowered P availability (Urabe and Sterner 1997; Elser et al. 2001) and excess C (Hessen et al. 2008) cannot fully explain the negative effects of P limited food (Rothhaupt 1995). In **chapter 2**, we confirmed the strong impacts of indirect non-stoichiometric effects of P limitation on rotifer consumers, and showed that the relative importance of direct and indirect effects varies with different life history traits. Those indirect effects may result from multiple aspects. Firstly, algae with a growth history under P limited conditions are shown to be associated with changes in biochemical composition, e.g. reduction in polyunsaturated fatty acids (PUFA; Spikerman and Wacker 2011; Challagulla et al. 2015), which has also been proved to be an important determinant for the quality of algae as food to consumers (Brett et al. 1997; Anderson and Pond 2000; Park et al. 2002; Acharya et al. 2005). Possibly, the variable strength of the direct and indirect effects on different traits may result from the fact that organisms have distinct

requirements for P (Urabe and Sterner, 2001; Villar-Argaiz et al., 2002; Færøvig and Hessen 2003) and essential biochemical components (Martin-Creuzburg and von Elert 2004; Boëchat and Adrian 2006; Wacker and Martin-Creuzburg 2007) during different life stages. For example, it has been showed that P (Elser et al. 2003) and cholesterol (Wacker and Martin-Creuzburg 2007) are important for somatic growth, whereas PUFAs are primarily needed for reproduction (Wacker and Martin-Creuzburg 2007). Alternatively, indirect effects may also result from morphological changes of algal cells under P limited conditions (van Donk and Hessen 1993; van Donk 1997). However, we are not aware of how the decrease in food palatability may affect herbivorous consumers throughout their ontogenetic phases.

The ways through which P limited food affect consumers seems to be complex. The shortage of one single biochemical component has been shown to strongly affect consumer performance (von Elert 2001; Ravet et al. 2003; Ravet and Brett 2006). However, the magnitude of these effects may depend on the type of the limiting components, because some limiting biochemical components are able to be compensated by the internal retro-conversion from another type of component (von Elert 2002; Martin-Creuzburg et al. 2010). Food with altered biochemical composition has also been shown to result in changes in the elemental metabolism of consumers, e.g. C in *Daphnia* (Lukas and Wacker 2014). Although we do not know about how these biochemical components may affect P metabolism in consumer bodies, the reduction in consumer performance could also be a consequence of the interactive effects among P deficiency and alterations in the biochemical composition.

Challenges and responses of consumers along a stoichiometric knife edge

Stoichiometric mismatch reflects a bidirectional deviation of elemental ratios, either to the side of nutrient limitation or to the side of nutrient excess. In nature, the detrimental effects of eutrophication on primary consumers are thought to mainly result from its indirect effects, e.g. toxic algal blooms (Carpenter 2008; Schindler et al. 2008) or fish stock enhancements (Sereda et al. 2008). In **chapter 3**, we showed that in addition to P limitation, food with excess P also caused strong reductions in consumer performance. These results therefore confirmed the existence of a stoichiometric knife edge (Elser et al. 2006; Bullejos et al. 2014; Benstead et al. 2014; Laspoumaderes et al. 2015; Elser et al. 2016). Given that the C:P range we worked on is highly ecological relevant (**Figure 6.1**), these findings may highlight the importance of excess P itself in determining the biomass and composition of primary consumers in eutrophic systems.

In addition, these negative effects may further affect ecosystem functions through the physiological adjustments of consumers to P rich food, such as decreased grazing rates and increased P release rates.

