ABSTRACT

Title of Document: PHYSIOLOGICAL RESPONSES OF *ACARTIA* AND *EURYTEMORA* SPP. TO CHANGES IN THE NITROGEN:PHOSPHORUS QUALITY OF THEIR FOOD

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This study addressed how copepods respond to varying nutrient content in their prey. Copepod physiological responses were measured along a gradient of prey nitrogen:phosphorus (N:P) ratios created by altering the P content in diatom prey grown at a constant rate. *Acartia tonsa*, a broadcast spawner, and *Eurytemora carolleeae*, a brood spawner, increased excretion of P as prey N:P declined (i.e. P increased). *E. carolleeae* had higher somatic tissue nutrient content, while *A. tonsa* had higher egg nutrient content overall and higher P in eggs as N:P decreased. *E. carolleeae* egg production was greatest when eating high N:P prey while *A. tonsa* showed the opposite. Egg viability declined at high N:P for both copepods, yet *A. tonsa* viability was always greater than *E. carolleeae* viability. Both copepods responded physiologically to food of varying quality, yet regulated their homeostasis differently. Prey nutrient content may be significant in the environmental selection of different copepods.

PHYSIOLOGICAL RESPONSES OF *ACARTIA* AND *EURYTEMORA* SPP. TO CHANGES IN THE NITROGEN:PHOSPHORUS QUALITY OF THEIR FOOD

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2014

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Acknowledgements

I would like to acknowledge the support of my advisor, Patricia Glibert, and committee members, James Pierson and Frances Wilkerson. I would also like to acknowledge the input and assistance of Horn Point Laboratory Analytical Services, Jeff Alexander, Eric Kiss, and Catherine Fitzgerald. The assistance of and input from all listed was invaluable in designing these experiments and analyzing them. Further, I would like to acknowledge the support of the Horn Point Laboratory Education Committee and the State and Federal Water Contractors Agency for funding and financial support.

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Physiological responses of *Acartia* and *Eurytemora* spp. to changes in the nitrogen:phosphorus quality of their food

Introduction

Copepod growth and reproductive success (egg production rates and egg viability or hatching success) have often been interpreted as a response to food quantity (measured as carbon, C), where increased rates are related to increasingly sufficient food quantity (Kleppel 1992, White and Roman 1992, Boersma and Kreutzer 2002, Acheampong et al. 2011, Kimmerer et al. 2014). However, food quality, the nutritional value of food to a consumer to meet its physiological needs, may also constrain copepod physiological processes (Checkley 1980, Kiørboe 1989, Jónasdóttir 1994, Koski et al. 1998, Jones et al. 2002, Breteler et al. 2005, Nobili et al. 2013). Studies on food quality have mainly emphasized nutrient-rich biomolecules in phytoplankton cells such as phosphorus (P)-rich lipids (Kleppel 1993, Kleppel and Burkart 1995, Kleppel et al. 1998, Arendt et al. 2005), and nitrogen (N)-rich proteins (Checkley 1980), or amino acids (Kleppel et al. 1998, Guisande et al. 2000, Müller-Solger et al. 2002, Helland et al. 2003), as measures of food quality. P is an important mineral element required for growth and reproduction because it is a major component of ribosomal RNA, and ribosomes are the machinery of growth (Sterner and Elser 2002). N is a major component of amino acids and proteins (Sterner and Elser 2002). Effects of food quality as measured by mineral N content have been reported for copepods (Kiørboe 1989, Jones et al. 2002, Nobili et al. 2013). However, there have been comparatively few studies of the effect of variable mineral N:P ratios of prey on estuarine copepods. Therefore this study was undertaken to determine the effects of altered N:P, as a measure of nutritional quality, on the physiology of estuarine copepods by altering the mineral P content in one food type provided at a constant and saturating level of food quantity.

Over the past several decades rising inputs of N to estuaries and coastal waters throughout the world have outpaced inputs of P, leading to changing ecosystems, largely as a result of single-nutrient management strategies that reduce potential P inputs while N inputs continue to rise (Nixon 1995, Howarth et al. 2000, Howarth and Marino 2006, Bricker et al. 2008, Glibert et al. 2014). For example, P removal from detergents has been a common management action (Glibert et al. 2011). Cultural eutrophication in estuaries and coastal waters, mainly caused by the development of synthetic N fertilizers and their application to the land, discharge of human waste, animal and aquaculture production, and the increased combustion of fossil fuels, are largely responsible for these increases (Cloern 2001, Boesch 2002, Howarth 2008). This unequal nutrient loading has largely resulted in rising N:P ratios in coastal and estuarine waters, making them proportionally more N rich and P poor (Howarth et al. 2000, Cloern 2001, Glibert et al. 2013, 2014).

