Trophic interactions in warming waters

Aquatic plant-consumer interactions under climate change

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Trophic interactions in warming waters

Aquatic plant-consumer interactions under climate change

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Waterplant-herbivoor interacties onder klimaatverandering

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

Global climate change in shallow water bodies

Due to anthropogenic activities, our planet is undergoing rapid global climate change. The global average surface temperature has increased about 0.85 °C from the beginning of last century. Without any preventive measures, global average surface temperature is predicted to rise another 4 °C till the end of this century (IPCC 2014). Temperature is one of the key abiotic factors that can directly influence the metabolism of organisms (Gillooly et al. 2001, Brown et al. 2004), thereby influencing trophic interactions, food-web structures and the functioning of ecosystems (Traill et al. 2010, Cross et al. 2015).

Shallow water bodies, especially shallow lakes, are the most abundant freshwater ecosystems on earth (Verpoorter et al. 2014). Shallow lakes provide many functions, such as the provisioning of habitat for aquatic organisms, the retention of watershed nutrients and carbon burial. In addition, shallow water bodies provide many valuable services to human beings, such as drinking water supply, opportunities for recreation and providing water for agricultural and industrial activities (Williamson et al. 2008, Carpenter et al. 2011, Hilt et al. 2017). Global climate change, especially temperature rise, can affect multiple aspects of shallow lakes, for example, decreasing the duration of ice cover in cold regions, changing the distribution of species, favoring cyanobacterial dominance and stabilizing turbid ecosystems, and altering trophic interactions and food web structures (Mooij et al. 2005, Shurin et al. 2012, IPCC 2014). As such, shallow lakes are regarded to be sentinels of climate change (Williamson et al. 2008, Adrian et al. 2009).

Aquatic plants in warm waters

Aquatic plants, particularly submerged species, are vital components of shallow aquatic ecosystems, and provide multiple functions (Carpenter and Lodge 1986, Scheffer 2004). For instance, aquatic plants can stabilize sediment and reduce resuspension (Carpenter and Lodge 1986, Barko and James 1998, Horppila and Nurminen 2003), compete with phytoplankton to keep water clear (Scheffer et al. 1993, van Donk and van de Bund 2002, Hilt and Gross 2008, Bakker et al. 2010), provide surface for periphyton growth (Phillips et al. 2016, Grutters et al. 2017a), release oxygen to the sediment and the water layer to facilitate aquatic animals, provide habitat and shelter for small aquatic animals (Meerhoff et al. 2007), and serve as food for higher trophic levels (Hansson et al. 2010, Bakker et al. 2016).

Due to human activities, aquatic plant diversity has decreased, and plant communities have declined in many shallow water bodies (Phillips et al. 2016, Zhang et al. 2017, Zhang et al. 2018a). Many shallow water bodies have therefore shifted from aquatic plant-dominated clear systems to phytoplankton-dominated turbid systems (Scheffer et al. 1993, Sayer et al. 2010a, Hilt et al. 2017). Eutrophication, the inflow of large amount of nutrients into the shallow water bodies, is perceived as the main driver of the shift from the plant-dominated to the phytoplankton dominated system, as algae grow faster than aquatic plants with nutrient loading and outcompete aquatic plants (Scheffer et al. 1993, Sayer et al. 2010a). However, global climate change might also contribute to the decline of aquatic plants (Mooij et al. 2005, Mooij et al. 2007, Kosten et al. 2009).

Temperature can have a strong influence on the growth of aquatic plants (Haag and Gorham 1977, Barko and Smart 1981, Tobiessen and Snow 1984, Madsen and Brix 1997, Santamaría and van Vierssen 1997). Warming simulation studies in microcosms have shown that warming did not influence the total biomass of the aquatic plants, but changed the species composition (Mckee et al. 2002, Li et al. 2017). However, field studies showed that warm water might change the distribution and increase the production of aquatic plants (Haag and Gorham 1977, Rooney and Kalff 2000), or decrease the plant biomass (Kosten et al. 2009). These often contrasting results indicate that different aquatic plant species might have different adaptations to water temperature. Hence, warming might favor some species, but suppress others.

The stoichiometry of aquatic plants under warming

The stoichiometry of aquatic plants, i.e. the plant's elemental ratios (in this thesis, I mainly focus on carbon:nitrogen and carbon:phosphorus ratios), is crucial for their role in the ecosystem. As the stoichiometry of aquatic plants affects both decomposition and consumption by higher trophic levels, this has consequences for nutrient cycling and energy transfer through the aquatic food web (Sterner and Elser 2002). Plant stoichiometry can vary considerably with and among aquatic plants, and often reflects the nutrient conditions in the environment (Demars and Edwards 2007). A lower carbon:nutrient ratio of an aquatic plant generally means higher nutrient availability in the environment (Su et al. 2016), and more palatable food to herbivores (Dorenbosch and Bakker 2011, Bakker et al. 2016). Submerged rooted plants can take up nutrients from both the water layer and the sediment (Rattray et al. 1991) (Fig. 1.1). This unique property makes that submerged rooted plants act as a physical connector of the sediment, water

layer and their respective biota, which makes the shallow aquatic ecosystem function as a whole. The nutrient composition and stoichiometry of aquatic plants not only depends on the nutrient conditions in the environment, but also on water temperature (Velthuis et al. 2017). Rising temperature might increase the growth of aquatic plants, with faster growth leading to faster nutrient depletion in the environment, and therefore higher carbon:nutrient ratios of the plants. However, rising temperature might also increase the nutrient availability in the environment due to higher decomposition rates by microbes (Gudasz et al. 2010, Sobek et al. 2017), which could result in lower carbon:nutrient ratios in the plants. Therefore, there is an urgent need to clarify the effects of warming on aquatic plant stoichiometry.

In most of the shallow lakes, climate change is not occurring alone, but it is accompanied by eutrophication (Jeppesen et al. 2010b, Steffen et al. 2015, Sinha et al. 2017). High nutrient loading to shallow lakes can change the plant community composition or shift the ecosystem to a phytoplankton-dominated state (Sayer et al. 2010a), whereas, slight nutrient loading could have a direct influence on aquatic plant stoichiometry without directly shifting plant community composition. However, most studies to date focused on effects of eutrophication on aquatic plant community composition and the competition with algae (Hilt et al. 2017), very few studies focused on the effects of eutrophication on changes in internal stoichiometry in aquatic plants. Studies investigating the combined effects of warming and eutrophication on aquatic plant stoichiometry are even less (Cross et al. 2015).

Aquatic plant-herbivore interactions under global warming

Herbivores (such as birds, mammals, crustaceans, mollusks, insects and fish) can have tremendous impacts on aquatic plant abundance, which has largely been neglected before (Bakker et al. 2016, Wood et al. 2017). However, possible effects of temperature on freshwater plant-herbivore interactions are largely unknown. Latitudinal studies have shown that plants are more palatable at higher latitudes (Pennings et al. 2007, Morrison and Hay 2012). However, the underlying mechanisms are still not very clear, suggest that plants are better defended at lower latitudes (Bolser and Hay 1996), or plant nutrient content increases with latitude (Cebrian and Lartigue 2004, Schemske et al. 2009) or both (Grutters et al. 2017b). Not all studies find latitudinal gradients in plant palatability (Adams et al. 2009, Moles et al. 2011). Temperature has also been suggested to underlie the observed latitudinal gradients in plant palatability (Schemske et al. 2009). However, studies that aimed to test this by mimicking warming effects

on plant palatability in marine or terrestrial plants showed inconsistent results, with either decreasing palatability in marine plants (Rodil et al. 2015) or having no discernible effect in either terrestrial (Backhaus et al. 2014) or marine plants (Poore et al. 2016). Therefore, experiments that directly test the effect of temperature on plant palatability and the traits underlying palatability are urgently needed.

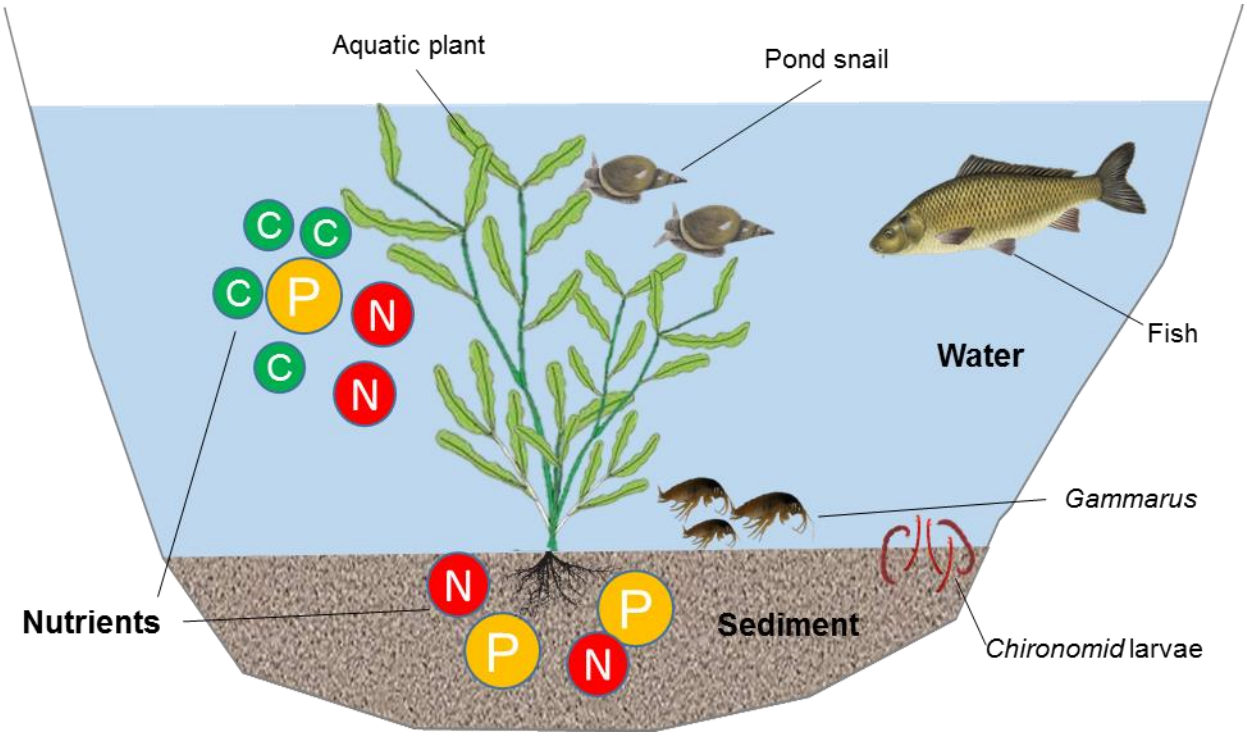

Figure 1.1 A simplified aquatic plant dominated freshwater ecosystem, depicting aquatic plants that extract nutrients from the water and sediment and are food for consumers such as pond snails and fish, which can also feed on *Gammarus* and *Chironomid* larvae. Submerged aquatic plants extract nutrients from both the water layer and the sediment, which together determines their stoichiometry and palatability to consumers.

The palatability of plants for herbivores is determined by three categories of plant traits: plant physical structure, plant nutrient level and plant secondary metabolism (PSM) (Hay 1996, Cronin et al. 2002, Elger and Lemoine 2005). Plant physical structure, namely, plant toughness or plant dry matter content as a proxy thereof, negatively affects plant palatability as softer plants or those with a lower dry matter content are preferred by herbivores (Pennings et al. 1998, Elger and Willby 2003). Plant nutrient levels are often expressed as plant nitrogen content,

protein content, or the relative nutrient content as the plant carbon:nitrogen ratio, which is also called plant stoichiometry. Generally, a higher nitrogen or protein content, or lower carbon:nutrient ratio, indicates a higher quality of the plant as food for consumers (Sterner and Elser 2002, Cebrian and Lartigue 2004, Bakker et al. 2016). Plant secondary metabolites (PSM) are compounds that the plant produces to deter animals from eating it. In aquatic plants, total phenolic compounds are most frequently used to indicate aquatic plant PSM (Smolders et al. 2000, Dorenbosch and Bakker 2011, Grutters et al. 2017b).

Aquatic plant palatability can be inferred from a single-date feeding trial by pond snails *Lymnaea stagnalis* (Elger and Barrat-Segretain 2004). This method was established by Elger and Barrat-Segretain (2002), and has been successfully used in many studies to test aquatic plant palatability (Elger and Willby 2003, Elger et al. 2004, Elger and Lemoine 2005, Grutters et al. 2017b). I use this interaction between aquatic plants and pond snails as part of my model system (Fig. 1.1).

Warming strengthens plant-herbivore interactions and has been found to enhance top-down control on aquatic plants (O'Connor 2009). That is because temperature rise generally increases animal ingestion rates faster than primary producer biomass accumulation rates (Gilbert et al. 2014, West and Post 2016). However, if plant quality would decline with rising temperature, then there might be a mismatch between plant food quality and herbivore nutrient demand. The question arises whether or not warming still enhances top-down control of plants if effects on plant quality are taken into account. Therefore, it is urgent to test the effects of warming on plant quality and how this affects palatability to aquatic animals. I predict that warming will enhance the interactions between aquatic plants and the herbivorous consumers (Fig. 1.2a).

With external nutrient loading to the water, plant nutrient content might increase and thereby also the palatability of the plant for herbivores, leading to enhanced top-down control on aquatic plant abundance (Bakker and Nolet 2014). This also might be the reason that aquatic plants disappear from shallow lakes with ongoing eutrophication (Hidding et al. 2016, van Altena et al. 2016). Therefore, it is also necessary to test the combine effects of warming and nutrient enrichment on aquatic plant quality and palatability.

Aquatic plant-omnivore interactions under ongoing warming

Omnivorous feeding modes are prevalent in aquatic ecosystems (Wootton 2017), including in birds, fish, crayfish, aquatic snails, tadpoles and others, from which many can consume both aquatic plant and animal food (Fig. 1.2a). Animal food has a higher nutrient content or lower carbon:nutrient ratio than plant food, whereas the stoichiometry of plants is more flexible than that of animals (Elser et al. 2000b, Van de Waal et al. 2010). Omnivorous animals have a more similar elemental composition compared to animal food than compared to plant material (Fig. 1.2b). Hence, I would expect that omnivores prefer animal food over plant material (Elser et al. 2000b, Sterner and Elser 2002).

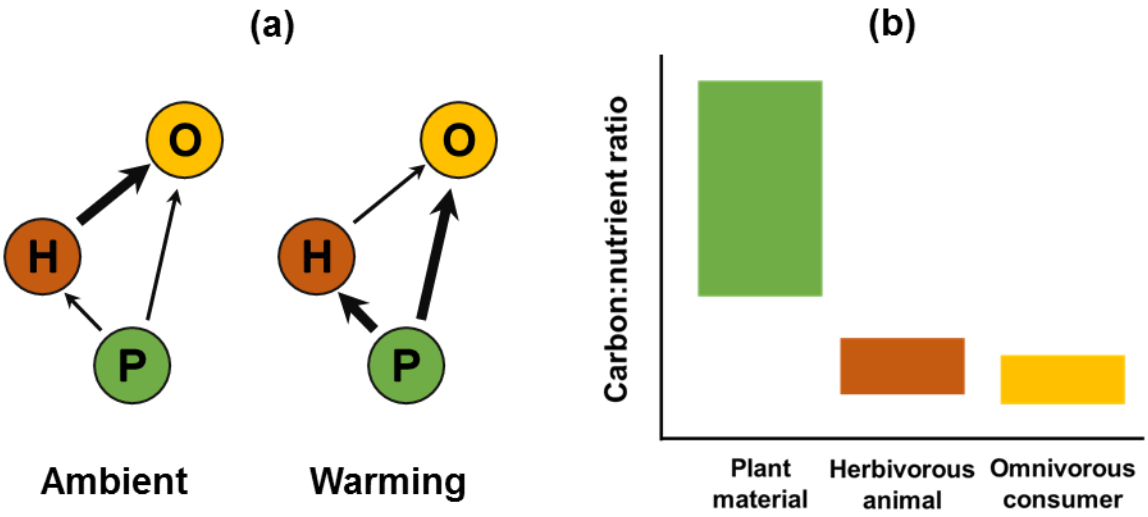

Figure 1.2 Conceptual omnivore-food interactions and their respective stoichiometry in freshwater ecosystems. (a) Typical omnivorous interaction in freshwater ecosystems under ambient and warming conditions. O = Omnivorous consumer, H = Herbivorous animal, P = Plant material. The arrows indicate the directions of energy flow. The width of the arrows indicates their strength. (b) Predicted carbon:nutrient stoichiometry of the different organisms involved. Boxes indicate relative ranges of the organisms' stoichiometry.

However, there is a latitudinal trend of increasing herbivory towards the equator in fish (Floeter et al. 2005, Moss 2010, Behrens and Lafferty 2012, González-Bergonzoni et al. 2012) and in invertebrates (Pennings et al. 2009, Boyero et al. 2012). Except for the evolutionary constraints, food quality and quantity explanations, direct temperature effects could also be a key driver of this observed gradient (Floeter et al. 2005, González-Bergonzoni et al. 2012).

Most aquatic animals are ectotherms (Isaak and Rieman 2013), which means that the metabolism of these aquatic omnivores will exponentially increase with rising temperature (Gillooly et al. 2001, Brown et al. 2004). If rising temperature would increase herbivory by aquatic omnivores, future global warming could have more impacts on aquatic ecosystems than anticipated, by altering the trophic level of aquatic animals and changing the abundance or composition of primary producers. This implies there is an urgent need to test if rising temperature would change diet selection of aquatic omnivores. I predict that omnivorous consumers will increase herbivory with warming (Fig. 1.2a).

Outline of the thesis

The aim of this thesis is to explore how warming will affect aquatic plants and their interactions with higher trophic levels. In my thesis, I mainly use submerged aquatic plants as model species. Here, I want to answer four questions:

(a) What is the effect of warming on aquatic plant growth?

(b) What is the effect of warming on aquatic plant stoichiometry?

(c) What is the effect of warming on aquatic plant-herbivore interactions?

(d) What is the effect of warming on aquatic plant-omnivore interactions?

To answer these questions I take three steps. First, I investigate the effects of warming on the growth and stoichiometry of aquatic plants (**Chapter 2, 3 and 4**). Second, I test how warming influences aquatic plant-herbivore interactions, and the underlying plant traits that affect plant palatability (**Chapter 3 and 4**). Third, I test plant quality and warming effects on plant-omnivore interactions (**Chapter 5, 6 and 7**).

In **Chapter 2**, I investigate how warming would influence aquatic plant growth and stoichiometry in mesocosms in a future warming scenario. In **Chapter 3**, I test the temperature effects on plant growth, chemical traits and palatability with multiple plant species to clarify the underlying mechanisms of warming effects on aquatic-plant herbivore interactions. In **Chapter 4**, I include another factor, nutrients, to test the combined effects of temperature and nutrient enrichment on aquatic plant growth, stoichiometry and palatability.

In **Chapter 5**, I test the diet selection by aquatic ectothermic omnivores for animal food and plant food, with a large gradient of differences in aquatic plant quality. And in **Chapters 6**

and 7, I investigate temperature effects on diet selection in aquatic omnivores feeding on animal food and plant material in a short-term and long-term experiment, respectively.

In **Chapter 8**, I integrate all the data and results from previous chapters to answer the main questions. I conclude how my findings advance our knowledge of warming effects on aquatic plants and their interactions with higher trophic levels, and the implications for aquatic ecosystems.

Chapter 2

Effects of warming on *Potamogeton crispus* growth and tissue stoichiometry in the growing season

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Abstract

Increased water temperature due to climate change may affect macrophyte phenology and nutrient content. In experimentally heated mesocosms the emergence and growth of *Potamogeton crispus* shoots under ambient and increased temperatures (+4.5 °C) were tracked over 55 days. At the end of the experiment we measured the C, N and P content of the *P. crispus* leaves. The results indicate that warming advanced the emergence of *P. crispus* shoots by approximately 10 days, whereas the final number of shoots and plant biomass were similar in ambient and heated tanks. Furthermore, warming influenced the ecological stoichiometry of this plant significantly. Leaf C and N content were both less in the heated tanks resulting in an increase in C:N ratios, whereas P content and C:P and N:P ratios were not affected.

Introduction

During the period 1905-2001, the temperature rise in China was 0.5-0.8 °C, which is slightly higher than the global warming range of 0.6 ± 0.2 °C in the same period (Ding et al. 2007). It is likely that the global temperature will increase by 1.1-6.4 °C in the 21st century compared to 1980-1999 on top of the warming in the last century (IPCC 2007). Warming affects a myriad of biological processes, including individual metabolic rates and growth rates (Yvon-Durocher et al. 2010), life history traits and phenology, which may induce trophic mismatches between consumer demands and prey availability (Winder and Schindler 2004).

Shallow lakes, with their low depth and large surface : volume ratio, are sensitive to warming (Coops et al. 2003, Meerhoff et al. 2012). Macrophytes are key components in the ecological functioning of shallow lake ecosystems and they are affected by warming in various ways (Kosten et al. 2009). At the level of the plant individual, warming may affect plant physiology (Madsen and Brix 1997, Rooney and Kalff 2000), growth (Haag and Gorham 1977, Short and Neckles 1999) and reproduction (Van Vierssen et al. 1984, Mckee et al. 2002). Furthermore, warming may result in alterations of trophic interactions (O'Connor 2009), which may have cascading effects throughout the food web (Shurin et al. 2012) due to a mismatch in timing (Winder and Schindler 2004) or change in food quality. Plant nutrient concentrations and the C:nutrient stoichiometry have been shown to be strong predictors of plant consumption rates across ecosystems (Elser et al. 2000a, Sterner and Elser 2002). High C:N and C:P ratios of primary producers indicate poor food, as N and P are needed by organisms to produce proteins and RNA (Elser et al. 2000b). The temperature-plant physiology hypothesis (Reich and Oleksyn 2004) predicts that plant N and P content should decline with increasing temperatures and indeed leaf N and P concentration of terrestrial plants decline towards lower latitudes as the average temperature goes up, but few studies have focused on aquatic plants. In aquatic plants at lower latitudes, temperature optima may be surpassed and growing season length may become limiting plant presence due to a shortening of their life cycle (Santamaría and van Vierssen 1997).

In the present study, we test how warming influences the growth and stoichiometry of a submerged aquatic macrophyte. We hypothesize that warming (1) advances the start of macrophyte growth and (2) increases macrophyte biomass. Furthermore, we hypothesize that (3) if the temperature-plant physiological hypothesis also applies to aquatic plants, the tissue concentrations of C, N and P will decrease as the temperature goes up resulting in (4) increasing

C: nutrient ratios of the plant with warming. We used the widespread submerged macrophyte *Potamogeton crispus* L. (Curlyleaf pondweed) as a model species and grew it under ambient and experimentally heated conditions (+ 4.5 °C higher than ambient).

Materials and Methods

Study species

P. crispus has a wide geographic distribution (Rogers and Breen 1980, Catling and Dobson 1985), and occurs in many freshwater ecosystems throughout China (Wu et al. 2009). It is most common in nutrient rich hard waters (Bolduan et al. 1994), and considered a pioneer species in the restoration of eutrophic lakes (Wu et al. 2009). Turions of *P. crispus* germinate in the fall when the temperature goes down (Catling and Dobson 1985, Nichols and Shaw 1986), then overwinter with little growth (Tobiessen and Snow 1984). *P. crispus* grow fast in early spring while the water temperature is still low (10 - 15 °C). Then during the late spring to the summer, *P. crispus* forms turions, flowers and seeds, and after that, senescence and decomposition of *P. crispus* progress rapidly (Tobiessen and Snow 1984, Hongda 1985). Temperature has a significant influence on the germination and growth of *P. crispus*. With warming from 15 °C to 30 °C, germination of turions decreases (Rogers and Breen 1980), whereas increased temperatures increase the number of turions formed (Sastroutomo 1980). From 5 °C to 10 °C, a significant increase in biomass was found with increasing temperature (Tobiessen and Snow 1984). The optimum temperature range for *P. crispus* appears to be 10 - 20 °C, above and below which growth is impaired (Ren et al. 1997).

Heating system

The experimental system was located at Wuhan (30°28′N, 114°21′E), in the center of China. Ten fiberglass tanks (1.6 m height and 1.5 m diameter) wrapped with insulation materials were used to simulate climate warming, equipped with a heating rod in the center of the water and a pump to promote the mixture of the water, connected to a computer control system. There were two temperature probes placed at two sides of each mesocosm which read one value every ten seconds. Temperature values of five ambient tanks were averaged and returned to the computer, and the computer determined how to heat the other five mesocosms for the heated treatment.

According to the prediction models provided by IPCC, the annual mean temperature in the middle and lower reaches of Yangtze River would be increased by 4.5 ℃ at the end of this century (Xu et al. 2004). Therefore, we selected the heated treatment to be 4.5 ℃ higher than the control exposed to ambient temperatures. All tanks were placed randomly to eliminate small spatial differences and evenly covered with 10 cm deep sediment (TN 5.3 ± 0.3 $mg \cdot g^{-1}$ dry weight, TP 0.41 ± 0.04 $mg \cdot g^{-1}$ dry weight) dredged from Lake Liangzi. The lake is located in Hubei, China (30°5′ - 30°18′N, 114°21′ - 114°39′E), with a surface area of 304 km^2 and an average depth of 4.2 m (Wang and Dou 1998). We dredged the sediment (location: 30°10′55″N, 114°37′40″E, with water depth 1.6 ± 0.3 (SD) m, secchi disk 1.5 ± 0.3 m, pH 8.8 ± 0.6, and conductivity 178 ± 3 $\mu s \cdot cm^{-1}$), where *P. crispus* was a dominate species. Then we filled each tank with tap water to yield 1m water depth. The heating system began to operate on 6 April 2013 before the emergence of *P. crispus*, and our first camera record was on 24 April when *P. crispus* could be seen in most of the tanks. The experiment ended on 31 May. The submerged macrophytes *Najas minor*, *Hydrilla verticillata* and *Ceratophyllum demersum* emerged in some of the tanks during the experiment, but *P. crispus* was the absolute dominant species during the entire experiment. We weekly monitored water quality in the tanks, which in the ambient tanks ranged from: NH_4^+: 0.30 ~ 0.74 $mg \cdot L^{-1}$, NO_3^-: 0.11 ~ 0.96 $mg \cdot L^{-1}$, PO_4^-: 1 ~ 26 $\mu g \cdot L^{-1}$, Chl-a: 0.54 ~ 2.1 $\mu g \cdot L^{-1}$, and in the heated mesocosms: NH_4^+: 0.36 ~ 1.53 $mg \cdot L^{-1}$, NO_3^-: 0.08 ~ 0.76 $mg \cdot L^{-1}$, PO_4^-: 2 ~ 33 $\mu g \cdot L^{-1}$, Chl-a: 0.91 ~ 3.29 $\mu g \cdot L^{-1}$ (see Fig. S2.1 in the Supplementary Material).

Camera record

We used a waterproof camera (Nikon COOLPIX AW100s) to record the growth of *P. crispus* every week. The camera was fixed in a steel framework with a long rod. There was a steel ruler (range 20 cm) fixed 30 cm from the camera lens. Recording in each tank followed a standard procedure: (1) record the label; (2) put the camera under the water just above the sediment, make the rod vertical and tract 360-degrees around; (3) with the back of the camera attached to the wall of the tank, walk along the side to record 360-degrees above the sediment; (4) if plants could be seen by eye, we placed the camera with the ruler attached to it on the sediment and tracked the shoots vertically from the bottom to the top; (5) as plants nearly reached the water surface, we used a long ruler to measure the height directly. All videos were analyzed in the computer, to read all recorded plant heights.

Sampling and Detecting

Attached filamentous algae and non-target floating-leaved macrophytes (especially *Trapa natans* and *Nymphoides peltata*) were removed from the mesocosms during the experiment. At the end of the experiment, we collected 3 to 20 individuals (according to the total amount of individuals in each tank) in each mesocosm of *P. crispus* with a height range from several to more than 100 centimeters. Attached filamentous algae and other debris were cleaned off the plants and the total above-ground height of each shoot was measured. Wet weight of each plant was measured, and then the plants were put in a plastic bag and brought to laboratory to dry. After complete drying in the oven at 60 ºC for 48 h, we collected the leaves from each shoot and used a vibration grinding machine (MiniBeadbeater-16) to grind the leaves to powder with two steel balls vibrating randomly in a special plastic tube. About 2~3 mg dry powder was used to detect the carbon and nitrogen content using the CN element analyzer (Flash EA 1112, CE Instruments, Italy). Phosphorus content of the leaves was measured by the ammonium molybdate ascorbic acid method (Sparks et al. 1996, Li et al. 2013).

Data analysis

We used the wet weight biomass and height of the plants collected at the end of the experiment to determine the relationship between plant height and wet weight biomass, which could be described by a power equation (Niklas and Enquist 2001, Brown et al. 2004). We used the equation $Y = 0.0535*X^{0.9602}$ ($F_{1,152} = 682$, $p < 0.001$, $r^2 = 0.82$), where Y is plant biomass in g wet weight and X is plant height in cm, to calculate plant wet weight biomass from the height measurements taken during the experiment both in ambient, and heated mesocosms. We used a Repeated measures ANOVA to test the effect of warming on shoot number, number of newly emerged shoots and biomass of *P. crispus* plants during the experiment. Where significant interactions were found between time of measurement and warming, the effect of warming was tested for each date using independent t-tests. To determine whether there was a difference in phenology between the warming treatments, we calculated the date at which half of all shoots had emerged in each tank. This was done by extrapolating between the nearest two dates on which the number of shoots was counted, assuming a linear increase in number of shoots between these two dates. The effect of warming on the day at which half of all shoots were present was tested with an independent t-test. The effect of warming on plant C, N and P concentration and C:N, C:P and N:P ratios ($g \cdot g^{-1}$) was tested with a nested ANOVA, with

warming treatment as a fixed and experimental tank as a random factor nested in warming treatment. Normality of the data distribution was tested with a Kolmogorov-Smirnov test. Spread versus level plots and residual plots were used to control other parametric assumptions. These indicated that no data transformations were necessary. The analyses were performed in SPSS 22.0 (IBM Corp. 2013) and Statistica 12 (StatSoft Inc. 2014).

Results

Temperature comparison

During our experiment, the average temperature in the heated mesocosms was 4.44 ± 0.28 °C higher than in the control (daily average in heated tanks minus average in ambient tanks, averaged over all days of the experiment). The daily average ambient water temperature varied from 13.9 °C to 27.1 °C (Fig. 2.1).

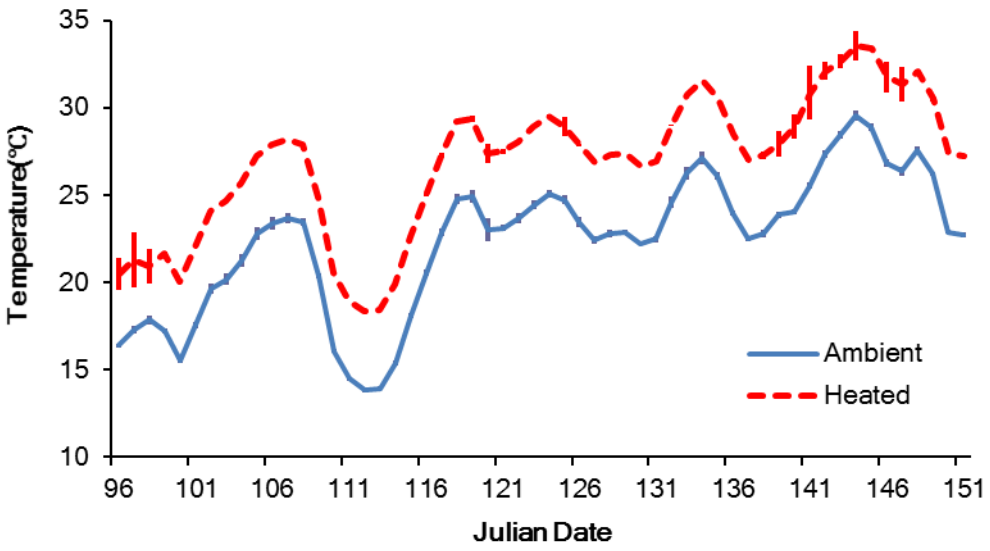

Figure 2.1 Mean daily temperature in the ambient and heated tanks (n=5 per treatment). Data are means ± SD.

Population growth

The number of shoots in the tanks increased significantly over time in both the ambient and heated tanks (Fig. 2.2a; Repeated measures ANOVA: time in experiment $F_{5,40} = 41.6, p < 0.001$).

There was no significant effect of heating treatment ($F_{1,8} = 0.08$, $p = 0.78$), whereas there was a marginal interaction between time and heating treatment ($F_{5,40} = 2.56$, $p = 0.042$), but further testing revealed no significant differences among heating treatments per date (independent t-tests, $p > 0.1$). The day at which 50% of the individuals had emerged was significantly affected by temperature treatment (independent t-test: $t_8 = 4.27$, $p = 0.003$). In the heated treatment 50% of the individuals was on average present on May 3 (day 123 ± 1 SE), whereas under the ambient temperatures 50% of the individuals was present ten days later on May 13 (day 133 ± 2 SE). The number of newly emerging shoots was not directly affected by the heating treatment ($F_{1,8} = 1.34$, $p = 0.28$), but there was a strong interaction between heating treatment and time in the experiment for the emergence of new shoots (interaction heating × time: $F_{4,32} = 7.02$, $p < 0.001$; effect of time: $F_{4,32} = 3.65$, $p = 0.015$). Only at the last day of measurement there was a significant difference among heating treatments: there were more new shoots in the ambient compared to the heated treatment (independent t-test: $t_8 = 2.94$, $p = 0.019$). On the other dates there were no significant differences among heating treatments (independent t-tests, $p > 0.1$) (Fig. 2.2b). The wet biomass of *P. crispus* strongly increased over time (Fig. 2.2c; Repeated measures ANOVA: effect of time $F_{5,40} = 32.25$, $p < 0.001$). There was no effect of the heating treatment on plant biomass during the experiment (effect of heating: $F_{1,8} = 0.30$, $p = 0.60$, time × heating: $F_{5,40} = 0.82$, $p = 0.54$).

Table 2.1 Effects of heating treatment on plant nutrient concentrations and stoichiometry. Results of nested ANOVA with heating treatment as fixed factor and tank nested in heating treatment as a random factor. Values are Mean ± standard error (SE).

Parameter	**Ambient**	**Heated**	**Heating treatment**		**Tank**	
			$\mathbf{F_{1,99}}$	***P***	$\mathbf{F_{8,99}}$	***P***
C ($mg \cdot g^{-1}$)	419 ± 4.7	407 ± 6.6	13.70	<0.001	3.59	0.001
N ($mg \cdot g^{-1}$)	41.4 ± 1.7	36.9 ± 1.9	19.99	<0.001	2.33	0.025
P ($mg \cdot g^{-1}$)	3.20 ± 0.48	2.75 ± 0.37	2.25	0.14	3.41	0.002
C:N ($g \cdot g^{-1}$)	10.2 ± 0.42	11.2 ± 0.47	15.83	<0.001	3.24	0.003
C:P ($g \cdot g^{-1}$)	145 ± 21	162 ± 23	1.06	0.31	4.03	<0.001
N:P ($g \cdot g^{-1}$)	14.0 ± 1.6	14.4 ± 1.6	0.05	0.83	4.49	<0.001

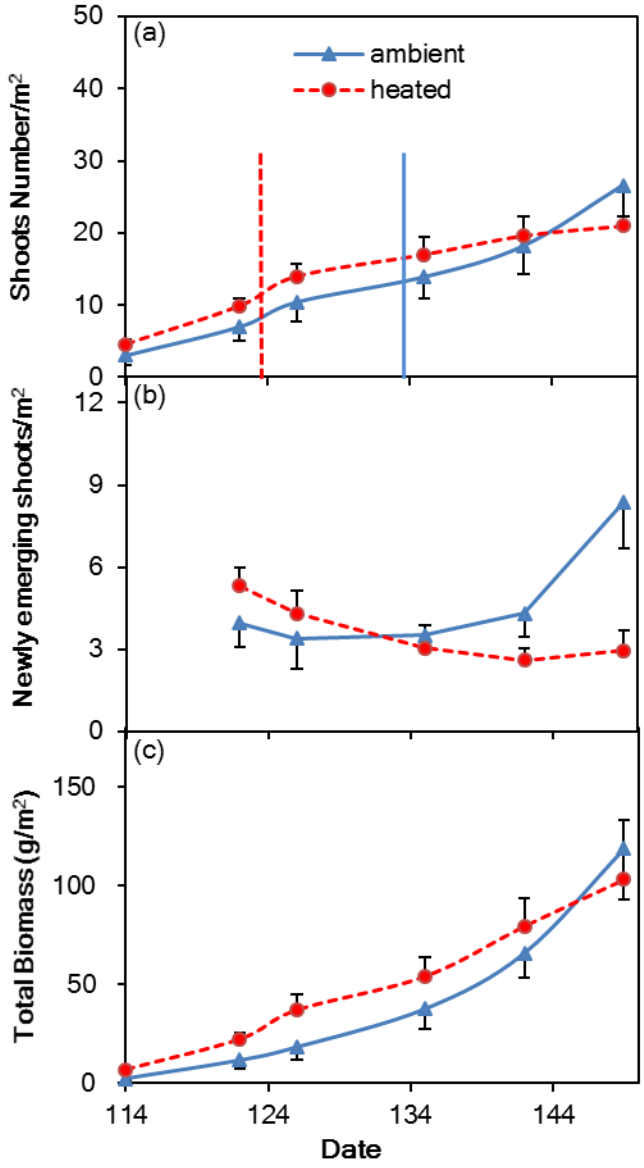

Figure 2.2 The average number of *P. crispus* shoots during the growing season (a), vertical lines represent emerging time of 50% of the total number of shoots. The average newly emerging number of shoots of *P. crispus* (b). The average wet weight biomass of *P. crispus* in each treatment (c). Error bars indicate ± standard error (SE).

Stoichiometric properties

Heating significantly affected the nutrient concentrations and ratios in the *P. crispus* leaves (Table 2.1). In total 109 individuals were measured, 54 for ambient tanks, and 55 for the heated tanks. Heating decreased the carbon and nitrogen concentration in the leaves, whereas there was no significant effect on phosphorus concentrations (Table 2.1). As a result the C:N ratio in the leaves was higher when heated, whereas there were no differences in C:P or N:P ratio (Table 2.1). There was considerable variation between the tanks, but the warming effect was significant when the variation among tanks was taken into account.

Discussion

Warming effects on growth

Warming significantly advanced the emergence of *P. crispus* shoots by ten days in our study. Hansson et al. (2012) found that a 3 °C warming will advance both phytoplankton and zooplankton spring peak abundances about two weeks. Previous studies also found that warming will advance the growth of aquatic plants (Haag and Gorham 1977, Rooney and Kalff 2000). Although some studies demonstrated that warming may increase total aquatic plant biomass (Barko and Smart 1981, Rooney and Kalff 2000, Feuchtmayr et al. 2009), others found no effect (Mckee et al. 2002, McKee et al. 2003) or a decreasing total plant biomass (Barko and Smart 1981). Our results are in line with the studies that did not observe a difference in total plant biomass in response to warming. The contrasting results of the impact of warming on aquatic plant biomass may be related to the optimal temperature range of the plant species studied. Within the optimal range, warming may enhance the growth of the plant, but if the elevated temperature exceeds the optimal range, then high temperatures may hinder the growth of the plant. At temperatures above 24 °C, the growth of *P. crispus* is inhibited, and when temperature surpasses 30 °C, the plant will start to senescence (Ren et al. 1997). During our experiment, the ambient water temperature ranged from 13.9 °C to 27.1 °C, so the growth of plants was probably inhibited more in the heated tanks during the second half of the experiment, as their temperatures were 4.5 °C higher than the tanks exposed to the ambient temperatures. Therefore we conclude that warming will more likely advance the life cycle of *P. crispus* earlier into the season, than increase its biomass in the subtropical middle and lower reaches of Yangtze River.

P. crispus stoichiometry

The nutrient concentration and stoichiometry of *P. crispus* leaves was significantly influenced by warming in the mesocosms. The concentration of C and N decreased in the warmed tanks but there was no significant effect on P concentration. This supports the temperature plant physiological hypothesis, that as the temperature goes up, plants invest less nutrients to produce proteins to sustain biochemistry reactions (Oleksyn et al. 1998, Tjoelker et al. 1999, Reich and Oleksyn 2004, Xia et al. 2014). The C:N ratio in the plants increased in the heated mesocosms, reflecting a lower N-based biomass per unit C in the plants. This indicates

that warming increases the nutrient use efficiency of this aquatic plant. A similar temperature-driven increase in nutrient use efficiency was observed for other angiosperms (Reich and Oleksyn 2004), and in phytoplankton communities (Domis et al. 2014). In our study, the highest total plant biomass in the heated tanks was similar to the biomass in the ambient ones. As the N concentration of each plant decreased with warming, as a consequence, the total amount of N in the plant population declined with warming. The lack of a significant response of P concentration to warming may be partly due to the larger variation in plant P concentration among replicates, as there was a tendency to lower P concentrations with warming. Alternatively, P concentration may be less responsive to warming.

The reasons for the considerable variation between tanks may be that the initial numbers of *P. crispus* propagules in each tank were small and may have differed, also, the turion sprouting time can vary significantly (Jian et al. 2003), and finally turion age may be a factor (Heuschele and Gleason 2014). As our turion were buried in the sediment, we could not establish their age. These may have been sources of variation in sprouting among the tanks.

Consequences for aquatic ecosystems

The response of *P. crispus* to warming can have several consequences for the aquatic ecosystem. With warming, fast growth of a *P. crispus* population could inhibit phytoplankton abundance in early spring resulting in clear water conditions. However, when the temperature further increases beyond the optimal range for *P. crispus* growth, the onset of senescence may also be earlier. During decomposition of this plant material, nutrients will be released to the water column (Bolduan et al. 1994), and, when no other submerged macrophytes dominate, a phytoplankton bloom may follow (Sayer et al. 2010b). As we only tested one macrophyte species, the response of other aquatic plants with different life styles and nutrient acquisition strategies need to be tested, to allow generalisation of these results.

Higher C:N ratios of *P. crispus* with warming reflect lower quality and palatability of food for herbivores (Mattson Jr. 1980, Demment and Van Soest 1985, Elser et al. 2000b, Dorenbosch and Bakker 2011). This may have a negative effect on the higher trophic levels and influence the whole aquatic food web. We conclude that warming alters the timing of *P. crispus* emergence and the nutrient composition of the plant. Therefore, we speculate that warming can affect the timing and duration of the clear water phase and the strength of aquatic food web interactions through its effect on freshwater macrophytes.

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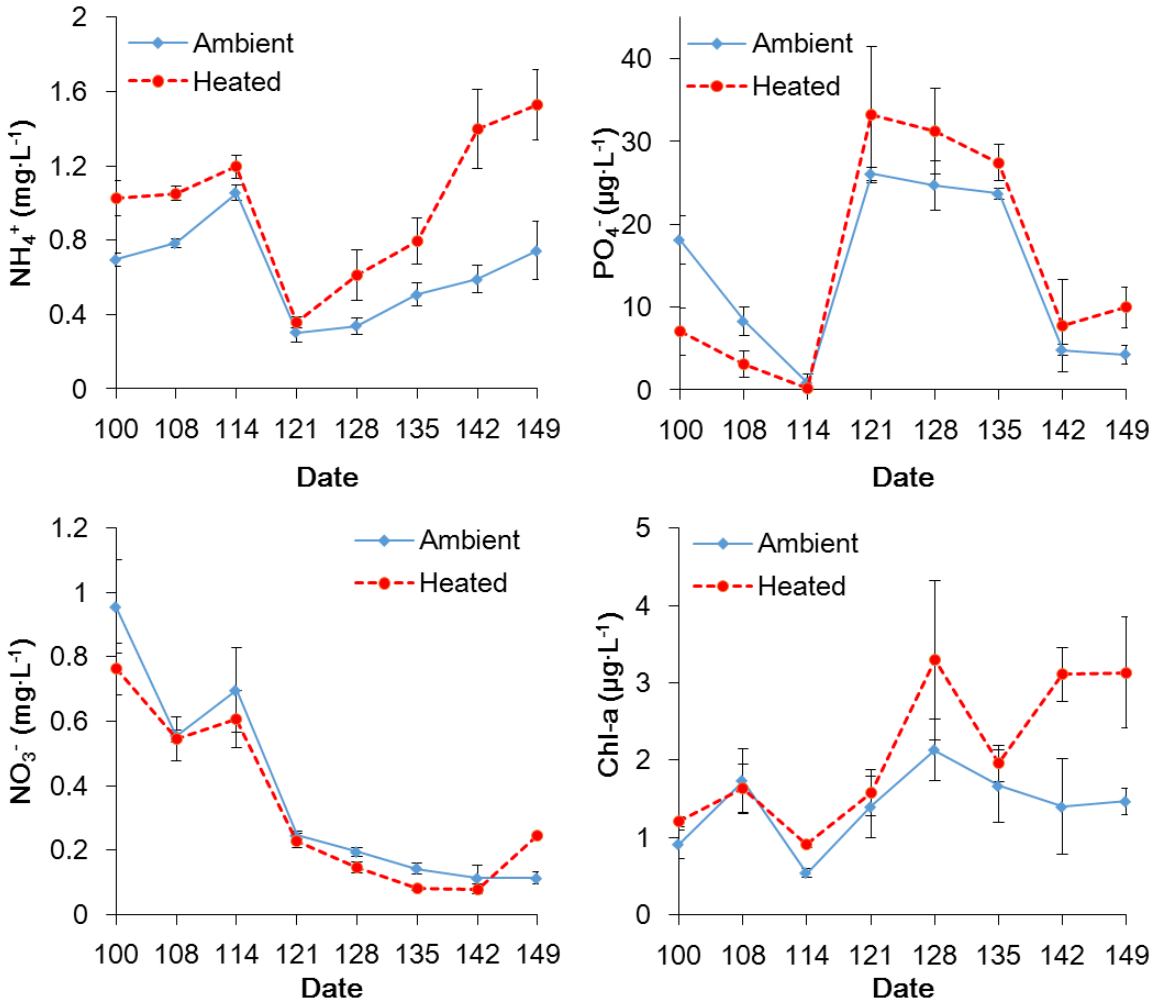

Figure S2.1 Water quality trends during the experiment in the ambient and heated mesocosm (Mean ± SE), n=5.