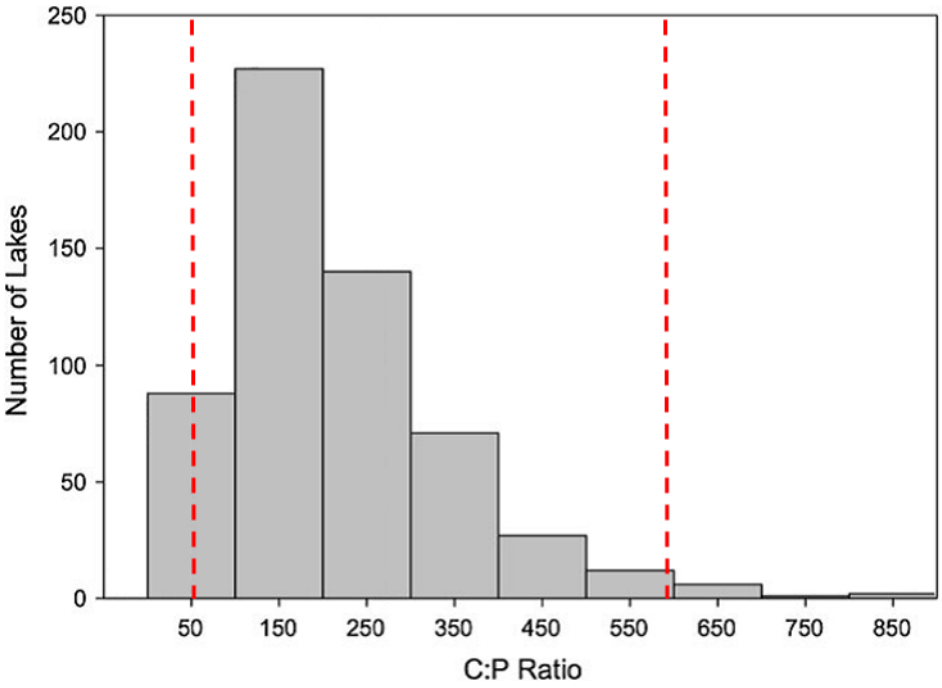


Figure 6.1 Histogram of freshwater lakes and ponds worldwide and seston C:P ratios from Sterner et al. (2008). The area between the two red dash lines represents the food C:P range in **chapter 3**. Modified from Morehouse et al. (2013).

The magnitude of negative effects of stoichiometric mismatch on consumers may depend on their ability to physiologically regulate and the costs associated with these regulations. Results in **chapter 3** suggest that consumers face different challenges at different sides of the stoichiometric knife edge. Interestingly, we found a complete homeostatic breakdown when food C:P doubled the optimum but this was not observed even when food P:C tripled the optimum. Rotifer consumers seem to be better at coping with excess P in low C:P food than dealing with deficient P and excess C in high C:P food. However, the strong reduction in growth in low C:P food suggests that dealing with excess P, e.g. storage (Persson et al. 2010) or elimination (Elser et al. 2006), is highly costly. In addition, alterations in body stoichiometry of primary consumers may also affect their quality as food for higher trophic levels. It has been shown that the negative effects of P limitation are able to travel up food chains (Boersma et al. 2008). Prey with very low C:P also reduces the performance of their predators (Benstead et al.

2014; Laspoumaderes et al. 2015). Based on our observations, such bottom–up cascades may be more pronounced in oligotrophic systems than eutrophic systems. To support this, more data about the response of other consumer species to such broad C:P gradient is nevertheless needed.

Results in **chapter 3** reflect the negative effects of direct stoichiometric aspects of low C:P food on consumers. One important question that arises from this is how low C:P food with a high P growth history may also affect consumers through indirect non-stoichiometric ways (as shown for P limited food in **chapter 2**). Currently, knowledge about how non-stoichiometric traits, such as biochemical composition or morphology of algae change under high P conditions is still lacking. Even less is known about whether these non-stoichiometric changes can further affect consumer performance. In addition, food consisting of a mixture of multiple algal species are thought to be better for consumers than that of a single species (Urabe et al. 2018). Although few recent attempts have been undertaken to test the stoichiometric knife edge (Currier et al. 2017) using lake seston, more studies are urgently needed.

Due to anthropogenic activities and climate change, stoichiometric imbalanced food in nature is generally not the only stressor that organisms have to cope with (Jackson et al. 2016). Another important but less addressed question in ecological stoichiometry is how stoichiometric mismatch may interactively affect consumers when accompanied with other stressors, e.g. increased temperature (Cole et al. 2002; Person et al. 2011). In **chapter 5**, we found evidence for the existence of antagonistic effects between P limitation and another common threat due to climate change, i.e. increased salinity. It has been shown that consumers fed P limited food have to cope with excess C content (Hessen et al. 2008). Possibly, high C:P food may provide an extra energy source for consumers to cope with increased ambient osmotic pressure and to neutralize ion toxicity caused by increased salinity. Based on this explanation, we would then expect that the combination of low C:P food and high salinity would result in synergistic effects on consumers, given that consumers decrease their C ingestion rates to avoid excess P intake (showed in **chapter 3**).

Effects of stoichiometric mismatch across generations

In addition to the interaction between genotype and environment, maternal effects have also been shown to affect the phenotype of organisms (Marshall and Uller 2007). Currently, how stoichiometric imbalanced food may affect consumers via maternal effects is poorly understood (but see Frost et al. 2010; He et al. 2016). Furthermore, nothing is known about how direct and indirect effects as described in **chapter 2** are able to transfer across generations. In **chapter 2** we showed that rotifers that are confronted with inferior food conditions produce larger eggs than those in benign conditions. Given the well accepted positive relationship between egg size and offspring quality (Dias and Marshall 2010; Krist et al. 2011; Segers and Taborsky 2011; Moore et al. 2015), one may expect that those larger eggs reflect an adaptive strategy of mothers in response to food P limitation. Results in **chapter 4**, however, did not support this expectation. Instead of being adaptive, both direct and indirect maternal effects of P limitation were shown to be transmissive in affecting offspring performance. The larger size of eggs produced under inferior conditions does not seem to reflect an increased allocation of essential nutrients, such as P or biochemical components but of energy rich substances instead. Indeed, larger eggs were found to be associated with higher starvation resistance of offspring (**chapter 4**).

The relative importance of direct and indirect maternal effects of P limitation varied with different life history traits. Generally, the quality of new born offspring highly depends on the essential resources that they receive from their mothers. It has been shown that for zooplankton consumers, either food P limitation (Urabe and Sterner 2001; Frost et al. 2010) or biochemical limitation (Wacker and Martin-Creuzburg 2007) result in a decreased allocation of the limiting materials to eggs. Altered nutrient allocation may reflect a selfish strategy of mothers when facing P limited food, and may explain the observed reductions in offspring quality.

In **chapter 3**, we showed negative effect of excess P on rotifer consumers. Whether these effects are also able to affect consumers across generations still needs to be investigated. Both storage (Persson et al. 2010) and metabolism of excess P (Elser et al. 2006) are suggested to be highly costly for consumers. Similar to the way by which how consumers deal with excess C in P limited food (Urabe and Sterner 2001), one possibility is that they may also allocate excess P to their eggs when fed P excess food.

Effects of stoichiometric mismatch on microevolutionary trajectories

The persistence of a species in nature may highly depend on how it is able to cope with multiple simultaneously occurring stressors, and whether it can rapidly adapt to new stressors. Increasing evidence has shown that evolutionary processes are able to happen at the same time scale of ecological processes (Hendry 2016; Govaert et al. 2019). It is therefore important to explore whether the evolutionary history of one consumer can be taken as an additional factor that interactively affect consumers with other ambient stressors. And if so, what is the consequence of such interactions. In **chapter 5**, we confirmed previous studies that consumers are able to rapidly adapt to P limited food (Declerck et al. 2015; Lemmen et al. in prep.) or increased salinity (Coldsnow et al. 2017; Hintz et al. 2018; Zhao et al. in prep.). Interestingly, adaptation to P limitation decreased the ability of consumers to cope with increased salinity. The underlying mechanism behind this observation are still unclear. Possibly, adaptation to P limited food may involve an increased ability to eliminate excess C (Darchanmbeau et al. 2003; Hessen et al. 2008). Although this should enable consumers to better cope with excess C in high C:P food, it may reduce the energy available to maintain osmotic homeostasis.

Given the specific design of the experiments, we only considered direct effects of P limitation in the study on adaptation to P limitation. However, adaptation to indirect effects, as demonstrated in **chapter 2**, could not be evaluated. An interesting question to future research is if and to what extent consumers may also adapt to biochemical deficiency (e.g. PUFAs, Spikerman and Wacker 2011; Challagulla et al. 2015) or low palatability (van Donk and Hessen 1993; van Donk 1997) associated with P-limited growth of algae. In such scenario, although we showed decreased ability of the adapted populations to cope with increased salinity, we may expect that adaptation still benefit them in dealing with food with low PUFA contents or with low palatability.