Chapter 3

Aquatic plant growth increases with temperature whereas plant stoichiometry and palatability show species-specific responses

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Submitted

Abstract

Global warming is expected to strengthen herbivore-plant interactions leading to enhanced top-down control of plants. However, if plant palatability would decline with temperature rise, then this may question the expectation that warming leads to enhanced top-down control. Latitudinal gradients in plant quality as food for herbivores suggest lower palatability at higher temperatures, but the underlying mechanisms are still unclear. Therefore, experiments that directly test the plant palatability and the traits underlying palatability along a temperature gradient are urgently needed. Here we experimentally tested the impact of temperature on aquatic plant growth, plant chemical traits, including stoichiometry, and plant palatability. We cultured three aquatic plant species at three temperatures (15, 20 and 25°C), measured growth parameters, determined chemical traits and performed feeding trial assays using the generalist consumer *Lymnaea stagnalis* (pond snail). We found that rising temperature significantly increased the growth of all three aquatic plants. Plant nitrogen and phosphorous content significantly decreased, and carbon:nutrient stoichiometry increased as temperature increased, especially for *Potamogeton lucens* and *Vallisneria spiralis*, but not for *Elodea nuttallii*. By performing the palatability test, we found a strong decreasing trend in palatability as temperature increased. In *P. lucens*, rising temperatures decreased plant palatability, which could be explained by changes in the underlying chemical plant traits. However, the palatability of *E. nuttallii* and *V. spiralis* was not affected by temperature. While *P. lucens* and *V. spiralis* were always more palatable than *E. nuttallii*. We conclude that warming generally stimulates plant growth, whereas the effects on chemical plant traits and plant palatability are species-specific. These results suggest that the outcome of the impact of temperature rise on macrophyte stoichiometry and palatability from single-species studies may not necessarily be generally valid. In contrast, the plant species tested consistently differed in palatability, regardless of temperature, suggesting that palatability may be more linked to species identity than to intraspecific variation in plant stoichiometry.

Introduction

Global warming is one of the most urgent threats to our ecosystems (IPCC 2014). The effect of warming has become visible in aquatic ecosystems by rising surface water temperatures and a reduction in ice cover over the last decades (Mooij et al. 2005, Woolway et al. 2017). Temperature rise is furthermore expected to lead to alterations in aquatic communities and their food web interactions (Meerhoff et al. 2012). Several of these changes are already observed: average fish size in temperate fish communities decreases with increasing water temperatures, and the communities tend to contain a higher proportion of omnivorous fishes at the expense of carnivory (Jeppesen et al. 2010a). Even without shifting their diet, warming increases the plant consumption rate of ectotherm omnivores and herbivores (Zhang et al. 2018b). Warming is thus expected to strengthen herbivore-plant interactions leading to enhanced top-down control of plants (O'Connor 2009, Gutow et al. 2016).

However, these predictions do not take into account that warming might also affect the plant traits that determine their palatability to herbivores. If plant palatability would decline with temperature rise, then this may question the expectation that warming leads to enhanced top-down control (O'Connor, 2009). Studies mimicking global warming showed inconsistent effects of temperature on plant palatability, either decreasing palatability in marine plants (Rodil et al. 2015), or having no discernible effect in either terrestrial (Backhaus et al. 2014) or marine plants (Poore et al. 2016). Temperature has been suggested to underlie latitudinal gradients in plant quality as food for herbivores: more palatable plants at higher latitudes suggest lower palatability at higher temperatures (Pennings et al. 2007, Morrison and Hay 2012). However, the underlying mechanisms are still unclear: this might be because plants are better defended at lower latitudes (Bolser and Hay 1996), or because plant nutrient content increases with latitude (Reich and Oleksyn 2004, Schemske et al. 2009) or both (Grutters et al. 2017b). In addition, not all studies find latitudinal effects on plant palatability (Adams et al. 2009, Moles et al. 2011), and other factors than temperature may be causing the observed patterns. Therefore, experiments that directly test the effect of temperature on plant palatability and the traits underlying palatability are urgently needed.

Plant palatability depends largely on three groups of plant traits: plant nutritional traits, plant physical structure and plant secondary metabolites (PSM) (Hay 1996, Cronin et al. 2002, Elger and Lemoine 2005). With rising temperature, aquatic plants grow faster (Madsen and Brix 1997, Short and Neckles 1999). With increased growth, there could be a nutrient dilution

effect: where nutrients become limited, the nutrient concentrations in the plant decrease and carbon:nutrient stoichiometry increases (Velthuis et al. 2017). A decrease in plant nitrogen content and increased carbon:nutrient stoichiometry correspond to reduced consumption rates by herbivores (Sterner and Elser 2002, Cebrian and Lartigue 2004, Bakker et al. 2016). Plant physical structure or toughness, here represented by leaf dry matter content (Pennings et al. 1998, Elger and Willby 2003), might also increase with rising temperature, as relatively more carbon accumulates in the tissue. PSM, in particular phenolic compounds, are produced by aquatic plants and can act as deterring compounds (Dorenbosch and Bakker 2011, Grutters et al. 2017b). However, the effect of warming on PSM in aquatic plants is unknown.

In this study, we tested the effect of temperature on aquatic plant growth, tissue stoichiometry, plant physical structure and PSM and the consumption rates of a generalist consumer. We performed two sequential experiments in which we first grew three common submerged freshwater plant species at three temperatures (15, 20, and 25°C). We determined the resultant plant growth and plant traits and subsequently performed a second experiment to test the feeding rates of a generalist consumer on the plants grown at different temperatures. We hypothesized that rising temperature will (1) increase plant growth, (2) decrease plant nutrient concentration and increase carbon:nutrient stoichiometry, (3) increase toughness, (4) decrease consumption rates. We did not have a hypothesis on the response of phenolic compounds, as there is no literature to base a hypothesis on.

Materials and Methods

Aquatic plant growth experiment

We selected three submerged aquatic plant species: *Elodea nuttallii* (Planch.) St. John, *Vallisneria spiralis* L. and *Potamogeton lucens* L. for the plant growth experiment. These species were chosen because of their wide distribution, representation of different plant genera and high palatability to the pond snail *Lymnaea stagnalis* L. (Elger et al. 2004, Grutters et al. 2017b). *E. nuttallii* and *P. lucens* propagules were collected near NIOO-KNAW, Wageningen, The Netherlands. *V. spiralis* seedlings were obtained from the local garden centre (Tuincentrum De Oude Tol, Wageningen, The Netherlands). For *E. nuttallii*, 7 cm apical shoots with 2 or 3 branches were chosen. For *V. spiralis*, plants were chosen with a shoot length of 24.2 ± 3.7 cm (mean ± SD, n = 45), from which the largest outer leaves were removed. This resulted in plants

with 5 to 7 young leaves in the rosette. For *P. lucens*, lower part of stems with 2 or 3 nodes to sprout new roots and leaves were selected, with a length of 23.7 ± 4.9 cm (mean ± SD, n = 45). Plant propagules were planted in the pots, first acclimated at 20°C for 2 weeks, and then assigned to their final controlled temperatures. We grew these plants in fifteen temperature-controlled aquaria (90 × 50 × 50 cm, l × w × h) at 15, 20 and 25°C, each with five replicate aquaria. These temperatures are within suitable ranges of these species in nature. In each aquarium, we had three replicates per species following a random design, totalling nine plants per aquarium. Fifteen un-sprouted *P. lucens* propagules were replaced at the beginning of the culturing (one pot in each aquarium).

Temperature was controlled by an automatic control system (Cascade Automation Systems, Ridderkerk, the Netherlands). A mixing pump was placed inside each aquarium to circulate the water. In order to reduce nutrient competition between different plants, each individual was cultured in a separate pot (top diameter 12.5 cm, bottom diameter 11 cm and height 11 cm). Each pot was filled to a depth of 7 cm with pond sediment (Pokon Naturado, Veenendaal, the Netherlands; rich in organic matter, but low in total nitrogen (TN) 8.5 ± 1.1 mg g^{-1} dry soil and total phosphorous (TP) 0.23 ± 0.05 mg g^{-1} (n = 3, mean ± SD)), and then covered with a 2 cm layer of sand to reduce nutrient release to the water layer. Each aquarium was filled with tap water (TN, 0.087 ± 0.004 mg L^{-1}; TP, 0.013 ± 0.0006 mg L^{-1}; n=3, mean ± SD) yielding a water depth of 30 cm. Demineralized water was added weekly to compensate for evaporation. Two great ramshorn snails *Planorbarius corneus* L. (shell length of 2.7 ± 0.1 cm, mean ± SD, n = 30) were added to each aquarium to control periphyton. They only consumed periphyton, not our plant species and equally consumed periphyton on all plants, as tested in pre-trials. Aquaria were individually lighted by lamps above each aquarium to reach a 16 : 8 h day : night cycle. And the aquaria were individually wrapped in aluminium foil to prevent light interference among aquaria and to increase light intensity within the aquaria by reflection. The light intensity at the water surface was 47.1 ± 3.2 μmol m^{-2} s^{-1} (a moderate light intensity, mean ± SD, n = 7).

Plants were grown in the experiment for 16 weeks from August 16th to December 6th 2015. During the experiment, the plants remained healthy and fresh, as they were kept indoors, and not exposed to natural daylight and the concomitant seasonality. Water quality parameters were measured five times during the experiment (Supplementary Material Fig. S3.1). Conductivity, pH and dissolved oxygen were checked with a multi–meter (Multi 350i/SET, Germany) (Fig. S3.1a,b,c). Alkalinity was measured by an auto-titration machine by adding

acid (0.1M HCl) until a pH of 4.2 was reached (TIM840 titration manager, Germany) (Fig. S3.1d). Chlorophyll a (Chl a) was determined by a phytoplankton analyser (PHYTO-PAM, WALZ, Germany). Before harvesting the plants, part of the water was replaced by tap water in some of the 20 and 25°C aquaria to decrease the phytoplankton biomass (Fig. S3.1e). Ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and orthophosphate (PO_4^{3-}) were analyzed by an AutoAnalyzer (QuAAtro, Seal Analytical, Fareham, UK) after filtering water samples over GF/F filter (Whatman, Maidstone, UK) (Fig. S3.1f,g,h,i). Periphyton was quantified by measuring the dry weight of algae removed from a selected plant surface area at the end of the experiment following (Zimba and Hopson 1997) (see Fig. S3.2 for the periphyton data). The algae were removed by cutting two pieces of *V. spiralis* leaves (leaf surface area of 23.88 ± 6.65 cm^2, mean ± SD, n = 15), shaking them in 30 ml of demineralized water for 30 s, filtering the periphyton onto a pre-weighed GF/F filter (Whatman, Maidstone, UK) and drying the material at 60°C over 48h. The leaf areas of the cut leaves were measured by first scanning the leaves on a piece of A4 paper, then calculating the surface area with ImageJ (Rasband 2015). To investigate how the availability of nutrients to the plants from the sediment in their pots was affected by plant species and temperature, sediment porewater was sampled using rhizons (Rhizosphere, Wageningen, the Netherlands) at the end of the experiment; porewater nutrient concentrations were assumed to be equal among the treatments at the start of the experiment. Total dissolved inorganic nitrogen (TIN: including NH_4^+, NO_3^-, NO_2^-) and PO_4^{3-} concentrations in porewater were determined in one pot per species per aquarium, in total 45 pots were measured. At the end of the experiment, part of the plant tissue in each pot was cut off for the feeding trials and the rest was harvested to quantify dry biomass. Shoots and roots were separately cleaned by high-speed rinsing water till no visible dirty material was attached, including periphyton and sediment particles, and dried in the oven at 60°C over 48 h. Plant relative growth rate was calculated according to the equation: Relative growth rate = ($\ln W_f - \ln W_i$)/Days; with W_f = final dry weight; W_i = initial dry weight. Plant initial dry weight was estimated by drying spare plants at the beginning of the experiment.

Snail feeding experiment

We selected *L. stagnalis* for our feeding trials, a generalist freshwater mollusk that has often been used in feeding trials (Elger and Barrat-Segretain 2002, 2004, Grutters et al. 2017b), and which feeds on a wide variety of vascular aquatic plants (Gaevskaia 1969, Elger et al. 2004).

Mollusks can have a large impact on aquatic plant abundance in the field (Lodge 1991, Newman 1991, Wood et al. 2017). For our feeding experiment, egg clusters from a single population (collected in a pond on the terrain of NIOO-KNAW) were hatched, after two weeks all the juveniles were transferred to plastic buckets, each filled with 15 liters of groundwater (20°C), and exposed to a 16 : 8 h day : night cycle. The snails were fed butterhead lettuce five days per week. Fish food pellets (Velda, Gold Sticks Basic Food, The Netherlands) and chalk were supplied once a week as food supplements. All water was fully replaced once a week. All snails were grown for two months before the feeding trials started. Snails used in the trials had an average shell length of 24.0 ± 1.7 mm (mean ± SD, n = 129).

No-choice feeding trials were carried out to assess whether the temperature at which the plants were grown affected their palatability. The trials followed the standard protocol developed for aquatic snails (Elger and Barrat-Segretain 2002, 2004, Grutters et al. 2017b). In total we used 270 plastic beakers (500 ml), each filled with 375 ml ground water. Prior to the feeding trials, all snails were starved for 48 h following the standard protocol, and all periphyton was removed from all plant material. The 270 beakers were divided in 135 experimental and 135 paired control beakers. Each experimental beaker received plant material from one plant pot, yielding fifteen replicates per plant species grown at each temperature, with in total 135 beakers containing both one snail and plant fragments. The 135 paired control beakers received plant fragments from the same pot as its experimental counterpart that weighed the same amount, to monitor potential autonomous changes in plant weight for each feeding trail during the 24h feeding experiment. Snails were offered approximately 0.1 g (wet weight) of apical shoot of *E. nuttallii*, about 0.4 g (wet weight) newly grown *V. spiralis* leaves, and for *P. lucens*, leaves lower than the third leave from the top were chosen, and cut into two equally sized portions with the midrib removed, and weighed about 0.12 g (wet weight) for each snail. The amount of plant materials offered to snails were the maximum amounts consumed per snail for each plant species as determined in pre-trials. All the plant materials (included the control portion) were cleaned to remove periphyton before being offered to the snails. Beakers were covered with mesh to prevent the snails from escaping. All the trials lasted 24 h and were performed with a 16 : 8 h day : night cycle at a water temperature of 20°C. All feeding trials were randomly divided into two sessions, for logistic reasons. After the feeding trials, snails were first frozen to death at -20°C, and the soft body was separated from its shell, then dried in the oven at 60 °C for at least 48 h. The mean snail dry weight without shell was 0.07 ± 0.01 g

(mean ± SD, n = 129). At the end of the feeding experiment all the remaining plant material was collected and also dried in the oven at 60 °C for at least 48 h and weighed.

Plant Relative Consumption Rate (RCR) (mg g^{-1} d^{-1}) was calculated following Elger and Barrat-Segretain (2002): $RCR = [(C_{fd} / C_{iw}) * F_{iw} - F_{fd}] / S_d /1day$, in which, C_{fd} is the final dry weight of the paired control plant, C_{iw} is the initial wet weight of the paired control plant, F_{iw} is the initial wet weight of the feeding trial plant, F_{fd} is the final dry weight of the feeding trial plant, and S_d is the snail dry weight without shell.

Plant chemical analyses

Plant fragments used as control in the feeding trials were analysed for their dry matter content and chemical composition. Plant dry matter content was determined as the dry weight divided by the wet weight and expressed as percentage. Each plant sample was ground individually in a 2ml tube on a Tissuelyser II (QIAGEN, Hilden, Germany). Plant carbon (C) and nitrogen (N) were determined on an elemental auto analyser (FLASH 2000, Thermo Scientific, Waltham, MA, USA). Phosphorus (P) content was determined by incinerating and digesting the sample, and then analyzing the phosphate concentration on an Auto Analyzer (QuAAtro method, Seal Analytical, Fareham, UK). For total phenolics analysis, between 2 and 4 mg of plant material was extracted with 1 ml of 80 % ethanol for 10 minutes at 80 °C before adding Sodium dodecyl sulfate solution and $FeCl_3$ reagent. The resulting reduction of Fe^{3+} to Fe^{2+} was measured at 510 nm on a spectrophotometer (Synergy HT Microplate Reader, BioTek, Winooski, VT, USA) against a tannic acid calibration curve (Hagerman and Butler 1989, Smolders et al. 2000). We expressed phenolic content as mg tannic acid equivalents per gram plant dry weight.

Data analyses

Data were analysed in multiple linear mixed-effects models. Dependent variables were the 4 plant growth parameters (Shoot biomass, Root biomass, Relative growth rate and Root:Shoot ratio), 2 nutrient parameters (Porewater TIN and PO_4^{3-} concentration), 7 plant traits (Plant dry matter content, N content, P content, C:N ratio, C:P ratio, Total phenolics concentration and N:Phenolics ratio) and Relative Consumption Rates by snails. Effects of temperature and differences between plant species were tested by including temperature as

continuous variable, species as fixed factor and their interaction. Aquarium was included as a random factor. The significance of included terms was tested by model selection based on AICc values (Burnham and Anderson 2002, Burnham et al. 2011). We discuss the contributions of all terms included in the top ranking models (ΔAICc < 2.0 from the best model, for model selection see Table S3.2). To compare and visualize effect sizes of temperature on the 14 dependent variables for individual species we used a second set of models, in which we included only temperature as a continuous predictor variable and aquarium as a random factor.

Three snails (2.2%) died during the feeding experiment, one snail per plant species treatment. Three *P. lucens* (each from one of the temperature treatments) did not have enough material for the feeding trials. These data points were excluded from the dataset. Pearson's correlations were used to test for correlations among all the different plant traits in all species simultaneously and separately within each species. For the mixed-modelling we used the package nlme (John and Sanford 2011, Pinheiro et al. 2016) in R version 3. 4. 1 (R Development Core Team 2017).

Results

Plant growth and sediment nutrients

In our experiment, the final shoot biomass, root biomass and relative growth rate increased significantly with temperature for all plant species (Table 3.1, Table S3.1, Fig. 3.1). The effect sizes differed only in magnitude among species, with *P. lucens* showing the largest response to temperature increase (Table 3.1, Fig. 3.1). The plant root:shoot ratio showed a species-specific response to increasing temperature: for both *P. lucens* and *E. nuttallii* root:shoot ratios decreased as temperature increased, whereas there was no temperature effect on *V. spiralis* (Fig. 3.1).

Sediment porewater total inorganic nitrogen (TIN) and PO_4^{3-} concentrations decreased as temperature increased. The effect sizes also differed among species, with *P. lucens* showing the largest decrease as temperature increased (Table 3.1, Fig. 3.1). During the experiment, alkalinity decreased from 1.5 to 1.0 meq L^{-1} (Fig. S3.1d). Nutrients (N and P) were limiting in the water layer (almost 0 after the first two weeks, Fig. S3.1f,g,h,i), but not limited in the sediment (Table S3.1).

Table 3.1 Linear mixed-effect model results for the effects of temperature and species on the plant growth and sediment pore water nutrient parameters, plant traits and RCR.

Category	Parameters	Factors	df	*F*	*p*
Plant growth	Shoot biomass	Temperature	1, 13	211.93	< **0.001**
		Species	2, 110	82.45	< **0.001**
		Temperature * Species	2, 110	43.68	< **0.001**
	Root biomass	Temperature	1, 13	56.99	< **0.001**
		Species	2, 110	131.39	< **0.001**
		Temperature * Species	2, 110	20.87	< **0.001**
	Growth rate	Temperature	1, 13	43.45	< **0.001**
		Species	2, 110	429.65	< **0.001**
		Temperature * Species	2, 110	8.47	< **0.001**
	Root:Shoot ratio	Temperature	1, 13	4.54	0.053
		Species	2, 110	419.52	< **0.001**
		Temperature * Species	2, 110	23.27	< **0.001**
Porewater nutrients	TIN[a]	Temperature	1, 13	3.68	0.077
		Species	2, 26	12.62	< **0.001**
		Temperature * Species	2, 26	2.05	0.149
	PO_4^{3-}	Temperature	1, 13	16.76	**0.001**
		Species	2, 26	8.78	**0.001**
		Temperature * Species	2, 26	3.17	0.059
Palatability	RCR[b]	Temperature	1, 13	4.01	0.067
		Species	2, 112	8.42	< **0.001**
Traits	Dry matter content	Temperature	1, 13	2.15	0.166
		Species	2, 110	448.82	< **0.001**
		Temperature * Species	2, 110	8.80	< **0.001**
	N content	Temperature	1, 13	5.57	**0.035**
		Species	2, 110	17.51	< **0.001**
		Temperature * Species	2, 110	6.85	**0.002**
	P content	Temperature	1, 13	4.82	**0.047**
		Species	2, 110	58.84	< **0.001**
		Temperature * Species	2, 110	17.33	< **0.001**
	C:N ratio	Temperature	1, 13	3.02	0.106
		Species	2, 110	9.37	< **0.001**
		Temperature * Species	2, 110	4.32	**0.016**
	C:P ratio	Temperature	1, 13	7.14	**0.019**
		Species	2, 110	68.50	< **0.001**
		Temperature * Species	2, 110	18.88	< **0.001**
	Total phenolics content	Temperature	1, 13	1.55	0.235
		Species	2, 110	318.23	< **0.001**
		Temperature * Species	2, 110	9.23	< **0.001**
	N:Phenolics ratio	Temperature	1, 13	4.13	0.063
		Species	2, 110	69.74	< **0.001**
		Temperature * Species	2, 110	6.51	**0.002**

[a] TIN means total dissolved inorganic nitrogen. [b] RCR means plant relative consumption rate. Bold numbers indicate a significance of $p < 0.05$.

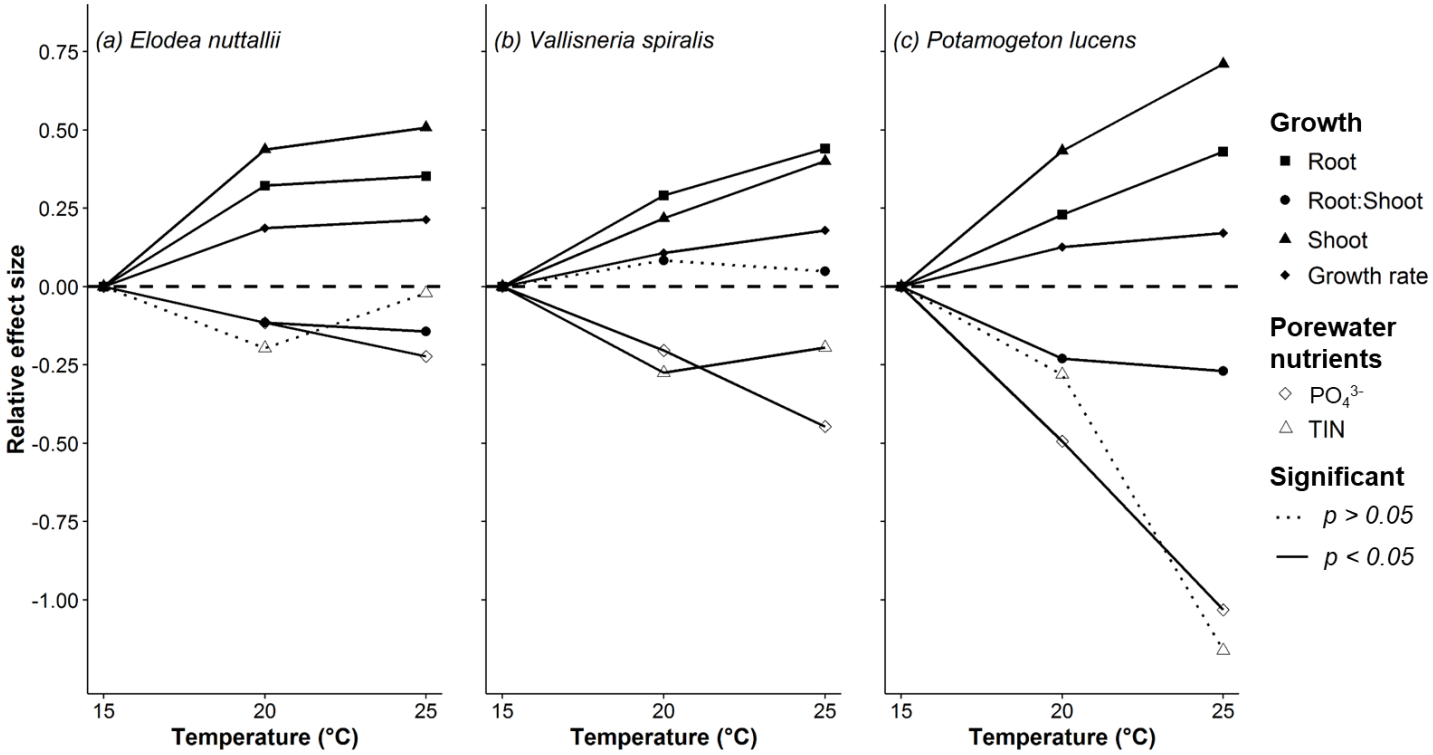

Figure 3.1 Relative effect sizes of the response of aquatic plant growth and available nutrients to rising temperature. Parameter estimates are indicated relative to the intercept formed by the 15°C treatment. Values in the plots were obtained by dividing the trait values of a temperature treatment by those of the 15°C treatment, and subsequently log10 transformed. (a) *E. nuttallii*; (b) *V. spiralis*; (c) *P. lucens*; TIN indicate sediment pore water total dissolved inorganic nitrogen concentrations. Significant effects of temperature are indicated by a solid line, non-significant temperature effects by a dotted line.

Plant traits

Plant stoichiometry traits showed a species-specific response to rising temperatures (Table 3.1, Fig. 3.2). Temperature had an effect on both plant N and P content, which showed a significant decrease with rising temperature for *P. lucens* and *V. spiralis*, though there was no significant influence on *E. nuttallii* (Fig. 3.2). The plant C:N ratio significantly increased for *P. lucens* but not for the other species (Fig. 3.2). The plant C:P ratio significantly increased with rising temperature in both *P. lucens* and *V. spiralis*, whereas there were no effects on *E. nuttallii* (Fig. 3.2, Table S3.1).

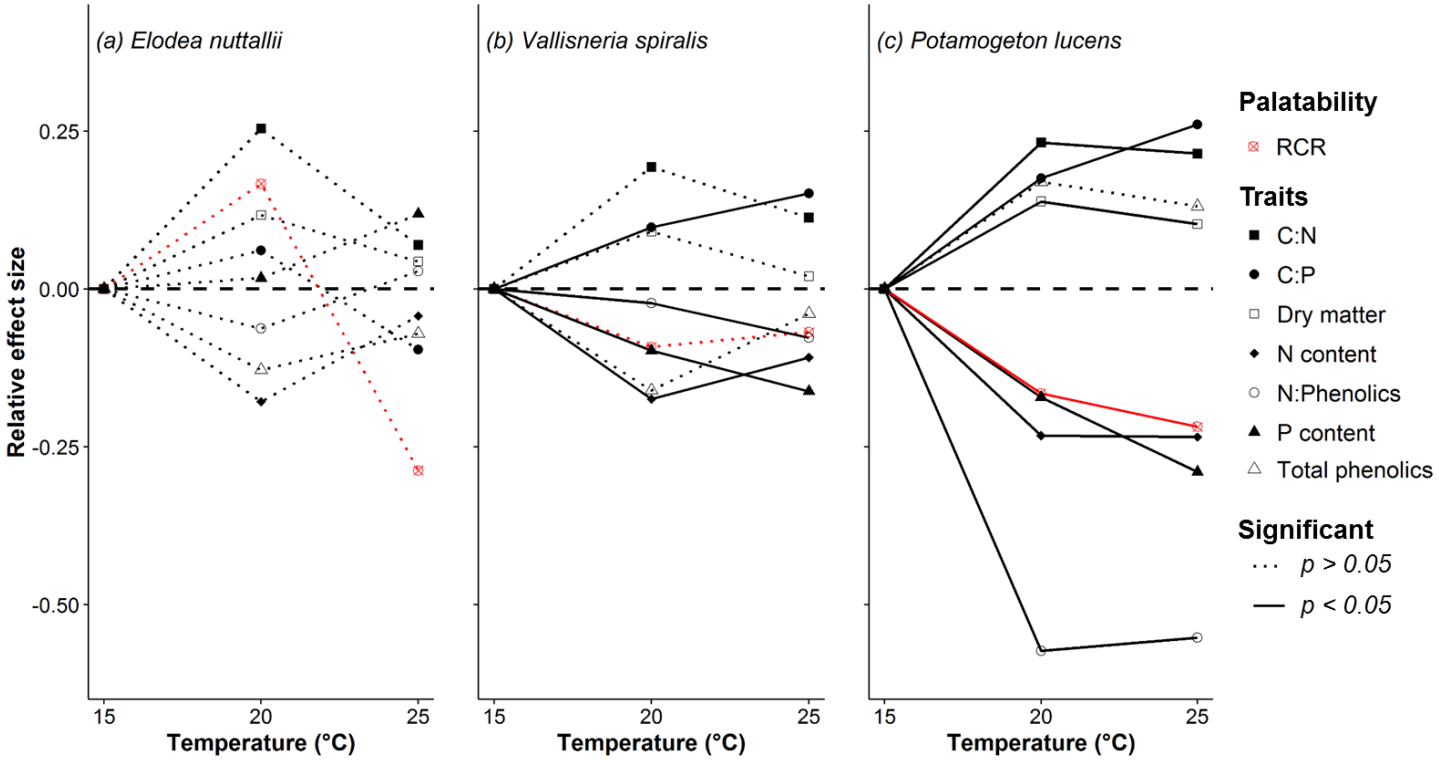

Figure 3.2 Relative effect sizes of the response of aquatic plant palatability and traits to rising temperature. Parameter estimates are indicated relative to the intercept formed by the 15°C treatment. Values in the plots were obtained by dividing the trait values of a temperature treatment by those of the 15°C treatment, and subsequently log10 transformed. (a) *E. nuttallii*; (b) *V. spiralis*; (c) *P. lucens*. RCR indicates plant relative consumption rate, which indicates palatability. Significant effects of temperature are indicated by a solid line, non-significant temperature effects by a dotted line.

In *P. lucens* and *V. spiralis*, we found that foliar N and P content were negatively correlated with plant total biomass (Fig. 3.3a,b) and were positively correlated with sediment porewater TIN concentration (Fig. 3.3c) and PO_4^{3-} concentration (Fig. 3.3d), respectively. The sediment porewater nutrient concentrations were negatively correlated with plant total biomass (Fig. 3.3e, f).

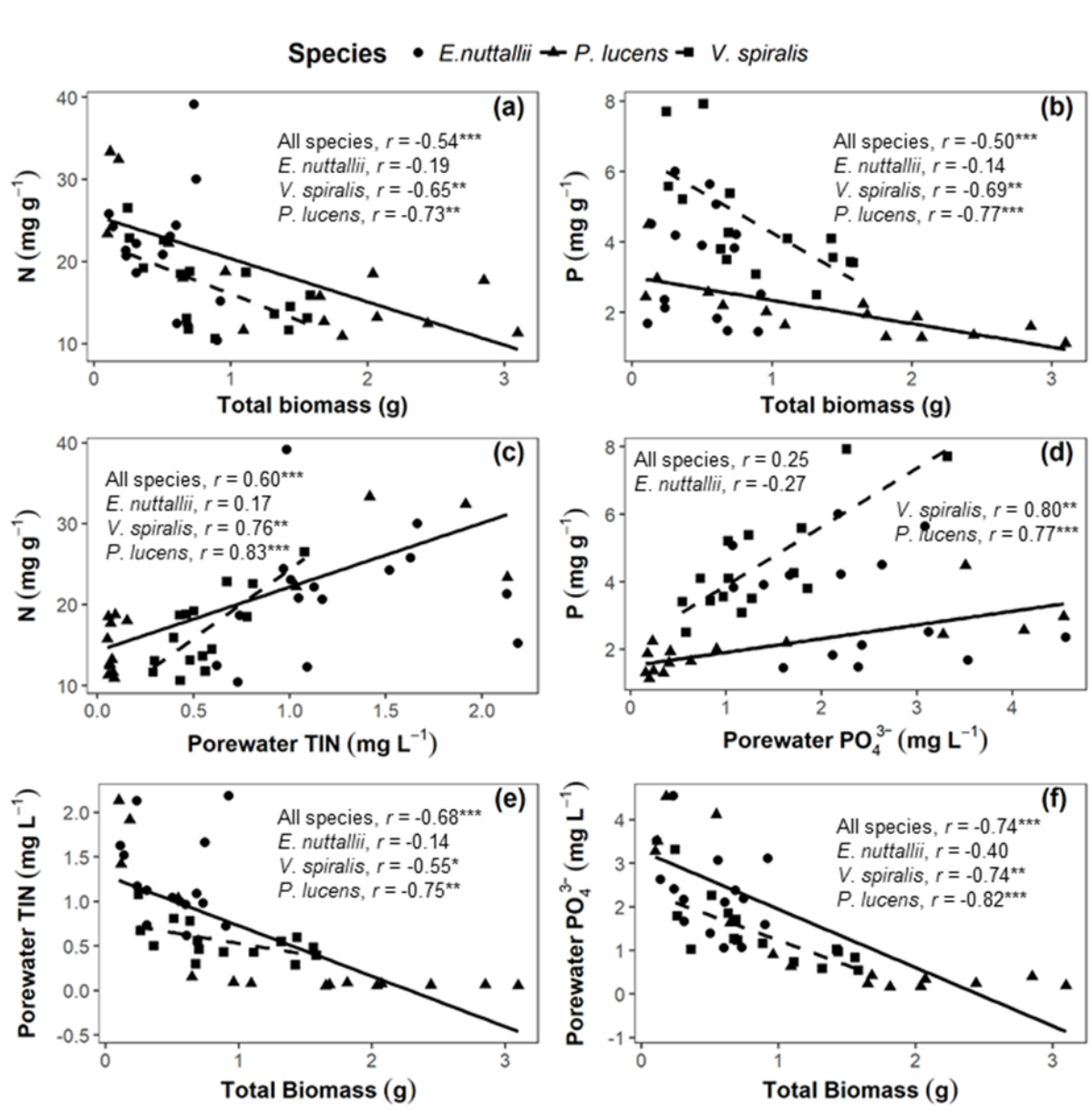

Figure 3.3 Pearson's correlations of plant total biomass, plant nutrient contents and porewater nutrient concentrations for the three tested species. Each species has 15 data points from every temperature treatment, in total 45 points for each panel. Lines in the graph indicate that factors are significantly correlated at the species level ($p < 0.05$). (a) N content in the plants in relation to the total plant biomass. (b) P content in the plants in relation to the total plant biomass. (c) N content in the plants in relation to the total inorganic nitrogen concentration in the porewater. (d) P content in the plants in relation to the phosphorous concentration in the porewater. (e) Total plant biomass in relation to the total inorganic nitrogen concentration in the porewater. (f) Total plant biomass in relation to the phosphorous concentration in the porewater. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

Table 3.2 Pearson's correlation coefficients among all the investigated plant quality traits for all three species pooled (n = 45), and for each species separately (n=15). The directions of the significant correlations that are the same in all three species are indicated as bold red in the table.

		RCR[a]	Dry matter[b]	N	P	C:N	C:P	Phenolics
All species	**RCR[a]**	1						
	Dry matter[b]	0.06	1					
	N	0.04	**-0.25**	1				
	P	0.04	-0.81***	**0.32***	1			
	C:N	0.03	**0.33***	**-0.91*****	**-0.41****	1		
	C:P	-0.03	0.86***	**-0.37***	**-0.93*****	**0.48*****	1	
	Phenolics	0.10	0.89***	-0.10	0.64***	0.11	0.69***	1
	N:Phenolics	-0.05	-0.87***	0.48***	**0.75*****	-0.45**	**-0.79*****	-0.86***
E.nuttallii	**RCR[a]**	1						
	Dry matter[b]	0.30	1					
	N	-0.17	-0.85***	1				
	P	-0.50	-0.70**	0.61*	1			
	C:N	0.34	0.93***	-0.88***	-0.64*	1		
	C:P	0.52*	0.81***	-0.68**	-0.92***	0.81***	1	
	Phenolics	-0.02	-0.80***	0.92***	0.39	-0.81***	-0.49	1
	N:Phenolics	-0.44	-0.74**	0.82***	0.77***	-0.82***	-0.81***	0.55*
V.spiralis	**RCR[a]**	1						
	Dry matter[b]	-0.08	1					
	N	0.27	-0.83***	1				
	P	0.21	-0.42	0.73**	1			
	C:N	-0.22	0.89***	-0.98***	-0.65**	1		
	C:P	-0.23	0.38	-0.69**	-0.96***	0.62*	1	
	Phenolics	0.14	-0.71**	0.80***	0.26	-0.81***	-0.25	1
	N:Phenolics	0.22	-0.30	0.41	0.78***	-0.38	-0.73**	-0.20
P.lucens	**RCR[a]**	1						
	Dry matter[b]	-0.69**	1					
	N	0.82***	-0.92***	1				
	P	0.79***	-0.79***	0.92***	1			
	C:N	-0.77***	0.87***	-0.97***	-0.86***	1		
	C:P	-0.76***	0.71**	-0.88***	-0.96***	0.87***	1	
	Phenolics	-0.57*	0.74**	-0.65**	-0.46	0.59*	0.45	1
	N:Phenolics	0.85***	-0.89***	0.90***	0.81***	-0.79***	-0.71**	-0.74**

[a] Plant relative consumption rate. [b] Plant dry matter content. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

Other plant traits also showed species-specific responses (Table 3.1, Fig. 3.2). Plant dry matter content, significantly increased with rising temperature in *P. lucens* (Fig. 3.2c), but not in the other species. For plant total phenolics, there was a significant difference among species, but no temperature effects for any of the species (Fig. 3.2). In contrast, for the plant N:Phenolics

ratio, both *P. lucens* and *V. spiralis* showed a significant decrease with rising temperature, whereas no significant influence was found on *E. nuttallii* (Fig. 3.2). Overall, *P. lucens* showed the largest response for all the plant traits, while none of the traits in *E. nuttallii* were significantly affected by temperature (Fig. 3.2).

There were several general correlations in chemical plant traits in all three species (Table 3.2). In all species, dry matter content correlated negatively with N content, and positively with plant C:N ratio. N content and P content were positively correlated with each other in all species. Most chemical plant traits were correlated with each other within the aquatic plant species, but there were differences between the species as to the strength and direction of the correlations (Table 3.2).

Plant palatability

Plant palatability (expressed as the relative consumption rate by the snails, RCR), generally showed a decreasing trend with increasing temperature ($p = 0.067$) (Table 3.1, Fig. 3.2), but differed among species ($p < 0.001$) (Table 3.1), and *P. lucens* and *V. spiralis* were always more palatable than *E. nuttallii* (followed by a post-hoc test). On a species level, the palatability of *P. lucens* decreased with rising temperature ($p < 0.05$). Palatability was not related to any of the measured plant traits when all species were pooled. Intraspecifically, in *P. lucens*, palatability was negatively correlated with dry matter content, C:N, and C:P ratio and total phenolics and it correlated positively with N and P content and the N:Phenolic ratio (Table 3.2). For *E. nuttallii*, only the plant C:P ratio correlated positively with palatability, while for *V. spiralis*, none of the measured plant traits correlated significantly with palatability (Table 3.2).

Discussion

In this study, we tested the effects of water temperature on the growth, chemical plant traits and the resultant palatability of three submerged aquatic plants. Temperature rise significantly increased plant growth, increased tissue C: nutrient ratios and there was a trend towards lower palatability, whereas interestingly, part of these effects were species-specific.

Plant growth

Rising temperatures enhanced plant growth in our experiment, confirming our first hypothesis, which has also been previously observed in the laboratory (Barko and Smart 1981, Madsen and Brix 1997, Velthuis et al. 2017) and the field (Rooney and Kalff 2000, Feuchtmayr et al. 2009). The optimum temperatures for photosynthesis for temperate submerged aquatic plants are usually located between 25 and 32°C (Barko et al. 1982, Santamaría and van Vierssen 1997, Pedersen et al. 2013), indicating that our highest temperature of 25°C was close to optimal for growth. The plants should have had enough carbon for growth during the experiment, as the alkalinity was always above 1.0 meq L^{-1} (Vestergaard and Sand-Jensen 2000a, b). The plant species that we used can take up nutrients from both sediment and water (Barko et al. 1986). Nutrients were limited in the water during the experiment (Fig. S3.1); there were much higher amounts of nutrients available in the sediment, hence this was the main source of nutrients for plant growth. It seemed that *P. lucens* had the best resource uptake strategy, as it rooted deep into the sediment, which we observed when washing the roots, and the nutrients in the sediment were depleted the fastest. *V. spiralis* developed roots only shallowly into the sediment, and *E. nuttallii* barely developed roots in the sediment, its roots were mainly in the water. Especially *E. nuttallii* can take up high amounts of nutrients from the water if these are available. That could also be the reason that *E. nuttallii* formed the lowest biomass of the three species, as this species may have suffered from the nutrient limitations in the water layer in this experiment. According to optimal partitioning theory, plants allocate more biomass to the roots when the available nutrients are lower in the sediment (Bloom et al. 1985). Indeed, as temperature increased, *E. nuttallii* and *P. lucens* showed a decrease in root:shoot ratio, which is consistent with the optimal partitioning theory. As we measured lower levels of nutrients in the sediment at higher temperatures in our experiment, and lower root:shoot ratios in two species, it seems that at higher temperatures, these plants can utilize nutrients better to accumulate biomass.

Plant traits

Our results showed that higher temperature led to faster growth and lower nutrient availability, which in turn led to lower tissue nutrients in two of the three plant species (*P. lucens* and *V. spiralis*). The observed shifts in nutrient content and stoichiometry follow the temperature-plant physiological hypothesis (Reich and Oleksyn 2004), which predicts that plant N and P content declines with increasing temperatures. At higher temperatures plants invest

less nutrients per carbon for their metabolism and growth (Reich and Oleksyn 2004, Zhang et al. 2016). This corresponds with our finding that there were lower levels of nutrients in the sediment at higher temperatures at which these plants can utilize nutrients better to accumulate biomass. We also found that there were strong negative correlations between macrophyte biomass and plant nutrient content and positive correlations between plant nutrient content and sediment porewater nutrient concentration. This means that there was a strong effect of nutrient dilution in plant tissue by increasing total biomass. This effect was not seen in *E. nuttallii*, which may have been less efficient in obtaining nutrients from the sediment and may have suffered nutrient limitation during the experiment. The rooted plants on the other hand, may eventually suffer from nutrient limitation at higher temperatures, but have more nutrients available from the sediment and at first overcome nutrient limitation by the physiological adaptation to invest less nutrients per carbon for their growth.

The tissue stoichiometry for *E. nuttallii*, and N content and C:N ratio for *P. lucens* and *V. spiralis* between 20 and 25°C, seemingly deviating from the trend, might have been caused by altered nutrient availability in the water layer at higher temperatures. Warming increases sediment respiration which probably increases the nutrient release from the sediment to the water (Liboriussen et al. 2011, Alsterberg et al. 2012, Zhang et al. 2012); this might result in higher nutrient availability at higher temperatures in the water layer (Ventura et al. 2008). These nutrients in the water could be taken up by aquatic plants, periphyton and phytoplankton (van Donk and van de Bund 2002).There was less periphyton at higher temperatures (Fig. S3.2; possibly related to an increased grazing pressure by the periphyton grazing snails at higher temperatures), and more phytoplankton accumulated at higher temperatures. All in all, the rising temperature might have affected the nutrient availability in the water, and resulted in the differential responses of tissue stoichiometry in the aquatic plants.

Dry matter content has been assumed to be negatively correlated with plant nutrient content (Elger and Willby 2003), which was true in all our three species. As temperature increased, plant nutrient content decreased, and then we can expect an increase in plant dry matter content. Rising temperature increased plant dry matter content in *P. lucens*, but not in the other two species.

There was no temperature effect on the total phenolics content, which is consistent with previous research on terrestrial plants (Jamieson et al. 2014). *P. lucens* had the highest total phenolics content among the three species, but was also preferred by *L. stagnalis*. This may have been due to the low total phenolic concentrations that we measured in our plants, even in

P. lucens, compared to other aquatic plants species (Grutters et al. 2017). In the comparison among 40 aquatic plants species of Grutters et al. (2017), 36 species have a higher phenolic concentration than *P. lucens* in our study. Hence, the total phenolic concentration may have been too low to deter snail feeding. Previous studies also showed that total phenolics could not adequately predict aquatic plant palatability (Elger and Lemoine 2005, Boiché et al. 2011). Furthermore, the correlation between N content and total phenolics concentration correlation showed different directions in the three species. This may also indicate that total phenolics can at best be considered a rough indicator of plant defense in aquatic plants (Gross and Bakker 2012), whereas there are specific phenolic compounds that determine anti-herbivore defenses (Bidart-Bouzat and Imeh-Nathaniel 2008, Harvey 2015), but the identity of these compounds is at present largely unknown in most freshwater plants.

Plant palatability

Because we observed the hypothesized changes in plant growth and in plant nutrient content and stoichiometry in two of our three tested plant species, we also expected that plant palatability would be reduced with increasing temperature. Indeed, aquatic plant palatability showed a decreasing trend as temperature increased, but this was at the species level only significant in *P. lucens*. Also among studies which each used different species did warming decrease marine plant palatability (Rodil et al. 2015), or had no effect (Poore et al. 2016). Therefore, we conclude that the effect of warming on plant palatability is to a certain extent species-specific, in our study depending on the plant species identity. In analogy, variation in the palatability of seaweeds across latitudes was recently found to vary with both plant and herbivore identity (Demko et al. 2017), and different generalist herbivores might respond differently to the same plant (Boiché et al. 2011). Here, it should be noted that we measured a plastic response of plants to temperature within a generation, whereas latitudinal gradients in plant traits and palatability are the result of selection pressures operating over generations. Similarly, the measured responses are short-term, whereas alterations in plant traits in response to climate change, including global warming, would be a slow process operating over generations.

Overall, in our study, plant palatability was significantly negatively correlated with plant dry matter content, C:nutrient ratio and total phenolics, and positively correlated with plant nutrient (N and P) content and N:Phenolics ratio in *P. lucens*, but not in the other two plant

species. Hence, all hypothesized relationships between plant traits and palatability, based on the literature, were true for *P. lucens*. *P. lucens* also responded to temperature rise as we expected both in its growth, chemical traits and palatability and moreover, we can understand the responses, as they are coherent with each other. However, *P. lucens* is clearly not representative for all aquatic plants, as the other two tested species responded differently and less consistently in their plant growth, chemical traits and palatability relationships. Possibly, the measured plant traits might be better in predicting plant palatability on an interspecies level, instead of intra specifically. Across a wide range of aquatic plant species palatability increased with decreasing dry matter content (Elger and Willby 2003, Elger and Lemoine 2005), and increasing N:phenolics ratio (Grutters et al. 2017b), and among different functional plant groups, consumption rates increased with N content (Cebrian and Lartigue 2004) and decreased with C:nutrient ratio (Elser et al. 2000b, Bakker et al. 2016, Grutters et al. 2016).

Implications for the aquatic ecosystem

The plant species tested differed strongly in resource uptake, which may give some species competitive advantages over other species in warming ecosystems. Consequently, warming might alter the aquatic plant community composition (Mckee et al. 2002, Zhang et al. 2015, Li et al. 2017). Similarly, under current global warming trends, the stoichiometry mismatch with higher trophic levels may enlarge with an increasing carbon:nutrient ratio in some plant species. As a consequence, the palatability difference between plant species may change, which may lead to a different pressure from herbivores on some species as compared to others, which may also change the aquatic plant community composition and abundance (Schiel et al. 2004, Harley et al. 2012).

Interestingly, the impact of herbivores on plants was found to increase with increasing temperature in a macro-algae which did not change in palatability to the tested herbivore (O'Connor 2009). However, our data show that the palatability did not change with temperature in two and decreased in one plant species. Hence an open question is how the balance between potentially reduced plant palatability and increased consumption rates of ectotherm animals with warming will play out. Since ectotherm animals will eat more with increasing temperatures (Zhang et al. 2018b), they may still induce a larger grazing pressure on the plants. However, if plant quality drops with temperature rise, the question is whether they remain good enough as

food to sustain the ectotherm consumer population. The combination of these two will determine future grazing pressure on plants in aquatic ecosystems with rising temperatures.

Acknowledgements

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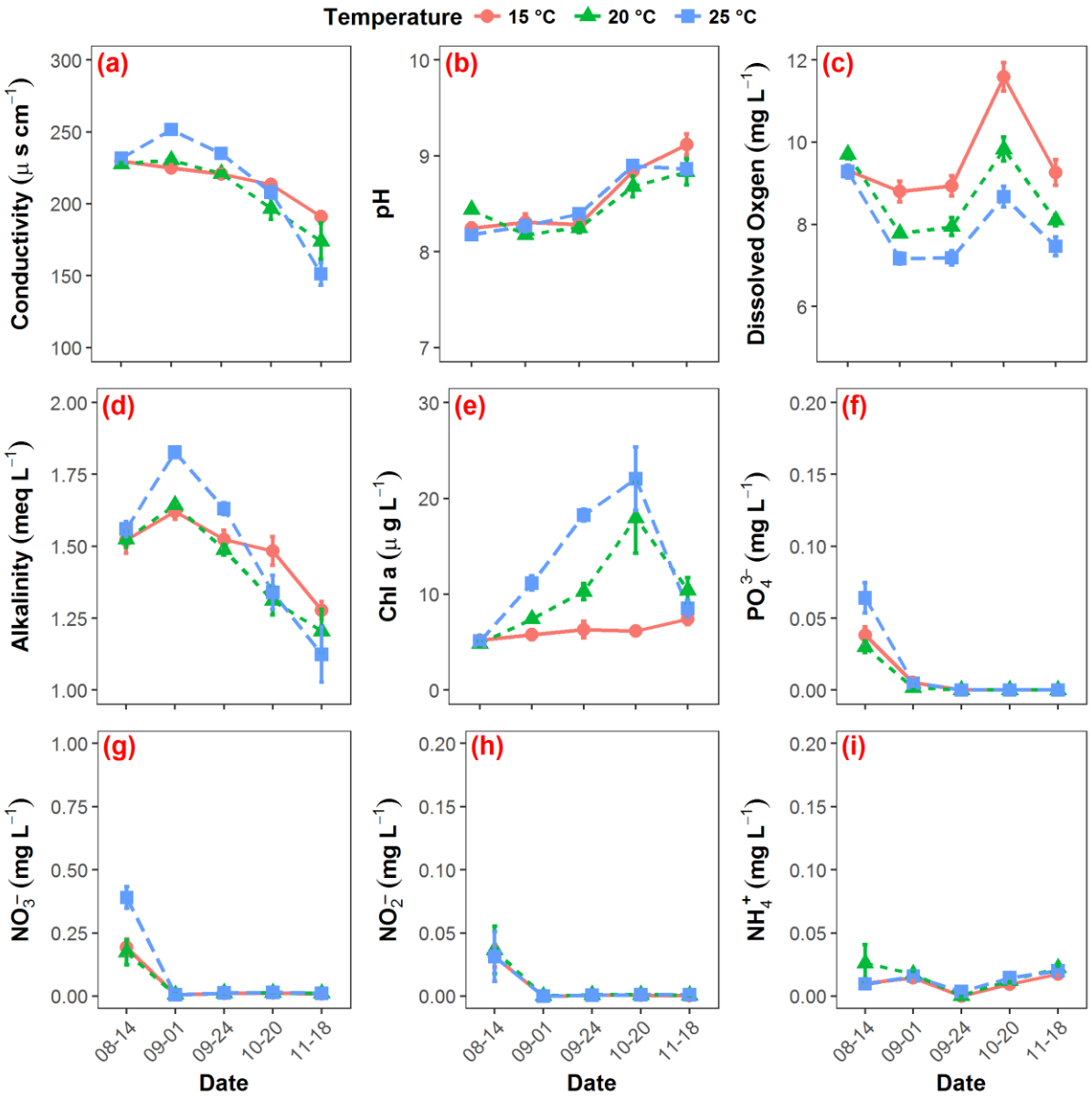

Figure S3.1 Water quality measurements during 16 weeks of the aquatic plants growth experiment, at three different water temperatures. (a) Conductivity; (b) pH; (c) Dissolved Oxygen; (d) Alkalinity; (e) Chlorophyll a; (f) PO_4^{3-}; (g) NO_3^-; (h) NO_2^-; (i) NH_4^+. Error bars are standard deviations.

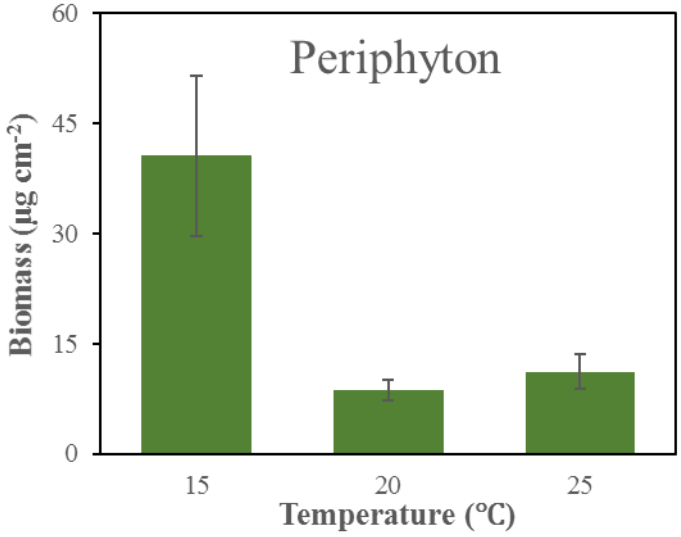

Figure S3.2 Final periphyton biomass that developed at the three different temperature treatments. One-way mixed effect model (with temperature as a continuous predictor and aquarium as a random factor) indicates a significant ($F_{1,13}$ = 7.42, p = 0.017) decrease of periphyton biomass with rising temperature. Error bars indicate standard errors. The amount of periphyton was very low (not much on the surface of all the plants was visible) in all the temperature treatments.

Table S3.1 Parameter values (mean ± standard error) and statistical results of temperature effect on each species. Weight values are grams dry weight in all cases. Ratios are based on g g^{-1} dry weight. Statistics report the results of one-way mixed effect models with temperature as a continuous predictor and aquarium as a random factor. TIN represents total dissolved inorganic nitrogen. TIN and PO_4^{3-} concentration were measured in sediment porewater. RCR represents plant relative consumption rate. Bold numbers indicate a significance of $p < 0.05$.

Species	Parameters	15 °C	20 °C	25 °C	$F_{1,13}$	p
E.nuttallii	Shoot (g)	0.19 ± 0.02	0.53 ± 0.05	0.62 ± 0.09	20.87	< **0.001**
	Root (g)	0.036 ± 0.005	0.076 ± 0.008	0.081 ± 0.011	8.67	**0.011**
	Growth rate (g g^{-1} d^{-1})	0.015 ± 0.001	0.023 ± 0.002	0.024 ± 0.001	8.98	**0.010**
	Root:Shoot	0.19 ± 0.02	0.15 ± 0.01	0.14 ± 0.01	6.32	**0.026**
	PO_4^{3-} (mg L^{-1})	2.96 ± 0.50	2.27 ± 0.24	1.77 ± 0.40	4.96	**0.044**
	TIN (mg L^{-1})	1.44 ± 0.23	0.91 ± 0.10	1.37 ± 0.24	0.05	0.834
	RCR (mg g^{-1} d^{-1})	29.20 ± 8.75	42.83 ± 12.13	15.07 ± 11.48	0.74	0.406
	Dry matter (%)	10.25 ± 0.25	13.41 ± 1.03	11.31 ± 0.45	0.39	0.544
	N (mg g^{-1})	24.45 ± 1.57	16.19 ± 1.86	22.14 ± 1.77	0.29	0.602
	P (mg g^{-1})	2.92 ± 0.28	3.04 ± 0.42	3.84 ± 0.32	2.33	0.151
	C:N (g g^{-1})	16.96 ± 0.84	29.94 ± 3.83	19.57 ± 1.29	0.18	0.680
	C:P (g g^{-1})	149.7 ± 13.0	172.3 ± 24.08	120.0 ± 15.2	0.70	0.418
	Total Phenolics (mg g^{-1})	10.53 ± 0.35	7.84 ± 0.56	8.94 ± 0.64	1.95	0.186
	N:Phenolics	2.32 ± 0.11	2.00 ± 0.12	2.47 ± 0.07	0.62	0.446
V.spiralis	Shoot (g)	0.31 ± 0.02	0.50 ± 0.03	0.77 ± 0.03	108.58	< **0.001**
	Root (g)	0.22 ± 0.02	0.43 ± 0.03	0.60 ± 0.03	81.11	< **0.001**
	Growth rate (g g^{-1} d^{-1})	0.020 ± 0.001	0.025 ± 0.001	0.030 ± 0.001	55.60	< **0.001**
	Root:Shoot	0.71 ± 0.04	0.86 ± 0.04	0.79 ± 0.03	1.30	0.274
	PO_4^{3-} (mg L^{-1})	2.05 ± 0.38	1.28 ± 0.12	0.73 ± 0.08	17.39	**0.001**
	TIN (mg L^{-1})	0.77 ± 0.09	0.41 ± 0.05	0.49 ± 0.04	5.95	**0.030**
	RCR (mg g^{-1} d^{-1})	71.01 ± 12.18	57.49 ± 13.08	60.66 ± 15.04	0.28	0.603
	Dry matter (%)	7.55 ± 0.30	9.30 ± 0.42	7.91 ± 0.23	0.23	0.639
	N (mg g^{-1})	18.70 ± 0.69	12.52 ± 0.63	14.56 ± 0.48	6.53	**0.024**
	P (mg g^{-1})	5.17 ± 0.32	4.13 ± 0.16	3.56 ± 0.11	28.09	< **0.001**
	C:N (g g^{-1})	21.29 ± 0.94	33.19 ± 1.48	27.58 ± 0.94	3.59	0.081
	C:P (g g^{-1})	79.65 ± 5.48	99.65 ± 3.95	112.9 ± 4.13	22.31	< **0.001**
	Total Phenolics (mg g^{-1})	8.62 ± 0.46	5.95 ± 0.18	7.87 ± 0.33	0.76	0.398
	N:Phenolics	2.24 ± 0.13	2.13 ± 0.12	1.88 ± 0.07	5.37	**0.037**
P.lucens	Shoot (g)	0.37 ± 0.06	0.99 ± 0.11	1.88 ± 0.11	132.98	< **0.001**
	Root (g)	0.24 ± 0.04	0.40 ± 0.06	0.63 ± 0.06	29.15	< **0.001**
	Growth rate (g g^{-1} d^{-1})	0.044 ± 0.003	0.058 ± 0.003	0.065 ± 0.002	38.39	< **0.001**
	Root:Shoot	0.62 ± 0.04	0.36 ± 0.04	0.33 ± 0.01	18.95	< **0.001**
	PO_4^{3-} (mg L^{-1})	2.94 ± 0.71	0.94 ± 0.59	0.27 ± 0.40	12.46	**0.004**
	TIN (mg L^{-1})	0.92 ± 0.36	0.48 ± 0.41	0.06 ± 0.004	4.04	0.066
	RCR (mg g^{-1} d^{-1})	83.39 ± 10.50	56.95 ± 8.74	50.42 ± 12.56	4.82	**0.047**
	Dry matter (%)	18.51 ± 1.15	25.46 ± 0.87	23.44 ± 0.54	7.43	**0.017**
	N (mg g^{-1})	24.48 ± 1.62	14.34 ± 1.05	14.26 ± 0.71	18.25	< **0.001**

Species	Parameters	15 °C	20 °C	25 °C	$F_{1,13}$	p
	P (mg g^{-1})	2.92 ± 0.24	1.97 ± 0.17	1.50 ± 0.07	34.33	< **0.001**
	C:N (g g^{-1})	17.96 ± 1.23	30.61 ± 1.84	29.40 ± 1.45	15.71	**0.002**
	C:P (g g^{-1})	152.6 ± 10.5	228.4 ± 16.7	277.8 ± 12.01	46.66	< **0.001**
	Total Phenolics (mg g^{-1})	26.0 ± 2.6	38.4 ± 2.7	35.1 ± 1.3	3.06	0.104
	N:Phenolics	1.45 ± 0.45	0.39 ± 0.03	0.41 ± 0.02	6.28	**0.026**

Table S3.2 Model selection based on AICc values. Fixed effects were fitted by backward selection based upon AICc values calculated using ML estimation. Aquarium is the random factor for all the models. Models with the lowest AICc value and those within 2.0 ΔAICc of this model are indicated in bold. An X indicates the factors included in each model.

Category	Parameters	Model	Plant species × Temperature	Plant species	Temperature	ΔAICc
Plant growth	Shoot	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +64.72
		3		x		Δ +105.89
		4			x	Δ +135.02
	Root	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +31.70
		3		x		Δ +55.97
		4			x	Δ +145.44
	Growth rate	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +11.88
		3		x		Δ +32.28
		4			x	Δ +251.93
	Root:Shoot	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +35.78
		3		x		Δ +38.08
		4			x	Δ +249.26
Porewater nutrients	TIN	**1**	**x**	**x**	**x**	**Δ +1.40**
		2		**x**	**x**	**Δ 0.0**
		3		**x**		**Δ +1.07**
		4			x	Δ +13.46
	PO_4^{3-}	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		**x**	**x**	**Δ +0.76**
		3		x		Δ +10.51
		4			x	Δ +8.56
Palatability	RCR	1	x	x	x	Δ +3.45
		2		**x**	**x**	**Δ 0.0**
		3		**x**		**Δ +1.77**
		4			x	Δ +11.93
Traits	Dry matter	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +12.40
		3		x		Δ +12.98
		4			x	Δ +247.96
	N	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +8.83
		3		x		Δ +12.05
		4			x	Δ +32.97
	P	**1**	**x**	**x**	**x**	**Δ 0.0**
		2		x	x	Δ +26.78

Category	Parameters	Model	Plant species × Temperature	Plant species	Temperature	ΔAICc
		3		x		Δ +29.57
		4			x	Δ +90.80
		1	**x**	**x**	**x**	**Δ 0.0**
	C:N	2		x	x	Δ +6.67
		3		x		Δ +8.98
		4			x	Δ +30.52
		1	**x**	**x**	**x**	**Δ 0.0**
	C:P	2		x	x	Δ +29.11
		3		x		Δ +33.92
		4			x	Δ +100.38
		1	**x**	**x**	**x**	**Δ 0.0**
	Total phenolics	2		x	x	Δ +13.13
		3		x		Δ +13.40
		4			x	Δ +223.15
		1	**x**	**x**	**x**	**Δ 0.0**
	N:Phenolics	2		x	x	Δ +8.21
		3		x		Δ +10.62
		4			x	Δ +91.52

Chapter 4

Interactive effects of rising temperature and nutrient enrichment on aquatic plant growth and stoichiometry

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Abstract

Anthropogenic activities have led to global climate change and eutrophication, which are strongly influencing aquatic ecosystems. Aquatic plants play a vital role in shallow aquatic ecosystems, because they maintain clear water and increase biodiversity. The abundance and stoichiometry of aquatic plants is thereby crucial for their role in the ecosystem, as the plant's elemental ratios affect both decomposition and consumption by higher trophic levels, with consequences for nutrient cycling and energy transfer through the aquatic food web. However, the effects of rising temperature and eutrophication on aquatic plant growth and resultant stoichiometry, and in particular their interaction, remain largely unknown. Here, we hypothesized that (1) both temperature rise and nutrient enrichment increase the growth of aquatic plants; (2) temperature effects on aquatic plant stoichiometry depend on environmental nutrient conditions; and that (3) changes in plant stoichiometry due to temperature and nutrient enrichment affect plant palatability to higher trophic levels. We used the common submerged aquatic plant *Vallisneria spiralis* as a model species to test the effect of temperature and nutrient availability in both the sediment and the water layer on plant growth and elemental composition and stoichiometry in a full-factorial two-month lab experiment. Plant growth increased with rising temperature and in nutrient-rich sediment, but decreased with external nutrient loading to the water layer likely due to competition by algae. Temperature effects on aquatic plant elemental composition and carbon:nutrient stoichiometry highly depended on the nutrient conditions in the environment. Plant carbon (C):nitrogen (N) and carbon:phosphorus (P) ratios were not affected by temperature when nutrients were either consistently abundant or limited. Alternatively, when rising temperature increased nutrient availability, plant C:N and C:P ratios decreased. Plant palatability tests with the generalist consumer *Lymnaea stagnalis* (Gastropoda) did not indicate either temperature or nutrient enrichment effects on the plant consumption rate. We conclude that rising temperature and nutrient enrichment can have strong effects on aquatic plant growth and stoichiometry, whereby the effects of temperature on stoichiometry depend on environmental nutrient conditions. Despite alterations in plant stoichiometry with temperature rise, we did not find evidence for altered plant consumption rates. This implicates that warming and eutrophication interact to alter plant abundance and stoichiometry, but may not directly alter plant consumption by higher trophic levels.

Introduction

Climate change and eutrophication are altering the ecosystem functioning and services of shallow water bodies globally (IPCC 2014, Steffen et al. 2015). In shallow aquatic ecosystems, aquatic plants are important components, as they stabilize a clear water state (Hilt and Gross 2008) and sustain high biodiversity (Declerck et al. 2005, Cronin et al. 2006). Generally, increasing temperature and nutrient availability increases submerged aquatic plant growth and abundance, until they reach their physiological temperature optimum, respectively become light limited due to algal growth, after which submerged plant growth declines (Barko et al. 1982, Bakker et al. 2013). Due to ongoing eutrophication, the abundance of submerged aquatic plants has declined in many shallow water bodies, resulting in a shift from a stable clear water state with abundant submerged vegetation to a turbid stable state dominated by phytoplankton (Scheffer et al. 1993, Sayer et al. 2010a). Global warming might also contribute to this disappearance of submerged aquatic plants by increasing phytoplankton dominance (Mooij et al. 2007, Kosten et al. 2009). However, even without disappearance, more subtle changes may occur in aquatic plants subjected to warming and eutrophication, which may still have far-reaching consequences for their role in the food web and the cycling of nutrients in plant-dominated shallow water bodies. In particular alterations in plant stoichiometry, i.e. the carbon (C):nutrient ratio, can affect plant decomposition and consumption by higher trophic levels (Sterner and Elser 2002, Bakker et al. 2016). However, studies on the impact of warming and eutrophication on aquatic plant stoichiometry are scarce and yield contradictory results (Cross et al. 2015, Velthuis et al. 2017). Furthermore, natural systems are commonly subjected to both temperature changes and nutrient enrichment (Jeppesen et al. 2010b, Cross et al. 2015). Hence, there is an urgent need to study the combined effects of temperature rise and eutrophication on aquatic plant stoichiometry.

Both warming (temperature rise) and eutrophication (nutrient enrichment) affect aquatic plant growth and subsequent stoichiometry. Nutrient enrichment significantly increases the plant nutrient content (Dorenbosch and Bakker 2011, Dülger et al. 2017), and decreases the C:nutrient ratio (Velthuis et al. 2017). However, the effects of rising temperature on the C:nutrient ratio of aquatic plants seem inconsistent: in experiments the C:nutrient ratio might decrease (Ventura et al. 2008, Velthuis et al. 2017), remain unaltered (Zhang et al. 2016), or even increase (Kaldy 2014, Zhang et al. 2016) in response to temperature rise. Similarly, field studies over a large temperature range also showed contradictory results, where the plant C:nutrient ratio either increased as temperature increased (Wang et al. 2015) (in the Tibetan

Plateau, with minor anthropogenic disturbance), or decreased as temperature increased (Xia et al. 2014) (in eastern China, with high nutrient loading to the water bodies). We hypothesize that these contradictory impacts of temperature are caused by variation in nutrient conditions among experimental studies or field sites and that the impact of temperature rise on aquatic plant stoichiometry depends on the nutrient availability in the plant's environment.

In this study, we test the interactive effects of increasing temperature and nutrient enrichment on the C:nutrient ratio of a model aquatic plant species, and assess the consequences for its palatability to a generalist herbivore. We propose three possible scenarios on how temperature might affect aquatic plant nutrient content and stoichiometry in interaction with environmental nutrient availability (Fig. 4.1). Firstly, plant C:nutrient ratios might decrease as temperature increases (Fig. 4.1a). In sediment with a high organic matter content, higher temperature might increase sediment organic matter mineralization (Gudasz et al. 2010, Sobek et al. 2017), leading to higher nutrient availability for plants (Fisher et al. 2005, Alsterberg et al. 2012). Similarly, with increased nutrient loading to the water layer, aquatic plants can also accumulate nutrients (Gu et al. 2016, Gu et al. 2018). Both forms of increased nutrient availability may lead to a decrease of the C:nutrient ratio with temperature rise as long as nutrients do not limit plant growth. Secondly, plant C:nutrient ratios might not change as temperature increases (Fig. 4.1b). With abundant availability of nutrients, plants may always have sufficient nutrients available for growth and grow faster with temperature rise without altering their C:nutrient ratio. Alternatively, with low availability of nutrients, plant growth may remain nutrient limited at all temperatures, with no changes in C:nutrient ratio. Thirdly, plant C:nutrient ratios might increase as temperature increases (Fig. 4.1c). Higher temperature can stimulate growth of plants, with fast growth leading to nutrient depletion and lower nutrient availability. This could result in the plants accumulating fewer nutrients. According to the temperature-plant physiology hypothesis (Reich and Oleksyn 2004), plants may invest less nutrients compared to C for their growth at higher temperature.

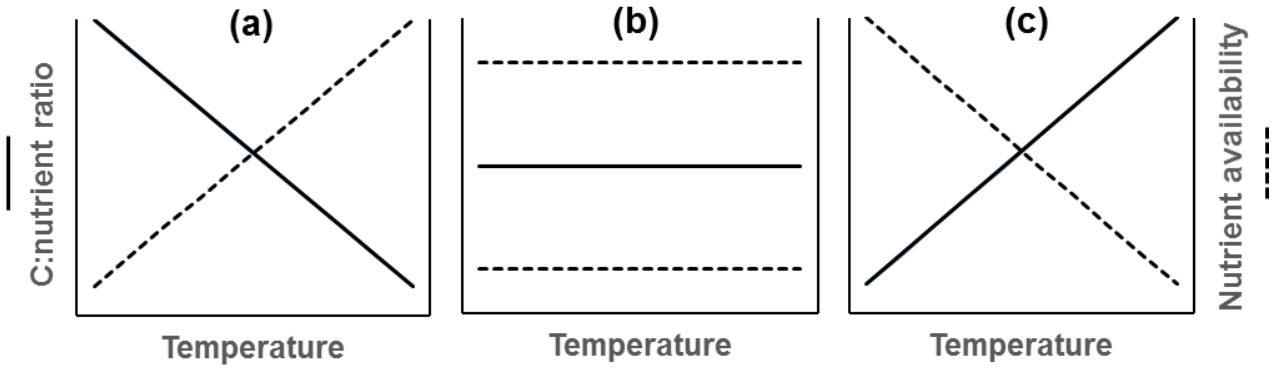

Figure 4.1 Schematic graph of hypothesized temperature effects on environmental nutrient availability and aquatic plant C:nutrient stoichiometry. Solid lines indicate C:nutrient ratios, and dashed lines indicate environmental nutrient availability. (a) Environmental nutrient availability increases and the plant C:nutrient ratio decreases as temperature increases. (b) The upper dashed line indicates consistently abundant nutrients, and the lower dashed line indicates consistently low nutrient availability limiting plant growth. As a result, the plant C:nutrient ratio might not change as temperature increases. (c) Nutrient availability decreases with increasing temperature, as a result, the plant C:nutrient ratio increases with temperature.

A higher nitrogen content or lower C:N ratio in plant tissue generally corresponds to a higher plant consumption by herbivores (Cebrian and Lartigue 2004, Bakker et al. 2016). Hence, as the stoichiometry of aquatic plants becomes altered by temperature rise and nutrient enrichment, we expect that plant palatability will change accordingly.

To contrast these three alternative scenarios, we selected the common aquatic plant *Vallisneria spiralis* as a model species and cultured it at different temperatures and nutrient conditions in a full-factorial design. Plants were grown at three different water temperatures. Nutrient conditions were experimentally manipulated in the water layer, the sediment, or in both the water and sediment, as in eutrophicated water bodies nutrient enrichment may result from external loading in the water column (Coppens et al. 2016), internal loading from the sediment (Fisher et al. 2005, Immers et al. 2015) or both. We tested how this affected plant growth, stoichiometry and subsequent palatability to the generalist consumer *Lymnaea stagnalis* (Gastropoda). We hypothesized that: (1) both rising temperature and nutrient enrichment increases the growth of aquatic plants; (2) temperature effects on aquatic plant stoichiometry depend on environmental nutrient conditions; and that (3) changes in plant stoichiometry due to temperature and nutrient enrichment affect plant palatability.

Materials and Methods

Plant culturing

Our model species was *V. spiralis*, a rooted submerged aquatic plant that is widespread and relatively palatable for generalist consumers such as the pond snail *Lymnaea stagnalis* (Elger et al. 2004, Grutters et al. 2017b). Rooted submerged aquatic plants can take up nutrients from both the sediment and the water layer (Rattray et al. 1991), which allows detailed manipulation of nutrient conditions for our model species. Ten original plants of *V. spiralis* were obtained from a local garden centre (Tuincentrum De Oude Tol, Wageningen, The Netherlands) and planted in one aquarium to produce vegetative tillers. 72 tillers (shoot length: 8.6 ± 2.0 cm, mean ± SD) were selected for the experiment. Each of these tillers was individually planted in a pot (top diameter 12.5 cm, bottom diameter 11 cm and height 11 cm) filled with 7 cm of sediment covered by a layer of 2 cm pure sand to limit a nutrient flux between the sediment and the water layer. Each pot was put in a transparent cylindrical vase (inner diameter of 18 cm and height of 50 cm) filled with tap water (Fig. S4.1).

A balanced full factorial design was applied where three temperature treatments were crossed with two sediment nutrient treatments and two external nutrient loading scenarios to the water layer. The three selected temperatures were: 20, 24 and 28°C with the steps of 4°C increase representing the predicted global temperature rise of 3-5°C over the next century (IPCC 2014). The optimum temperature for *Vallisneria* growth is around 28°C (Barko et al. 1982, Bartleson et al. 2014), hence the increase in temperature along the selected temperature range implies increasing plant growth. The two sediment types consisted of nutrient-rich sediment (S1) with 100% of artificial pond soil (Pokon Naturado, Veenendaal, the Netherlands), and nutrient-poor sediment (S0) with 25% of pond soil mixed with 75% of sand by volume. The pond soil contains 20% organic matter, 8.0 ± 0.48 mg g^{-1} (dry weight) and 1.1 ± 0.084 mg g^{-1} (dry weight) total nitrogen and total phosphorus respectively (mean ± SE, n = 5). The two external nutrient loading treatments were with external nutrient loading to the water layer (W1), and without external nutrient loading to the water layer (W0). The nutrient solution was made by dissolving NH_4NO_3 and KH_2PO_4 powder into demi water, and its addition simulated a high-level nutrient loading of 0.5 mg L^{-1} Nitrogen (N) and 0.05 mg L^{-1} Phosphorus (P) per week, the dosing level and ratio followed those of experiments in Sagrario et al. (2005), Jeppesen et al. (2007) and Coppens et al. (2016). In order to prevent that the plants would be outcompeted by phytoplankton at the start of the experiment, the treatment of nutrient loading to the water

started half way (after four weeks) of the culturing and was applied four times during the experiment.

In total, there were 12 treatments that each had six replicates. The vases were placed in six aquaria (180 × 50 × 50 cm, l × w × h) which served as water baths to regulate the water temperature in the vases. Every aquarium contained 12 vases, and every two aquaria had the same temperature treatment. Vases with different nutrient treatments were randomly placed in the aquaria (see Fig. S4.1 for a scheme of the experimental design). The experiment ran for two months from October 6th to December 5th of 2016. The plants were first acclimated in their vases for the first week at 20°C, and then assigned to the experimental temperatures. The day:night cycle was 16:8 hours, and light intensity on the water surface was 62 ± 17 µmol m^{-2} s^{-1} (mean ± SD, n = 72). Demi water was added twice a week to the vases to compensate for water evaporation. The water level was elevated from 25 cm to 30 cm in all vases halfway the experiment, as the plants grew rapidly at the high temperature treatment and almost reached the surface.

Water quality parameters were measured four times during the experiment, including conductivity; pH; chlorophyll a; alkalinity; NO_3^-; NH_4^+; PO_4^{3-} (the data are depicted in Fig. S4.2). At the end of the experiment, the seston concentration (mainly phytoplankton) was quantified by filtering a known volume of water (adapted to the concentration of the phytoplankton) over pre-weighed GF/F filters (Whatman, Maidstone, UK), filters were dried in the oven at 60°C for 48 h, and weighed. The seston concentration was expressed as mg dry weight per litre of water (Fig. S4.3 and Table S4.1). Periphyton growth was quantified by placing a 21 × 2 cm (l × w) plastic strip fixed to a stick in each vase at the start of the experiment, which was collected at the end of the experiment. The periphyton dry weight (µg dry weight per cm^2 area) was determined by cutting a certain size of the strip (from 4 to 21 cm^2, determined by the density of periphyton), cleaning it with a tooth brush in a beaker with demi water and filtering the water over pre-weighed filters (Whatman, Maidstone, UK). The filters were dried in the oven at 60°C for 48 h and weighed, the change in dry weight of the filter allowed quantification of the dry weight of the periphyton (Fig. S4.3 and Table S4.1). To determine the sediment nutrient availability for the plants, sediment porewater was sampled in each pot using rhizons (Rhizosphere, Wageningen, The Netherlands) at the end of the experiment. The porewater was then analysed for total dissolved inorganic nitrogen (DIN: including N from NH_4^+, NO_2^- and NO_3^-) and P-PO_4^{3-} concentrations on an auto analyser (QuAAtro method, Seal Analytical, Fareham, UK) (Fig. S4.4 and Table S4.1).

At the end of the experiment, about 0.4 g fresh plant material from each pot was collected for the feeding trials, and the rest of the plant material was harvested to quantify dry biomass. Shoots and roots were separated, cleaned carefully and dried in the oven at 60°C for 48 h. Plant relative growth rate was calculated according to the equation: Relative growth rate = $(\ln W_f - \ln W_i)$/days; with W_i = initial dry weight and W_f = final dry weight (including shoot biomass and root biomass, also including the weight of the plant parts used for feeding trials). Plant initial dry weight was determined by drying and weighing ten spare plants before the start the experiment.

Each dried plant sample was ground individually in a 2 mL tube on a ball mill Tissuelyser II (QIAGEN, Hilden, Germany). Plant carbon (C) and nitrogen (N) were determined on an elemental auto analyser (FLASH 2000, Thermo Scientific, Waltham, MA, USA). Phosphorus (P) content was determined by incinerating and digesting the sample, and then analyzing the phosphate concentration on an Auto Analyzer (QuAAtro method, Seal Analytical, Fareham, UK).

Snail culturing and palatability test

We tested for variation in palatability among the cultured plants using a generalist consumer, the pond snail *L. stagnalis*. This species can feed on a large variety of aquatic plants, and is frequently used as a model species for testing aquatic plant palatability (Elger and Barrat-Segretain 2002, 2004, Grutters et al. 2017b, Zhang et al. 2018b). We hatched snails from egg clusters from a pond of NIOO-KNAW (51°59'16.8"N, 5°40'24.7"E, Wageningen, The Netherlands). Juvenile snails were reared for two months in buckets at 20°C, filled with tap water and constant aeration, and exposed to a day:night cycle of 16:8 hours. Snails were fed with commercially obtained lettuce five times per week. Fish food (Velda, Gold Sticks Basic Food, The Netherlands) and chalk were supplied once a week to supplement for other nutrients. Snails of similar size (shell length 30.4 ± 0.9 mm, mean ± SD, n = 62) were selected for the palatability tests.

The palatability tests followed the protocol developed by Elger and Barrat-Segretain (2002, 2004). This test measures how much material is consumed by one individual snail over a certain time, using no-choice feeding trials. Snails were placed individually in a beaker (volume of 500 mL) with 375 mL tap water for 24 hours without food before the feeding trials. From each vase, approximately 0.2g wet weight of fresh plant leave was harvested, cleaned to

remove periphyton, and offered to each snail. The amount was the maximum of the plant material one snail could eat in one day as determined in pre-trials. As controls, another 0.2g leaves from the same vase was placed in a control beaker without a snail, to monitor possible weight changes in plant material as a result of decomposition or growth during the 24 h feeding trial. Each beaker was covered with a mesh to prevent the snail from escaping. After the feeding trials, leftover plant materials were weighed and dried in the oven at 60°C for 48 hours and weighed again. The snails were frozen, dried in the oven at 60°C with their shell separated from the soft body part, and weighed. Plant dry matter content was determined as the dry weight divided by the wet weight and expressed as percentage, using the control portion of the plant. Plant dry matter content can be used to indicate plant toughness, and has been shown to negatively correlate with aquatic plant palatability (Elger and Willby 2003). Plant palatability, indicated by plant Relative Consumption Rate (RCR) (mg g^{-1} d^{-1}), was calculated according to Elger and Barrat-Segretain (2002):

$$RCR = [(C_{fd} / C_{iw}) * F_{iw} - F_{fd}] / S_d / 1day,$$

Where C_{fd} is the final dry weight of the control plant, C_{iw} is the initial wet weight of the control plant, F_{iw} is the initial wet weight of the feeding trial plant, F_{fd} is the final dry weight of the feeding trial plant, and S_d is the snail dry weight without shell.

Data analysis

There were five vases in which the plant had died during the experiment, which were excluded from the dataset (dead plants were spread over the treatments: one in the 20°C W1S1 treatment, one in 24°C W0S0, one in 24°C W1S0, one in 24°C W0S1 and one in the 28°C W1S0 treatment). This resulted in 67 individual plants that were available for the analysis of four plant growth parameters (plant shoot biomass, root biomass, relative growth rate and root:shoot ratio), three plant elemental compositions (plant C, N and P content) and three plant stoichiometry traits (C:N, C:P and N:P ratio). The palatability test was performed on 62 individual plants, as another five vases (mainly at low temperatures and low nutrients: four at 20°C W0S0 and one at 24°C W1S0) did not contain enough plant material for the feeding trials as plants grew slowly under these conditions and were therefore excluded. Linear Mixed-Effect Models, using the package nlme (Pinheiro et al. 2017), were used to analyse the effects of temperature, sediment type, external nutrient loading and their interactions on all the parameters. Aquarium was set as a random factor in all the models to account for the dependency structure

in our experimental design. QQplot and residual plot were used to test the normality of data, if data were not normally distributed, data were transformed (data transformation is added in Table 4.1). Best models were selected based on model AICc values. If multiple models were within 2.0 ΔAICc from the best model, the presented estimates were based on the averaging over these models within 2.0 ΔAICc (details on model selections can be found in Table S4.2) (Burnham and Anderson 2002, Burnham et al. 2011). Following the overall test, temperature effects were tested separately in all four nutrient conditions (W0.S0, W1.S0, W0.S1 and W1.S1), with temperature as a fixed factor and aquarium as a random factor in Linear Mixed-Effect Models. These tests provided the formulas, r^2 values and *p*-values as presented directly in the figures. Simple linear regression tests (lm) were applied to test the correlation between sediment nutrient concentration and plant nutrient content, and plant palatability with plant dry matter content, elementary composition or stoichiometry. All analysis were performed in R version 3.4.3 (R Development Core Team 2017).

Results

Plant growth and culturing conditions

Sediment type, external nutrient loading to the water column and temperature all affected plant growth parameters (Fig. 4.2 and Table 4.1). Plant shoot biomass and plant root biomass both were significantly higher in nutrient-rich sediment, whereas no effects of external nutrient loading on plant shoot or root biomass were observed. Rising temperature significantly increased plant shoot biomass in three nutrient treatments (W0S0, W0S1 and W1S1), but not in the treatment with only external nutrient loading (W1S0). Rising temperature significantly increased plant root biomass in the treatments without external nutrient loading (W0S0 and W0S1), but not with external nutrient loading (W1S0 and W1S1). With rising temperature, both plant shoot and root biomass increased faster in nutrient-rich sediment (W0S1) than nutrient-poor sediment (W0S0) (Fig. 4.2 and Table 4.1).

Table 4.1 Effects of temperature, sediment type, external nutrient loading and their interactions on plant growth, elemental composition, stoichiometry, and plant palatability. Effects were analysed by Linear-Mixed Effect models after model selection. It is indicated in case data were transformed to meet model requirements.

Category	Dependent variable	Explanatory variable	Estimate	SE	*Z*-value	*p*-value
Plant growth	**Shoot biomass**	Intercept	0.098	0.013	7.33	<**0.001**
		Sediment	0.099	0.016	5.98	<**0.001**
		Temperature	0.009	0.003	1.79	0.074
		Water	4.0E-04	0.016	0.03	0.980
		Sed × Temp	0.015	0.003	4.68	<**0.001**
		Sed × Water	-0.041	0.020	1.96	0.050
		Temp × Water	-0.004	0.003	1.15	0.249
	Root biomass	Intercept	0.067	0.006	11.72	<**0.001**
		Sediment	0.022	0.006	3.36	<**0.001**
		Temperature	0.004	0.002	1.85	0.064
		Water	-0.002	0.006	0.25	0.800
		Sed × Temp	0.003	0.001	2.17	**0.030**
		Sed × Water	-0.020	0.009	2.14	**0.032**
		Temp × Water	-0.002	0.001	1.27	0.203
	Growth rate	Intercept	0.021	0.001	18.60	<**0.001**
		Sediment	0.009	0.001	8.68	<**0.001**
		Temperature	0.002	3.4E-04	3.43	<**0.001**
		Water	-0.003	0.001	2.67	**0.008**
		Sed × Temp	0.001	3.2E-04	3.87	<**0.001**
		Temp × Water	-0.001	3.2E-04	1.78	0.075
	log(Root:Shoot ratio + 0.001)	Intercept	-0.337	0.041	8.13	<**0.001**
		Sediment	-0.433	0.045	9.48	<**0.001**
		Temperature	-0.037	0.009	2.92	**0.003**
		Water	-0.076	0.045	1.68	0.093
		Sed × Temp	-0.018	0.013	1.29	0.198
Plant elemental composition	**Carbon**	Intercept	379.455	1.011	368.46	<**0.001**
		Sediment	-1.341	1.446	0.91	0.361
		Temperature	-1.236	0.215	4.05	<**0.001**
		Water	-6.737	1.471	4.51	<**0.001**
		Sed × Temp	0.794	0.304	2.55	**0.011**
		Sed × Water	3.116	2.005	1.52	0.128
	log(Nitrogen)	Intercept	2.185	0.042	50.59	<**0.001**
		Sediment	0.531	0.046	11.28	<**0.001**
		Temperature	0.021	0.012	1.28	0.201
		Water	0.392	0.047	8.23	<**0.001**
		Water × Temp	-0.032	0.012	2.60	**0.009**
		Sed × Temp	0.016	0.012	1.32	0.188
		Sed × Water	-0.078	0.079	0.96	0.338
		Intercept	1.244	0.040	30.44	<**0.001**

Category	Dependent variable	Explanatory variable	Estimate	SE	*Z*-value	*p*-value
Plant stoichiometry	log(Phosphorous)	Sediment	-0.175	0.046	3.69	<**0.001**
		Temperature	0.060	0.011	3.95	<**0.001**
		Water	0.219	0.046	4.61	<**0.001**
		Sed × Temp	-0.060	0.014	4.18	<**0.001**
		Water × Temp	-0.012	0.014	0.82	0.410
	C:N ratio	Intercept	43.839	1.333	32.18	<**0.001**
		Sediment	-18.729	1.584	11.57	<**0.001**
		Temperature	-0.800	0.342	1.67	0.095
		Water	-15.514	1.611	9.42	<**0.001**
		Sed × Water	7.940	2.263	3.43	<**0.001**
		Water × Temp	0.972	0.341	2.79	**0.005**
		Sed × Temp	-0.286	0.341	0.82	0.412
	C:P ratio	Intercept	113.045	4.616	24.00	<**0.001**
		Sediment	18.144	5.596	3.18	**0.001**
		Temperature	-6.267	1.271	3.60	<**0.001**
		Water	-23.483	5.628	4.09	<**0.001**
		Sed × Temp	5.401	1.447	3.65	<**0.001**
		Water × Temp	1.975	1.441	1.34	0.180
		Sed × Water	-9.379	9.627	0.95	0.340
	sqrt(N:P ratio)	Intercept	1.606	0.037	42.24	<**0.001**
		Sediment	0.695	0.045	15.02	<**0.001**
		Temperature	-0.040	0.011	2.70	**0.007**
		Water	0.168	0.046	3.61	<**0.001**
		Sed × Temp	0.068	0.011	6.11	<**0.001**
		Water × Temp	-0.021	0.011	1.90	0.058
		Sed × Water	-0.087	0.072	1.18	0.237
Plant palatability	RCR	Intercept	32.620	2.657	12.01	<**0.001**
		Temperature	-1.482	0.737	1.42	0.156

"Temp" represents temperature treatment. "Sed" represents sediment treatment. "RCR" represents plant relative consumption rate. "log" and "sqrt" indicate the data are natural log and square root transformed respectively. Bold numbers indicate $p < 0.05$.