Another intriguing question is to what extent natural populations may be able to rapidly adapt to low C:P food. Results in **chapter 3** suggest that the negative effects of low C:P food mainly result from excess P. It would be interesting to explore if adaptation to low C:P food results in consumers that are able to eliminate excess P more efficiently. Also, the question to what extent populations are able to adapt to possible indirect effects of excess P remains an open question.

The interactive effects of altered stoichiometric ratios and other stressors to consumers in freshwater systems are currently rarely studied (but see Person et al. 2011; Cross et al. 2015 for temperature). To better address these questions, rapid adaptation should be incorporated more explicitly in related studies. This may especially important to understand the ecological consequences of stoichiometric mismatch in nature systems. For example, the feedbacks between ecology and evolution under P limited condition, i.e. eco-evolutionary dynamics (Declerck et al. 2015), may potentially be affected by the presence of another stressor such as increased salinity (**chapter 5**), or warming (Kaspari et al. 2016). In addition, the effects of evolutionary history may vary with type of stressors, based on the observations that adaptation to P limitation reduced the ability of rotifers to cope with increased salinity, but not vice versa. This also elicits the necessity to investigate how evolution can mediate the performance of organisms in the face of other combinations of stressors in future studies, such as combinations among varied salinity, temperature, and food quantity.

Concluding remarks

The research presented here contributes to a better understanding of the consequences of stoichiometric mismatch between producers and their consumer. I mainly focused on investigating how food with imbalanced C:P ratios affect consumer performance at contemporary, intergenerational, and evolutionary time scales. Firstly, I observed strong indirect non-stoichiometric effects of P limited food on consumers, and showed that the relative strength of direct and indirect effects varies among different fitness components (**chapter 2**). In **chapter 3**, I confirmed the existence of a stoichiometric knife edge, and further demonstrated that challenges to consumers differ at different sides of this knife edge. Particularly, I found a complete homeostatic breakdown of consumers at high food C:P but not at low food C:P ratios, suggesting that consumers are better able to cope with low C:P than high C:P food. In **chapter 4**, I found that direct and indirect effects of P limitation are able to affect consumers through maternal effects, but again showed these effects to vary strongly among different fitness components. Results in **chapter 5** confirmed the findings that rotifers are able to rapidly adapt to either P limitation or increased salinity, and demonstrated that being adapted to one stressor may affect the ability to deal with another stressor.

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Summary

Human activities have strongly altered the biogeochemical cycles of essential elements, such as carbon (N), nitrogen (N), and phosphorus (P). Ecological stoichiometry provides a powerful tool for investigating the ecological causes and consequences of alterations in the ratios of these elements that are available to biota. In freshwater systems, P is considered as a key limiting element due to its scarcity and the fact that it is unsubstitutable for the growth of producers and consumers.

In the field of ecological stoichiometry, there is a long tradition of studies exploring how P limitation of producers may affect the performance of consumers. However, many knowledge gaps still exist. For example, it is not very clear yet how P limitation of producers through indirect non-stoichiometric effects may affect their quality as food for consumers (e.g. through changes in their palatability or biochemical composition). Furthermore, it remains unclear what is the relative importance of such indirect effects compared to the direct effects of P-shortage. In **chapter 2**, we experimentally disentangled the direct and indirect effects of P limited food, and found that both had a strong negative influence on the performance of the herbivorous rotifer *Brachionus calyciflorus*. Interestingly, the relative strength of these two types of effects varied among different life history traits. For example, egg size and development time were found to be almost entirely determined by indirect effects in contrast to adult body size that was mainly determined by the direct effects of P limitation.