Plant relative growth rate increased in nutrient-rich sediment, but decreased with external nutrient loading. Rising temperature significantly increased plant growth rate in all the nutrient treatments (Fig. 4.2 and Table 4.1). Plant root:shoot ratio decreased in nutrient-rich sediment, whereas external nutrient loading had no significant effects on plant root:shoot ratio. Rising temperature significantly decreased plant root:shoot ratio in the nutrient-rich sediment treatments (W0S1 and W1S1), but not in the nutrient-poor sediment treatments (W0S0 and W1S0) (Fig. 4.2 and Table 4.1).

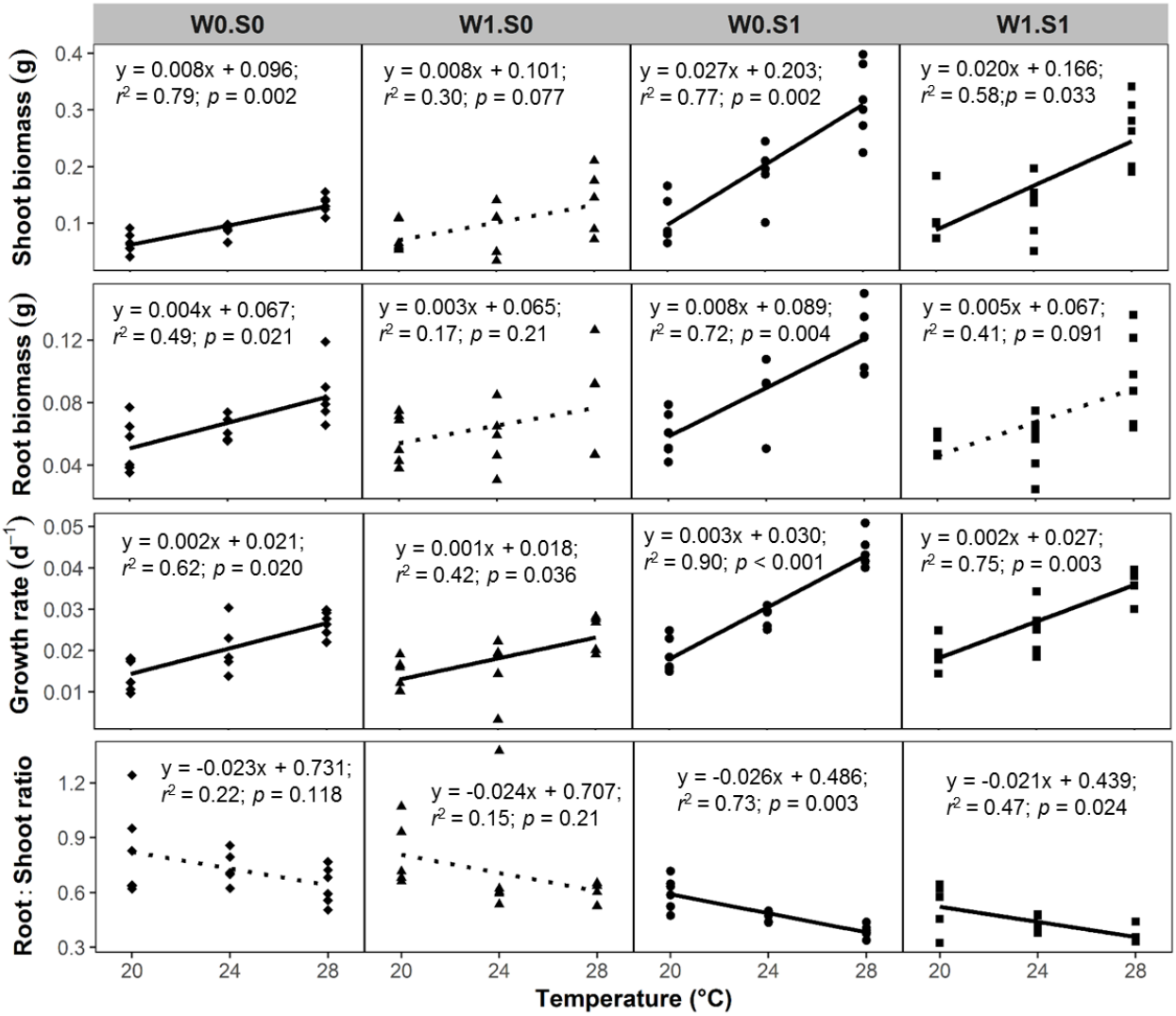

Figure 4.2 Temperature effects on plant growth parameters indicated per nutrient treatment. S1 indicates nutrient-rich sediment, S0 indicates nutrient-poor sediment, W1 indicates with external nutrient loading to the water, and W0 indicates without external nutrient loading. A dotted line indicates $p > 0.05$, and a solid line indicates $p < 0.05$. Intercepts of the formulas are all at 24°C.

The seston concentration significantly increased with external nutrient loading, whereas it was not affected by sediment type. Rising temperature significantly increased the seston concentration in the treatment with external loading and nutrient-rich sediment (W1S1), but not in the other nutrient treatments (Fig. S4.3 and Table S4.1). External nutrient loading also significantly increased periphyton concentration, whereas there were no effects of sediment type and temperature (Fig. S4.3 and Table S4.1). There was a negative correlation between the

periphyton concentration and plant shoot biomass (Fig. S4.4a), whereas no significant correlation of the seston concentration and plant shoot biomass was found (Fig. S4.4b).

Both sediment porewater DIN and $P\text{-}PO_4^{3-}$ concentrations were much higher in nutrient-rich sediment than nutrient-poor sediment (Fig. S4.3 and Table S4.1). Rising temperature significantly increased the DIN concentration in W0S1 treatment, but not in the other nutrient treatments. There were no temperature effects on the porewater $P\text{-}PO_4^{3-}$ concentration (Fig. S4.3 and Table S4.1). At the end of the experiment, external nutrient loading significantly increased DIN concentration in the water column, whereas no effects of temperature and sediment type were observed. Water column $P\text{-}PO_4^{3-}$ concentration was lower above nutrient-rich sediment than nutrient-poor sediment; no effects of temperature and external nutrient loading were found (Fig. S4.3 and Table S4.1). DIN and $P\text{-}PO_4^{3-}$ concentrations were much lower in the water column than in the sediment porewater.

Plant elemental composition and stoichiometry

Sediment type, external nutrient loading and temperature all affected plant elemental composition and stoichiometry (Fig. 4.3 and Table 4.1). Plant C content decreased with external nutrient loading, but was not affected by sediment type. Rising temperature significantly decreased plant C content in the treatments with nutrient-poor sediment (W0S0 and W1S0), not in nutrient-rich sediment (W0S1 and W1S1) (Fig. 4.3 and Table 4.1).

Plant N content increased in the nutrient-rich sediment treatment, and increased with external nutrient loading. Rising temperature significantly increased plant N content in the treatment with nutrient-rich sediment and no external nutrient loading (W0S1), but not in the other nutrient treatments (Fig. 4.3 and Table 4.1).

Plant P content increased with external nutrient loading, but decreased in nutrient-rich sediment. Rising temperature significantly increased plant P content in the nutrient-poor sediment treatments (W0S0 and W1S0), but not in nutrient–rich sediment treatments (W0S1 and W1S1) (Fig. 4.3 and Table 4.1). The plant N content was positively correlated with the porewater DIN concentrations (Fig. S4.5a). In contrast, the plant P content was negatively correlated with the porewater $P\text{-}PO_4^{3-}$ concentrations (Fig. S4.5b).

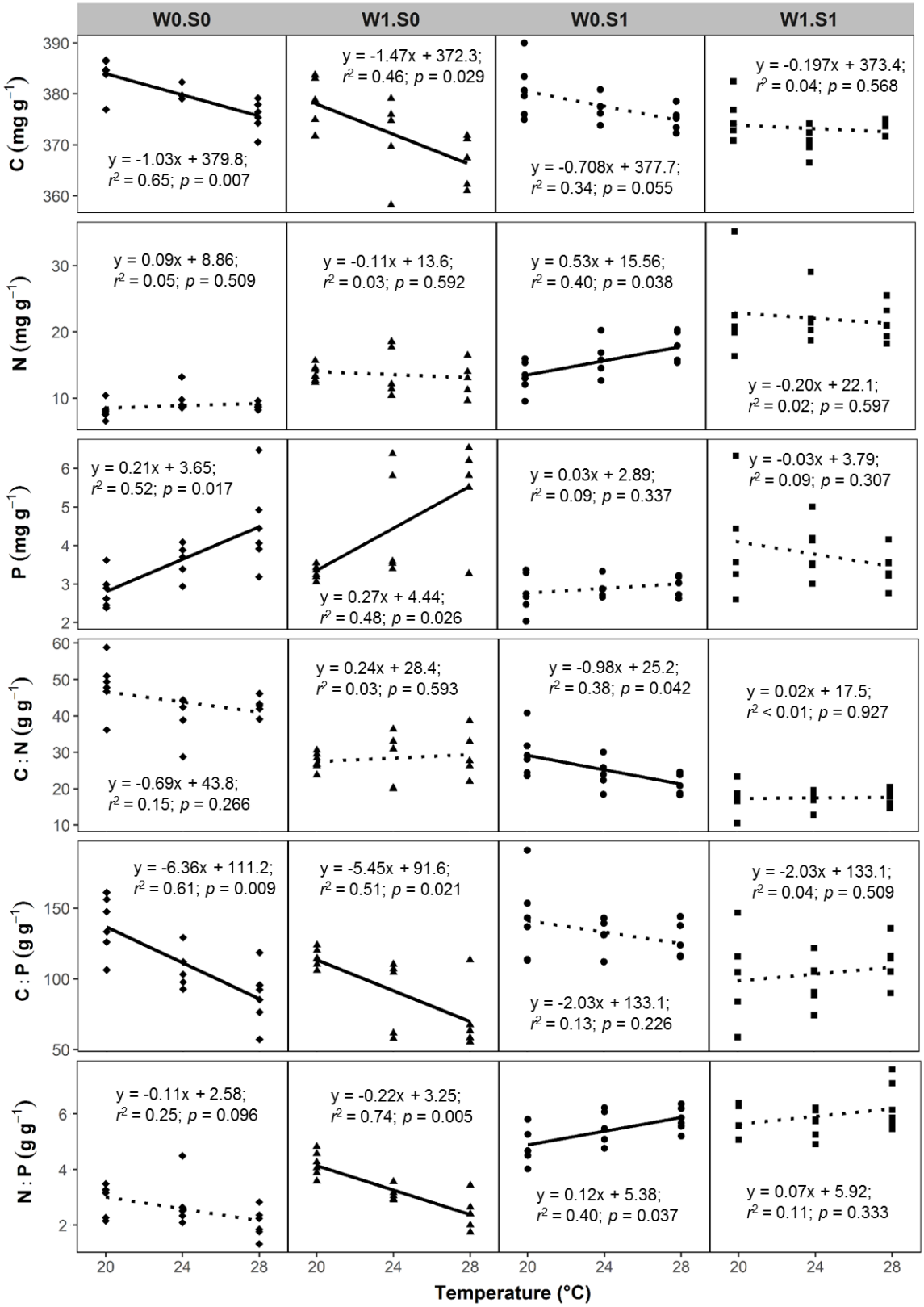

Figure 4.3 Temperature effects on plant elemental composition (C, N and P) and stoichiometry (C:N, C:P and N:P ratio) indicated per nutrient treatment. S1 indicates nutrient-rich sediment, S0 indicates nutrient-poor sediment, W1 indicates with external nutrient loading to the water, and W0 indicates without external nutrient loading. A dotted line indicates $p > 0.05$, and a solid line indicates $p < 0.05$. Intercepts of the formulas are all at 24°C.

The plant C:N ratio decreased in nutrient-rich sediment, and with external nutrient loading. Rising temperature significantly decreased the plant C:N ratio in the nutrient-rich sediment treatment without external nutrient loading (W0S1), but not in the other nutrient treatments (Fig. 4.3 and Table 4.1).

The plant C:P ratio decreased with external nutrient loading, but increased in the nutrient-rich sediment treatment. Rising temperature significantly decreased the plant C:P ratio in nutrient-poor sediment treatments (W0S0 and W1S0), but not in nutrient-rich sediment treatments (W0S1 and W1S1) (Fig. 4.3 and Table 4.1).

The plant N:P ratio increased in nutrient-rich sediment, and with external nutrient loading. Rising temperature significantly decreased the plant N:P ratio in the treatment with only external nutrient loading (W1S0), and increased the plant N:P ratio in the treatment with only enriched sediment (W0S1), whereas there were no effects in the other nutrient treatments (Fig. 4.3 and Table 4.1).

Plant palatability

Plant palatability, measured as the relative consumption rate (RCR), varied from 0 to 77 mg g^{-1} d^{-1}, and was not affected by any of the temperature or nutrient treatments (Fig. 4.4 and Table 4.1). Plant palatability did not correlate with plant dry matter content, nor with any of the elemental composition and stoichiometry parameters that were measured. (Linear regression testing palatability (RCR) with respectively dry matter content, $r^2 = 0.04$, $p = 0.14$; C content, $r^2 = 0.01$, $p = 0.498$; N content, $r^2 = 0.01$, $p = 0.577$; P content, $r^2 = 0.02$, $p = 0.324$; C:N ratio, $r^2 = 0.01$, $p = 0.551$; C:P ratio, $r^2 = 0.002$, $p = 0.737$; N:P ratio, $r^2 = 0.01$, $p = 0.544$.)

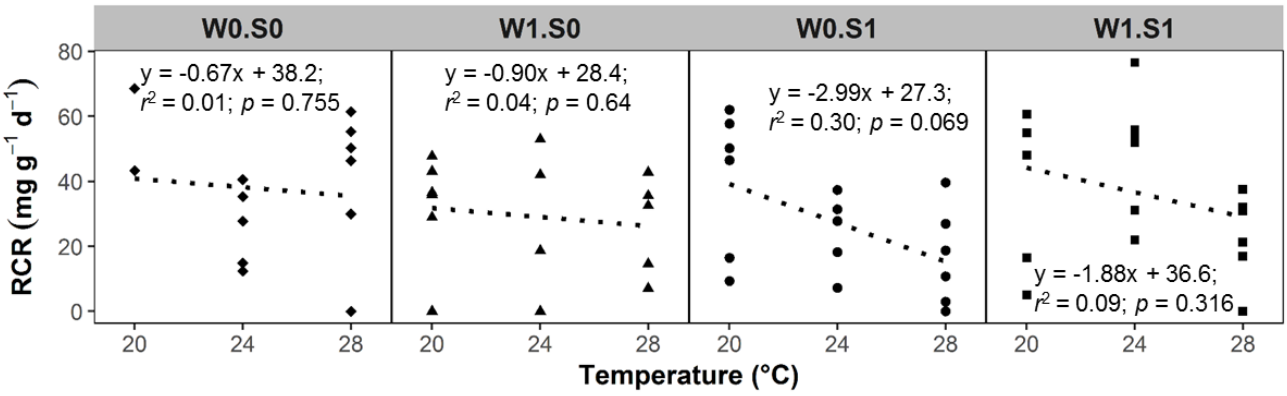

Figure 4.4 Temperature effects on plant palatability to the pond snail *L. stagnalis* expressed as Relative Consumption Rate (RCR), indicated per nutrient treatment. S1 indicates nutrient-rich sediment, S0 indicates nutrient-poor sediment, W1 indicates with external nutrient loading to the water, and W0 indicates without external nutrient loading. A dotted line indicates $p > 0.05$. Intercepts of the formulas are all at 24°C.

Discussion

In this study, we tested how temperature rise and nutrient enrichment interactively affect aquatic plant growth, stoichiometry and palatability. By culturing a submerged aquatic plant under different temperatures and nutrient conditions, we found that temperature and nutrient availability in the sediment and the water column all influenced plant growth and stoichiometry. Plant growth was enhanced by rising temperature, and by nutrient enrichment of the sediment thus confirming our first hypothesis. However, it did matter where the nutrients were available, as plant growth was enhanced with nutrient enrichment in the sediment, whereas external nutrient loading inhibited plant growth. Temperature effects on plant stoichiometry highly depended on environmental nutrient conditions, which is in accordance with our second hypothesis. Even though plant stoichiometry changed in the temperature and nutrient treatments, we did not detect changes in plant palatability and thus reject our third hypothesis. We discuss the mechanisms and implications of our findings below in more detail.

Plant growth

Both temperature rise and nutrient enrichment in the sediment enhanced the growth of *V. spiralis*, whereas external nutrient loading had a negative effect on the growth of the plant.

Rising temperature can stimulate the growth of aquatic plants in their suitable temperature range, which has been shown in a large variety of aquatic plant species (Barko et al. 1982, Madsen and Brix 1997, Kaldy 2014, Velthuis et al. 2017). However, whether rising temperature results in enhanced plant growth also depends on the availability of nutrients to realize growth (Cross et al. 2015). In our study, plant growth rate increased with rising temperature, but this increase was faster at high sediment nutrient availability, demonstrating the interactive effect of temperature and nutrient enrichment on plant growth. Furthermore, it depends whether the added nutrients are available for plant growth. *V. spiralis* is a submerged aquatic plant, which can take up nutrients from both sediment and water layer (Rattray et al. 1991). However, nutrient enrichment in the sediment increased plant growth, whereas nutrient loading to the water layer inhibited the growth of plants. Hence, though *V. spiralis* could take up the added nutrients from the water column, algae can do this as well (van Donk and van de Bund 2002, Yu et al. 2015), and they were faster than *V. spiralis*, indicated by the enhanced seston and periphyton biomass that we found in our experiment under external nutrient loading. As a result, *V. spiralis* profited from nutrient enrichment in the sediment, but suffered from competition from phytoplankton and periphyton under external nutrient loading to the water column, as also illustrated by the negative relationship between periphyton and *V. spiralis* shoot biomass. Plant root:shoot ratio decreased with rising temperature and nutrient enrichment in the sediment. Possibly, plant nutrient uptake efficiency increases at higher temperatures, and thus plants need to invest less biomass in root formation (Barko and Smart 1981, Barko et al. 1982, Riis et al. 2012). Furthermore, in nutrient-rich sediment, there were more nutrients available, hence plants allocated less biomass to roots (Olsen and Valiela 2010). These shifts in plant root:shoot ratio can be explained by the optimal partitioning theory (Bloom et al. 1985), and is an adaptive strategy for plants to cope with environmental change.

Plant elemental composition and stoichiometry

We hypothesized that temperature effects on plant stoichiometry depended on environmental nutrient conditions. The relative variance of plant C content (CV, coefficient of variation, 1.6%) was much lower than the variance of N (CV, 38.0%) and P content (CV, 27.0%). Therefore, the temperature and nutrient enrichment effects on C:N and C:P ratios were mainly determined by the effects on N and P content respectively.

Generally, plant N content is related to the environmental nitrogen availability (Cronin and Lodge 2003, Demars and Edwards 2007, Cao et al. 2011). In our study, plant N content was also higher and the C:N ratio was lower both in the nutrient-rich sediment treatment and with external nutrient loading. Furthermore, plant N content positively correlated with sediment porewater DIN concentration, which indicates that sediment DIN might be a major source for the plant's nitrogen acquisition in our experiment. As we hypothesized, temperature effects on plant N content and C:N ratio depended on the environmental nutrient conditions. In nutrient-poor sediment, the porewater DIN concentration was always low and not affected by temperature. Hence, without external nutrient loading, plant N content was always low and plant C:N ratio was always high across the whole temperature range (W0S0 treatment). With the same amount of external nutrient loading, plant N content and C:N ratio were still constant across the tested temperatures (W1S0 treatment). These results are in accordance with our second scenario (Fig. 4.1b). In contrast, in the nutrient-rich sediment and without external nutrient loading treatment (W0S1 treatment), rising temperature increased the inorganic nitrogen availability in the sediment, probably due to increased sediment organic matter mineralization with rising temperature (Gudasz et al. 2010, Sobek et al. 2017), and the plant N content increased and C:N ratio decreased. This is in accordance with our first scenario (Fig. 4.1a). With external nutrient loading to the nutrient-rich sediment treatment (W1S1 treatment), no temperature effects on porewater inorganic nitrogen availability were found, resulting in no temperature effects on plant N content and C:N ratio, which is also in line with our second scenario (Fig. 4.1b). Therefore, temperature effects on the plant C:N ratio depended on the environmental nitrogen conditions.

In our study, the plant P content was negatively correlated with the porewater $P\text{-}PO_4^{3-}$ concentration. This indicates that the water column phosphorus might be a major source for plant phosphorus. Normally, we would assume that aquatic plants take up most of their phosphorus from the sediment by their roots (Carignan and Kalff 1980), but some aquatic plant species showed a higher uptake rate of phosphorus by shoots than roots, such as *Myriophyllum alterniflorum* (Christiansen et al. 2016). This might be due to their adaptive strategy of a large surface area to volume ratio of the shoot (Carignan and Kalff 1980, Christiansen et al. 2016). Our model species *V. spiralis* also has a large surface area compared to other submerged plants (Grutters et al. 2015), which means the species might take a large portion of phosphorus from the water. The plant P content increased and C:P ratio decreased with external nutrient loading in our study also supporting that the plant took up most of the phosphorus by its shoot. However,

the plant P content decreased and C:P ratio increased with nutrient enrichment in the sediment. The reason could be that the plants accumulated more biomass in nutrient-rich sediment, which then dilutes the P content in the shoots and leads to a higher C:P ratio. Furthermore, the temperature effects on plant P content and C:P ratio also depended on the nutrient conditions. In nutrient-poor sediment, rising temperature increased plant P content and decreased plant C: P ratio. The reason could be that rising temperature increased the phosphate release from the sediment to the water column and plants accumulated more phosphorous at high temperature, which fit in our first scenario (Fig. 4.1a). In nutrient-rich sediment, rising temperature did not increase the nutrient release from sediment to water layer, probably due to periphyton growth on the sediment inhibiting phosphate release to the water, leading to no temperature effects on plant P content or C:P ratio. These results fit in the second scenario (Fig. 4.1b).

Previous studies have observed that the plant C: N ratio decreases with rising temperature (Ventura et al. 2008, Velthuis et al. 2017), which fit in our first scenario (Fig. 4.1a). Several other studies did not find temperature effects on plant C:N ratio (Touchette et al. 2003) nor C:P ratio (Ventura et al. 2008, Zhang et al. 2016, Velthuis et al. 2017), which fit in our second scenario (Fig. 4.1b). And there are studies which found that the C:N ratio of aquatic plants increased as temperature increased (Kaldy 2014, Zhang et al. 2016), probably due to decreased nitrogen availability for the plant with rising temperature, which fit in our third scenario (Fig. 4.1c). Hence, all three scenarios have been observed to occur. However, it is not possible to directly link these alternative scenarios to our hypothesis that differences in environmental nutrient availability are causing these alternative outcomes, as they did not directly quantify nutrient availability in the environment. In contrast, our hypothesis can explain the different trends in field studies across large temperature gradients, where plants had an increasing C:N ratio as temperature increased in the Tibetan Plateau, which is little human-disturbed thus with limited human-induced nutrient enrichment (Wang et al. 2015), and a decreasing C:N ratio as temperature increased in eastern China with high external nutrient loading (Xia et al. 2014). Therefore, combining these results with our experiment, we can conclude that temperature effects on aquatic plant C:nutrient stoichiometry are not uniformly consistent but highly dependent on the nutrient conditions in the environment in a predictable way.

Even though the variance in plant C content was much lower than the variance in plant N and P contents, we did observe temperature and nutrient treatment effects on plant C content. In our study, plant C content decreased with rising temperature, especially in nutrient-poor

sediment. The reason could be that there was less of a carbon source in nutrient-poor sediment than in nutrient-rich sediment. Rising temperature increased the growth of the plant and could have depleted the carbon source in nutrient-poor sediment, resulting in less inorganic carbon being available and less carbon in the plant tissue. Furthermore, with external nutrient loading, the growth of algae (both phytoplankton and periphyton) increased, and the algae can compete for inorganic carbon with submerged plants (Jones et al. 2002), thus leading to less inorganic carbon being available for the plants, resulting in a lower C content with external nutrient loading. In addition, temperature and nutrient treatments also influenced the plant N:P ratio in our study, which reflected the relative availability of nitrogen and phosphorus in the environment, as the plant is quite flexible with N and P compositions.

Plant palatability

We could not detect any effects of temperature or nutrient treatments on plant palatability. Furthermore, plant palatability was not correlated with any of the plant parameters that we measured. The plant feeding by herbivores can be determined by plant physical structure, plant nutrient level, and plant defence compounds (Cronin et al. 2002, Elger and Lemoine 2005, Dorenbosch and Bakker 2011). Even though a large amount of studies have shown that aquatic plant palatability might increase as plant N increases or C:N ratio decreases (Dorenbosch and Bakker 2011, Bakker and Nolet 2014, Bakker et al. 2016), some other studies also did not find a correlation of aquatic plant palatability with plant nutrient contents or stoichiometry (Cronin et al. 2002, Cronin and Lodge 2003). Our result is in accordance with the latter. It might be that secondary metabolites which deterred the animals from feeding on the plants played a role in the feeding choice (Gross and Bakker 2012, Agrawal and Weber 2015, Grutters et al. 2017b). However, submerged plants are generally low in phenolic compounds (Smolders et al. 2000), the most common group of herbivore deterrent compounds in aquatic plants (Gross and Bakker 2012). As the specific secondary compounds are largely unknown in freshwater aquatic plants (Gross and Bakker 2012), we cannot further elaborate on their impacts on plant palatability here. Studies which find a correlation of plant palatability with plant physical and chemical traits are across a range of species (Elger and Willby 2003, Elger and Barrat-Segretain 2004, Dorenbosch and Bakker 2011, Grutters et al. 2017b). In contrast, studies that test one, or a few plant species did not find a relationship between palatability and plant physical or chemical traits (Cronin et al. 2002, Cronin and Lodge 2003).

It seems that those traits of aquatic plants can better predict plant palatability at an inter-species level than an intra-species level.

Under current global environmental change, different species might respond different to warming and eutrophication, leading to change in aquatic plant communities (Mckee et al. 2002, Feuchtmayr et al. 2009, Li et al. 2017). Furthermore, although we did not observe plant palatability change under rising temperature and nutrient enrichment, nutrient enrichment has been shown to increase top-down control on aquatic plants (Bakker and Nolet 2014), and plant-eating ectotherm herbivores might increase their consumption rate with warming (Zhang et al. 2018b), leading to enhanced top-down control on plants (O'Connor 2009, Schaum et al. 2018).

Conclusions

We conclude that temperature rise and nutrient enrichment can have strong effects on aquatic plant growth and stoichiometry. Aquatic plant growth increased with rising temperature and nutrient enrichment in the sediment, whereas nutrient loading to the water column could inhibit the growth of the plant. The effects of temperature on stoichiometry highly depend on environmental nutrient conditions. Despite alterations in plant stoichiometry with rising temperature, we did not find evidence for altered plant consumption rates. This implicates that warming and eutrophication interact to alter plant abundance and stoichiometry, but may not directly alter plant consumption by higher trophic levels.

Acknowledgements

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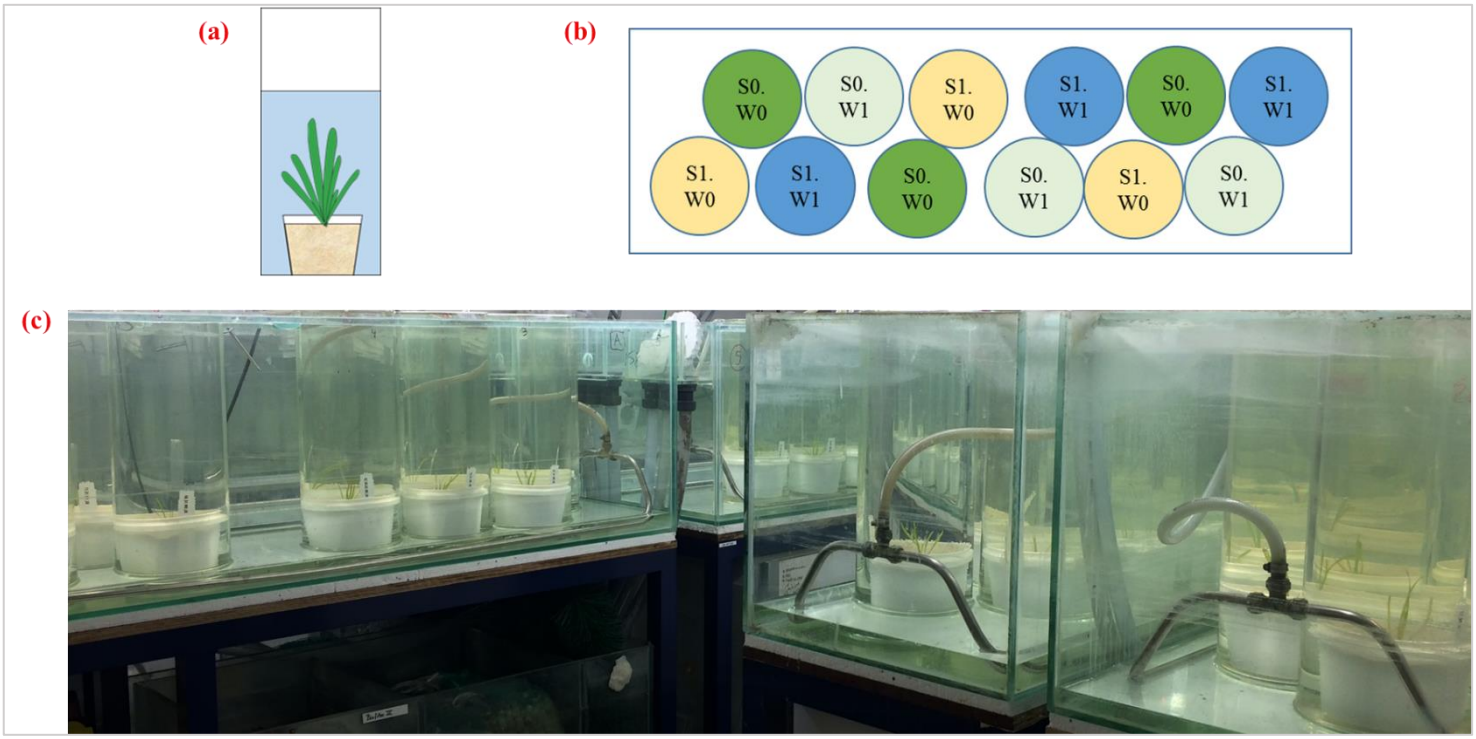


Figure S4.1 Experimental design and layout. (a) Plant in each vase; (b) Layout of the four different nutrient treatments in one aquarium, the distribution was random and different in each aquarium. S1 indicates nutrient-rich sediment, S0 indicates nutrient-poor sediment, W1 means with external nutrient loading to the water, and W0 means without external nutrient loading. (c) Side view of the set-up at the beginning of the experiment.

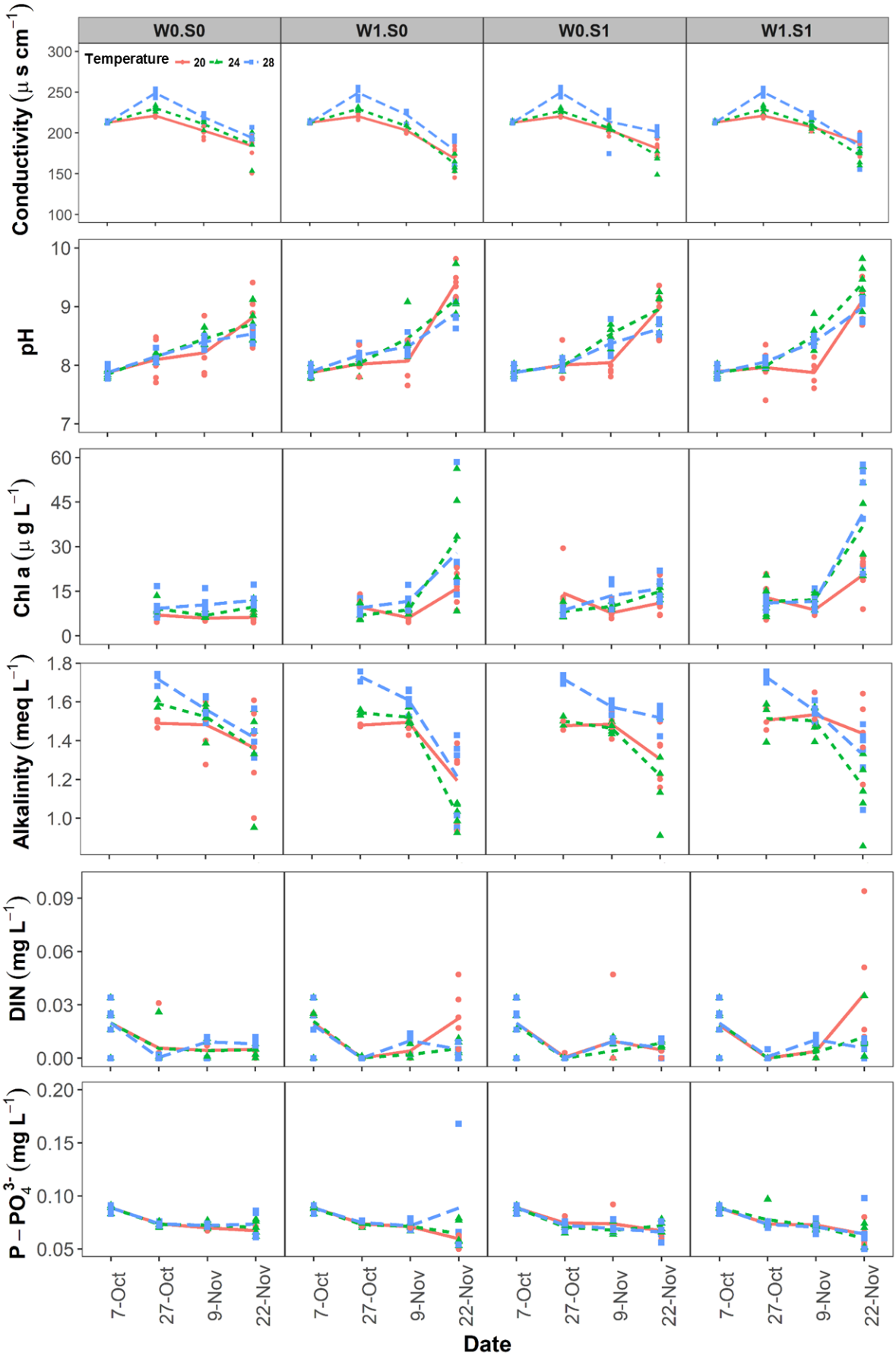
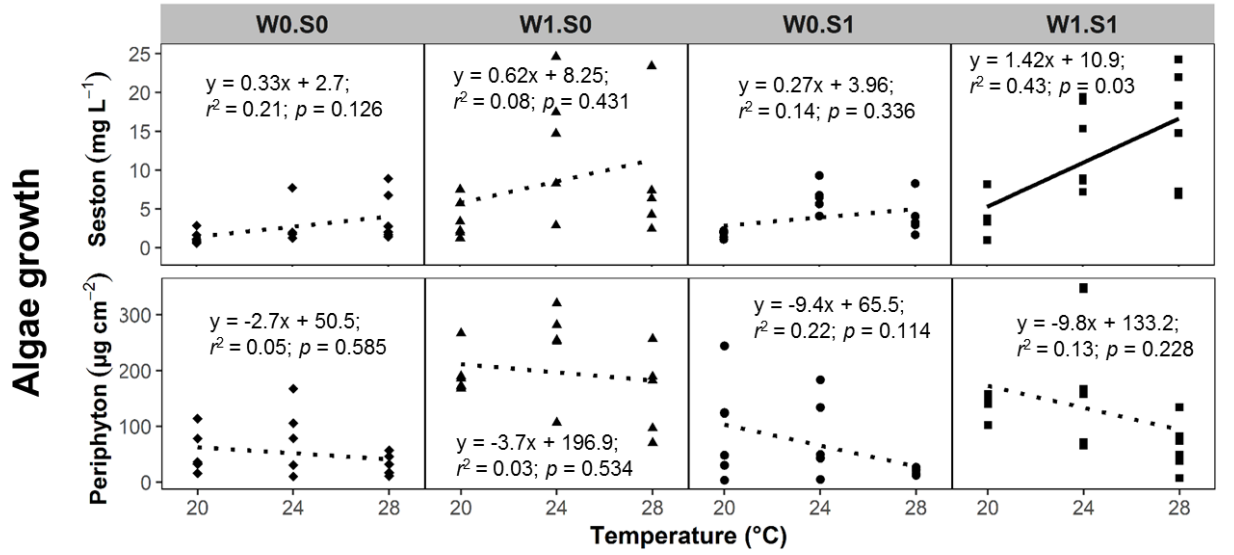
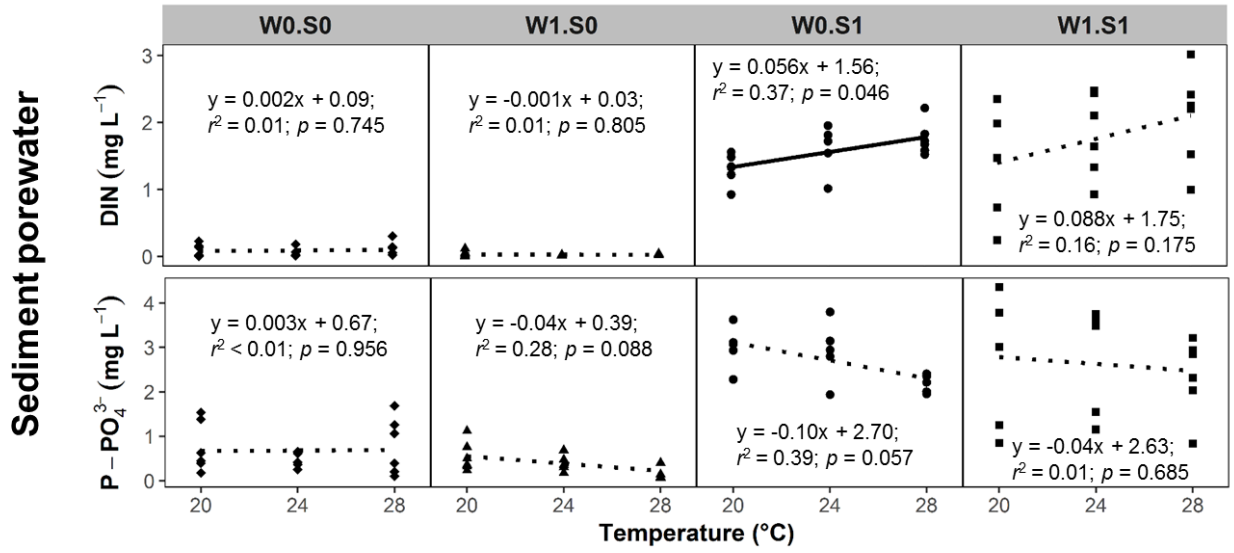

W0.S0
W1.S0
W0.S1
W1.S1
Temperature
20
24
28
Conductivity (µ s cm^{-1})
pH
Chl a (µ g L^{-1})
Alkalinity (meq L^{-1})
DIN (mg L^{-1})
P – PO$_4^{3-}$ (mg L^{-1})
7-Oct
27-Oct
9-Nov
22-Nov
Date

Figure S4.2 Water quality measurements in the water column over time during the experiment indicated per nutrient treatment. Parameters include Conductivity; pH; Chlorophyll a; Alkalinity; DIN (the sum of N from NH_4^+, NO_2^- and NO_3^-); P-PO_4^{3-} (the P from PO_4^{3-}). S1 indicates nutrient-rich sediment, S0 indicates nutrient-poor sediment, W1 indicates with external nutrient loading to the water, and W0 indicates without external nutrient loading.

Conductivity and pH were measured with a multi–meter (Multi 350i/SET, Germany). Alkalinity was measured by an auto-titration machine (TIM840 titration manager, Germany). Chlorophyll a (Chl a) concentration in the water column was determined by a phytoplankton analyser (PHYTO-PAM, WALZ, Germany). Ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and orthophosphate (PO_4^{3-}) were analyzed by an AutoAnalyzer (QuAAtro, Seal Analytical, Fareham, UK) after filtering water samples over prewashed GF/F filter (Whatman, Maidstone, UK).


Algae growth
W0.S0
W1.S0
W0.S1
W1.S1
Seston (mg L^{-1})
y = 0.33x + 2.7; r^2 = 0.21; p = 0.126
y = 0.62x + 8.25; r^2 = 0.08; p = 0.431
y = 0.27x + 3.96; r^2 = 0.14; p = 0.336
y = 1.42x + 10.9; r^2 = 0.43; p = 0.03
Periphyton (µg cm^{-2})
y = -2.7x + 50.5; r^2 = 0.05; p = 0.585
y = -3.7x + 196.9; r^2 = 0.03; p = 0.534
y = -9.4x + 65.5; r^2 = 0.22; p = 0.114
y = -9.8x + 133.2; r^2 = 0.13; p = 0.228
Temperature (°C)


Sediment porewater
W0.S0
W1.S0
W0.S1
W1.S1
DIN (mg L^{-1})
y = 0.002x + 0.09; r^2 = 0.01; p = 0.745
y = -0.001x + 0.03; r^2 = 0.01; p = 0.805
y = 0.056x + 1.56; r^2 = 0.37; p = 0.046
y = 0.088x + 1.75; r^2 = 0.16; p = 0.175
P – PO_4^{3-} (mg L^{-1})
y = 0.003x + 0.67; r^2 < 0.01; p = 0.956
y = -0.04x + 0.39; r^2 = 0.28; p = 0.088
y = -0.10x + 2.70; r^2 = 0.39; p = 0.057
y = -0.04x + 2.63; r^2 = 0.01; p = 0.685
Temperature (°C)

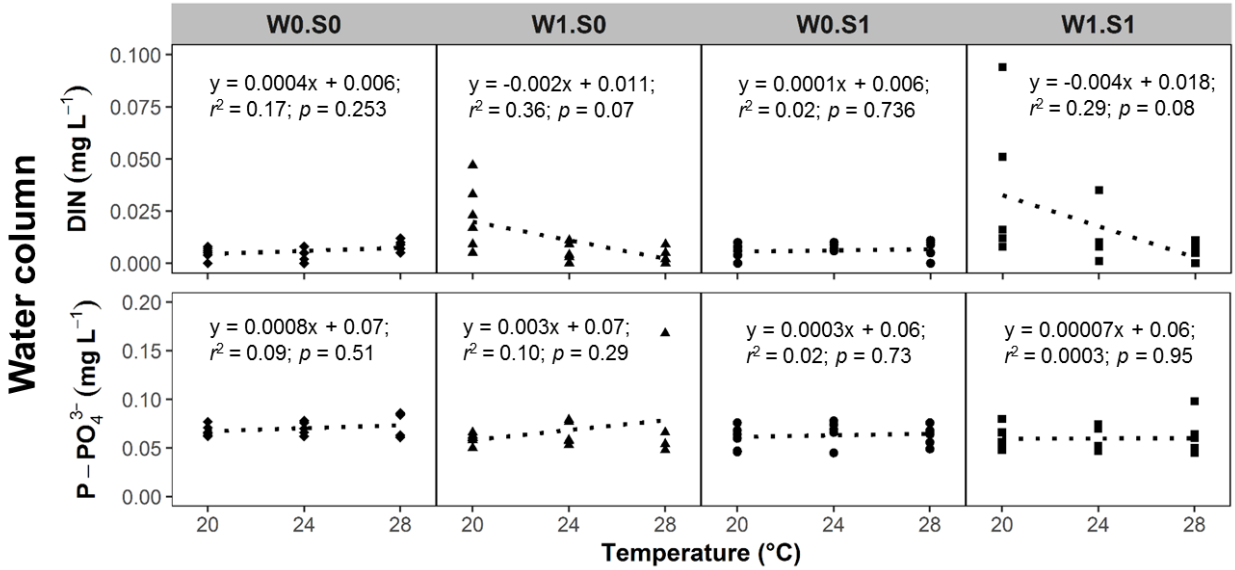

Water column
W0.S0
W1.S0
W0.S1
W1.S1
DIN (mg L^{-1})
y = 0.0004x + 0.006; r^2 = 0.17; p = 0.253
y = -0.002x + 0.011; r^2 = 0.36; p = 0.07
y = 0.0001x + 0.006; r^2 = 0.02; p = 0.736
y = -0.004x + 0.018; r^2 = 0.29; p = 0.08
P – PO_4^{3-} (mg L^{-1})
y = 0.0008x + 0.07; r^2 = 0.09; p = 0.51
y = 0.003x + 0.07; r^2 = 0.10; p = 0.29
y = 0.0003x + 0.06; r^2 = 0.02; p = 0.73
y = 0.00007x + 0.06; r^2 = 0.0003; p = 0.95
Temperature (°C)

Figure S4.3 Temperature effects on algae growth, nutrient concentrations in the sediment porewater, and nutrient concentrations in the water column at the end of the experiment, indicated per nutrient treatment. DIN indicates total dissolved inorganic nitrogen (including N from NH_4^+, NO_2^- and NO_3^-). S0 indicates nutrient-poor sediment, W1 indicates with external nutrient loading to the water, and W0 indicates without external nutrient loading. A dotted line indicates $p > 0.05$, and a solid line indicates $p < 0.05$. Intercepts of the formulas are all at 24℃. Please notice that the y-axis range for sediment porewater nutrients are much larger than that of water column nutrients.

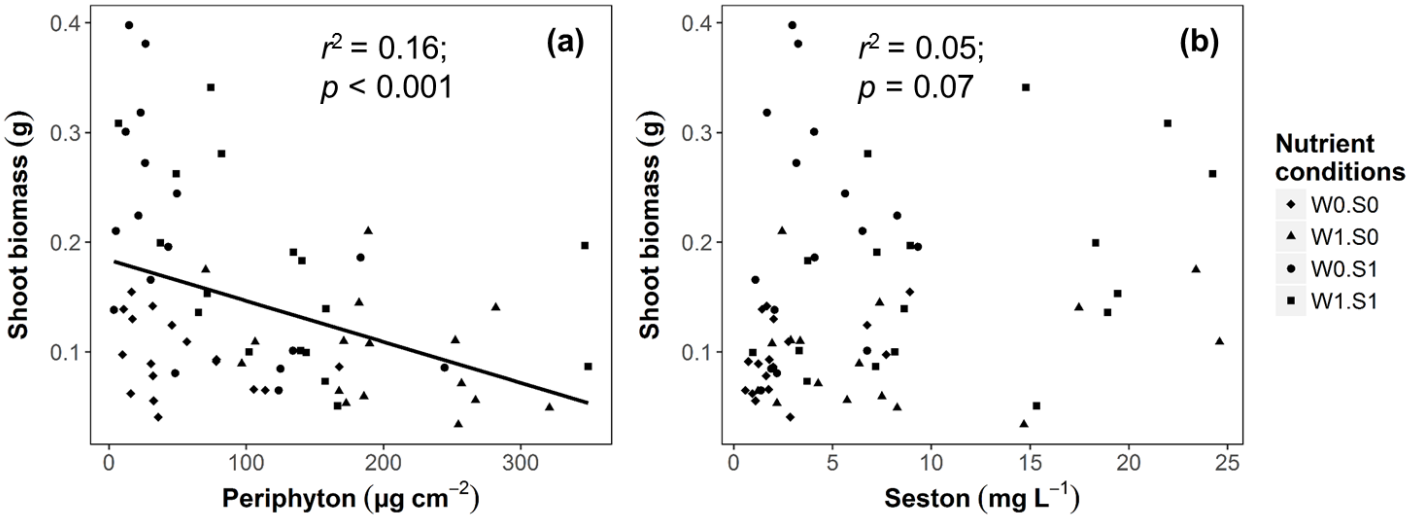


Figure S4.4 The relationship between algae growth and plant shoot biomass at the end of the experiment. (a) Periphyton biomass density (dry weight) and plant shoot biomass (dry weight per vase); (b) Seston concentration (dry weight) and plant shoot biomass. Linear regression test results are shown in the figures. See caption of Figure S4.1 for an explanation of the abbreviations of the nutrient treatments.

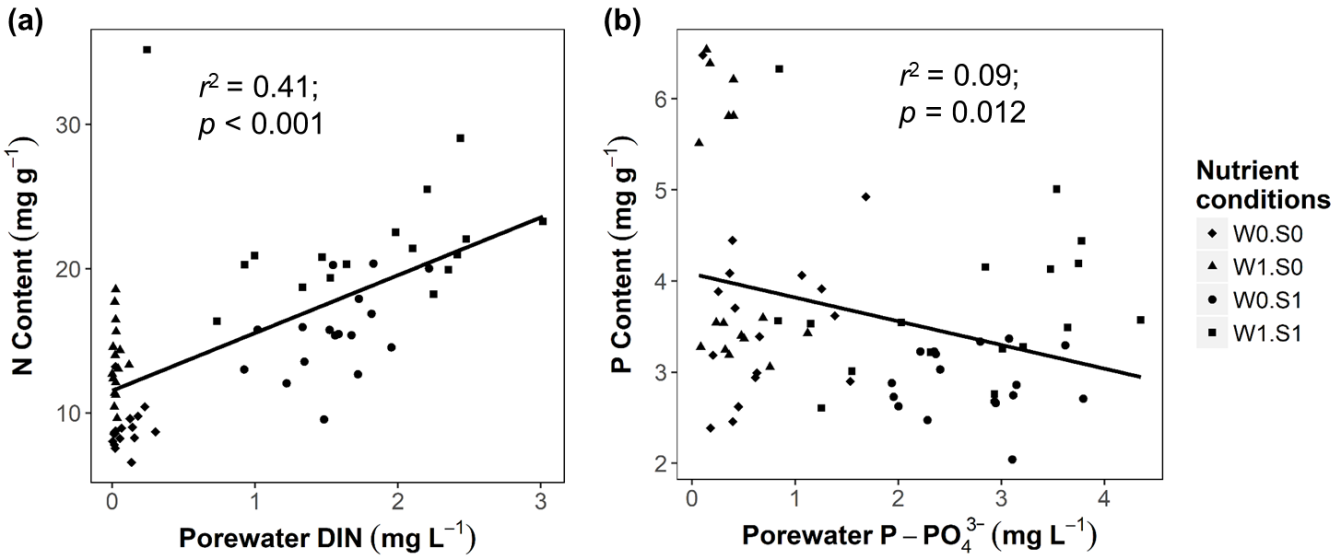


Figure S4.5 The relationship between sediment porewater nutrient concentrations correlation and plant nutrient contents. (a) porewater DIN concentration and plant N content, DIN indicates total dissolved inorganic nitrogen (including N from NH_4^+, NO_2^- and NO_3^-). (b) porewater P-PO_4^{3-} concentration and plant P content. Linear regression test results are shown in the figures. See caption of Figure S4.1 for an explanation of the abbreviations of the nutrient treatments.

Table S4.1 Effects of temperature, sediment type, external nutrient loading and their interactions on algae growth, nutrient concentrations in the sediment porewater and nutrient concentrations in the water column at the end of the experiment. Effects were tested by Linear-Mixed Effect models after model selection. It is indicated in case data were transformed to meet model requirements.

Category	Dependent variable	Explanatory variable	Estimate	SE	*Z*-value	*p*-value
Algae growth	**Seston**	Intercept	2.876	1.317	2.14	**0.032**
		Sediment	1.702	1.076	1.55	0.121
		Temperature	0.260	0.371	0.50	0.617
		Water	6.334	1.081	5.74	<**0.001**
		Water × Temp	0.767	0.326	2.30	**0.021**
		Sed × Temp	0.349	0.324	1.05	0.292
	sqrt(Periphyton)	Intercept	6.623	1.013	6.40	<**0.001**
		Sediment	0.643	0.969	0.65	0.516
		Temperature	-0.267	0.260	0.74	0.458
		Water	7.312	0.986	7.26	<**0.001**
		Sed × Water	-3.801	1.384	2.69	**0.007**
		Sed × Temp	-0.338	0.207	1.60	0.110
Sediment porewater nutrient concentrations	**log(DIN + 0.01)**	Intercept	-2.735	0.170	15.79	<**0.001**
		Sediment	3.162	0.216	14.31	<**0.001**
		Water	-0.691	0.220	3.08	**0.002**
		Temperature	0.045	0.029	1.08	0.281
		Sed × Water	0.714	0.309	2.27	**0.024**
	P-PO_4^{3-}	Intercept	0.575	0.167	3.38	<**0.001**
		Sediment	2.129	0.160	13.07	<**0.001**
		Water	-0.199	0.159	1.23	0.220
		Temperature	-0.045	0.036	0.88	0.380
Nutrients in the water column	**log(DIN + 0.01)**	Intercept	-4.556	0.552	8.08	<**0.001**
		Temperature	0.016	0.023	0.49	0.621
		Water	2.953	0.622	4.65	<**0.001**
		Sediment	-0.112	0.026	4.28	0.361
		Temp × Water	0.080	0.085	0.91	<**0.001**
	log(P-PO_4^{3-} +0.001)	Intercept	-2.664	0.060	43.673	<**0.001**
		Sediment	-0.109	0.042	2.519	**0.012**
		Water	-0.077	0.042	1.805	0.071

"Temp" represents temperature treatment. "Sed" represents sediment treatment. DIN means total dissolved inorganic nitrogen (including N from NH_4^+, NO_2^- and NO_3^-). "log" and "sqrt" indicate the data are natural log and square root transformed respectively. Bold numbers indicate $p < 0.05$.

Table S4.2 Model selection for all the parameters. The parameters are plant growth, plant elemental composition, plant stoichiometry, plant palatability, algae growth, sediment porewater nutrient concentration and water column nutrient concentration. For each parameter, all models with a ΔAICc < 2 were selected and averaged. Only selected models are shown in the table. "+" indicates the variable that was included in the model. "Temp" represents temperature treatment. "Sed" represents sediment treatment.

Category	Parameters	Sediment	Temperature	Water	Sed × Temp	Sed × Water	Temp× Water	Sed × Temp× Water	AICc	ΔAICc	Weight
Plant growth	Shoot biomass	+	+	+	+	+			-216.11	0.00	0.34
		+	+	+	+	+	+		-214.96	1.14	0.19
		+	+		+				-214.39	1.71	0.14
		+	+	+	+				-214.37	1.74	0.14
	Root biomass	+	+	+	+	+			-325.60	0.00	0.33
		+	+	+	+	+	+		-324.78	0.81	0.22
	Growth rate	+	+	+	+		+		-523.42	0.00	0.43
		+	+	+	+				-522.46	0.96	0.27
	Root:Shoot ratio	+	+	+					-28.16	0.00	0.24
		+	+						-27.46	0.70	0.17
		+	+	+	+				-27.44	0.72	0.17
		+	+		+				-26.91	1.25	0.13
Plant elemental composition	C	+	+	+	+	+			391.38	0.00	0.28
		+	+	+	+				391.39	0.02	0.28
	N	+	+	+			+		-38.19	0.00	0.31
		+	+	+	+		+		-37.55	0.64	0.23
		+	+	+		+	+		-36.64	1.55	0.14
	P	+	+	+	+				-21.79	0.00	0.50
		+	+	+	+		+		-19.98	1.81	0.20
Plant stoichiometry	C:N	+	+	+		+	+		412.89	0.00	0.60
		+	+	+	+	+	+		414.79	1.90	0.23
	C:P	+	+	+	+				601.78	0.00	0.37

Category	Parameters	Sediment	Temperature	Water	Sed × Temp	Sed × Water	Temp× Water	Sed × Temp× Water	AICc	ΔAICc	Weight
Plant palatability	N:P	+	+	+	+		+		602.33	0.55	0.28
		+	+	+	+	+			603.33	1.55	0.17
		+	+	+	+		+		-50.76	0.00	0.39
		+	+	+	+	+	+		-49.69	1.07	0.23
		+	+	+	+				-49.32	1.44	0.19
	RCR		+						548.08	0.00	0.26
									549.24	1.16	0.15
Algae growth	Seston	+	+	+			+		407.23	0.00	0.25
			+	+			+		407.38	0.15	0.23
		+	+	+	+		+		408.56	1.33	0.13
	Periphyton	+		+		+			349.90	0.00	0.27
		+	+	+	+	+			350.04	0.14	0.25
		+	+	+		+			350.32	0.42	0.22
Sediment porewater nutrient concentration	DIN	+		+		+			141.69	0.00	0.33
		+	+	+		+			142.13	0.44	0.26
	$P\text{-}PO_4^{3-}$	+							145.58	0.00	0.24
		+		+					146.28	0.70	0.17
		+	+						146.45	0.88	0.15
		+	+	+					147.27	1.69	0.10
Nutrients in the water column	DIN		+	+			+		62.49	0.00	0.52
		+	+	+			+		64.06	1.56	0.24
	$P\text{-}PO_4^{3-}$	+		+					-27.80	0.00	0.30
		+							-26.70	1.11	0.17

Chapter 5

Aquatic omnivores shift their trophic position towards increased plant consumption as plant stoichiometry becomes more similar to their body stoichiometry

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Abstract

Human induced eutrophication has strongly altered aquatic ecosystems. With increasing eutrophication, plant nutrient concentrations increase, making them more attractive as food for herbivores. However, most aquatic consumers are omnivorous. Ecological stoichiometry theory predicts that animals prefer to consume food which has a similar nutrient (N and P) composition or C:nutrient ratio compared to their own bodies, hence omnivorous animals may prefer to eat animal prey instead of plants. We ask whether aquatic omnivores would shift their diet towards more plant consumption when plants are more nutritious and their stoichiometry becomes more similar to the stoichiometry of the omnivore. We hypothesized that: (1) the omnivore increases plant consumption as the plant C:nutrient ratio decreases when there is only plant material available; (2) the omnivore generally prefers animal food over plant material; (3) the omnivore will increase its relative plant consumption as the plant C:nutrient ratio decreases, in the presence of animal food. As a model system, we used the pond snail *Lymnaea stagnalis* (omnivorous consumer), the aquatic plant *Potamogeton lucens* (plant food to the consumer, cultured at different nutrient regimes to obtain different plant C:nutrient ratios), and the crustacean *Gammarus pulex* (animal food to the consumer, using freshly dead individuals). When there was only plant material available, the consumers increased their relative consumption rate with decreasing plant C:nutrient ratio from no measurable amount to about 102 mg g^{-1} day^{-1}. When plant material was offered simultaneously with animal food, even though the omnivores always preferred animal food over plant material, the omnivores still increased their relative intake of plant material as plant C:nutrient ratio decreased, from virtually nothing at the highest to on average 16% of their diet at the lowest plant C:nutrient ratio, with a maximum of 28%. Therefore, we conclude that as nutrient loading increases in aquatic ecosystems, plants are flexible in taking up nutrients which can lower their C:nutrient ratio. As a result, plant-eating omnivorous animals may shift their trophic position towards increased plant consumption and alter the food web structure, resulting in increased top-down control on aquatic plants.

Introduction

Nutrient loading caused by anthropogenic activities has strongly altered the structure and functioning of aquatic ecosystems (Scheffer et al. 1993, Carpenter et al. 2011, Hilt et al. 2017). In shallow aquatic systems, aquatic plant communities are important components because they stabilise clear water states (Bakker et al. 2010) and sustain high biodiversity (Declerck et al. 2005, Cronin et al. 2006). Submerged aquatic plant communities have rapidly declined because of eutrophication (Sand-Jensen et al. 2000, Zhang et al. 2017). The classical underlying mechanism for rapid plant declines is the fast growth of algae that outcompete plants at high nutrient loadings (Scheffer et al. 1993, Sayer et al. 2010a). However, more recent insights also point to increased herbivory on aquatic plants as a reason for their decline, as herbivores can have a large impact on aquatic plants (Bakker et al. 2016, Wood et al. 2017).

More specifically, it has been hypothesized that the impact of herbivores on aquatic plants increases as plant quality increases (Bakker and Nolet 2014, van Altena et al. 2016). The underlying mechanism is that, when plant nutrient concentration increases with nutrient loading to a water body, these plants would be more attractive to herbivores, experience more grazing, resulting in enhanced top-down control under eutrophic conditions (van Altena et al. 2016). However, most aquatic plant-consuming animals are omnivores (Bakker et al. 2016, Wootton 2017), which means that they feed on both plant and animal material. Omnivores can actively select preferred food types if both types are available. Ecological stoichiometry theory predicts that animals prefer to consume food which has similar nutrient (N and P) composition or C:nutrient ratio compared to their own bodies (Elser et al. 2000b, Sterner and Elser 2002). Animal prey has a more similar C:nutrient ratio with omnivores compared to plant material, which generally has much higher C:nutrient ratio than its animal consumers (Elser et al. 2000b, Van de Waal et al. 2010). Hence, according to ecological stoichiometry theory, omnivorous animals would generally prefer animal prey over plant material. In this scenario plant material would only be eaten, when not enough animal material is available (Dorenbosch and Bakker 2011, Guinan Jr et al. 2015). However, we hypothesize that this may change when the stoichiometry of plant material becomes more similar to the stoichiometry of animal prey.

Whereas animal food has a more stable stoichiometric composition than plants (Mattson Jr. 1980, Sterner and Elser 2002, Andersen et al. 2004), plants are more flexible, meaning that their quality as food for consumers may increase as a result of eutrophication. If plants become more nutrient rich under eutrophic conditions, this could decrease the Carbon: nutrient ratio gap

between plant material and animal food. This could be an underlying mechanism explaining patterns of selective foraging such as previously found in grazing experiments with ducks (Bakker and Nolet 2014). Indeed, aquatic animals prefer plants with a higher nitrogen concentration and lower C:N ratio (Dorenbosch and Bakker 2011, Grutters et al. 2017b). However, it is unknown whether aquatic omnivores would shift to plant consumption when plants are more nutritious, or still prefer their animal food. Whereas terrestrial omnivores have been recently shown to shift their trophic position towards more plant consumption upon eutrophication (Liman et al 2017), there are no studies to date which have directly tested the effects of intraspecific variation in plant quality on plant consumption by aquatic omnivores. The consequences of eutrophication for the impact of omnivorous animals on aquatic plants remain therefore largely unknown for aquatic ecosystems.

Here we use an aquatic plant-animal prey-omnivore system to experimentally test the consumption rates of omnivorous aquatic animals in response to changing plant quality, expressed as nutrient concentration or C:nutrient ratio, in the presence and absence of animal food. We hypothesise that: (1) the omnivore increases plant consumption as plant quality increases when there is only plant material available; (2) the omnivore generally prefers animal food over plant material; (3) the omnivore will increase its relative plant consumption as plant quality increases, in the presence of animal food.

Methods and Materials

Model omnivore

We used the generalist omnivore *Lymnaea stagnalis* L., the great pond snail, because it represents a common, generalist omnivorous consumer in aquatic systems and aquatic molluscs can have large impacts on aquatic plant abundance (Lodge 1991, Newman 1991, Wood et al. 2017). *L. stagnalis* has been previously used for plant feeding trials in aquatic settings (Elger and Barrat-Segretain 2002, 2004, Grutters et al. 2017b, Zhang et al. 2018b). *L. stagnalis* feeds on vascular plants (Reavell 1980, Elger et al. 2004) as well as carrion, such as dead insects, crayfish, frog tadpoles, fish and even other dead snails (Bovbjerg 1968). It can distinguish high and low quality food by perception of volatile organic compounds released by the food (Moelzner and Fink 2014).

Egg clusters from captive *L. stagnalis* were collected in a pond on the terrain of NIOO-KNAW, Wageningen, The Netherlands (51°59'14.8"N, 5°40'14.8"E) and hatched, after two weeks the juveniles were transferred to square plastic buckets (0.38 × 0.26 × 0.27 m, L × W × H), each filled with 15 liters of groundwater, and exposed to a 16 : 8 h day : night cycle at a constant temperature of 20°C. Snails were reared on a diet of butterhead lettuce five days per week, and fish food pellets (Velda, Gold Sticks Basic Food) and chalk (ensured sufficient calcium for shell development) were supplied once a week to provide other nutrients. Culturing water was replaced weekly and constantly aerated. All snails were cultured for over 100 days before performing the feeding trial. Snails used in the trials had an average shell length of 30.2 ± 2.4 mm (mean ± SD, n = 94).

Plant food

Potamogeton lucens L. was chosen as the plant material, as it is a common native plant in The Netherlands and one of the most preferred submerged aquatic plants by *L. stagnalis* (Elger et al. 2004, Grutters et al. 2017b). *P. lucens* rhizomes were collected from a ditch to the west of Wageningen, The Netherlands (51.966484°N, 5.620158°E). To obtain plant material of varying nutrient contents, 76 individual rhizomes were planted individually in 76 square bins (20.5 × 19 × 27cm) and placed in 19 blocks in a single climate-controlled room. Four different nutrient loadings (Table S5.1) were applied to each block of 4 bins to create plant material with a wide range of nutrient contents. Nutrient solutions were made by dissolving NH_4NO_3 and KH_2PO_4 in deionized water and added to the bins to reach the targeted nutrient loading. The plants were cultured from July 16th to October 1st 2015, after which plant material was harvested from 38 bins that had enough material for feeding trials (at least 0.3 g of fresh leaves was needed for each pair of feeding tests). To increase the power of the experiment, several plants (n = 6) of the treatments which produced a limited amount of plant material provided leaf material for multiple feeding trials.

Animal food

We chose the crustacean *Gammarus pulex* L. as our animal food source. *G. pulex* is one of the most important invertebrate species in temperate streams, and widely distributed throughout Europe (Holdich and Pöckl 2007). Populations can reach a density of 10000 m^{-2} and

it has a continued mortality throughout the year (Welton 1979). *G. pulex* feeds on a variety of debris, such as oak and elm leaves (Sutcliffe et al. 1981). Ditches with oak trees along the banks are common in The Netherlands, providing suitable habitat, and there are also plenty of macrophytes and *L. stagnalis* in many of these ditches. The habitat of *G. pulex* largely overlaps with suitable habitat for *L. stagnalis*, as both thrive in macrophyte-dominated ditches and other shallow waters. Therefore, *L. stagnalis* can be expected to frequently encounter *G. pulex* carrion as possible food source in its natural habitat.

G. pulex were procured three days before the start of the feeding trial from a ditch close to Wageningen University, The Netherlands (51.989674°N, 5.648653°E). Individuals were placed in continuously aerated groundwater in a plastic tank (0.38 × 0.26 × 0.27 m, L × W × H) and fed detritus from the same ditch. For the experiment, only *G. pulex* exceeding 1.4 cm in body size were selected for the feeding trials. Shortly before the feeding trials, *G. pulex* were killed in 45°C water before being offered to the snails as snails cannot capture the living *G. pulex*, and the *G. pulex* would not structurally degrade when killed at this temperature, as was shown from pilot trials.

Feeding trials

The feeding trials followed standard protocols developed for snails (Elger and Barrat-Segretain 2002, 2004, Grutters et al. 2017b). To test our first hypothesis, we performed feeding trials in which snails were fed plant material of varying nutrient status (the no-choice experiment). To test our second and third hypotheses, we performed feeding trials in which snails were offered both plant and animal food simultaneously (the choice experiment). In total for both experiments, 94 snails were randomly divided into two groups: snails that were to be fed only *P. lucens* of varying nutrient status (no-choice plant group, n=47), and snails which were offered a choice between *P. lucens* and *G. pulex* (choice group, n=47). Additionally, for each plant a portion of the leaves was introduced to a cup without snail, to act as a control (plant control, n=47). The same was done with *G. pulex* (animal food control, n=12). Pilot trials demonstrated that a snail consumed a maximum of 0.15g (wet weight) plant food per 24 hours. Leaf portions were sampled as follows: for every plant, all leaves were cut from the stem, including their petiole but excluding stipules. Every leaf had its midrib removed, as this part is not preferred by the snail, and the remaining two halves of lamina were further cut into 6 equally sized pieces, 3 portions for the no-choice plant feeding trial, 2 portions for the choice feeding

trial, and 1 portion as plant control. This distribution was expected to minimize or at least randomize the possible differences between leaves within a plant. For the no-choice feeding trials, we therefore weighed approximately 0.15g wet weight plant material from one bin for each snail. For the choice experiments, we combined 0.10g wet weight plant material (from the same bin as the no-choice feeding trial) with 0.19g wet weight animal material for each snail. This is the maximum animal food one snail could eat during 24 h as was tested by pre-trials. For the choice experiment, the amount of plant material and animal food were different, but both were always present in excess amounts for snails to choose from. We weighed 0.05g plant material and 0.19g animal food for the controls to monitor weight change over 24 h in water without snails. All snails were starved for 48 h before the start of the trials and the feeding lasted for 24 h. Each snail was fed individually in a plastic cup (top diameter 9 cm, and height 11.5 cm) filled with 375 ml groundwater, covered with a mesh at the top to prevent snail escape.

After all feeding trials, the dry weights of all remaining food as well as uneaten plant material was collected, measured, and dried in an oven at 60°C for over 48 h. All snails were first frozen to death at -20°C, the soft body of the snail was separated from its shell, and then the snail was dried in an oven at 60°C for over 48 h. We measured carbon (C), nitrogen (N), and phosphorus (P) contents of random samples of *G. pulex*, n = 12, and *L. stagnalis* bodies, n = 13, as well as all 47 plant control portions. Dried samples were ground into fine powders in a 2ml tube on a Tissuelyser II (QIAGEN, Hilden, Germany). C and N contents were determined by an auto elemental analyser (FLASH 2000, Thermo Scientific, Waltham, MA, USA). P content was determined by first incinerating, digesting, and analyzing in an Auto Analyzer (QuAAtro method, Seal Analytical, Fareham, UK).

Data analyses

Food palatability, represented by food Relative Consumption Rate by the snails (RCR) (mg g^{-1} d^{-1}) was calculated by the following formula (after Elger & Barrat-Segretain, 2002):

$RCR = (F_{id} - F_{fd}) / S_d / 1day$

Where F_{id} is the initial dry weight of the offered food, F_{fd} is the final dry weight of the retrieved food, and S_d is the dry weight of the snail body without shell. To back-calculate the initial dry weight that was offered to the snails from the wet material that was offered, we used extra *G. pulex* and *P. lucens* leaves to establish dry weight – wet weight regression lines. The

regression line for *G. pulex* was y = 0.2107*x (r^2 = 0.99, p < 0.001, n = 27). For *P. lucens* the regression line was y = 0.2477*x (r^2 = 0.97, p < 0.001, n = 25), with y giving dry weight in mg, and x being wet weight in mg. Pairwise t-tests showed that the control portion of plant and animal food lost some weight after soaking in the water for 24 h. Plant material average wet weight loss was 0.0019 g (t_{46} = 5.05, p < 0.001), and animal food average wet weight loss was 0.021 g (t_{11} = 7.76, p < 0.001). We used this to calibrate the food consumption in the feeding trials by accounting for the lost weight of the control portions of food.

Three snails (2.8%) died during the feeding trials and were excluded from the dataset. During feeding trials in which snails ingested very little material, measurement errors on the wet weight of the offered food can explain why we occasionally report negative plant palatability values. To test whether plant palatability was affected by plant nutrient status (hypothesis 1), we used linear regressions to relate plant palatability to plant nutrient content and carbon:nutrient ratios of the food. To test for snail diet selection in the choice feeding trials (hypotheses 2 and 3), we calculated the plant material : animal food consumption ratio and used this in linear regression analyses. Differences in consumption rates between plant and animal matter were calculated by Students t-tests. Pearson's correlation was used to test the relation between plant N and P content. One-way Anova was used to test nutrient level differences among the three organisms. All statistics were performed in R (R version 3.4.2).

Results

Plant leaf N content varied from 10.4 mg g^{-1} to 35.7 mg g^{-1}, P content varied from 0.6 mg g^{-1} to 4.4 mg g^{-1} and C content varied from 347.7 mg g^{-1} to 407.3 mg g^{-1}. Pearson's correlations showed significant correlations between plant N content and P content (t_{45} = 3.55, r = 0.48, p < 0.001). The C:N ratio of plant leaves varied over 3-fold (Fig. 5.1a), and the C:P ratio varied over 7-fold (Fig. 5.1b). In contrast, the C:N and C:P ratios of the animal food and the omnivore all varied less than 1.5-folder (Fig. 5.1, Table 5.1). Organism stoichiometry properties differed among the species, such that the omnivore had a C:N and C:P ratio of its body similar to the animal food, whereas the plant material had an over 4-fold higher C:N and C:P ratio compared to the omnivorous consumer (Fig. 5.1, Table 5.1).

Table 5.1 Elemental composition and stoichiometry of the study organisms. Different letters in the same column indicate that there is a significant difference between the two organisms. Ratios are presented by first calculating the ratio for each individual data point, and thereafter calculating the means. All numbers are presented as means ± SD.

Type	Species	C (mg g^{-1})	N (mg g^{-1})	P (mg g^{-1})	C:N (g g^{-1})	C:P (g g^{-1})	N:P (g g^{-1})
Omnivore	*L. stagnalis* (n=13)	440.6 ± 6.4[a]	100.6 ± 5.1[a]	8.8 ± 0.8[a]	4.4 ± 0.2[a]	50.2 ± 4.3[a]	11.5 ± 1.0[a]
Animal food	*G. pulex* (n=12)	340.0 ± 8.0[b]	68.4 ± 2.4[b]	12.2 ± 0.5[b]	5.0 ± 0.2[a]	27.8 ± 1.4[b]	5.6 ± 0.3[b]
Plant material	*P. lucens* (n=47)	390.4 ± 11.6[c]	22.9 ± 5.1[c]	1.9 ± 0.9[c]	18.0 ± 4.8[b]	247.2 ± 120.3[c]	14.0 ± 6.5[a]

Plant material consumption rates were higher in the no choice trial than in choice trial (in the no-choice trial, 43.4 ± 40.7 mg g^{-1} d^{-1}; in the choice trial, 11.0 ± 18 mg g^{-1} d^{-1}, mean ± SD, t-test, $t_{60} = 4.89$, $p < 0.001$, Fig. 5.2). However, animal food consumption rates were lower in the no choice trial than in the choice trial (in no-choice, 151.7 ± 32.5 mg g^{-1} d^{-1}; in choice, 180.4 ± 47.7 mg g^{-1} d^{-1}, mean ± SD, t-test, $t_{25} = -2.44$, $p = 0.02$, Fig. 5.2). In the no-choice feeding trial, plant relative consumption rates of snails increased with increasing plant N content ($F_{1,42} = 19.93$, $p < 0.001$) and plant P content ($F_{1,42} = 11.77$, $p < 0.01$), and decreased with increasing of C:N ratio ($F_{1,42} = 16.76$, $p < 0.001$, Fig. 5.3a) and C:P ratio ($F_{1,42} = 8.101$, $p < 0.01$, Fig. 5.3b). In the choice feeding trials with both plant and animal matter present, animal material was consumed in much larger quantities than plant material (pairwise t-test, $t_{45} = -26.56$, $p < 0.001$, Fig. 5.2). Animal consumption rate was not affected by plant N content ($F_{1,44} = 0.44$, $p = 0.53$), plant P content ($F_{1,44} = 0.66$, $p = 0.42$), plant C:N ratio ($F_{1,44} = 0.92$, $p = 0.36$, Fig. 5.3), nor plant C:P ratio ($F_{1,44} = 1.21$, $p = 0.28$, Fig. 5.3). In the choice trials, plant consumption rate shows a strong increasing trend as plant N content increased ($F_{1,44} = 3.90$, $p = 0.06$) and as P content increased ($F_{1,44} = 3.60$, $p = 0.06$), as C:N ratio decreased ($F_{1,44} = 3.97$, $p = 0.057$, Fig. 5.3c) and as C:P ratio decreased ($F_{1,44} = 3.93$, $p = 0.054$, Fig. 5.3d). The plant:animal food consumption ratio in the choice feeding trials increased as plant N content increased ($F_{1,44} = 4.72$, $p = 0.035$) and as plant C:N ratio decreased ($F_{1,44} = 4.58$, $p = 0.038$, Fig. 5.3e). The plant:animal food consumption ratio also showed an increasing trend as plant P

content increased ($F_{1,44}$ = 3.38, p = 0.071) and as plant C:P ratio decreased ($F_{1,44}$ = 3.09, p = 0.086, Fig. 5.3f).

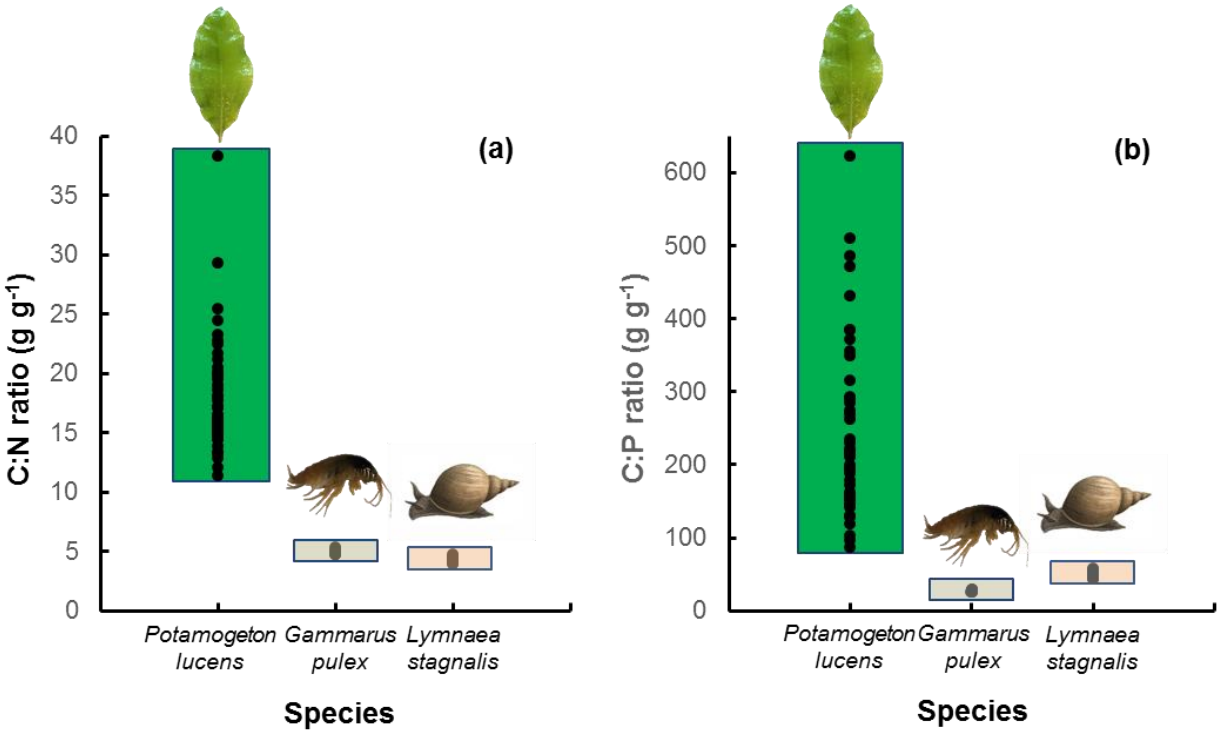

Figure 5.1 Stoichiometry properties of the organisms used in the study. (a) C:N and (b) C:P stoichiometry for respectively leaves of the plant food *Potamogeton lucens* (n = 47), the animal food *Gammarus pulex* (n = 12) and the omnivorous consumer *Lymnaea stagnalis* (n = 13). Dots in the graph reflect the values measured in the experiment. The plants have been cultured at different nutrient loadings to create a range of plant nutrient contents (see main text).

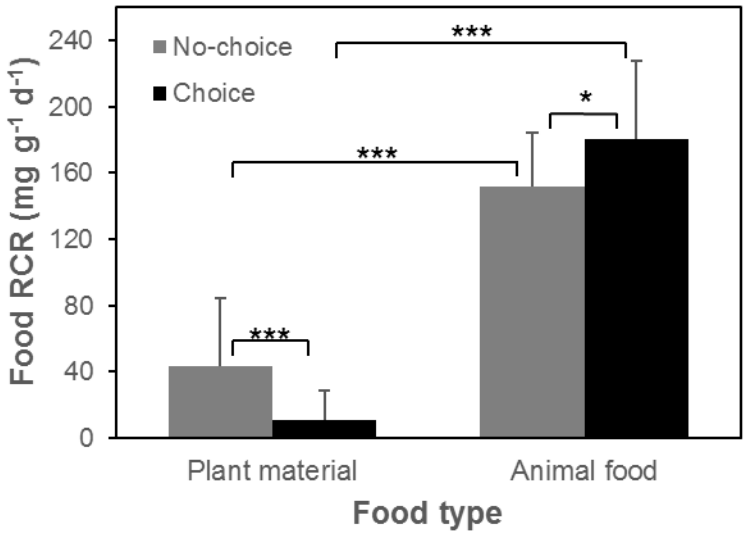

Figure 5.2 Relative consumption rates of plant material and animal food by the omnivore *L. stagnalis* in both choice and no-choice trials. RCR indicates relative consumption rates in mg dry weight consumed per gram dry weight snail body mass per day. * indicates $p < 0.05$, and *** indicates $p < 0.001$.

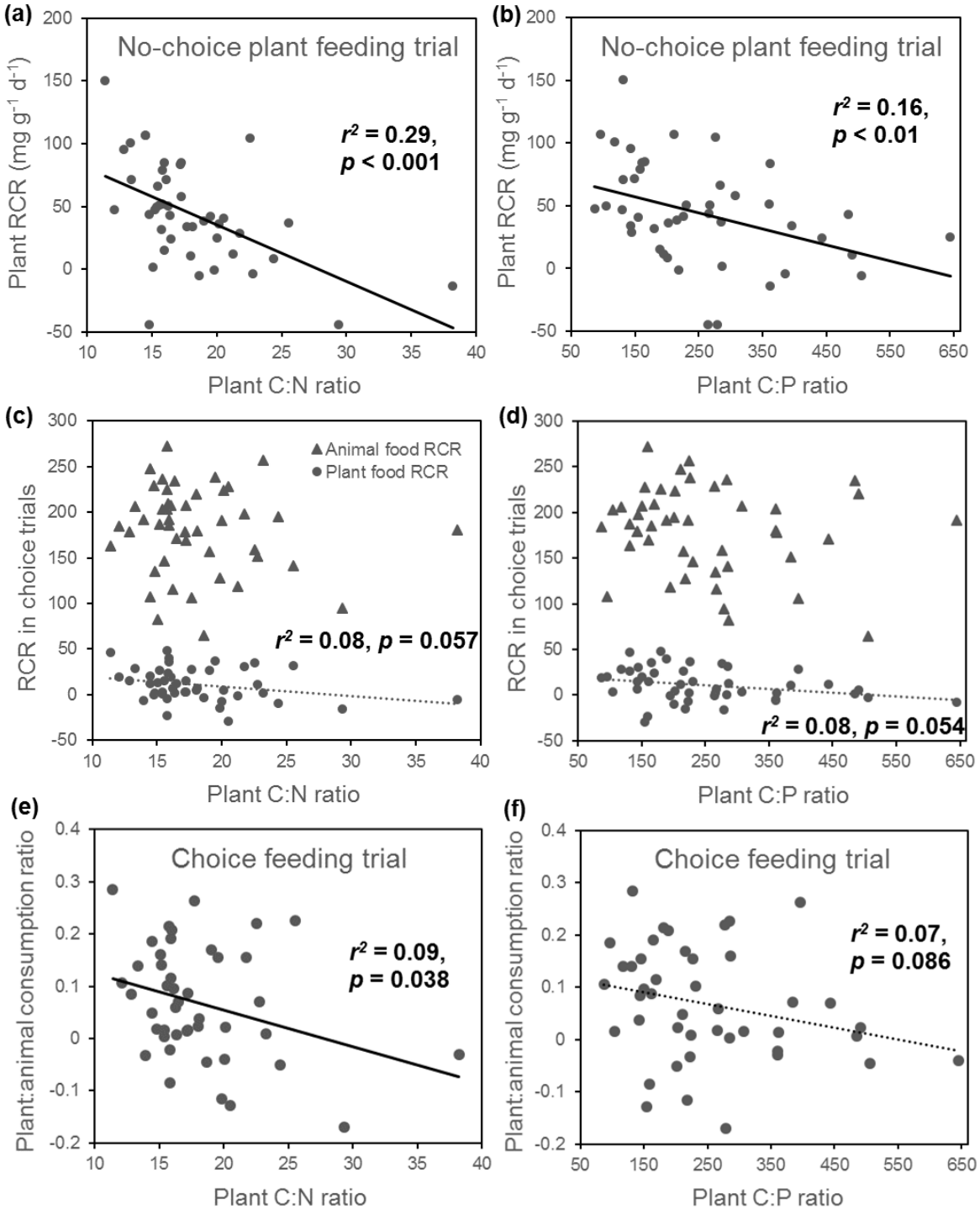

Figure 5.3 Plant relative consumption rate in no-choice trials changed with plant C:N ratio (a) and with plant C:P ratio (b). Plant material and animal food relative consumption rates in choice trials changed with plant C:N ratio (c) and C:P ratio (d). Plant material : Animal food consumption ratio in choice trials changed with plant C:N ratio (e) and C:P ratio (f). Solid regression lines indicate $p < 0.05$ and dotted lines indicate $0.05 < p < 0.1$.

Discussions

In this study we experimentally tested whether aquatic omnivores increase plant consumption as plant quality, expressed as nutrient content and C:nutrient stoichiometry, increases. We found that when there was only plant material available, the omnivore increased plant consumption as plant quality increased from no measurable amount to about 102 mg g^{-1} day^{-1}. When plant material was offered simultaneously with animal food, the omnivores strongly preferred animal food, a result which mirrored the intake rates of plant and animal food in the no-choice tests. Despite this preference for animal food, the omnivores increased their relative intake of plant material as plant quality increased from virtually nothing at the lowest plant quality to on average 16% of their diet at the highest plant quality, with a maximum of 28%. We conclude that as nutrient loading increases in aquatic ecosystems, plants may increase in nutrient content and omnivorous animals may shift their trophic position towards more plant consumption and alter the food web structure, resulting in increased top-down control on aquatic plants.

When there was only plant material available, the omnivore consumed more plant material as the plant C:nutrient ratio decreased. Hence, this confirms our first hypothesis. Generally, this is a classic observation that herbivore removal of plant standing biomass increases as plant N content increases (Mattson Jr. 1980, Cebrian and Lartigue 2004, Shurin et al. 2006). The increased consumption might be more than the re-growth of the plant, leading to enhanced top-down control on plant standing biomass. This has been demonstrated by fertilization experiments with mallard duck (Bakker and Nolet 2014), and is also supported by modelling studies (Hidding et al. 2016, van Altena et al. 2016). In contrast, higher consumption rates in no-choice feeding trials have also been interpreted as compensatory feeding, where the consumer needs to feed more on a poor quality resource to meet its nutrient or energy demands (Cruz-Rivera and Hay 2000, Fink and Von Elert 2006). However, by comparing our no-choice and choice feeding trials we can demonstrate that this is not the case in our experiment. The snails ate 4-fold more animal food compared to plant food in 24 hour no-choice feeding trials, whereas plants were of lesser quality than animal food. Hence for compensatory feeding they should have eaten much more plant food to compensate for the low nutrient levels in plant food. Furthermore, the outcome of the no-choice tests reflected those of the choice tests very well, where the snails similarly consumed much more animal food. When there was animal food available, the omnivore always showed a much stronger preference for animal food, thus

confirming our second hypothesis. Yet, the snails showed an increased preference for plant material as the plant C:nutrient ratio decreased, confirming our third hypothesis.