Stoichiometric mismatch does not only apply to situations where P is limiting, but may also exists when P is available in excess. Either too high or too low food C:P ratios have been found to result in reductions in consumer growth, a phenomenon that is referred to as the 'stoichiometric knife edge'. In **chapter 3**, we experimentally subjected rotifer populations to a broad food C:P gradient and assessed responses in their growth performance, elemental composition, and two key physiological rates (e.g food and P ingestion rates and P loss rates). We confirmed the existence of a stoichiometric knife edge by showing that the highest growth was observed at an intermediate food C:P level of approximately 170. Above the optimal molar food C:P, the rotifers had to cope with P shortage and excess C simultaneously. Thanks to compensatory feeding and reduced P loss rates, they were able to remain homeostatic until a food C:P value up to 391, beyond which homeostasis broke down completely. Below the optimal food C:P, consumers had to deal with excess P. They remained homeostatic in the face of a three-fold C:P reduction by decreasing their food ingestion rates and increasing their P loss

rates. In summary, these results indicate that rotifers deal with different challenges along opposite sides of the food stoichiometry gradient. At odds with the common belief about metazoan consumers, we also show that the strength of rotifer elemental homeostasis is limited and variable across the food C:P gradient.

Although poorly studied, P limitation may affect consumers via maternal effects. In **Chapter 2,** we observed that rotifers fed low P food produced larger eggs than rotifers fed with P sufficient food. This may indicate the existence of anticipatory maternal effects, where maternal generations enhance the fitness of their offspring by allocating more resources to their eggs. In **Chapter 4,** we performed a life history experiment studying maternal effects related to the direct and indirect consequences of P limitation (see also **Chapter 2**). However, we found no fitness advantages of offspring hatched from such larger eggs. Furthermore, both direct and indirect maternal effects were important in determining consumer performance. The relative strength of these two effects varied with different life history traits. Surprisingly, maternal diets were found to be more important in determining the somatic growth of consumers than the contemporary diet, indicating that maternal effects have the potential to decouple the relationship between food stoichiometry and observed consumer performance. This highlights the need of taking maternal effects into account in ecological stoichiometry.

Consumers have been demonstrated to shown rapid evolutionary adaptation to P limitation within an ecological time scale. In nature, P limitation may also be accompanied by other stressors. In **chapter 5**, we investigated how adaptation to P limitation may affect rotifer growth performance in the presence of an additional stressor, i.e. increased salinity. Similarly, we studied how adaptation to salinity may affect the ability to cope with P-limitation. Interestingly, when fed P limited food, a simultaneous moderate increase in salinity had no impact on the growth of non-adapted reference populations. This can be explained by the idea that consumers fed P limited food have to cope with C in excess supply, whereas under saline conditions they have to spend energy to keep osmotic homeostasis and to neutralize the toxic effects of ions. Possibly, consumers may be able to use the excess C in P limited food as an extra energy source to cover the increased energy requirements needed to cope with increased salinity. Furthermore, we found that adaptation to P limitation reduced the ability of rotifer consumers to cope with increased salinity. Possibly, adaptation to P limitation may enable consumers to better eliminate excess C when fed P limited food. This ability may nevertheless be counter-adaptive under conditions of increased salinity given that less energy may be available to maintain osmotic

homeostasis and mitigating ion toxicity. These results thus suggest that the evolutionary history of a population may strongly determine how it responds to different environmental stressors.

Samenvatting

Door menselijk toedoen zijn er tijdens de laatste decennia belangrijke veranderingen opgetreden in de biogeochemische cycli van voor organismen essentiële elementen, zoals koolstof (C), stikstof (N) en fosfor (P). Ecologische stoichiometrie biedt een krachtig conceptueel raamwerk die ons in staat stelt de oorzaken en gevolgen te bestuderen van veranderingen in de verhoudingen van dergelijke essentiële elementen. Fosfor wordt beschouwd als één van de belangrijkste limiterende factoren van zoetwatersystemen, doordat het relatief zeldzaam is én onvervangbaar voor de groei van planten en dieren. Als primaire producenten groeien onder fosforlimiterende omstandigheden dan kan een stoichiometrische discrepantie ontstaan tussen de voedselnoden van consumenten en de verhouding van fosfor met andere elementen in het voedsel van deze consumenten.