As the plant nutrient content increased and the C:nutrient ratio decreased, the quality difference compared to animal food decreased, thus making plant food relatively more attractive. In our study plant consumption increased from about zero at low plant quality to a maximum of 28%. This indicates that the snail was still highly omnivorous, with a preference for animal food, but does include a substantial amount of plant food in its diet. These results are in line with the notion that most of the generalist feeders try to consume a mixed diet to balance their nutrition intake (Behmer 2009, Raubenheimer 2011, Lihoreau et al. 2015, Zhang et al. 2018b). Food searching is a very cost-intensive process for the snails due to their low motility. In order to maximize the fitness of the feeding (Pyke et al. 1977), the snails include relatively more plant material in their diet as plant quality is getting closer to their nutrient demand. Similarly, omnivorous fish (Dorenbosch and Bakker 2012, Gu et al. 2018) and mallard duck (Bakker and Nolet 2014), increased plant consumption with increasing plant quality, or shifted their diet to alternative prey when aquatic plants were not palatable. Furthermore, the no-choice feeding trials demonstrate that when the snails have no animal food available they ingest even more plant material. Therefore, with eutrophication, aquatic plants are likely to have more top-down pressure, from both aquatic herbivores and omnivores.

In this study, we employed nutrient contents and C:nutrient stoichiometry as a proxy for plant quality for omnivores. The C:nutrient stoichiometry of food has been shown to be a good indicator of food quality to aquatic animals, where a lower C:nutrient ratio represents higher quality (Elser et al. 2000b, Dorenbosch and Bakker 2011, Bakker et al. 2016). Even though plant secondary metabolites might deter animals from feeding on the plant (Dorenbosch and Bakker 2011, Gross and Bakker 2012), this is not the case for *P.lucens*, as it contains very low total phenolic concentrations compare to other aquatic plants (Grutters et al. 2017b). Furthermore, the concentration of plant phenolic compounds might decrease as plant nutrient content increases, as has been shown in seagrass (Goecker et al. 2005). *P.lucens* is a moderate palatable aquatic plant species based on palatability rankings among a wide range of species from Elger et al. (2004) and Grutters et al. (2017b), which indicates that this species can well represent many other aquatic plant species. In our study the variation in plant C:nutrient ratios was much larger than the variation in C:nutrient ratio of the animal food and the omnivore. Whereas the sample size was larger for plants than the animals that we tested and the plants received different nutrient addition treatments, generally, plants have a much broader range of

C:nutrient ratios than animals (Elser et al. 2000b, Van de Waal et al. 2010). Recent studies show that the C:nutrient stoichiometry of aquatic invertebrates can also vary in eutrophic conditions (Cai et al. 2016). However, with C:N ratios varying from 3.8 to 7.7 g g-1 (Cai et al. 2016), the variation is still much smaller than the C:N ratio of the plants in our study and the C:N ratio in other aquatic plants (Bakker et al. 2016). Therefore, our study still has implications for aquatic plant-omnivore interactions in general.

Implication for ecosystems

In shallow aquatic ecosystems, the growth of aquatic plants is also inhibited by shading of phytoplankton and periphyton (Scheffer et al. 1993, Hidding et al. 2016, Phillips et al. 2016). There is a pervasive top-down pathway through which omnivores can influence aquatic plants from omnivores (fish and birds) to invertebrates (both zooplankton and macroinvertebrates) to algae (both phytoplankton and periphyton) to aquatic plants. The omnivores can inhibit the growth of aquatic plants indirectly by feeding on invertebrates, which graze on algae, thereby releasing the algae from grazing pressure and subsequently, the algae can inhibit the growth of aquatic plants (Brönmark and Weisner 1992, Scheffer et al. 1993, Jeppesen et al. 1997, Hidding et al. 2016, Phillips et al. 2016). Under eutrophication, as primary producer increase, the omnivores abundance increase, imposing more pressure on invertebrates, leading to more biomass of algae, and resulting in more shading pressure and less aquatic plant abundance (Jones and Sayer 2003, Hidding et al. 2016). A similar phenomenon has also been observed in terrestrial ecosystems that increased plant quality can stabilize an omnivore population, and keep the pest animal prey at a low level (Liman et al. 2017). On the other hand, some aquatic omnivores can also directly affect aquatic plants by consuming them (Jones and Sayer 2003, Bakker and Nolet 2014, Hidding et al. 2016). Here, plant quality can increase with eutrophication and the omnivores increase their consumption of aquatic plants. In addition, if the animal prey is not available, the omnivore might feed more on aquatic plants. Therefore, under eutrophication, omnivores are expected to impose a stronger top-down control on aquatic plant standing biomass both indirectly by increasing the shading pressure by algae and directly by increased plant consumption (Hidding et al. 2016). However, whereas the former mechanism has been well documented, the latter, which we demonstrate here, has largely been overlooked. We conclude that omnivores increase their impact on aquatic plants under eutrophication by shifting their trophic position towards enhanced plant consumption. The combined stress of shading by algae and grazing pressure by omnivores and herbivores under eutrophication can

lead to disappearance of submerged aquatic vegetation and a shift to a turbid state dominated by phytoplankton (Hidding et al. 2016, van Altena et al. 2016).

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Table S5.1 Nutrient addition treatments in the plant culture. Each block had four nutrient addition treatments (N+P+; N-P-; N+P-; N-P+) to maximize differences in plant nutrient composition. Nutrients were added once every two weeks. Each bin was filled with 4 cm sand and filled up with 7 L of tap water. Deionized water was added during the culturing to compensate for evaporation. The climate-controlled room was kept at a constant temperature of 20°C, a day:night cycle of 16:8 h, and light intensity at the water surface was approximately 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$. *Daphnia magna* were introduced to control phytoplankton growth in the water, and a single pulmonate snail *Planorbarius corneus* was added to each bin to control periphyton growth. The snail species does not consume our target plant as determined in pre-trials. The nutrient treatments were designed for a one-way Anova test, whereas plants did not produce enough leaf biomass for the feeding trials in multiple bins, therefore we decided to analyse the relation between the resulting plant nutrient contents with plant consumption rates using a regression approach.

Treatment	N adding ($mg\ L^{-1}$)	P adding ($mg\ L^{-1}$)
N+P+	1	0.14
N-P-	0.1	0.014
N+P-	1	0.014
N-P+	0.1	0.14

Chapter 6

The effect of temperature on herbivory by the omnivorous ectotherm snail *Lymnaea stagnalis*

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Abstract

Rising temperatures likely affect trophic interactions in temperate regions as global warming progresses. An open question is how a temperature rise may affect consumer pressure and plant abundance in shallow aquatic ecosystems, where most consumers are omnivorous. Interestingly, herbivory (plant-eating) is more prevalent towards low latitudes in ectotherms such as fish and aquatic invertebrates, and this may be temperature driven. We used pond snails (*Lymnaea stagnalis* L.) as a model aquatic ectotherm species and tested their consumption of both animal prey (*Gammarus pulex* L.) and plant material (*Potamogeton lucens* L.) at three different temperatures (15, 20 and 25 °C). Higher temperatures led to higher consumption rates by the omnivore on both plant food and animal prey, when fed separately. When the food was offered simultaneously, the pond snails consistently preferred animal prey over plant material at all tested temperatures. However, the omnivore did consume plant material even though they had enough animal prey available to them. Based on our experiments, we conclude that with increasing temperatures, *L. stagnalis* will only increase their consumption rates but not change food preference. Further studies are needed to test the generality of our findings across aquatic species to predict the effect of warming on aquatic plant consumption.

Introduction

Average water temperatures are increasing in many temperate lakes as a consequence of climate change (Mooij et al. 2008, Adrian et al. 2009). Biological responses to this temperature rise have already been observed: mean fish size decreases in temperate freshwater systems (Jeppesen et al. 2010a, Meerhoff et al. 2012, Edeline et al. 2013), water bird migration is changing (Van Eerden et al. 2005, Van der Jeugd et al. 2009) and fish communities may be changing towards increasing abundance of fish with wide temperature tolerances (Jeppesen et al. 2012, Meerhoff et al. 2012) as well as fish becoming more omnivorous, e.g. including more plants in their diets (Jeppesen et al. 2010a). An open question is how these changes may affect consumer pressure and plant abundance in shallow aquatic ecosystems. Whereas the temperature rise may generally enhance the growth rates of macrophytes and thus initially stimulate plant abundance (Rooney and Kalff 2000, Feuchtmayr et al. 2009), increased consumption on plants may offset this benefit for the plants (O'Connor 2009). Trophic downgrading, i.e. the shift towards more macrophyte consumption in food webs, can have a very strong impact on the vegetation, the organisms depending on it, and ultimately on ecosystem functions when temperatures increase (Estes et al. 2011, Strickland et al. 2013).

In aquatic systems, most animals consuming vascular plants are omnivorous, including both plant and animal material in their diet (González-Bergonzoni et al. 2012, Gross and Bakker 2012, Wood et al. 2012). The prevalence of herbivory (plant-eating) varies with latitude: in fish communities, the level of herbivory strongly increases towards the equator (Floeter et al. 2005, Moss 2010). This pattern is consistent with increased plant consumption at lower latitudes by invertebrates as well (Pennings et al. 2009, Schemske et al. 2009, González-Bergonzoni et al. 2012). However, it is debated which factors drive this pattern (González-Bergonzoni et al. 2012, Ho and Pennings 2013). Ambient temperature, the evolutionary time to adapt to plant consumption, and food availability have been evoked as important explanatory factors (González-Bergonzoni et al. 2012). At higher temperatures ectotherm animals, such as fish, can better digest plant material (Clements et al. 2009). Indeed, plant consumption by fish shows a strong positive correlation with ambient temperature (Behrens and Lafferty 2007, 2012), and above a threshold of 16°C plant consumption is found to be more common in freshwater fish (Prejs 1984, Lake et al. 2002). Alternatively, more rapid evolution and shorter generation times (due to warmer temperatures) and absence of strong seasonal temperature fluctuations have contributed to the radiation of tropical species (Allen and Gillooly 2006, Mittelbach et al. 2007) and may have allowed adaptation to plants as a food source. Another possibility is that there is

simply less animal food available at low latitudes, resulting in increased plant consumption (Meerhoff et al. 2012). Therefore, it remains unclear whether temperature, evolutionary constraints on plant consumption or food availability are driving the latitudinal gradient in plant consumption by fish. If temperature is an important driver of plant consumption, then this has important implications for the impact of a temperature rise on plant consumption in temperate regions. Therefore experiments that test the effect of temperature on plant consumption are urgently needed.

In this study, we test whether and how different temperatures affect the consumption rates and diet selection of aquatic omnivorous ectotherms. Based on the literature, we hypothesize that: the omnivore will select more plant relative to animal food at higher temperatures (Behrens and Lafferty 2007, 2012). We chose the generalist omnivore *Lymnaea stagnalis* L. as a model aquatic omnivore species and conducted no-choice and choice feeding trials with plants (*Potamogeton lucens* L.) and animal prey (*Gammarus pulex* L.) at different temperatures.

Materials and methods

Model system

We chose *L. stagnalis* for our experiment, an omnivorous molluscan species that has often been used in feeding trials (Elger and Barrat-Segretain 2002, 2004), and which is reported to feed on a variety of benthic and periphytic algae (Brönmark 1989), vascular plants (Gaevskaia 1969, Elger et al. 2004) as well as carrion, such as dead crayfish, insects, frog tadpoles, fish and even snails (Bovbjerg 1968). In the field, molluscs can have a large impact on macrophyte abundance (Lodge 1991, Newman 1991). *P. lucens* is one of the most preferred submerged macrophytes by *L. stagnalis* (Elger et al. 2004) and also a common macrophyte in the Netherlands. The crustacean *G. pulex* is one of the most important invertebrate species in temperate streams, which is widely distributed throughout Europe (Holdich and Pöckl 2007), and can reach a density of 10,000 m^{-2} and has a continued mortality throughout the year (Welton 1979). *G. pulex* feeds on a variety of debris, such as oak and elm leaves (Sutcliffe et al. 1981). Ditches with oak trees along the banks are common in Netherlands, and there are also plenty of macrophytes and *L. stagnalis* in many of these ditches. The snails live in these ditches together with *G. pulex*, which is present at a high density. The snails may intentionally or unintentionally

incorporate some dead *G. pulex* in their diet. Considering their abundance and sympatry, we chose these three species as a snail-animal prey-plant model system to study the effect of changing temperatures on omnivorous diet choice.

Experimental subjects preparation

P. lucens shoots were sampled in a ditch close to Wageningen, the Netherlands (51.97°N, 5.62°E) and then acclimated at 20°C in the laboratory to prevent the decay of plant tissues during the one week storage period preceding the feeding trial. The *G. pulex* were collected in another small ditch close to Wageningen. *G. pulex* were sieved and pipetted into a groundwater filled bucket (15L with aeration) with some degraded plant material from the same ditch to keep them alive. 131 *L. stagnalis* were collected from the ponds at the NIOO-KNAW, Wageningen, the Netherlands (51.99°N, 5.68°E) in June 2015. Snails were evenly divided into 3 plastic buckets (26 * 38 * 26.5cm), each filled with 15 liters of groundwater, and then put in temperature-controlled (15, 20 and 25°C) aquaria (50 * 185 * 50 cm) with a 16:8 day : night cycle to acclimatize 10 days before the feeding trials. The snails were fed butterhead lettuce five days per week. Fish food pellets (Velda, Gold Sticks Basic Food) were supplied once a week to provide other nutrients, and half a piece of chalkboard chalk was supplied to provide calcium, as the concentration of calcium in the ground water (36 mg L^{-1}) may be low for the calciphile species *L. stagnalis* (Van der Borght and Van Puymbroeck 1966, Dalesman and Lukowiak 2010). The water used to culture the snails was fully replaced once, and halfway through the culturing period several indicators of water quality were checked with a multi – meter (Multi 350i/SET, Germany). Average pH was 7.9 ± 0.2 (mean ± SD, n=3), and average conductivity was 307 ± 25.3 µS/cm (mean ± SD, n=3). Snails used in the trials had an average shell length of 27.1 ± 2.2 mm, wet weight with shell of 1.80 ± 0.44 g, and dry weight without shell of 0.12 ± 0.03 g (mean ± SD, n = 108).

Feeding trials

The feeding trials followed standard protocols developed for snails (Elger and Barrat-Segretain 2002, 2004). Both no-choice (only one type of food) and choice (both types of food) trials were performed at 3 different temperatures. Each treatment (9 treatments in total) had 12 replicates and in total 108 snails were used. The test temperatures 15, 20 and 25°C were chosen

because the optimal temperature for the growth and reproduction of *L. stagnalis* is around 20°C and the snail will not feel stress at both 15°C and 25°C (Van der Schalie and Berry 1973). Fresh undamaged *P. lucens* leaves with their midrib removed were offered to the snails as snails prefer the soft parts of the leaves over the midrib. Sub-adults and adults of *G. pulex* were killed in 45°C water before being offered to the snails as snails cannot catch the living *G. pulex*, and the *G. pulex* would not structurally degrade when killed at this temperature, as was shown from pilot trials. Pre-trial pilots showed that no measurable plant growth nor animal prey weight loss occurred when these were left in water for 24 hours at different temperatures, apart for the plant material at 25°C (Table S6.1). At this temperature the plants lost on average almost 2% of weight, which was just significant (Table S6.1). Since this was such a low amount of weight loss, we decided to not further correct for it. Further pilot trials demonstrated that a snail consumed at maximum 0.15 g (wet weight) food in 24 hours' feeding. So both plant material and animal prey were weighed to approximately 0.18 g (wet weight) per portion to allow the snails ad libitum access to food during the feeding trials. For the no-choice trial the amount of food was 0.18 g (wet weight) in each cup, but for the choice trial this was double the amount, about 0.36 g (wet weight) in each cup, because the snails needed ad libitum food for each food type. The mean number of *G. pulex* individuals offered in each cup was 9.2 ± 2.2 (mean ± SD, n = 72) throughout the entire experiment, and pre-trial pilots showed that there was no relationship between consumption of animal prey and the number of animal prey individuals offered, as long as there were enough individuals offered to allow ad libitum feeding. Each snail was fed individually in a plastic cup (top diameter 9 cm, and height 11.5 cm) filled with 375 ml groundwater, which was acclimated to the experimental temperature (Fig. S6.1). The cups were covered with mesh to prevent the snails from escaping. Floating polystyrene foam platforms were used to hold the cups on top of the water in the aquarium. Less than one hour was spent in both putting the snails in the cups before the feeding trials and taking the snails out of the cups after the feeding trials, all the feeding trials were performed simultaneously. All snails were starved for 48 h before the start of the trials and the feeding lasted for 24 h. After the feeding, leftover food was dried in an oven at 60°C for at least 48 h. All snails were first frozen to death at -20°C, the soft body of the snail was separated from its shell, and then the whole snail was dried in an oven at 60°C for at least 48 h. We measured carbon (C), nitrogen (N), and phosphorus (P) contents of random samples of *P. lucens*, *G. pulex*, and *L. stagnalis* bodies, n = 3 for each species. Dried samples were ground into fine powders. C and N were determined by an auto elemental analyser (FLASH 2000, Thermo Scientific, Waltham, MA, USA). P was

determined by first incinerating, digesting, and analyzing in an Auto Analyzer (QuAAtro method, Seal Analytical, Fareham, UK) (Grutters et al. 2015).

Data analysis

We followed the procedures of Elger and Barrat-Segretain (2002) to calculate the snail consumption rates of plant and animal prey. Extra *G. pulex* and *P. lucens* leaves were used to establish dry weight – wet weight regression lines, from which the initial dry weight of the food was back-calculated. The regression line for *G. pulex* was $y = 0.2005*x$ ($r^2 = 0.99$, $p < 0.001$, $n = 14$), with y giving dry weight in mg, and x being wet weight in mg. For *P. lucens* the regression line was $y = 0.2061*x$ ($r^2 = 0.98$, $p < 0.001$, $n = 30$), with y giving dry weight in mg, and x being wet weight in mg. Consumption rate was described as milligram dry weight of food per gram dry weight of snail (without shell) consumed per day (Elger and Barrat-Segretain 2002). The amount of food consumed was calculated by subtracting the dry weight of the left-over food from the calculated initial dry weight of food offered. One-way ANOVA was used to test the mean difference in consumption rate among temperature treatments and the difference in nutrient concentration and stoichiometry between the food items and the consumer. Two-way ANOVA was used to test the interaction between temperature and the presence/absence of an alternative food source (choice and no-choice trials). Plant consumption rates and animal prey consumption rates were tested separately. Pearson correlation was used to test the relationship between plant material consumption and animal prey consumption in the choice trial. There were 4 negative values out of 108 feeding trials, the negative values were most likely due to slight differences in the wet-dry weight ratio calculated from the calibration line, and we kept these values in the statistics. We used the ratio of the plant : animal consumption rate at each temperature to test the diet selection by the snails. To test for differences in plant consumption rate in the no-choice trials at different temperatures by a one-way ANOVA, we transformed the data by add a value then $\log_{10}$ to make the variance homogeneous, which was confirmed by a Levene's test. Data were tested for normality using a Kolmogorov-Smirnov test. All tests were performed in SPSS 22.0 (IBM 2013).

Results

The food items differed significantly in nutrient concentrations and most nutrient ratios from each other and in several of these traits from the body composition of the consumer (Table 6.1). The plant leaves had an almost three times higher C : N and C : P ratio compared to both the animal prey and the snail body composition, the latter not being significantly different from each other. This was mainly driven by lower N and P concentrations in the plant material (Table 6.1).

Table 6.1 Nutrient content and stoichiometry in the food items (plant leaves and animal prey) and the consumer. Values are mean ± standard deviation (SD). Different superscript letters in the same row indicate a significant difference between the items (one-way ANOVA). For all the nutrient measurements n = 3. Dry matter content for plant leaves n = 30, for animal prey n = 14.

	Plant leaves (*P. lucens*)	**Animal prey (*G. pulex*)**	**Omnivore (*L. stagnalis*)**	***F***	***p***
C (mg g^{-1})	397 ± 2.5^{a}	347 ± 18^{b}	430 ± 6.6^{c}	290.1	< 0.001
N (mg g^{-1})	30.8 ± 0.62^{a}	77.8 ± 5.8^{b}	100 ± 3.8^{c}	236.6	< 0.001
P (mg g^{-1})	3.17 ± 0.33^{a}	8.53 ± 1.3^{b}	7.59 ± 0.59^{b}	34.10	0.001
C:N (g g^{-1})	12.9 ± 0.33^{a}	4.48 ± 0.32^{b}	4.29 ± 0.17^{b}	928.6	< 0.001
C:P (g g^{-1})	126 ± 13^{a}	41.4 ± 6.2^{b}	56.8 ± 3.8^{b}	87.17	< 0.001
N:P (g g^{-1})	9.76 ± 1.1^{a}	9.23 ± 1.3^{a}	13.3 ± 0.89^{b}	11.93	0.008
Dry matter content (g g^{-1})	0.21 ± 0.02	0.20 ± 0.02	-	0.0002	0.476

During the feeding trials, we observed the snails eating, they were enthusiastically feeding, they scraped the leaves, wrapped the whole *G. pulex* by their soft bodies. Pieces of animal prey bodies or scraps of plant leaves were found in almost all the cups after the feeding trials. During the feeding trial, the snails consumed large parts of the animal prey but less plant material. In the no-choice feeding trials, the wet weight of the consumed plants decreased from 0.19 ± 0.01 g to 0.15 ± 0.03 g (mean ± SD, n = 35), and for the animal prey from 0.18 ± 0.01 g to 0.08 ± 0.04 g (mean ± SD, n = 36), or in number from 8.2 ± 2.3 to 2.2 ± 1.7 (mean ± SD, n = 36) of undamaged *G. pulex* individuals left at the end of the feeding trials. In the choice trials, the wet weight of the consumed plants decreased from 0.19 ± 0.01 g to 0.18 ± 0.02 g (mean ± SD, n = 36), and for the animal prey from 0.18 ± 0.01 g to 0.08 ± 0.04 g (mean ± SD, n = 36), or undamaged individual in number from 10.1 ± 1.7 to 2.1 ± 1.8 (mean ± SD, n = 36). Snail

consumption rates significantly increased as temperature increased in the no-choice feeding trials, both when the snails were fed with only plants ($F_{2,32} = 3.37$, $p < 0.05$) and only animal material ($F_{2,33} = 12.69$, $p < 0.001$) (Fig. 6.1a,b). Similarly, snail consumption rates increased with temperature when both food types were offered simultaneously in the choice feeding trials ($F_{2,33} = 6.06$, $p < 0.01$) (Fig. 6.1c). However the diet selection by the snails, expressed as the plant : animal consumption ratio, was not different among the temperature treatments ($F_{2,33} = 0.649$, $p = 0.53$) (Fig. 6.1d).

In the presence of animal prey, plant consumption rates were consistently lower compared to when plants were the only food type, and this was true at all tested temperatures (two-way ANOVA: effect of temperature, $F_{2,65} = 2.42$, $p = 0.10$; effect of choice / no-choice test, $F_{2,65} = 23.53$, $p < 0.001$; interaction, $F_{2,65} = 0.31$, $p = 0.97$). In the choice feeding trial, there was no difference in plant consumption between different temperature treatments ($F_{2,33} = 1.012$, $p = 0.38$) (Fig. 6.1c). The snails consumed equal amounts of animal prey both in the absence and presence of plants as alternative food source, independent of temperature (two-way ANOVA: effect of temperature, $F_{2,66} = 20.88$, $p < 0.001$; effect of choice / no-choice, $F_{2,66} = 0.17$, $p = 0.68$; interaction, $F_{2,66} = 2.27$, $p = 0.11$). Animal prey consumption rates significantly increased with increasing temperature in the choice trials ($F_{2,33} = 9.89$, $p < 0.001$). There was a significant positive correlation between the consumption of animal prey and plant material in the choice trial across all temperature treatments ($r = 0.43$, $p < 0.01$, n = 36) (Fig. 6.2).

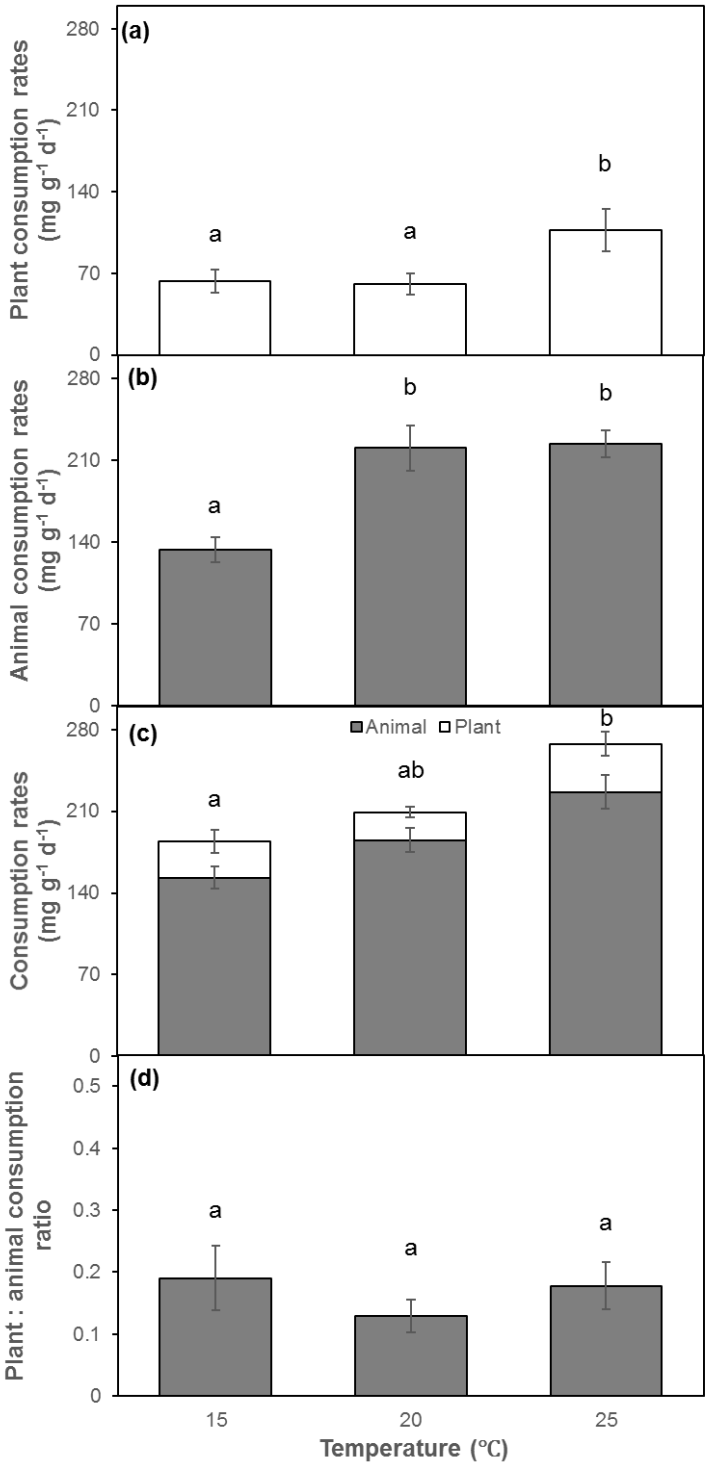

Figure 6.1 Food consumption rates and diet selection. (a) Plant material consumption rates in the no-choice trial, (b) animal prey consumption rates in the no-choice trial, (c) both plant material and animal prey consumption rates in the choice trial, and (d) plant consumption : animal consumption ratio in the choice trial. Different letters indicate a significant difference between the two bars. Error bars indicate ± standard error (SE).

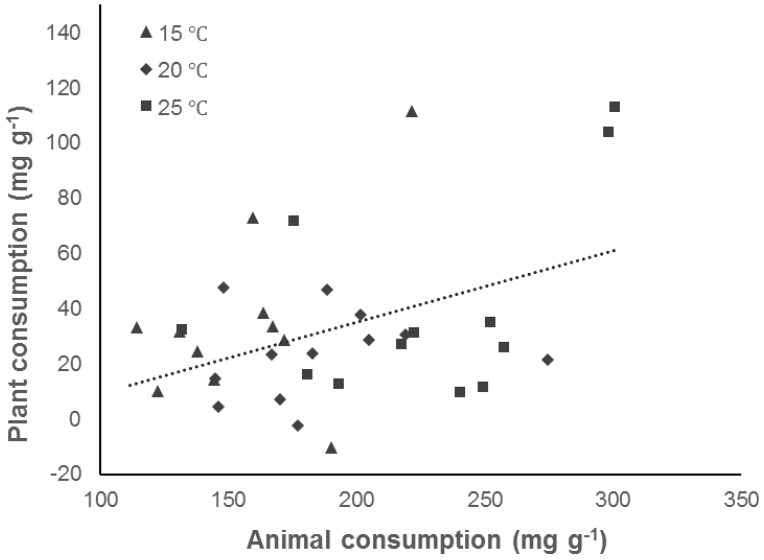

Figure 6.2 Correlation between plant material consumption and animal prey consumption in the choice trial at all temperatures (Pearson correlation: $r = 0.424$, $p = 0.01$, n = 36). Consumption described as milligram food (dry weight) consumed by per gram snail (dry weight without shell).

Discussion

As temperature increased, both plant material and animal prey consumption increased in our study. Generally, within the tolerable temperature range, the metabolic rates of ectotherms increase exponentially with rising temperature and the consumption rates also increase (Gillooly et al. 2001). Previous studies have shown that metabolic rates increase faster than consumption rates in many ectotherms with a temperature rise (Kingsolver and Woods 1997), which may lead to mismatch between consumption and digestion in ectotherms (Lemoine and Burkepile 2012). One potential strategy to reduce the mismatch is to consume a diet with more carbohydrates, which can be easily utilized for energy (Lee et al. 2015). This would imply selection for a more carbon-based diet with increasing temperatures and, hence, more plants and less meat for omnivorous ectotherms (Boersma et al. 2016).

Indeed, several studies support this hypothesis. Caterpillars increased their preference for carbohydrates at higher temperatures (Lee et al. 2015), whereas omnivorous fish consume proportionally more plant material with increasing temperatures (Prejs 1984, Behrens and

Lafferty 2007, 2012, González-Bergonzoni et al. 2016). Similarly, the herbivorous amphipod *Ampithoe longimana* Smith, 1873, collected in a cold-temperate environment, consumed more low organic and protein content seaweeds at higher temperatures (Sotka and Giddens 2009). However, our results are in line with studies showing that a temperature rise does not alter food preference in ectotherms. For example, increased temperature did not alter the protein : carbohydrate consumption ratio by locusts (Miller et al. 2009, Clissold et al. 2013), and beetles consumed plants with higher, not lower, N content at higher temperatures (Lemoine et al. 2013). In line with these studies, we reject our hypothesis and conclude that in our study snails did not change their diet with increasing temperature.

The snails in our experiment consistently preferred animal prey over plants as food, regardless of temperature. The preference for animal prey could be explained by its stoichiometry with C : N and C : P ratios being much more similar to the body composition of the consumer than the offered plant material (Table 6.1). This was particularly due to the increased concentrations of N and P, whereas the C concentration was somewhat lower in the animal prey compared to the plant material, but the difference was not as large as in N or P. Consumers are predicted to preferentially eat food with a composition similar to their own bodies (Elser et al. 2000b). A similar result was found in an experiment with fish where rudd and grass carp consistently preferred animal prey over plant food, both in short-term feeding trials at 18°C and in 10-week pond experiments with water temperatures varying from 16-24°C (Dorenbosch and Bakker 2011, 2012).

Even though the omnivores showed a consistent strong preference for animal prey, they did consume plant material in the choice trial at all temperatures where both food types were presented ad libitum. Also, we found a significant correlation between animal prey consumption and plant material consumption when both food types were offered to the omnivorous consumer (Fig. 6.2). Most generalist consumers have a strategy involving feeding on mixed food to obtain a balanced nutrition intake (Behmer 2009, Raubenheimer 2011, Lihoreau et al. 2015). The snails in our experiment may have been balancing their nutrient intake by mixing animal prey with plant food.

There are some limitations to our study. We tested consumption rates and diet selection at three temperatures (15-25°C), which may be a relatively narrow range of temperatures compared to temperatures naturally experienced by aquatic snails. However, our chosen testing temperatures lie well within the range of those of other experimental studies revealing a diet switch from animal prey to plant food in aquatic ectotherms, for instance a marine fish tested

at 12-27°C (Behrens and Lafferty 2007) and copepods tested at 10-24°C (Boersma et al. 2016). In both studies, the observed diet switch was also visible within the 15-25°C temperature range. Therefore, the limitations of our experimental design do not explain the lack of diet switch by the snails. Possibly, not all ectotherms are capable of adjusting their diet with changing temperatures.

The study of the effects of rising temperature on trophic interactions becomes more urgent as global warming progresses. An increasing strength of plant-herbivore interactions has been found to coincide with rising temperatures (Barton et al. 2009, O'Connor 2009, Shurin et al. 2012). Compared to the amount of studies currently investigating the effects of global warming on trophic interactions, there seems to be only relatively few studies investigating the role of omnivores in this situation (e.g. Boersma et al. (2016)). Our study indicates that with increasing temperatures more plant consumption is expected by ectotherm omnivores due to increased consumption rates, not increased plant preference. However, to further generalize from our snail experiments to patterns of plant consumption by ectotherm omnivores in response to global warming, more experimental studies are needed, especially as the ones available yield contrasting results.

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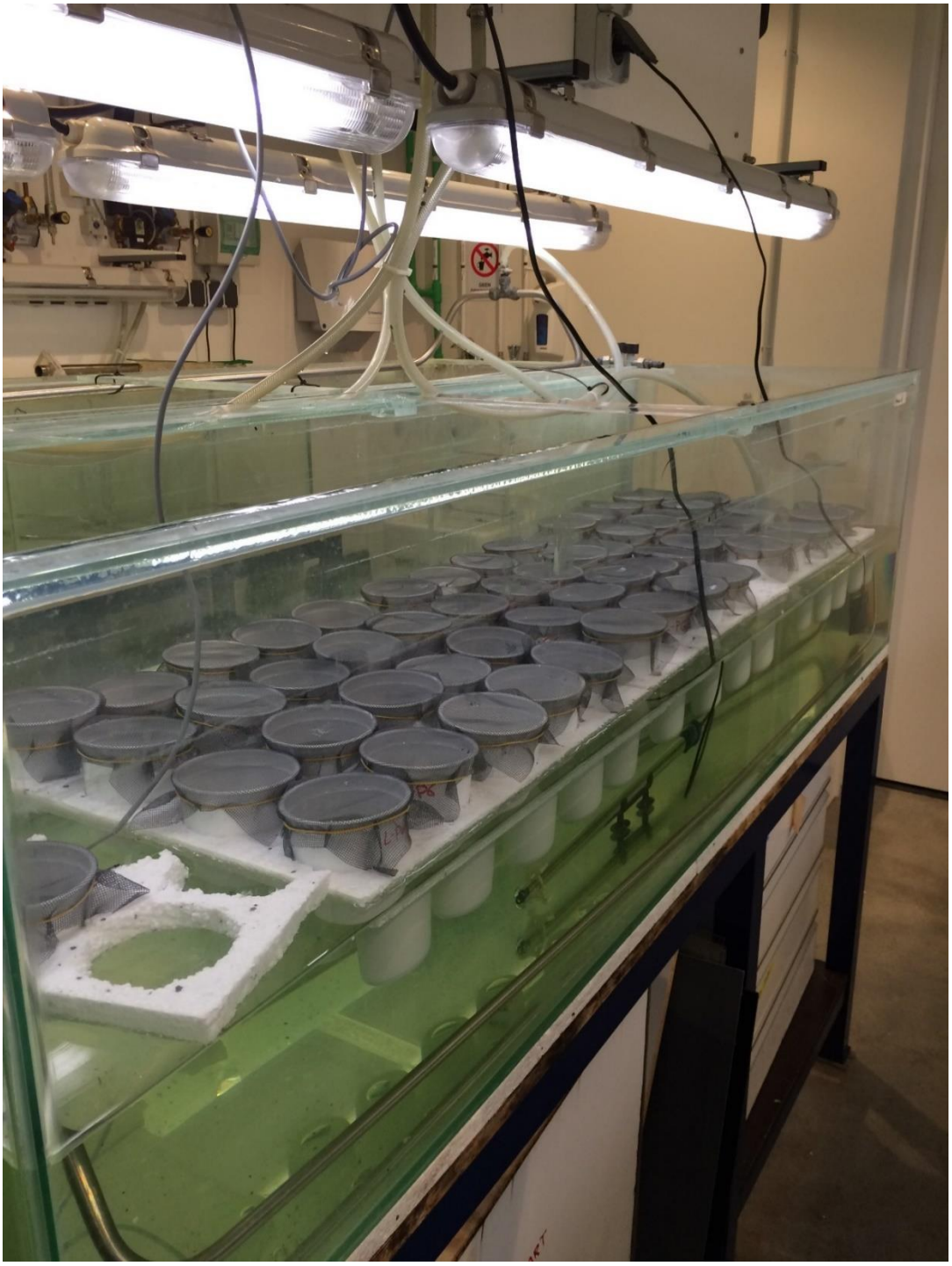

Figure S6.1 Experimental design of the feeding trials. Floating platforms hold the experimental cups in which one individual snail fed on its food during a 24 hour feeding trial. The temperature of the water in the aquarium was controlled by means of a computer-guided temperature systems.

Table S6.1 Control of both food items when kept in water of different temperatures for 24 hours without snails. Paired-Samples t-test were used to compare the difference between start and end wet weight. Values are mean ± standard deviation (SD). N = 12 for each group.

Treatment	*P. lucens* wet weight				*G. pulex* wet weight			
	Start (g)	End (g)	t_{11}	*p*	Start (g)	End (g)	t_{11}	*p*
15 °C	1.07 ± 0.17	1.07 ± 0.18	0.149	0.885	0.11 ± 0.05	0.11 ± 0.06	-1.898	0.084
20 °C	0.92 ± 0.09	0.91 ± 0.09	1.409	0.186	0.13 ± 0.04	0.13 ± 0.04	0.365	0.722
25 °C	1.01 ± 0.21	0.99 ± 0.21	2.246	0.046	0.07 ± 0.04	0.07 ± 0.03	1.388	0.193

Chapter 7

Perspectives on herbivory by aquatic omnivores in globally warming freshwater

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Submitted

Abstract

Aquatic plants are vital components in shallow aquatic ecosystems, because they can keep water clear and sustain high biodiversity. Anthropogenic activities have led to collapse of aquatic plant abundance and loss of plant diversity. While omnivorous feeding modes are prevalent in aquatic ecosystem, latitudinal diet component analysis showed an increased herbivory at low latitude. Temperature might be the key factor underlying these trends. We hypothesize that rising temperature will increase herbivory of aquatic ectothermic omnivores. We tested this hypothesis by applying two approaches, temperature manipulation experiments and literature study. We performed feeding trials by two aquatic ectothermic omnivores (pond snail, *Lymnaea stagnalis* and common carp, *Cyprinus carpio*) at different temperatures, supplying them with both animal food and plant materials. And we directly quantified the consumption rates by the omnivore over time. The results showed that snails cultured at high temperatures increased the proportion of plant materials in their diets after 17 days, which support our hypothesis. In contrast, there was no temperature effect on common carp diet selection, probably due to food limitation. In the literature study, we found that rising temperature increased herbivory in multiple taxa of aquatic animals, including zooplankton, amphibian, crayfish, fish and snail. Therefore, we conclude that increased herbivory of aquatic omnivores with rising temperature might be a common phenomenon in aquatic ecosystems. However, the reasons why aquatic omnivores increase herbivory with rising temperature are not clear. We propose that an ontogenetic diet shift might also contribute to the observed shift towards more plant material. Future warming might increase the pressure on aquatic plants by strengthening the top-down control of both aquatic herbivores and omnivores. More studies are needed to assess the warming induced pressure on aquatic plants at a more complex level and in a more realistic scenario.

Introduction

Aquatic plants play a vital role in shallow aquatic ecosystems, because they can compete with algae to keep water clear (Hilt and Gross 2008), form habitats for aquatic animals which increases biodiversity (Declerck et al. 2005, Meerhoff et al. 2007), and provide food for higher trophic levels (Bakker et al. 2016). However, with anthropogenic activities resulting in global environmental change, many submerged aquatic plant communities have collapsed following eutrophication, resulting in a decrease in plant abundance and diversity (Zhang et al. 2017, Zhang et al. 2018a). Climate change might further contribute to a decrease in aquatic plant abundance, as temperature rise of freshwater ecosystems can enhance phytoplankton dominance, which compete with submerged aquatic plants for light and nutrients (Mooij et al. 2007, Kosten et al. 2009). However, although the effects of eutrophication on submerged plant abundance are well known, the effects of temperature rise are much less clear.

An often overlooked but potentially important hypothesis on why aquatic plant abundances may decline with rising temperatures is an enhanced grazing pressure of animals on plants. Aquatic animals can have huge impacts on aquatic plant abundance through herbivory (Bakker et al. 2016, Wood et al. 2017). Most aquatic animals are ectotherms (Isaak and Rieman 2013), and therefore their metabolism will increase with temperature (Brown et al. 2004), resulting in increased food consumption. In the case of herbivores this implies enhanced grazing pressure at higher temperatures. However, most aquatic plant consumers are omnivorous (Wootton 2017), hence the amount of plant consumption can change with temperature when omnivores alter their diet selection towards more or less plant material relative to animal food items. Across latitudinal gradients, there is a prevalence of herbivory towards lower latitudes, especially in fish communities (Floeter et al. 2005, Jeppesen et al. 2010a, Moss 2010, Behrens and Lafferty 2012, González-Bergonzoni et al. 2012), and in invertebrates (Pennings et al. 2009, Schemske et al. 2009, Boyero et al. 2012). There are many potential explanations for these latitudinal trends, from the perspectives of evolutionary constraints, food availability and food quality, including the hypothesis that temperature might be the key factor underlying these latitudinal trends in herbivory (Floeter et al. 2005, González-Bergonzoni et al. 2012).

If the latitudinal patterns of increasing herbivory at lower latitudes are driven by temperature then omnivorous animals may shift towards a more plant-based diet at warmer temperatures. As a consequence, increasing temperatures may result in enhanced top-down

control on plants, not only by herbivores through increased consumption rates by ectotherm animals, but also by omnivorous animals which may shift towards a more plant-based diet at warmer temperatures. Experimental evidence testing this hypothesis is growing, yet still scarce. Here, we (1) perform experiments with a common aquatic snail and common fish species to test their diet selection at different temperatures, (2) provide an overview of available experimental evidence on effects of temperature on omnivore diets integrating our own data, (3) outline and evaluate the existing proposed mechanisms possibly causing temperature-induced diet shifts, and (4) provide perspectives on how to proceed and improve future work on this timely topic.

Methods and Materials

Experimental design

We test potential effects of water temperature on diet selection for two different types of aquatic ectothermic omnivores: the pond snail *Lymnaea stagnalis* L. and common carp *Cyprinus carpio* L., representing taxa of gastropods and fish respectively. We offered plant and animal food simultaneously to individually kept juvenile animals and quantified their consumption rates. The temperature range for the experiments were chosen from 12 to 27ºC to represent a maximum range of temperatures at which the animals would still be feeding enough (the lowest temperature) and would survive (the maximum temperature). The experiments lasted for three weeks to allow the animals to select their preferred food.

Food preparation

Elodea nuttallii (Planch.) St.John was chosen as our plant material, as it is a cosmopolitan aquatic plant and also a palatable plant food for aquatic animals, which has been demonstrated using the pond snail *L. stagnalis* (Elger and Barrat-Segretain 2004, Grutters et al. 2017b), fish (Dorenbosch and Bakker 2011) and waterfowl (Bakker and Nolet 2014). *Chironomidae* larvae were selected as our animal food, as it is a common animal food eaten by many aquatic animals (Armitage 1995). Carbon (C) and nitrogen (N) content were measured in samples from dried plant material and animal food (Table S7.1) and determined by an auto elemental analyzer (FLASH 2000, Thermo Scientific, Waltham, MA, USA). Both types of food

were ground to powder through a 0.5 mm sieve and mixed into agar (P1001, Duchefa biochemie) to standardize the texture and concentration, using ratios determined by pre-trials (Table 7.1).

Table 7.1 Original nutrient concentration and stoichiometry of the food and the agar food composition. All data are means ± SD, n = 3. Significant differences between the food components (independent t-test) are indicated by different letters.

Food type		Original food nutrient composition			Agar food composition		
		C (mg g^{-1})	N (mg g^{-1})	C:N (g g^{-1})	Agar	Demi-water	Food materials
For snail	Plant	334.9 ± 2.1[a]	32.5 ± 1.4[a]	10.3 ± 0.45[a]	1g	50ml	2.5g
	Animal	355.6 ± 6.7[b]	76.3 ± 1.3[b]	4.66 ± 0.03[b]	1g	50ml	4g
For fish	Plant	354.4 ± 4.1[a]	37.7 ± 0.75[a]	9.39 ± 0.24[a]	3g	100ml	4g
	Animal	402.5 ± 13[b]	59.6 ± 1.1[b]	6.75 ± 0.21[b]	3g	100ml	8g

Agar food was made according to the following procedure (Crenier et al. 2017): the agar was cooked in a microwave until the agar completely dissolved in demi water; stirring the agar solution when it cooled down to 55ºC; adding a predetermined amount of ground plant or animal material into the agar and keep stirring until well mixed; pouring the mixture onto a polyethylene mould with holes (diameter of 7mm, height of 5mm); leaving the mould in the fridge at 4°C for 2 hours; taking out the mould and flatly cutting off the top layer by a blade; poking out the agar pellets and storing them in the fridge until use (maximum preservation of 3 days). For the fish, the agar food pellets were created slightly different: after cooking the agar and mixing it with the ground food material, the mixture was poured into petri dishes which were used as moulds, and stored at 4°C until use. The agar food was sliced into pieces of approximately 0.8 × 0.4 × 0.4 cm, weighed into portions and offered to the fish. The plant and animal agar food can be easily sorted by eye, as plant food is green and animal food is brown.

Snail experiment

E. nuttallii was collected in the front pond of NIOO, Wageningen, The Netherlands (51°59'12.7"N, 5°40'15.4"E). Plants were thoroughly cleaned and freeze-dried. Freeze-dried *Chironomidae* larvae were commercially obtained (https://www.goedkoopvisvoer.nl/). Pond snails were originally obtained from another pond at the terrain of NIOO-KNAW

(51°59'17.5"N, 5°40'28.5"E). Egg clusters were hatched, and reared in plastic buckets at 20°C, filled with tap water and constantly aerated, and exposed to a day:night cycle of 16:8 hours. Hatched snails were fed *ad libitum* with commercially obtained lettuce and Fish food (Velda, Gold Sticks Basic Food, The Netherlands). After culturing for one month, 48 juvenile snails with a shell length of 1.21 ± 0.07cm (mean ± SD) were selected for the experiment. Each snail was put in a plastic beaker (top diameter 12.5 cm, bottom diameter 11 cm and height 11 cm), filled with 500ml temperature-acclimated tap water and covered with mesh to prevent the snails from escaping. Four beakers were set in one styrofoam platform, and put in one aquarium (90×50×50cm, l×w×h) in 30cm deep of water. Six temperatures were chosen: 12, 15, 18, 21, 24 and 27°C. Every two aquaria were set at the same temperature, and the temperatures were randomly assigned to 12 aquaria in the climate room. The light intensity on the water surface was 10.35 ± 0.28 μmol m^{-2} s^{-1} (mean ± SD, n = 5), with a day:night cycle of 12:12h.

Snails were offered both plant-based and animal-based agar pellet food ad libitum. Leftover food was checked every day for each snail to make sure they had enough of both types of food for the next day, if not, new agar food pellets were added. Every three days, water was replaced and the bucket was cleaned for each snail, meanwhile, leftover food was retrieved, put in a pre-weighed aluminium cup, dried in the oven at 60°C for 48h and weighed to quantify final food dry weight. New food pellets of both types of plant and animal food were supplied to each snail, and quantities were increased if necessary. Agar food can lose some weight over time as parts of it may dissolve in the water. Therefore, we performed dissolving trials at different temperatures and initial dry weight of an agar food pellet in the snail feeding trial was estimated based on these dissolving trials (Table S7.1). The food consumption rate was expressed as how much food was consumed by each snail per day: (Food initial dry weight – Food final dry weight) / days. Snails were cultured for 24 days from October 20th to November 12th 2017 and their wet weight was measured at the start and end of the experiment. Snail relative growth rate was expressed as: [ln (Final snail weight) – ln (Initial snail weight)] / days (Fig. S7.1).

Fish experiment

The plant *E. nuttallii* was collected in September 2017 from a pond in Wageningen, The Netherlands (51°58'49.4"N, 5°39'20.2"E). The plants were sorted and cleaned with high speed flush water, first air-dried at room temperature, followed by oven-drying at 40°C until dry. For

animal food, sun-dried *Chironomidae* larvae were commercially obtained (https://www.goedkoopvisvoer.nl/), also further dried in the oven at 40°C. Due to logistic reasons of limited freeze-drying capacity, we could not use freeze-dried materials for the fish trials. We tried to preserve the original chemical composition as much as possible by drying at low temperature (40°C).

24 juvenile common carp (*C. carpio*) with a tail length of 10-12 cm, were obtained from CARUS-ARF (Wageningen, The Netherland). The fish were housed in eight 450-liter aquaria (185×50×50 cm, l×w×h) that were pairwise connected to four 150 liter biological filters. The aquaria were filled with tap water and kept at a day:night cycle of 12:12 (light intensity at the surface was 17.8 ± 3.1 µmol m^{-2} s^{-1} (mean ± SD, n = 8)). Water temperatures were the same with the room temperature (20°C) at the beginning. Biological filters regulated the water quality to keep NH_4^+ < 0.5 mg L^{-1}, NO_2^- < 0.25 mg L^{-1}, NO_3^- < 200 mg L^{-1}. The pH (7.0 – 8.2) and conductivity (500-3500 uS cm^{-2}) were checked twice per week and adjusted if necessary throughout the experiment. On a daily basis, all aquaria were cleaned by syphoning out fish feces and leftover food.

Initially, all 24 fish were divided over four aquaria in even groups. Fish were kept in this group situation for 21 days to acclimate them to the new environment. During this period, the fish were fed regular fish food (Skretting MP presta 1.8 mm), mixed with increasing proportions of the two types of agar food. When the fish were used to feeding on the agar food, we divided each aquarium into three equal compartments (61×50×50 cm, l×w×h) using vertical frames with mesh. This way each fish was housed individually but with at least visual contact with conspecifics. After two days of allowing the fish to acclimatize to the situation, water temperatures in all aquaria were adjusted by maximum 1°C per day to one of four experimental temperatures: 12, 17, 22 and 27 °C. After eight days the experimental temperatures were reached and we started the experiment.

At the start of the experiment all fish were weighed to the nearest gram, and their tail length was estimated from photographs taken from the top in a bucket of water with a 1.0 × 1.0 cm raster on the bottom. The experiment lasted for 23 days from November 15th to December 7th 2017. On the first day, a restricted amount of animal food and plant food was offered to the fish in order to check the possibility of plant food consumption (Fig. S7.2c). From day 2 to 19, the fish were fed an abundance of both types of food in a 1:2 (plant food : animal food) weight ratio in a certain time, and quantity was increased if necessary (plant agar food ranged from 0.75g to 2.75g wet weight per day, animal agar food ranged from 1.5g to 5.5g wet weight per

day, warmer treatments were offered more food but at equal ratios). In the snail experiment we provided a continuous supply with ad libitum food of both types, but due to logistic reasons of making and re-collecting agar food, we could not supply enough for ad libitum feeding for the fish, which consumed much more than the snails. Therefore, we decided to supply abundant food during 45 minutes for the fish to feed, to make the work logistically possible. Leftover food for every individual was syphoned out of the water in random order and caught in a 1220 µm sieve. Collected leftover plant and animal food were separated, dried at 60 °C for 48 h and weighed (leftover food in dry weight). At day 20, 21 and 22, a possible influence of offering the food only for 45-minutes to the fish was investigated. For three days, an abundance of food was offered in a ratio of 1:4 (plant food : animal food) instead of 1:2, and the fish were allowed to forage for 2h instead of 45 min (plant agar food ranged from 0.75g to 2.75g wet weight per day, animal agar food ranged from 3g to 11g wet weight per day, Fig. S7.2a,b). On the last day (day 23), animal food was limited again to demonstrate that fish still consumed plant material (Fig. S7.2d). After the experiment the weight and length of all fish was measured again using the same methods and all fish were moved to a local fish supplier.

Dissolving trials at different temperatures showed no temperature effects on agar food for fish after 45 minutes' soaking in the water. Therefore, we used the linear regression functions from the dissolving trials to calculate the initial agar food dry weight. For animal food, $y = 0.0724*x$ ($r^2 = 0.99$, $p < 0.001$, $n = 48$) and for plant food, $y = 0.0548*x$ ($r^2 = 0.99$, $p < 0.001$, $n = 36$), where x is the agar food wet weight in g and y is the agar food dry weight in g. The food consumption rate was expressed as how much food was consumed by each fish per day: (Food initial dry weight – Food final dry weight)/day.

Data analysis

Linear Mixed Effects models, using package nlme (Pinheiro et al. 2017), were employed to analyse temperature effects on plant food consumption rates, animal food consumption rates and plant:animal consumption ratio of the omnivores over time. Both temperature (ranging from 12 to 27 °C) and time (in days) were included in the models as continuous explanatory variables, aquarium was included as a random factor to account for the dependency structure in our experimental design. Normality and homoscedasticity of the dependent variables was assessed visually in histograms, and verified by plotting model residuals versus fitted values

and quantile-quantile plots of the model residuals. Plant food consumption rate for snails was log-transformed to improve its normal distribution before performing the analysis.

Due to the acclimation, the first three days of the feeding data were excluded from the data set for both snail and fish. To measure food consumption in dry mass we had to convert measured wet weights into dry weights, which introduced slight measurement errors. These small errors explain why we occasionally report negative consumption rates if the animals ingested very little material. Diet selection was indicated by the ratio of plant food consumption rate to animal food consumption rate. The negative animal food consumption rates would lead to nonsensical ratios, therefore those data were removed from the data set (for both snail and fish data this was < 3% of all the data during the whole experiment respectively). Due to the very little consumption of plant material for the fish (plant consumption rate 1.67 ± 5.62 mg d^{-1}, mean ± SD, n=366), there is no need to calculate the consumption ratio for the fish (average plant:animal consumption ratio was less than 2%). All statistics were performed in R 3.4.3 (R Development Core Team 2017).

Literature study

To provide an overview of available experimental evidence on effects of temperature on omniovore diets, we surveyed the peer-reviewed literature for published experiments. We used the searching terms: "temperature" OR "warm*" OR "heat" in the title, "omnivor*" and "diet" OR "food" in the topic to search in ISI: Web of Science, and checked for cross-references in the obtained literature. In total, 49 publications were found, of which 11 (including our own data) had data on aquatic omnivorous diet selection with changing temperature, which were selected. This resulted in 15 cases, covering 5 taxa and 11 species of aquatic animals (including this study).

Results

Feeding experiments

The animal food had a higher nitrogen content and a lower carbon:nitrogen ratio than the plant food in both experiments (Table 7.1). Pond snails increased their plant food consumption rates in response to rising temperature, and when temperatures were ≥ 15°C pond

snails increased plant consumption rates over time (Fig. 7.1a and Table 7.2). Animal food consumption rates by pond snails increased with temperature rise and increased over time at all temperatures (Fig. 7.1b and Table 7.2). Temperature effects on the plant:animal consumption ratios emerged after 17 days, and rising temperature significantly increased plant:animal consumption ratios afterwards. However, plant:animal consumption ratios increased over time only when temperatures were ≥ 21ºC (Fig. 7.1c and Table 7.2). The average plant:animal food consumption ratio over the entire experiment was 15.23 ± 14% (mean ± SD, n=327). Snail relative growth rate also significantly increased as temperature increased (Fig. S7.1).

Table 7.2 Time and temperature effects on pond snail and common carp food consumption. Bold numbers indicate $p < 0.05$.

Animal type	Parameters	Factors	numDF	denDF	*F*	*p*
Pond snail	Plant food consumption rate	Time	1	313	35.6	< **0.001**
		Temperature	1	10	2.3	0.157
		Time × Temperature	1	313	121.6	< **0.001**
	Animal food consumption rate	Time	1	313	60.3	< **0.001**
		Temperature	1	10	158.1	< **0.001**
		Time × Temperature	1	313	6.0	**0.015**
	Plant:animal consumption ratio	Time	1	313	64.7	< **0.001**
		Temperature	1	10	39.3	< **0.001**
		Time × Temperature	1	313	71.5	< **0.001**
Common carp	Plant food consumption rate	Time	1	356	0.8	0.376
		Temperature	1	6	1.0	0.346
		Time × Temperature	1	356	4.9	**0.027**
	Animal food consumption rate	Time	1	356	8.5	**0.004**
		Temperature	1	6	44.5	**0.001**
		Time × Temperature	1	356	27.8	< **0.001**

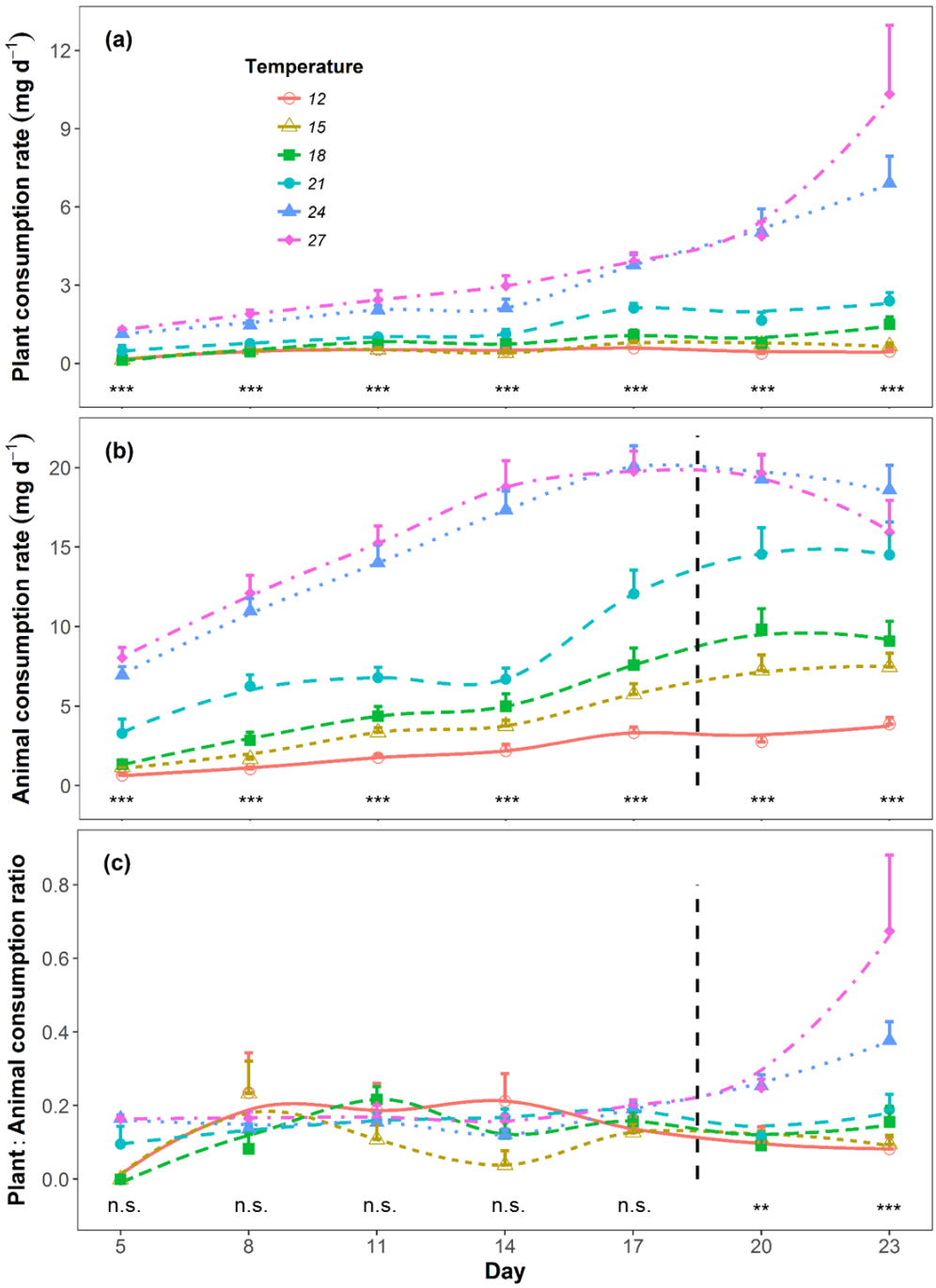

Figure 7.1 Temperature effects on pond snail food consumption rates and diet selection during the experiment. (a) Plant food consumption rates by snails; (b) Animal food consumption rates by snails; (c) Plant:animal food consumption ratio during the experiment. Standard errors are indicated by vertical bars. Dashed vertical lines indicated the diet shift time point. Each data point represents the mean value over the three days of measurement and is displayed at the middle day. Temperature effects on food consumption and diet selection at each time point was tested by Linear-mixed effects models. Statistic results are also shown in the figures. "n.s." represents no significant effects, $p > 0.05$; "**" indicates $p < 0.01$; "***" indicates $p < 0.001$.

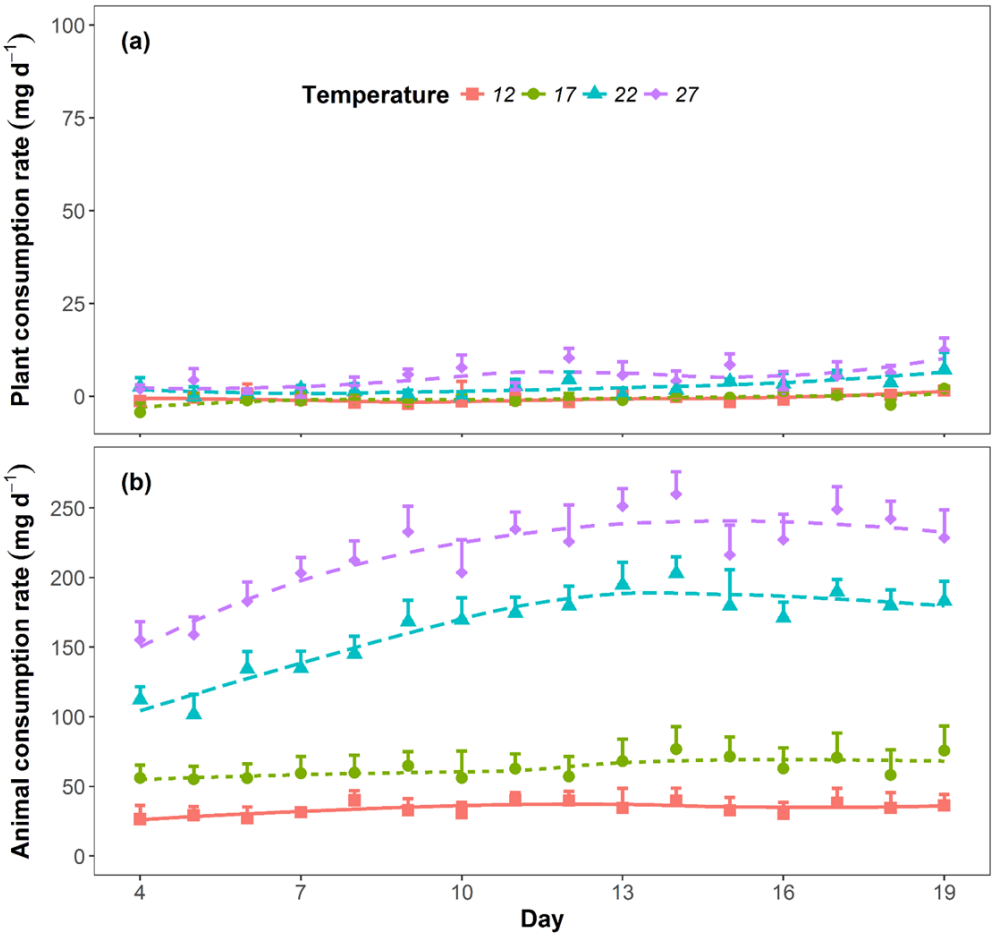

Figure 7.2 Temperature effects on common carp food consumption rates during the feeding period. (a) Plant food consumption rates by common carp; (b) Animal food consumption rates by common carp. Standard errors are indicated by vertical bar. Due to the very little consumption of plant material for the fish (plant consumption rate 1.67 ± 5.62 mg d^{-1}, mean ± SD, n=366), there is no need to calculate the consumption ratio for the fish (average plant:animal consumption ratio was less than 2%).

Table 7.3 Studies which investigated rising temperature effects on aquatic omnivorous diet selection.

Taxon	Omnivore	Plant food	Animal food	Experiment approach	Quantified methods	Time	Temperature range	Results	Reference
Zooplankton	Copepod (*Temora longicornis*)	Cryptophyte (*Rhodomonas salina*)	Dinoflagellate (*Oxyrrhis marina*)	Seasonal comparison	Stable isotope method	-	3 - 23 ℃	Decreased trophic level with rising temperature	Boersma et al. 2016
				Temperature manipulation	Quantify grazing rate	24 h	8 - 26 ℃	Preferred plant food at high temperature	Boersma et al. 2016
Amphibian	Tadpole (*Discoglossus galganoi*)	Macrophyte (*Juncus heterophyllus*)	Chironomid larvae	Temperature manipulation	Stable isotope method	2 months	17 - 25 ℃	Increased herbivory with rising temperature	Carreira et al. 2016
	Tadpole (*Hyla arborea*)	Macrophyte (*Ranunculus peltatus*)	Chironomid larvae	Temperature manipulation	Stable isotope method	2 months	17 - 25 ℃	Increased herbivory with rising temperature	Carreira et al. 2016
	Tadpole (*Hyla meridionalis*)	Macrophyte (*Ranunculus peltatus*)	Ephemeroptera (mayfly) larvae	Temperature manipulation	Stable isotope method	2 months	17 - 25 ℃	Increased herbivory with rising temperature	Carreira et al. 2016
Crayfish	*Procambarus clarkii*- Juvenile	Macrophyte (*Juncus heterophyllus*)	Chironomid larvae	Temperature manipulation	Stable isotope method	2 months	17 - 25 ℃	Tendency of increasing herbivory with warming	Carreira et al. 2017
	Procambarus clarkii- Adult	Macrophyte (*Juncus heterophyllus*)	Chironomid larvae	Temperature manipulation	Stable isotope method	2 months	17 - 25 ℃	No temperature effects on preference	Carreira et al. 2017
Fish	Rudd (Scardinius erythrophthalmus)	Macrophytes	-	-	Gut content analysis	-	15 - 23 ℃	Increased plant consumption as temperature increased	Prejs 1984
	Opaleye (*Girella nigricans*)	Algae (Ulva spp.)	Squid pieces	Temperature manipulation	Quantify performance	28 days	12 - 27 ℃	Increased performance at low temperature feeding on animal food	Behrens & Lafferty 2007

Taxon	Omnivore	Plant food	Animal food	Experiment approach	Quantified methods	Time	Temperature range	Results	Reference
Snail	*Bryconamericus iheringii*	Filamentous algae	Tubificid worms	Seasonal comparison	Gut content analysis	4 days	10 - 22 ℃	Increased herbivory in summer	González-Bergonzoni et al. 2016
	Rudd (*Scardinius erythrophthalmus*)	-	-	Seasonal comparison	Stable isotope method	-	9 - 25 ℃	Increased herbivory in summer	Guinan Jr et al. 2015
	Rudd (*Scardinius erythrophthalmus*)	Macrophyte (*Potamogeton pectiatus*)	*Chironomus*larvae	Temperature manipulation	Gut content analysis	24 and 168 h	13 - 24 ℃	Increased herbivory with rising temperature	Vejříková et al. 2016
	Cichlids (*Amatitlania nigrofasciata*)	Plants	Molluscs, insects, crustaceans	Field comparision	Gut content analysis	-	27 - 31℃	Increased herbivory at higher temperature	Emde et al. 2016
	Comon carp (*Cyprinus carpio*)	Macrophyte (*Elodea nuttallii*)	*Chironomid* larve	Temperature manipulation	Quantify feeding rate	19 days	12 - 27℃	No temperature effects on preference	Zhang et al. (This study)
	Pond snail (*Lymnaea stagnalis*)	Macrophyte (*Potamogeton lucens*)	Amphipod (*Gammarus pulex*)	Temperature manipulation	Quantify feeding rate	24 h	15 - 25 ℃	No temperature effects on preference	Zhang et al. 2018
	Pond snail (*Lymnaea stagnalis*)	Macrophyte (*Elodea nuttallii*)	*Chironomid* larve	Temperature manipulation	Quantify feeding rate	23 days	12 - 27 ℃	Increased herbivory at higher temperature after 17 days	Zhang et al. (This study)

Common carp showed a strong preference for animal food at all experimental temperatures. The fish consumed very little plant material (1.67 ± 5.62 mg d^{-1}, mean ± SD, n = 366), and increased their consumption rates over time when temperatures were ≥ 17ºC (Fig. 7.2a and Table 7.2). Animal food consumption rates increased with temperature and increased over time (Fig. 7.2b and Table 7.2). When the feeding period was extended to two hours, fish increased animal food consumption compared to the 45 minutes' feeding period (mean percentage increased 66.7% from day 19 to day 20), but still ingested almost no plant material (2.69 ± 5.49 mg d^{-1}, mean ± SD, n = 72, Fig. S7.2). However, the fish ingested significant amounts of plant material both in the beginning and the end of the experiment, when we supplied limited animal food (Fig. S7.2). During the experimental period, the fish did not increase in weight (initial weight 51.4 ± 6.3 g; final weight 52.2 ± 5.1 g, mean ± SD, pairwise t-test, $t_{23} = -0.80$, $p = 0.43$) and only slightly in length (increment of 0.73 cm, initial length, 14.9 ± 0.6 cm; final length 15.6 ± 0.6 cm, mean ± SD, pairwise t-test, $t_{23} = -4.58$, $p < 0.001$), but there was no temperature effect on fish length increments ($F_{3,4} = 1.13$, $p = 0.44$).

Literature study

Rising temperature increased herbivory of aquatic omnivores in 12 of the 15 cases found, which includes taxa in zooplankton, amphibians, crayfish, fish and aquatic snails (Table 7.3). The two most common approaches to study potential effects of temperature on omnivore diets were temperature manipulation experiments (12 cases) or seasonal comparison approaches (3 cases) to compare temperature effects on omnivore diet selections. Differences between the lowest and highest temperature in each case ranged from 4 to 20 °C. Among the 11 cases which employed temperature manipulation, the time that the experiment ran ranged from 24 h to 2 months for the different organisms. There are seven cases that focused on fish, of which six found temperature effects on diet selection, such that rising temperatures increase the share of plant material in the fish diets.

Discussion

Through a combination of controlled experiments and a literature study, we found that most aquatic omnivores increased herbivory when water temperatures increased. There are

three cases which are not in line with our expectation. Below we will discuss possible reasons why there were no diet shift found in these studies, and we will elaborate on the mechanisms why and how the omnivores might increase herbivory when temperature increases. Furthermore, we predict the possible effects of warming on plant-herbivory interactions and implications for the study design to test temperature impacts on possible diet shift of ectothermic omnivores.

Increased herbivory with rising temperature

Studies which support that rising temperature increased herbivory of aquatic omnivores covered five taxa, including zooplankton (Boersma et al. 2016), tadpoles (Carreira et al. 2016), crayfish (Carreira et al. 2017), aquatic snail (Zhang et al. this study) and fishes (Prejs 1984, Behrens and Lafferty 2007, Guinan Jr et al. 2015, Emde et al. 2016, González-Bergonzoni et al. 2016, Vejříková et al. 2016). Therefore, it seems a common phenomenon for aquatic ectothermic omnivores to increase herbivory as temperature increases. However, there are still three cases which did not find temperature effects on aquatic omnivorous diet selection.

In contrast to our snail experiment and the majority of the published studies, we did not find temperature effects on fish diet preference in our experiments. Fish showed a strong preference for animal food at any temperature. There could be several reasons for this. First of all, the fish in our experiment could have disliked the plant food and only eat animal food. However, pre-trials showed that the fish did eat the plant food when animal food was limited. Secondly, the experimental period of 45 minutes could have been too short so the fish possibly only went for the high quality food in this limited time. However, the fish did not increase plant consumption as feeding time extended to two hours. Thirdly, the fish grew only little in our experiment which indicated that they were likely limited in food supply. Because the fish were likely under nutrient limitation, they always selected for high quality food. To avoid this effect we conclude that it is important to provide both types of food ad libitum during the whole experimental periods.

The few published cases that did not find temperature effects on omnivorous diet selection could also be due to a bias against publishing experiments that did not find temperature effects on animal diet selection. Compared to other studies, our study was performed in a more direct way. We measured the omnivore consumption rates on both type of food over time, whereas other studies employed stable isotope methods (Guinan Jr et al. 2015, Boersma et al. 2016, Carreira et al. 2016, Carreira et al. 2017). The stable isotope method has

been used to indicate the food assimilation, which is not equal to consumption (Boecklen et al. 2011). Whereas isotope studies can indicate better that which type of food could be utilized better by omnivores, our study can indicate better the direct top-down grazing pressure of omnivores on aquatic plants.

Compared to plant material, animal food is rich in nutrients (Nitrogen, N; and Phosphorus, P), and has a lower Carbon (C):nutrient ratio (Elser et al. 2000b, Van de Waal et al. 2010), which we also observed in our experiment. Animal food has a nutrient composition more similar to the body composition of omnivore, compared to plant material (Elser et al. 2000b, Zhang et al. 2018b). From this viewpoint, animal food is more suitable for omnivores. Indeed, in our experiment, both snails and fish strongly preferred animal food over plant food. However, with a changing temperature, in the longer term the animals will choose the most suitable combination of food items to meet their requirements (Raubenheimer 2011, Lihoreau et al. 2015).

Possible mechanisms causing temperature induced diet shifts

There are several possible mechanisms that may underlie temperature-induced diet shifts in aquatic omnivores that have led to the development of different hypotheses, which relate to the physiology, metabolic, stoichiometry demand and ontogeny of ectothermic omnivores (Fig. 7.3). Firstly, the *Temperature Physiological Constraint Hypothesis* (TPCH), which was initially developed for fish, seems to be an acceptable explanation. This hypothesis indicates that omnivores cannot meet their energetic demands by only consuming plant materials at low temperatures (Gaines and Lubchenco 1982, Floeter et al. 2005). Although metabolic rates and energy requirements generally decrease with decreasing temperatures, feeding rates decrease faster than metabolic rates as temperature decreased (Floeter et al. 2005). Utilizing cellulose in plant materials require microorganisms growing above certain temperatures to produce enzyme cellulase (Vejříková et al. 2016). Compared to plant materials, animal food items are higher in nutrient contents, hence ectothermic omnivores ingest more animal food at low temperatures (Behrens and Lafferty 2007). However, there are also some herbivorous fish in cold regions which could digest plant material as they have long digestive tract to increase the food retention time for digestion (Targett and Arnold 1998, Clements et al. 2009), and herbivorous fish population abundance was found to be higher at high latitudes than at low latitudes in the southern hemisphere, which do not support the TPCH (Trip et al. 2014).

Therefore, the TPCH alone might not be sufficient to explain why the omnivores increase herbivory with rising temperature.

An alternative explanation might be achieved under the framework of Ecological stoichiometry theory (Sterner and Elser 2002) and metabolic ecology (Brown et al. 2004). The *Temperature Metabolic Stoichiometry Hypothesis* (TMSH), predicts that ectotherms at warmer temperatures need to consume more carbohydrates (or Carbon) (Croll and Watts 2004, Acheampong et al. 2014, Lee et al. 2015, Malzahn et al. 2016). As temperature increase, animals need to obtain more energy to sustain their increased metabolism, resulting in an increased ingestion rate. However, respiration rate of animals increases faster than their growth rate as temperature increases (Karl and Fischer 2008, Forster et al. 2011). Therefore, ectothermic animals need to consume more carbohydrates to sustain the increased energy demand, as carbohydrates are easy to utilize. However, research also found that rising temperature increased the preference for protein (N rich) in grasshoppers (Schmitz et al. 2016), or the preference of carbohydrates and protein changed with temperatures in mealworm beetles (Rho and Lee 2017). Furthermore, model simulation showed that the requirement for the food C:N ratio would not change as temperature increases if the increased amount of food is taken into consideration (Anderson et al. 2017). Together, these observations suggest that more work is needed before we can conclude the importance of the TMSH hypothesis.

Surprisingly, the life stage and ontogenetic stoichiometry demand variation has been completely neglected in the previous hypothesises. Animals might have different nutrient demands at different life stages (Stockhoff 1993, Urabe and Sterner 2001, Claes and Maarten 2003, Bullejos et al. 2014, Richard et al. 2018). Juveniles might need lower C:nutrient ratio food than the adults of the same species, as they need more nitrogen and phosphorous for their growth, leading to the ontogenetic stoichiometry demand shift (Nakazawa 2011, Bullejos et al. 2014, Boros et al. 2015). Many aquatic omnivores increase the proportion of plant material during their ontogenetic development (Werner and Gilliam 1984, German and Horn 2006, Burgett et al. 2018). At high temperatures, animals grow faster and mature earlier, resulting in a smaller body size (Kingsolver and Huey 2008, Daufresne et al. 2009), and accrue relatively less nutrients in their bodies (Woods et al. 2003). Thus, they might shift their diet earlier from lower C:nutrient ratio food to higher C:nutrient ratio food with increasing temperature, namely by increasing herbivory at higher temperatures. We here coin this hypothesis the *Temperature Ontogenetic Stoichiometry Hypothesis* (TOSH). This hypothesis can also explain why Carreira et al. (2017) found that there was a tendency of increasing herbivory with rising temperature

only in juvenile crayfish but not in the adult, as crayfish increased herbivory with increasing age (Momot 1995). The snails in our experiment grew faster and had larger sizes at higher temperatures and increased herbivory, whereas the fish did not grow and did not change their diet preference with rising temperature, which also support this hypothesis. More studies are needed to verify this hypothesis.

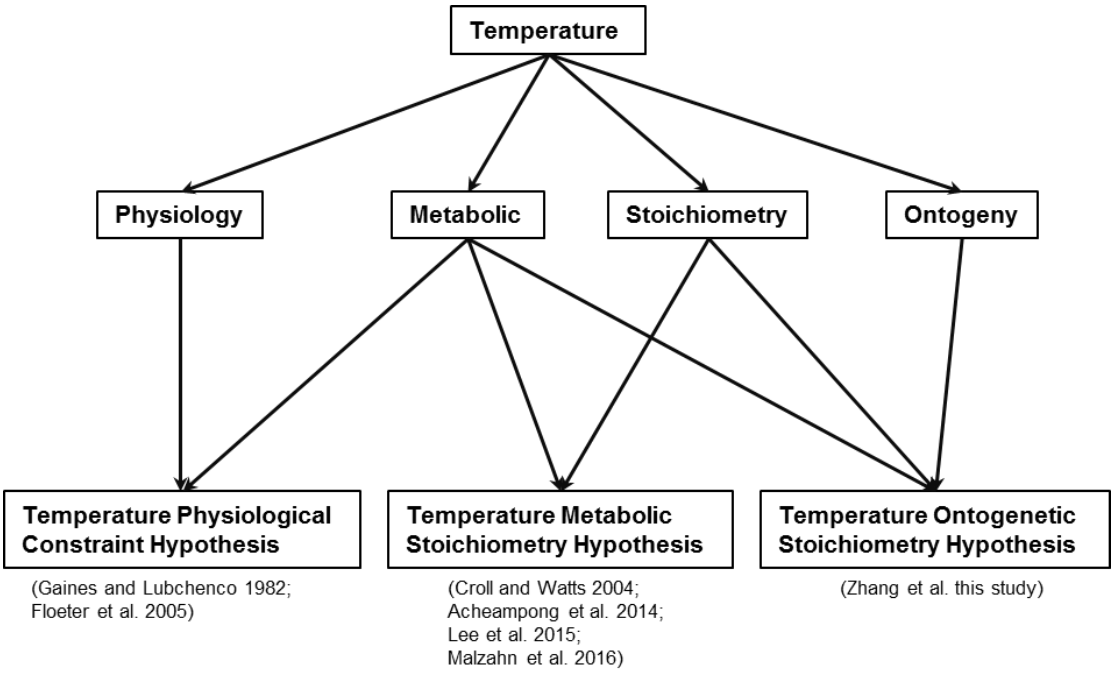

Figure 7.3 Schematic graph of possible mechanisms for increased herbivory by aquatic omnivores with rising temperature. Temperature influences different aspects of the omnivores and the hypotheses are based on these effects.

Implications of increased herbivory under global warming

Under the ongoing climate change (IPCC 2014), ectothermic animals might increase their metabolism resulting in higher consumption rates, and leading to enhanced top-down control on plants by herbivores (O'Connor 2009), as the consumption rate of animals increases faster than the biomass accumulation rate of primary producer with rising temperature (Gilbert et al. 2014, West and Post 2016, Schaum et al. 2018). Furthermore, with rising temperature, aquatic omnivores might increase herbivory in their diet (Fig. 7.4), and omnivores might grow faster and establish larger population sizes leading to more consumption of plants (Meerhoff et al. 2007, Jeppesen et al. 2010a, González-Bergonzoni et al. 2012). Together this suggests that

future climate change might impose stronger pressures on aquatic plant communities by both herbivores and omnivores (Fig. 7.4).

In nature, omnivorous diet selection also depends on food availability (Guinan Jr et al. 2015, Vejříková et al. 2016). Animal food is not always as abundant as plant food, therefore, omnivorous animals might become more herbivorous, which means they need to consume more plant material to compensate for their nutrient demand (Cruz-Rivera and Hay 2000, Fink and Von Elert 2006). The average plant material consumption percentage in our experiment is less than 15% for snails, whereas in other studies the proportion of aquatic plants in the diet of the omnivores could reach more than 50% (Carreira et al. 2016, Emde et al. 2016, González-Bergonzoni et al. 2016, Vejříková et al. 2016), which indicates that the increased top-down control on aquatic plant by herbivory might be even stronger in nature under warming.

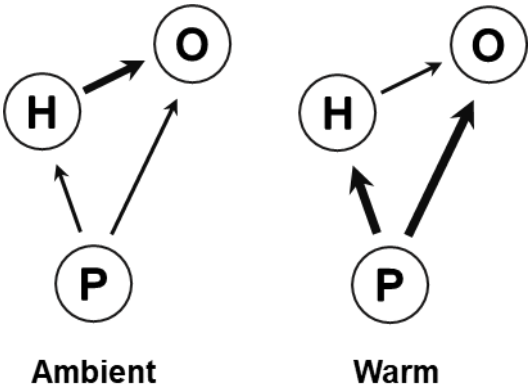

Figure 7.4 Possible warming effects on aquatic omnivore-herbivore-plant interactions. Omnivore and herbivore share the same plant material. Arrows indicate the energy transfer direction. Width of the arrow lines indicates the strength. O represents omnivore, H represents herbivore and P represents Plant material.

Future studies

(1) Although ectothermic omnivores seem to generally increase herbivory with rising temperature, the underlying mechanisms are still unclear. Explanations from the aspects of temperature effects on animal physiology, metabolism, and stoichiometric demands could not fully clarify why ectothermic omnivores increase herbivory with rising temperature. We here propose to additionally take animal ontogenetic diet shifts into consideration when interpreting diets shifts by omnivores. However, clearly more studies are needed to contrast the various hypotheses.

(2) Future studies should test more ectothermic omnivores in long-term diet-selection experiments at multiple life-stages at different temperatures. Furthermore, multiple

plant species should be tested to generalize the conclusions. The use of agar pellets is a suitable approach in feeding trials, as it can standardize the physical structure and composition of the food. However, the effort to make large amounts of agar food should not be underestimated and should not lead to food shortages during feeding trials.

(3) Apart from experiments, another helpful approach is modelling, which could help to better understand and predict the influence of rising temperature effects on diet selection in omnivores.

(4) Even though so many studies have found that warming might increase herbivory of aquatic omnivores, there is still no study that explored the effects of warming on omnivore impacts on aquatic plant abundance or diversity at the community or ecosystem level. Therefore, more studies are needed to test the effects of warming on aquatic plant by herbivory in a more realistic scenario.

Acknowledgements

We sincerely thank Xianghong Kong, Erik Reichman, Dennis Waasdorp, Suzanne Wiezer and Nico Helmsing for their assistance in preparing and performing the experiments. Peiyu Zhang acknowledges the China Scholarship Council (CSC) for funding his scholarship to study at NIOO-KNAW. Permission for the fish experiment was obtained from NIOO-KNAW (Supplementary material 7.1).

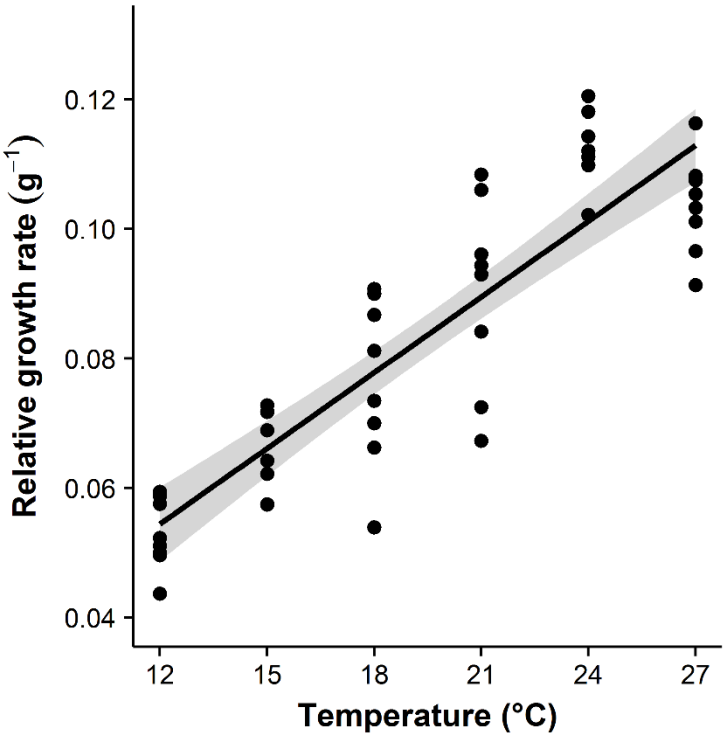

Figure S7.1 Snail relative growth rate at different temperatures during the experiment. Linear Mixed-Effects models showed that the snail relative growth rate increased with temperature increase ($F_{1,10}$ = 94.66, r^2 = 0.78, p < 0.001).