Ondanks de lange traditie van onderzoek naar de gevolgen van fosforlimitatie op de groei van consumenten, bestaan er nog steeds belangrijke kennishiaten. Het is bijvoorbeeld nog onduidelijk hoe en in welke mate fosforlimitatie van producenten via indirecte niet-stoichiometrische effecten gevolgen heeft voor hun kwaliteit als voedsel voor consumenten, zoals bijvoorbeeld via veranderingen in hun verteerbaarheid of biochemische samenstelling. Vraag is ook hoe belangrijk de impact van dergelijke indirekte effecten is op de performantie van consumenten in vergelijking met de direkte effecten van fosfortekorten. In Hoofdstuk 2 waren we in staat op experimentele manier de impact van beide types effecten van mekaar te onderscheiden en vonden we dat beide effecten een erg negatieve invloed hadden op zowel de populatie- als lichaamsgroei van het herbivore raderdier *Brachionus calyciflorus*. Interessant was ook de waarneming dat het relatieve belang van deze effecten sterk afhing van het beschouwde kenmerk. Hoewel de grootte en ontwikkelingstijd van eieren vooral bleken beïnvloed door indirekte effecten, werd adulte lichaamsgrootte vooral door direkte effecten van fosforlimitatie bepaald.

Een stoichiometrische discrepantie tussen voedsel en consumenten doet zich niet enkel voor bij fosforlimitatie, maar kan ook voorkomen wanneer fosforconcentraties in het voedsel veel hoger zijn dan de noden van de consumenten. Recente studies hebben het bestaan van een ‘stoichiometric knife edge’ gesuggereerd, waarbij de groei van consumenten optimaal is wanneer de koolstof *vs.* fosforverhouding (C:P-verhouding) van het voedsel intermediair is, terwijl de groei sterk afneemt bij sterk toenemende of afnemende C:P-verhoudingen. Voor Hoofdstuk 3 onderwierpen we *B. calyciflorus* populaties aan een brede voedsel C:P gradiënt en

bestudeerden we de respons van verschillende van hun kenmerken, zoals groeiperformantie, de C:P-verhouding van hun lichaam en de snelheid van een aantal belangrijke fysiologische processen, zoals de voedsel- en fosforopname en het verlies van fosfor aan de omgeving. We vonden een bevestiging voor het bestaan van de 'stoichiometric knife edge' en observeerden een maximale groei wanneer het voedsel een molaire C:P-verhouding had van ongeveer 170. Boven deze optimale voedsel C:P werden de raderdieren geconfronteerd met de combinatie van een tekort aan fosfor en een teveel aan koolstof. Dankzij een toegenomen voedsel- en dus fosforopnamesnelheid en een verminderde fosforafgifte bleken ze in staat hun somatische C:P-verhouding constant te houden tot aan een voedsel C:P niveau van 391. Boven dit niveau bleken ze evenwel niet meer in staat homeostase te handhaven en varieerde hun somatische C:P proportioneel met dat van het voedsel. Beneden het voedsel C:P optimum werden de raderdieren vooral geconfronteerd met een teveel aan fosfor. Ondanks een sterke afname in groeiperformantie bleken ze bij een afnemende voedsel C:P -verhouding grotendeels in staat hun stoichiometrische homeostase te behouden dankzij een afname van de voedselopnamesnelheid en een toename van fosforafgifte. Samenvattend tonen deze resultaten aan dat raderdieren langs de verschillende uiteinden van de voedsel C:P gradient met verschillende types uitdagingen worden geconfronteerd en dat de sterkte van hun stoichiometrische homeostase beperkt en variabel is.