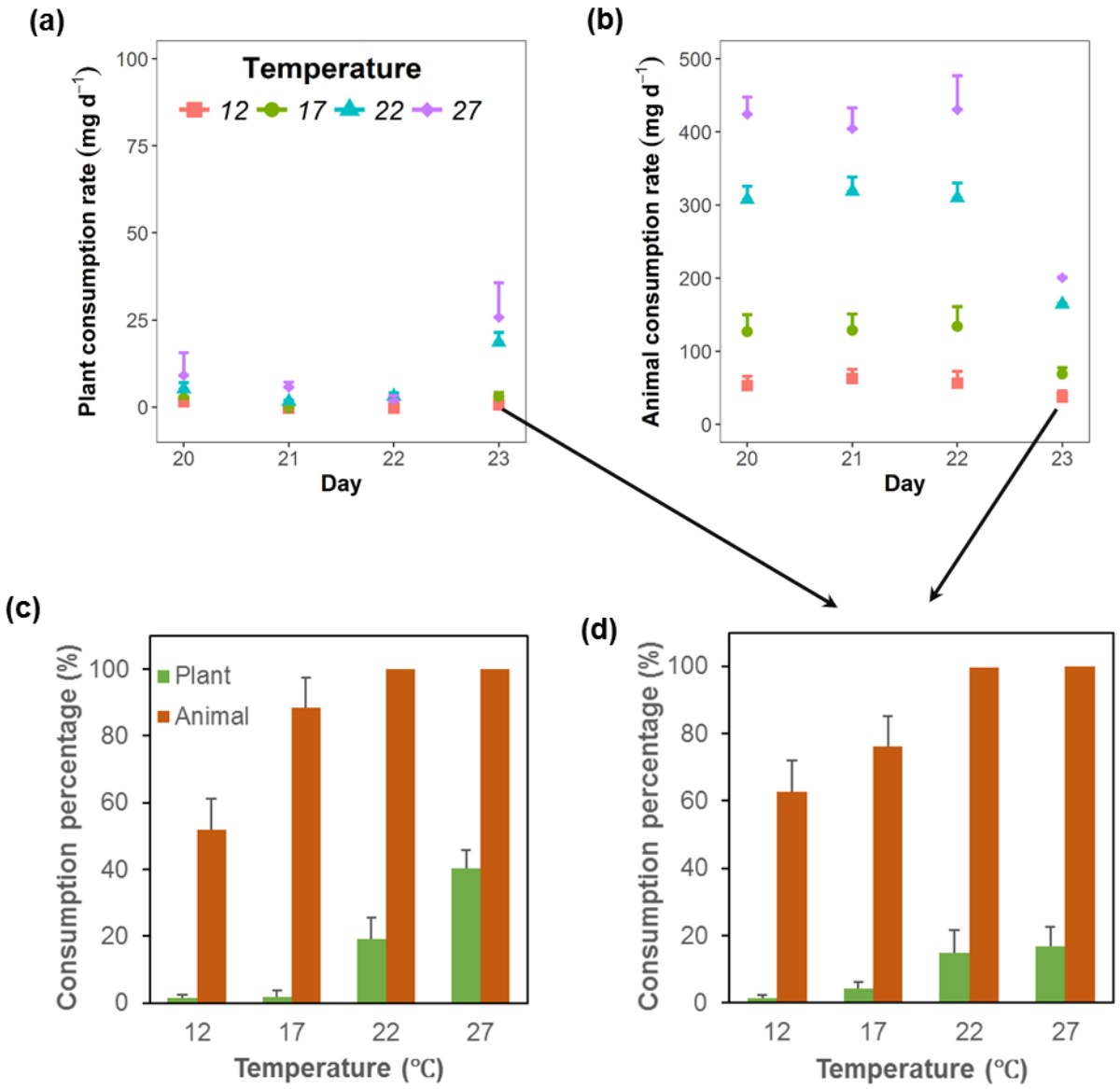

Figure S7.2 Fish consumption rate in the first day and the last four days at different temperatures. Fish were offered food for a two hours' feeding trial in the last four days to test whether feeding time is a limited factor. (a) Plant food consumption and (b) animal food consumption in the last four days. (c) Consumption percentage of the two types of food at different temperatures in the first day. Restricted animal food was offered to the fish to demonstrate their consumption of plant material. (d) Consumption percentage of the two types of food in the last day, restricted animal food was offered to the fish to demonstrate their consumption on plant material at the end. Bars indicate standard error. Fish at high temperatures first finished the animal food, then started to eat plant material.

Table S7.1 Dissolving test to calibrate the loss of agar food pellet for snails during the feeding period at different temperatures in the absence of snails. Agar food pellets were put in buckets at different temperatures for three days without snails and the dry weight of the pellets was quantified at the end. Mean ± SD, n = 6 for each temperature tested. A Linear mixed model test showed that temperature had effects on both dissolving of plant agar food ($F_{1,10} = 11.3$, $p = 0.007$) and dissolving of animal agar food ($F_{1,10} = 204.7$, $p < 0.001$). Therefore, we calibrated the dissolved amount at each temperature separately in the feeding trials with snails by subtracting the dissolved part. A plant agar pellet food without dissolving weighed 12.73 ± 0.37 mg (n =6), average dissolving rate was 19.5%. An animal food pellet without dissolving weighed 19.87 ± 1.05 mg (n =6), the average dissolving rate was 43.2%.

Temperature	**12 (°C)**	**15 (°C)**	**18 (°C)**	**21 (°C)**	**24 (°C)**	**27 (°C)**
Plant (mg)	10.57 ± 0.35	10.43 ± 0.13	10.22 ± 0.52	10.12 ± 0.20	10.11 ± 0.16	10.04 ± 0.13
Animal (mg)	12.01 ± 0.12	11.96 ± 0.35	11.49 ± 0.11	11.21 ± 0.08	10.71 ± 0.17	10.34 ± 0.17

Supplementary material 7.1 Permission for the fish experiment from NIOO-KNAW.

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

Liesbeth Bakker

NIOO-KNAW
Wageningen

Amsterdam, 22 September 2017

Date meeting IvD-NIOO: -

File: 677-Reaction IvD-NIOO

Concerns: Reaction on Study Proposal

Dear Dr Bakker,

The IvD-NIOO discussed your document concerning the experimental design and background of the project "Effects of climate warming on dietary preferences of omnivorous freshwater fish".

The conclusion of the IvD is that the planned experiments with fish do not involve animal experiments as is defined in the Dutch Law on animal experimentation (Wod).

The IvD encourages you to take all possible efforts to release the fish in the wild or to return them to the aquarium shop. Take note that when euthanizing the fish it is not allowed to take tissue samples for experimental purposes because such a procedure would make it an animal experiment requiring a CCD license.

On behalf of the IvD,

Dr. W. Kamphuis,
Secretary IvD-NIOO.

cc: Ruben de Wit

Het Trippenhuis
Postadres: Dierexperimentencommissie KNAW
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Telefoon 020 566 5504 . Fax 020 566 6121
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Chapter 8

Synthesis

In this thesis, I studied the effects of rising temperature on aquatic plant growth, plant stoichiometry and plant palatability, and the interactions of plants with higher trophic levels. Based on the results from all the different chapters, I answer the four questions which I raised in the introduction of this thesis. I discuss how warming affects the growth, the stoichiometry, the plant-herbivore interactions and plant-omnivore interactions of aquatic plants, and conclude what the implications for aquatic-plant dominated ecosystems are.

Warming effects on aquatic plant growth

In this thesis, I tested the effects of rising temperature on the growth of four aquatic plant species. In a mesocosm experiment with simulated warming, I found that warming did not increase the total biomass of *Potamogeton crispus*, but advanced the phenology of the plant during the growing season. This result is in accordance with previous experiments, which also did not find warming effects on total biomass over the growing season (Mckee et al. 2002, Li et al. 2017). However, in other microcosm experiments (Chapters 3 and 4), where plants were cultured at different constant temperatures, I found that the biomass and growth rates of all three species (*Elodea nuttallii*, *Vallisneria spiralis* and *Potamogeton lucens*) increased with rising temperature. These results are in accordance with previous studies that found increased growth of aquatic plants with rising temperature (Barko and Smart 1981, Rooney and Kalff 2000, Feuchtmayr et al. 2009). This suggests that changes in fluctuating temperatures as well as changes in constant temperatures have the potential to affect plant growth and total biomass, but that not all plant species respond similarly. Species-specific responses of aquatic plants to rising temperature can be better understood by looking in more detail to the variation in thermal tolerances among different plant species.

Temperature is a fundamental abiotic factor, which regulates the metabolism of all organisms. The metabolic rate increases exponentially with rising temperature in ectotherms within a certain temperature range (Gillooly et al. 2001, Brown et al. 2004). However, every species has its thermal tolerance, the range of temperatures at which an organism can grow, and an optimum temperature (Pörtner and Farrell 2008, Padfield et al. 2016, Sinclair et al. 2016, Payne and Smith 2017) (Fig. 8.1). Below the optimum temperature, growth rates increase with rising temperature. However, when exceeding the optimum temperature, growth rates will decline rapidly if the temperature continues to rise (Fig. 8.1). In our study, the optimum temperature for the growth of *P. crispus* is between 10 and 20 °C. When temperatures are above

24 °C, the plant stops growing (Ren et al. 1997). In contrast, for *E. nuttallii*, *V. spiralis* and *P. lucens*, the optimum temperatures are probably between 25 °C and 32 °C (Barko et al. 1982, Santamaría and van Vierssen 1997, Bartleson et al. 2014). Hence, under natural conditions, *P. crispus* is an early emerging species, that starts to grow when the temperature is still too low for the other species to grow (Tobiessen and Snow 1984). Later, when temperature rises and exceeds the optimum temperature for *P. crispus*, the species starts to senesce (Hongda 1985). By that time, the other aquatic plant species can establish. Temperature change might be the reason for the seasonal community succession in aquatic plants (Sayer et al. 2010a, Sayer et al. 2010b). Warming can therefore affect plant community composition directly because of species-specific thermal tolerances (Mckee et al. 2002, Feuchtmayr et al. 2009, Li et al. 2017). Under ongoing global warming, species with different thermal tolerance might respond differently at different regions. Cold-adapted species might just advance the growing season under warming, with an early emergence and early senescence. However, warm-adapted species might have a longer growing season in cold regions, and advance the growing season in warm regions under warming.

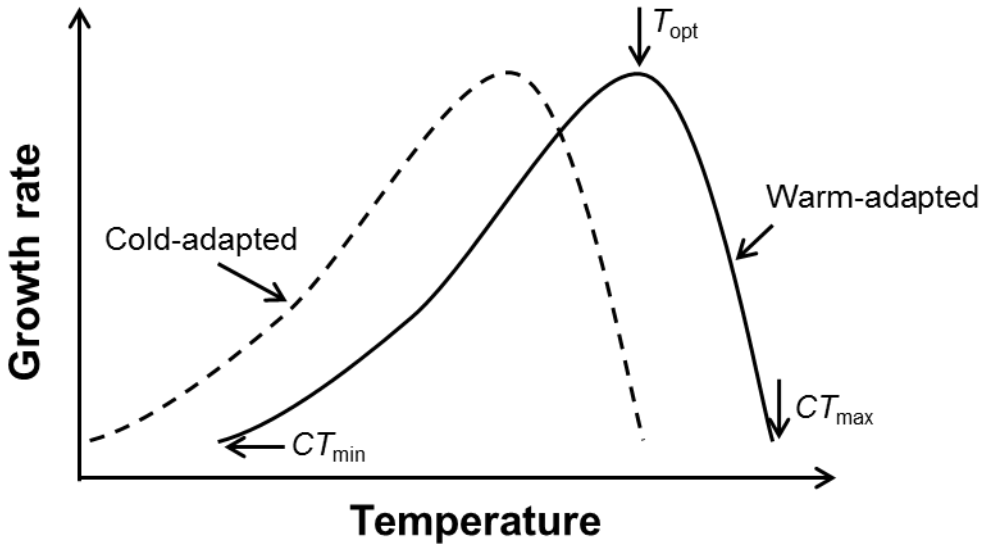

Figure 8.1 Thermal growth rate curves for two different types of species, cold-adapted and warm-adapted. T_{opt}: thermal optimum, CT_{min}: critical thermal minimum, CT_{max}: critical thermal maximum. The figure is modified after Figure 1 in Sinclair et al. (2016).

In Chapter 4, I found that environmental nutrient conditions affect the temperature effects on aquatic plant growth. The plant growth rate increased faster with rising temperature in nutrient-rich sediment than in nutrient-poor sediment. Submerged aquatic plant can take up nutrients from both the sediment and the water layer (Rattray et al. 1991). Nutrient enrichment in the sediment to a certain extent has synergistic effects with rising temperature on aquatic plant growth. However, external nutrient loading to the water layer had negative effects on aquatic plant growth (Chapter 4). External nutrient loading to the water increased the growth of phytoplankton and periphyton, which inhibited the growth of the aquatic plants (Scheffer et al. 1993, Bakker et al. 2010, Sayer et al. 2010b). However, Kosten et al. (2011) found that submerged plants would tolerate shading better in warm waters. Probably warming might enhance the growth of submerged plants under eutrophication, but if exceeding the optimum temperature, aquatic plants might suffer more from eutrophication. In many shallow lakes, climate change is often accompanied by local nutrient loading, and both affect the growth of the aquatic plants.

Warming effects on aquatic plant stoichiometry

In my experiments, I found that rising temperature significantly affected the growth of aquatic plants, and also influenced plant elemental composition and stoichiometry. In the first two experiments (Chapter 2 and Chapter 3), aquatic plant C:nutrient ratio (the nutrients I consider here are N and P) either increased or remained unaltered with rising temperature. An increase of plant C:nutrient ratio with rising temperature could be explained by the Plant Temperature Physiology Hypothesis (Reich and Oleksyn 2004), which predicts that plants invest less nutrients compared to carbon for their growth at warmer conditions. Autotrophs can increase their nutrient use efficiency at higher temperature, which has been demonstrated in terrestrial plants (An et al. 2005) and phytoplankton communities (Toseland et al. 2013, Domis et al. 2014). When the nutrient availability is the same, plants at higher temperatures can invest less nutrients to sustain growth and reach a higher growth rate. Hence, direct temperature effects on plant physiology might result in higher C:nutrient stoichiometry.

However, not only temperature could affect aquatic plant C:nutrient stoichiometry, but also nutrient conditions in the environment could affect plant stoichiometry (Cross et al. 2015, Xing et al. 2015, Su et al. 2016, Velthuis et al. 2017). For submerged plants, nutrient enrichment in both the sediment and water layer might decrease the C:nutrient stoichiometry of the plant

(Gu et al. 2016). Therefore, I performed an experiment to test the interaction of rising temperature and nutrient enrichment on aquatic plant stoichiometry (Chapter 4). I found that the effects of rising temperature on aquatic plant stoichiometry highly depend on environmental nutrient conditions (Fig. 8.2). Nutrient availability will also change with the growth of the plant, as the plant might deplete the nutrients while growing. Hence, the plant C:nutrient ratio would change with rising temperature and the growth of the plant (Fig. 8.2a).

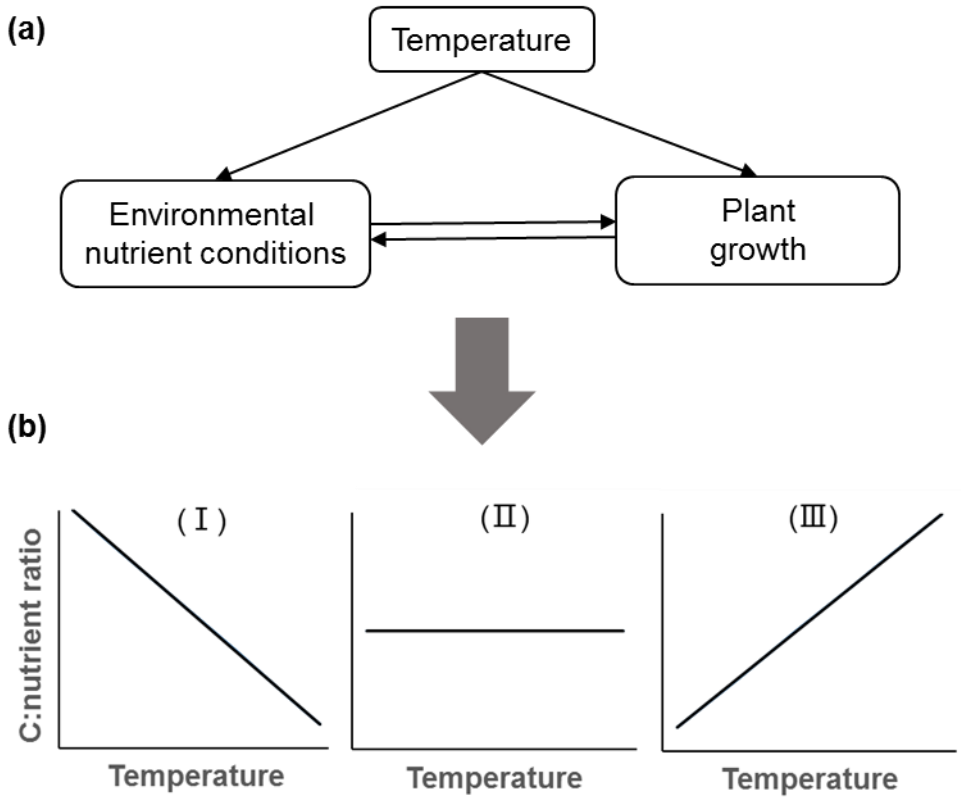

Figure 8.2 Mechanism and scenarios of temperature effects on aquatic plant C:nutrient stoichiometry. (a) Mechanism of temperature effects on aquatic plant stoichiometry. Temperature impacts on environmental nutrient conditions and plant growth; environmental nutrient conditions and plant growth also interact with each other. The arrows indicate the direction of impact. (b) Different scenarios of temperature effects on aquatic plant C:nutrient stoichiometry. (I) The C:nutrient ratio decreases with rising temperature. (II) The C:nutrient ratio remains unaltered with rising temperature. (III) The C:nutrient ratio increases with rising temperature.

I introduce three different scenarios for how temperatures may affect plant stoichiometry. Firstly, in rich organic matter sediment, rising temperature would increase the

decomposition rate of organic matter (Gudasz et al. 2010, Sobek et al. 2017), which would increase nutrient availability for the growth of aquatic plants (Fisher et al. 2005, Alsterberg et al. 2012) and decrease the plant C:nutrient ratio (Fig. 8.2b, scenario I). Secondly, in nutrient poor sediment, rising temperature might not increase nutrient availability and plant stoichiometry might not change with rising temperature (Fig. 8.2b, scenario II). Alternatively, in a nutrient rich environment, the available nutrients may always be abundantly available for aquatic plant growth, implying that plant stoichiometry does not change with rising temperature either (Fig. 8.2b, scenario II). Thirdly, it could be that there is a certain amount of nutrients in the sediment, rising temperature increases the growth of the aquatic plant, nutrients are depleted with the growth of the plant, the faster growth leads to faster depletion, and overall this results in less nutrients in plant tissue and a higher C:nutrient ratio (Fig. 8.2b, scenario III). I observed all three different responses of aquatic plant stoichiometry to temperature rise in my experiments. Hence, I conclude that temperature effects on aquatic plant stoichiometry are complex and interact with the nutrient conditions and plant growth in the environment, but the effects are predictable.

Under the current global climate change and eutrophication, the abundance and diversity of aquatic plants has declined. However, even before the decline of aquatic plants, plant stoichiometry might already be altered by climate change and eutrophication. Warming might have inconsistent effects on aquatic plant C:nutrient stoichiometry, but eutrophication will certainly decrease the C:nutrient ratio of the plants. The combination of warming and eutrophication will therefore most likely lead to a decrease of plant C:nutrient stoichiometry. This makes plant C:nutrient stoichiometry a possible indicator or early warning signal of the shift from a stable state dominated by submerged plants to a state dominated by phytoplankton. More studies are needed to verify this extrapolation.

Warming effects on aquatic plant-herbivory interactions

The feeding preference of herbivores is a multiple choice, determined by three categories of plant traits: plant toughness, plant nutrient level and plant secondary metabolism. My experiments showed that rising temperature effects on aquatic plant toughness (indicated by dry matter content) and nutrient level (nutrient content or C: nutrient stoichiometry) were species-specific, with no significant effects of temperature on plant secondary metabolism (indicated by total phenolic compounds) (Table 8.1). Plant palatability was decreased with

rising temperature in *P. lucens*, but no effects were found in *E. nuttallii* and *V. spiralis*. In *P. lucens*, plant palatability could be inferred by plant dry matter content, plant N and P content, plant C:N and C:P ratio, plant total phenolic compounds, and the plant N:phenolic compounds ratio. However, the palatability of the other plant species could not be inferred from those plant traits. These plant traits might be better to predict the feeding preference of herbivores at an inter-specific level (Elger and Willby 2003, Grutters et al. 2017b) than at an intra-specific level (Cronin et al. 2002, Cronin and Lodge 2003).

Table 8.1 Summarized warming effects on traits of aquatic plants and ectothermic herbivores which relate to their interactions.

	Traits	**Warming effects**	**Reference**
Aquatic plant	Toughness	Species-specific, not well studied	Chapter 3,4
	Nutrient content	Depends on environmental nutrient condition	Chapter 2,3,4
	Stoichiometry	Depends on environmental nutrient condition	Chapter 2,3,4
	Secondary metabolism	No effects, not well studied	Chapter 3
	Palatability	Species-specific, not well studied	Chapter 3,4
	Growth rate	Increases in the suitable physiological range	Chapter 3,4
Herbivore	Consumption rate	Increases in the suitable physiological range	Chapter 6,7
	Growth rate	Increases in the suitable physiological range	Chapter 6,7

These species-specific effects of warming on plant physical and chemical traits and palatability make it difficult to generalize the warming effects on plant-herbivore interactions from the perspective of aquatic plants. However, if we take the temperature effects on organism metabolism into consideration, the pattern becomes clear. The plant consumption rates of pond snails significantly increased as temperature increased (Chapter 6). In the suitable physiological temperature range, metabolism rates of both plants and snails increase exponentially with rising temperature (Gillooly et al. 2001, Brown et al. 2004), hence the animals ingest more food to sustain their increased metabolism. Furthermore, the animal consumption rates increase faster than plant growth rate with rising temperature, leading to enhanced top-down control on plants (O'Connor 2009, West and Post 2016, Schaum et al. 2018). This implies that warming might impose strong pressure on aquatic plant standing biomass. However, the top-down pressure

may first lead to a shift in species composition, as warming may have species-specific effects on palatability (Chapter 3).

Under eutrophication, plant nutrient content increases and C:nutrient ratio decreases, plant palatability might increase in some species (such as *P. lucens*), while it might not change in other species (such as *V. spiralis*). Therefore, eutrophication might also alter plant species composition, which is similar to the warming effects on plant-herbivore interactions. Together with warming, we can expect that aquatic plant communities first shift in species composition and then decline in abundance.

Warming effects on aquatic plant-omnivore interactions

Most aquatic plant consuming animals are omnivores (Wootton 2017). I tested effects of temperature rise on two omnivorous consumers, the pond snail and the common carp, in this thesis. The omnivores were simultaneously offered both plant material and animal food. In a short-term (24h) diet selection experiment, the pond snail did not change diet preference and always preferred animal food (Chapter 6). In a long-term (three weeks) diet selection experiment, the pond snail cultured at high temperature increased herbivory after 17 days (Chapter 7), and snails grew faster at high temperatures than at low temperatures. In contrast, fish did not shift their diet with rising temperature during a three weeks' feeding trial, probably due to the fact that the fish did not grow during the experiment. Through a literature study, I found that during my PhD project an increase in herbivory with rising temperature has been observed in multiple taxa, including zooplankton, amphibians, crayfish, fish and aquatic snails (Guinan Jr et al. 2015, Boersma et al. 2016, Carreira et al. 2016, Emde et al. 2016, González-Bergonzoni et al. 2016, Vejříková et al. 2016, Carreira et al. 2017). Therefore, I conclude that it might be a common phenomenon for aquatic omnivores to increase herbivory with rising temperature. However, the mechanisms behind this phenomenon are not clear. Both the *Temperature Physiological Constraint Hypothesis* (Gaines and Lubchenco 1982, Floeter et al. 2005, Trip et al. 2014) and the *Temperature Metabolic Stoichiometry Hypothesis* (Malzahn et al. 2016, Anderson et al. 2017, Rho and Lee 2017) could not fully explain why omnivores increase herbivory with rising temperature. I therefore propose to include the animal ontogenetic stoichiometry demand into consideration, resulting in the *Temperature Ontogenetic Stoichiometry Hypothesis*, which indicates that aquatic omnivores increase herbivory with

rising temperature because of an ontogenetic diet shift of the animals that mature faster and earlier at higher temperatures (Chapter 7).

Similarly to warming effects on plant-omnivore interactions, with eutrophication, aquatic omnivores increased plant consumption as plant quality increased (Chapter 5). Omnivorous animals have a more similar elemental composition compared to animal food than compared to plant material. Under eutrophication, the plant C:nutrient ratio decreases, and the difference with animal food becomes smaller, leading to increased consumption of plant material by omnivores under eutrophication (Bakker and Nolet 2014, Gu et al. 2018). Therefore, I conclude that both warming and eutrophication might lead to increased herbivory by aquatic omnivores.

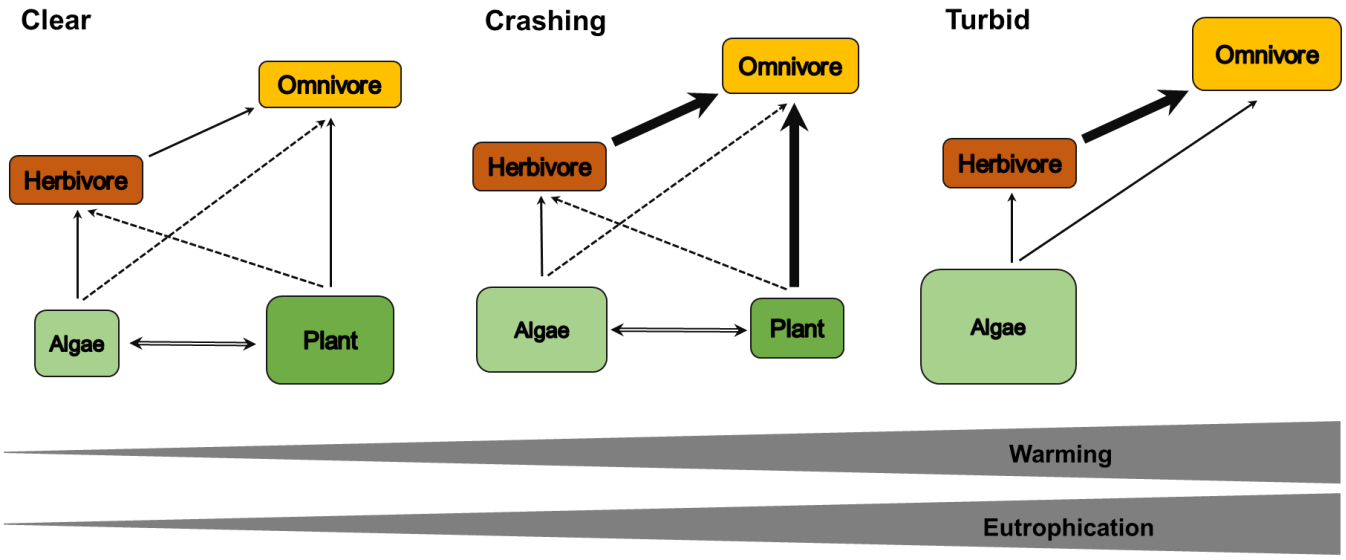

Figure 8.3 Omnivorous trophic interactions in shallow aquatic ecosystems during warming and eutrophication. The ecosystem shifts from an aquatic plant dominated clear state, to a crashing state and to an algae dominated turbid state. The algae represent both phytoplankton and periphyton. Warming and eutrophication have similar effects. The arrows indicate the direction of matter transfer. A two-way arrow line indicates competition. A solid line indicates a known interaction. A dashed line indicates a possible interaction. The width of the solid line indicates the strength of the interaction. The terminology and figure structure refer to Hidding et al. (2016).

However, in shallow aquatic plant-dominated ecosystems, there is a pervasive top-down pathway: aquatic plant-eating omnivores – invertebrates – algae – aquatic plants, which could influence the growth of the aquatic plants (Brönmark and Weisner 1992, Scheffer et al. 1993,

Jeppesen et al. 1997). The omnivores include fish, crayfish and water birds; invertebrates can be both zooplankton and macroinvertebrates, and algae represents both phytoplankton and periphyton. Aquatic plant-eating omnivores feed on both aquatic plants and some invertebrates (Jones and Sayer 2003, Bakker and Nolet 2014, Hidding et al. 2016). The invertebrates feed on algae, and the algae can inhibit the growth of the aquatic plants (Phillips et al. 2016, Mormul et al. 2017) (Fig. 8.3). With increasing eutrophication, the primary production increases, leading to a higher abundance of aquatic omnivores (Jones and Sayer 2003). The aquatic omnivores impose stronger top-down pressures on aquatic plants and invertebrates, resulting in decreased invertebrate abundances and more competition of algae with the plants, leading to the decline of aquatic plants. Therefore, aquatic plants could suffer from direct grazing and indirect shading effects from eutrophication (Hidding et al. 2016). Similarly, with ongoing global warming, the abundance of aquatic omnivores might also increase (Meerhoff et al. 2007, Jeppesen et al. 2010a, González-Bergonzoni et al. 2012, O'Gorman et al. 2017), resulting in stronger top-town control on aquatic plants and invertebrates and more competition of algae with the plants, leading to an increase of direct grazing and indirect shading pressure on aquatic plant (Mooij et al. 2007, Kosten et al. 2009). Therefore, I conclude that global warming and eutrophication have synergistic effects on aquatic plant-dominated ecosystems, which both could lead to the decline and collapse of aquatic plant standing biomass (Fig. 8.3).

Perspectives on alternative stable states

Globally, many shallow lakes have shifted from an aquatic plant dominated clear state to a phytoplankton dominated turbid state (Scheffer et al. 1993, Sayer et al. 2010b, Zhang et al. 2017). The most acceptable explanation is the anthropogenic activities resulting in eutrophication, leading to the fast decline of aquatic plants, and this explanation has lots of theoretical and experimental support (Scheffer et al. 1993, Sayer et al. 2010a, Phillips et al. 2016, Hilt et al. 2017). In contrast, another factor, global warming, might also contribute to this stable state shift, which thus far only was supported by modelling and indirect latitudinal observation (Mooij et al. 2007, Kosten et al. 2009). Mesocosm warming simulation studies to date all failed to observe the transit shift, as they only found a change in plant species composition with warming (Mckee et al. 2002, Feuchtmayr et al. 2009, Li et al. 2017). However, none of the simulation studies have taken into consideration the role of plant-eating consumers in their systems. Both plant-eating ectothermic herbivores and omnivores increase plant consumption with warming, leading to enhanced top-down control on aquatic plant

communities. Furthermore, aquatic plant quality increases with eutrophication, aquatic herbivores and omnivores increase plant consumption which leads to enhanced top-down control on plant standing biomass. The increased herbivory under warming and eutrophication might contribute to the stable state shift in shallow lakes. Therefore, future studies simulating global warming effects on a stable state shift in aquatic ecosystems should incorporate plant-eating herbivores and omnivores into these systems.

Summary

Anthropogenic activities have led to global environmental change, resulting in the deterioration of shallow aquatic ecosystems. This includes the decline of submerged aquatic plant communities, which provide vital functions in shallow aquatic ecosystems by maintaining a clear water state and sustaining high biodiversity. Eutrophication has been perceived as the main driver of these plant declines. However, the hypothesis that climate change might further exacerbate the decline of aquatic plant abundance remains largely untested. In this thesis, I aimed to clarify the effects of global warming on aquatic plants. I studied the effects of warming on aquatic plants with regard to four aspects: the effects of warming on plant growth, plant stoichiometry, plant-herbivore interactions, and plant-omnivore interactions. By manipulating temperature, environmental nutrient availability or both in experiments, I investigated their effects on aquatic plant growth, stoichiometry, and palatability to the generalist consumer *Lymnaea stagnalis*. I also tested the effects of temperature on plant consumption rates of aquatic herbivores and omnivores.

First, I tested how warming would affect the growth of aquatic plants. In Chapter 2, warming advanced the growing season of the submerged plant *Potamogeton crispus*. In Chapters 3 and 4, rising temperature increased the growth of three submerged aquatic plants (*Elodea nuttallii*, *Vallisneria spiralis* and *Potamogeton lucens*). Therefore, I conclude that temperature rise increases plant growth when within the physiological thermal tolerance range of the plant species. I also tested whether the effects of rising temperature on plant growth depended on environmental nutrient availability in Chapter 4, using *V. spiralis*. Results showed that the plants grew faster in nutrient-enriched sediment, whereas the plant growth was inhibited by external nutrient loading to the water layer, probably due to competition by algae. The plants grew faster with rising temperature in nutrient-rich sediment than in the nutrient-poor sediment. Hence, warming and eutrophication can interactively influence the growth of aquatic plants.

Secondly, I tested how warming would affect the stoichiometry of aquatic plants. The stoichiometry of aquatic plants affects the consumption by higher trophic levels, and generally, a lower carbon (C):nutrient (nitrogen or phosphorus) ratio indicates a higher quality food. I found that rising temperature increased the C:nutrient ratio in *P. crispus*., *V. spiralis* and *P. lucens*, but not in *E. nuttallii* (Chapters 2 and 3). Subsequent exploration, in Chapter 4 revealed that, the C:nutrient ratio of *V. spiralis* was either decreased or unaltered with rising temperature

at different environmental nutrient conditions. Therefore, I conclude that warming effects on aquatic plant stoichiometry depend on the environmental nutrient conditions. Warming could therefore either decrease the plant C:nutrient ratio if warming increases environmental nutrient availability for the plants, it might not affect the plant C:nutrient ratio if warming does not change the nutrient availability for aquatic plants, or warming could increase the plant C:nutrient ratio if it results in depleted nutrient availability for aquatic plants.

Thirdly, I tested how warming would affect the plant-herbivore interactions of aquatic plants. In Chapters 3 and 4, I tested the effects of rising temperature on aquatic plant palatability to a generalist consumer, the pond snail *L. stagnalis* offered only plant food. Results showed that the palatability of *P. lucens* decreased with warming, but no temperature effects were observed on *E. nuttallii* and *V. spiralis*. Therefore, from the perspective of aquatic plants, warming might decrease plant palatability in some species, but not in others. In Chapter 6, I tested the temperature effects on the consumption rate of aquatic plants by herbivory and found rising temperature to significantly increase the consumption rates of aquatic ectotherm animals. Hence, from the perspective of ectothermic herbivores, warming will lead to an increased plant consumption and further impose top-down grazing pressure on aquatic plant species. Similarly, I also found that plant palatability increased under eutrophication in *P. lucens* (Chapter 5) but not in *V. spiralis* (Chapter 4), which could lead to enhanced top-down grazing pressure on some aquatic plant species as well. Therefore, I conclude that both warming and eutrophication could lead to a stronger top-down grazing pressure on aquatic plants by herbivory, but this varies among plant species.

Finally, I tested how warming would affect the plant-omnivore interactions of aquatic plants. In Chapter 6 and 7, I found that pond snails increased the proportion of plants in their diet at higher temperatures in three-week's feeding trials, but not in 24 hour's feeding trials. A test with common carp did not reveal a change in their diet preference at higher temperatures during a three-week's feeding trial. In a literature study I found that multiple taxa of aquatic omnivores (including fish, tadpole, crayfish and snail) increased the proportion of plants in their diet under warming. As warming accelerates the ontogeny of ectothermic omnivores, and larger organisms consume more food, this may increase plant consumption by ectothermic omnivores in warming waters. In addition, I found that aquatic omnivores increased aquatic plant consumption as plant quality increased with eutrophication (Chapter 5). Therefore, I conclude that warming might increase herbivory of aquatic ectothermic omnivores, and eutrophication might strengthen this effect.

In the last chapter (Chapter 8), I synthesize the results and conclude that although plants initially grow faster with increasing temperature and nutrient enrichment, this eventually also resulted in an increased shading pressure on aquatic plants by algae. Both warming and eutrophication could lead to an increased grazing pressure on aquatic plants by ectothermic herbivores and omnivores. Therefore, the combination of eutrophication and warming will first cause aquatic plant communities to shift in species composition, followed by a decline in their overall abundance. Furthermore, both warming and eutrophication could lead to stable states shift from an aquatic plant-dominated clear system to a phytoplankton-dominated turbid system.

Samenvatting

Menselijke activiteiten hebben geleid tot wereldwijde verandering van het milieu, met als gevolg de achteruitgang van de ecologische kwaliteit van ondiepe aquatische ecosystemen. Een sterke achteruitgang is onder anderen te zien in gemeenschappen van onderwaterplanten, die belangrijke functies in ondiepe aquatische ecosystemen vervullen door zowel een heldere toestand van het water als een hoge biodiversiteit in stand te houden. Eutrofiëring (hoge nutriëntenbelasting) wordt gezien als de belangrijkste oorzaak van deze afname van ondergedoken waterplanten. De hypothese dat klimaatverandering de achteruitgang van waterplanten verder zou kunnen verergeren, is echter niet getoetst. Het doel van dit proefschrift is om de effecten van wereldwijde temperatuur-stijging op waterplanten te ontrafelen. Ik bestudeerde daarvoor de effecten van opwarming op waterplanten met betrekking tot vier aspecten: plantengroei, planten stoichiometrie (nutriëntenverhoudingen), plant-herbivoor interacties en plant-omnivoor interacties. Door de temperatuur en de beschikbaarheid van nutriënten te variëren in experimenten, onderzocht ik hun effecten op de groei van waterplanten, stoichiometrie en eetbaarheid voor de generalistische consument *Lymnaea stagnalis* (de gewone poelslak). Ik testte ook de effecten van temperatuur op de mate van plantenconsumptie door aquatische herbivoren en omnivoren.

Eerst heb ik getest hoe opwarming de groei van waterplanten beïnvloedt. In hoofdstuk 2 bleek het groeiseizoen van de ondergedoken waterplant *Potamogeton crispus* te worden vervroegd door opwarming. In de hoofdstukken 3 en 4 versnelde de stijgende temperatuur de groei van drie onderwaterplanten (*Elodea nuttallii, Vallisneria spiralis* en *Potamogeton lucens*). Daarom concludeer ik dat temperatuurstijging de plantengroei stimuleert binnen het fysiologische thermische tolerantiebereik van de plantensoort. Ik heb ook getest of de effecten van stijgende temperatuur op plantengroei afhankelijk waren van de beschikbaarheid van nutriënten in hoofdstuk 4, met de soort *V. spiralis*. De resultaten toonden aan dat de planten sneller groeiden in met voedingsstoffen verrijkt sediment, terwijl de plantengroei werd geremd door externe nutriëntbelasting van de waterlaag, waarschijnlijk als gevolg van competitie met algen. De planten groeiden sneller door de stijgende temperatuur in voedselrijk sediment dan in het voedselarme sediment. Hieruit blijkt dat opwarming en eutrofiëring gezamenlijk de groei van waterplanten kunnen beïnvloeden.

Ten tweede heb ik getest hoe opwarming de stoichiometrie van waterplanten beïnvloedt. De stoichiometrie van waterplanten beïnvloedt de consumptie door hogere trofische niveaus, en in het algemeen duidt een lagere koolstof (C): nutriënten (stikstof of fosfor) verhouding op voedsel van hogere kwaliteit. Ik ontdekte dat stijgende temperatuur de C: nutriënten ratio verhoogde in *P. crispus.*, *V. spiralis* en *P. lucens,* maar niet in *E. nuttallii* (Hoofdstukken 2 en 3). Daaropvolgend onderzoek, in hoofdstuk 4, onthulde dat de C: nutriënten ratio van *V. spiralis* ofwel verlaagd of ongewijzigd was met stijgende temperatuur bij verschillende beschikbaarheid van nutriënten in de omgeving. Daarom concludeer ik dat de effecten van opwarming op stoichiometrie van waterplanten afhankelijk zijn van de beschikbaarheid van nutriënten. Opwarming zou daarom de plant C: nutriënten ratio kunnen verlagen als opwarming de beschikbaarheid van nutriënten voor de planten verhoogt, het zou de plant C: nutriënten ratio niet kunnen beïnvloeden als opwarming de beschikbaarheid van nutriënten voor waterplanten niet verandert, of opwarming kan de plant C: nutriënten ratio verhogen als dit resulteert in een uitgeputte nutriënten voorraad voor waterplanten.

Ten derde heb ik getest hoe opwarming de plant-herbivoor-interacties van waterplanten beïnvloedt. In de hoofdstukken 3 en 4 testte ik de effecten van stijgende temperatuur op de eetbaarheid van waterplanten voor een generalistische consument, de gewone poelslak *L. stagnalis,* die ik alleen planten als voedsel aanbood. De resultaten toonden aan dat de eetbaarheid van *P. lucens* afnam met het verwarmen, maar er werden geen temperatuureffecten waargenomen op *E. nuttallii* en *V. spiralis*. Opwarming kan de eetbaarheid van planten in sommige soorten verminderen, maar dit geldt zeker niet voor alle soorten. In hoofdstuk 6 heb ik de effecten van verschillende temperaturen op de snelheid van het consumeren van waterplanten getest en vond dat een temperatuurstijging de consumptie snelheid van aquatische koudbloedige dieren significant verhoogt. Vandaar dat opwarming, vanuit het perspectief van koudbloedige herbivoren, zal leiden tot een verhoogde plant-consumptie en daardoor een grotere top-down begrazingsdruk op waterplanten zal veroorzaken. Op dezelfde manier vond ik ook dat de eetbaarheid van de plant toenam onder eutrofiëring in *P. lucens* (Hoofdstuk 5) maar niet in *V. spiralis* (Hoofdstuk 4), wat ook zou kunnen leiden tot verhoogde top-down begrazingsdruk op sommige waterplanten. Daarom concludeer ik dat zowel opwarming als eutrofiëring kan leiden tot een hogere top-down begrazingsdruk op waterplanten door herbivorie, maar dat dit verschilt per plantensoort.

Ten slotte heb ik getest hoe opwarming de plant-omnivoor-interacties van waterplanten beïnvloedt. In hoofdstuk 6 en 7 ontdekte ik dat poelslakken het aandeel planten in hun dieet verhoogden bij hogere temperaturen in drie weken durende voedingsexperimenten, maar niet

in 24-uurs voedingsexperimenten. Een test met de Europese karper (*Cyprinus carpio*) onthulde geen verandering in hun dieetvoorkeur bij hogere temperaturen tijdens een voedingsexperiment van drie weken. In een literatuurstudie ontdekte ik dat meerdere taxa van aquatische omnivoren (waaronder vissen, kikkervisjes, rivierkreeften en slakken) het aandeel planten in hun dieet bij opwarming verhoogden. Omdat opwarming de ontogenese van koudbloedige omnivoren versnelt, en grotere organismen meer voedsel consumeren, kan dit de consumptie van planten door koudbloedige omnivoren in opwarmende wateren verhogen. Bovendien ontdekte ik dat aquatische omnivoren de consumptie van waterplanten verhoogden naarmate de plantkwaliteit toenam met toenemende nutriëntenbelasting (hoofdstuk 5). Daarom concludeer ik dat opwarming de mate van herbivorie door aquatische koudboedige omnivoren zou kunnen doen toenemen, en eutrofiëring zou dit effect kunnen versterken.

In het laatste hoofdstuk (hoofdstuk 8) voeg ik de resultaten samen en concludeer dat hoewel planten aanvankelijk sneller groeiden met toenemende temperatuur en verrijking van voedingsstoffen, dit uiteindelijk ook resulteerde in een verhoogde beschaduwing van waterplanten door algen. Zowel opwarming als eutrofiëring kunnen leiden tot een verhoogde begrazingsdruk op waterplanten door koudbloedige herbivoren en omnivoren. Daarom zal de combinatie van eutrofiëring en opwarming de aquatische plantengemeenschappen als eerste van soortensamenstelling doen veranderen, gevolgd door een afname van hun algehele talrijkheid. Hierdoor kunnen zowel opwarming als eutrofiëring tenslotte leiden tot stabiele verschuivingen van een door waterplanten gedomineerd helder systeem naar een door fytoplankton gedomineerd troebel systeem.

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Peiyu Zhang, Wageningen

About the author

Peiyu Zhang (1989-09) was born in a rural area in Wuhan, China. There is a river around his village, which is a branch of a branch of……Yangtze River. He had a wonderful childhood in the village playing with nature. The two most awesome things for him to do were swimming and fishing. Meanwhile, there were a lot of globalizations happening in the river, such as eutrophication, species invasion, biodiversity loss, climate change, and so on. At that time, he only knew that it was environmental degradation, and many years later, he knew those terminology. He was so curious about those things. And then, he chose to study environment science at Wuhan University of Technology in 2007. After four years' bachelor study, he got a bit lost and still could not find his way. And he continued his master on limnology at Institute of Hydrobiology, Chinese Academy of Science. During his master, he was lucky to join projects to sample many lakes in China. He was fascinated by those lakes. He felt that his knowledge was so limited. Thanks to the Chinese Scholarship Council (CSC), he could come to NIOO at 2014 to work with a group of enthusiastic ecologists. He gained a lot by working with those people. He also had a wonderful time in this small and quiet town, Wageningen. Four years are too short, so, to be continue……

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Peer-reviewed

Zhang, P., B. A. Blonk, R. F. van den Berg, and E. S. Bakker. 2018. The effect of temperature on herbivory by the omnivorous ectotherm snail *Lymnaea stagnalis*. Hydrobiologia 812:147-155.

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Zhang, P., A. Kuramae, C.H.A. van Leeuwen, M. Velthuis, E. van Donk, E.S. Bakker. Interactive effects of rising temperature and nutrient enrichment on aquatic plant growth and stoichiometry. (Submitted)

Zhang, P., R.F. van den Berg, C.H.A. van Leeuwen, B.A. Blonk, E.S. Bakker. Aquatic omnivores shift their trophic position towards increased plant consumption as plant stoichiometry becomes more similar to their body stoichiometry. (Submitted)

Zhang, P., C.H.A. van Leeuwen, D. Bogers, M. Poelman, E.S. Bakker. Perspectives on herbivory by aquatic omnivores in warming freshwater. (Ready to submit)