Fosforlimitatie kan een generatie raderdieren ook beïnvloeden via maternale effecten. In Hoofdstuk 2 vonden we dat fosforlimitatie van het voedsel resulteerde in de vorming van grotere eieren. Deze observatie suggereert de mogelijkheid dat de moedergeneratie anticipeert op de negatieve gevolgen van P-limitatie door extra bronnen in haar nakomelingen te investeren. Voor Hoofdstuk 4 werd een levensgeschiedenisexperiment uitgevoerd om na te gaan of maternale effecten inderdaad het gevolg zijn van zo'n adaptieve strategie en ook om het relatief belang van direkte en indirekte effecten van fosforlimitatie in maternale effecten te evaluëren. We vonden evenwel geen voordelen geassocieerd met de productie van grotere eieren, in tegendeel. Fosforlimitatie van de ancestrale generaties resulteerde in een sterk verminderde groei van hun nakomelingen en hierbij speelden zowel direkte als indirekte effecten een rol. Verder bleek het relatief belang van direkte en indirekte maternale effecten sterk af te hangen van het kenmerk. Maternale effecten veroorzaakt door fosforlimitatie bleken zelfs een grotere impact te hebben op de somatische groei van raderdieren dan de kwaliteit van het voedsel waaraan de dieren direkt onderhevig waren. Dit suggereert dat maternale effecten tot een tijdelijke ontkoppeling kunnen leiden tussen de waargenomen performantie van populaties en

de kwaliteit van het voedsel waar ze afhankelijk van zijn. De sterkte van de maternale effecten geeft ook aan dat dergelijke effecten meer expliciet in stoichiometrisch onderzoek dienen te worden geïntegreerd.

Van zooplankton is geweten dat ze zich snel evolutionair kunnen aanpassen aan voedsel met tekorten aan fosfor. In de natuur kan fosforlimitatie zich evenwel simultaan in combinatie met andere stressfactoren voordoen. Voor Hoofdstuk 5 onderzochten we of een historiek van adaptatie aan fosforlimitatie de capaciteit van raderdieren beinvloedt om om te gaan met een verhoogd zoutgehalte. Omgekeerd onderzochten we ook hoe aan zout aangepaste populaties omgaan met fosforlimitatie in hun voedsel. Eerst onderzochten we hiervoor wat de gecombineerde effecten zijn van verhoogde zoutgehaltes en fosforlimitatie op de populatiegroei van raderdieren. We vonden dat toediening van fosforgelimiteerd voedsel de negatieve effecten van zout neutraliseert. Dit kan verklaard worden aan de hand van het feit dat fosforgelimiteerd voedsel heel koolstof- en dus energierijk is. Mogelijks stelt deze verhoogde energieinhoud van het voedsel de raderdieren in staat beter om te gaan met de energiekosten die gepaard gaan met het handhaven van osmotische homeostase en het neutraliseren van de toxische effecten van zout. Verder vonden we dat populaties die evolutionair aangepast zijn aan fosforgelimiteerd voedsel een verminderd vermogen hebben om te gaan met zout. Het is onze hypothese dat de aanpassing aan fosforgelimiteerd voedsel onder meer inhoudt dat populaties meer efficient het teveel aan koolstof in hun voedsel kunnen elimineren. Wanneer geconfronteerd met verhoogde zoutgehaltes zou dergelijke aanpassing niettemin kunnen resulteren in een verminderd energiebudget dat kan aangewend worden om de negatieve gevolgen van de hogere zoutconcentraties tegen te gaan. In het algemeen tonen deze resultaten aan dat de recente evolutionaire historiek van populaties in sterke mate het success kan beinvloeden van populaties om te gaan met verschillende stressoren.

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About the author

Libin Zhou was born on the 20th of March 1989 in Anhui, China. In 2008, he started his career in Anhui University (AHU) to study environmental science. Thereafter, he continued his science career by doing a master in the field of freshwater ecology in Nanjing Institute of Geography and Limnology, Chinese Academy of Science (NIGLAS, China). During his master, his first met those tiny but lovely creatures in the waters, named 'Zooplankton', and started knowing more about it. He was surprised by the fact that these visually small animals are so important in contributing the stability and function of the whole lake system. That is the time when he initiated his relationship with zooplankton. After he successfully defended his master thesis, he continued his career as a PhD at a place that is so far away from his home. But actually, it is the place where his is writing these words now. During his PhD, he mainly worked on the topic of ecological stoichiometry, but meanwhile he also developed multidisciplinary research interests, such as rapid evolution, cross protection, and nutrient geometry. Time flies, it is a time that this period needs to be stopped here, but also a time to start a new page in his life. He wants to say he likes pretty much this small but nice town (city?), Wageningen, but his journey will continue.

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