Phytoplankton elemental content tends to reflect ambient nutrient conditions. Phytoplankton cellular nutrient stoichiometry (as mineral C, N, or P content) can vary significantly with nutrient concentration and ratio (Hecky and Kilham 1988, Sommer 1989, Kilham et al. 1997, Glibert et al., 2011, 2013). Consumers, in contrast to phytoplankton, are generally more homeostatic, maintaining relatively constant cellular elemental stoichiometry (Sterner and Elser 2002). Ecological stoichiometry is the study of the balance of multiple chemical elements in ecological interactions and how they vary through the food web (Sterner and Elser 2002). It is based on the idea that the mass of chemical elements must balance, and thus elements can be traced through ecological interactions (Sterner and George 2000, Sterner and Elser 2002). Ecological stoichiometry suggests that consumers can compensate for eating prey of varying quality by using physiological mechanisms such as excretion, egestion, and the

allocation of nutrient into eggs, to selectively retain or release nutrient (Sterner and Hessen 1994, Sterner and Elser 2002, Hessen and Anderson 2008, Hessen et al. 2013, Chrzanowski and Foster 2014), but ultimately the success of consumers depends on the availability of critical elements for their biomass and growth. How consumers vary their physiology in response to food quality has implications for nutrient release to the environment and copepod reproductive success.

In this study the small centric diatom *Thalassiosira pseudonana* was grown across a gradient of dissolved molar N:P ratios and served as prey of varying food quality for grazing and viability experiments with the calanoid copepods *Acartia tonsa* and *Eurytemora carolleeae*. Previous experiments with *T. pseudonana* have shown that their cellular N:P content varies in relation to available nutrient supply (Glibert et al. 2013). Further, diatoms have been considered an important food source thought to largely support the traditional pelagic food web (Cushing 1989, Legendre 1990). The copepods *A. tonsa* and *E. carolleeae*, a sibling species to the cosmopolitan *E. affinis* (Lee 2000, Alekseev and Souissi 2011), were selected because both are important estuarine copepods with widespread distribution (Ambler 1986, Mauchline 1998, Kimmel and Roman 2004, Devreker et al. 2012, Lloyd et al. 2013). *A. tonsa* is a broadcast spawner while *E. carolleeae* is a brood spawner (Mauchline 1998) thus presenting contrasting metabolic pathways. The broadcast spawner, *A. tonsa*, is considered an opportunistic producer, increasing and decreasing egg production rates in more and less favorable environments, respectively (Dagg 1977, Jonasdotttir 1994). This study was designed to ask how copepod somatic tissue nutrient stoichiometry, excretion rates, egg stoichiometry, egg production rates, and egg viability respond to eating food of varying quality (and not quantity) when the only variable in food quality is the N:P ratio. In this study the N:P ratio of the prey was varied by varying the P availability, not the N availability of its growth media, to explore how alterations

in P loads may alter copepod consumer physiological responses to varying N:P ratios in their environments. We hypothesized that 1) copepod stoichiometry and nutrient content should not vary significantly as food stoichiometry varies, 2) excretion rates of P (or N) when eating a P-rich (or N-rich) food should increase as the concentration of that nutrient in the food increases, 3a) egg production rates, egg nutrient content, and egg viability should decline as P content in food decreases, and 3b) *A. tonsa,* broadcasting copepods, should respond to declining P in food by decreasing egg production and viability, while *E. carolleeae*, the brood spawner, should maintain consistent egg viability although egg production may decline when grazing on P-poor food.

Methods

Overview

The diatom *T. pseudonana* was grown in turbidostat continuous culture systems to maintain constant and exponential growth, on media of varying dissolved N:P ratios. This diatom was then provided as prey in equal amounts and at saturating levels (in terms of C) for the calanoid copepods *A. tonsa* and *E. carolleeae*. For each N:P condition of the prey, copepod somatic tissue nutrient stoichiometry, excretion rates, egg stoichiometry, egg production and egg viability were assessed following both a 4 h (day 1) and a 7-day period of feeding (Figure 1).

Algal Growth

The diatom *T. pseudonana* clone 3H was obtained from the Oyster Hatchery at the Horn Point Laboratory in Cambridge, MD. Cells were grown in f/2 media (Guillard and Ryther, 1962) for all constituents except for PO_4^{3-}, the concentration of which was adjusted to achieve molar N:P ratios ranging above and below Redfieldian proportions (N:P=4, 16, 24, 32). Media was made using artificial seawater with a salinity of 12.

The diatoms were grown in turbidostat continuous culture (Li et al. 2011) in which growth was exponential and near constant for all nutrient conditions. Four 2 L turbidostat chambers were set up in an environmental chamber with temperature held at 14-16 °C and a 14:10 light:dark cycle for all experiments. For each experiment 2 turbidostats were set to one N:P ratio (either N:P 4 or 16) and 2 were set to a higher N:P ratio (either 24 or 32, respectively).

In turbidostat systems cells grow exponentially and this rate is maintained by dilution of the culture with fresh media (Li et al. 2011). During growth of the diatom in turbidostat continuous culture systems, turbidity readings, recorded every minute, were used as a proxy for growth rate (Figure 2). As cells grow, turbidity increases. Once turbidity reaches a preset threshold value, new media is automatically delivered, resulting in a 5% reduction in optical signal, corresponding to an approximate 10% volume dilution (Li et al. 2011). The rate of dilution, or number of dilutions over time, is proportional to growth rate. The numbers of dilutions per day were counted for the 3 days prior to the start of an experiment for each experiment. Where data were unavailable for this time period, data from 3 consecutive days nearest to the experiment were used. These values were averaged to obtain the average number of dilutions for each experiment at each ratio; although not an actual growth rate, the consistency in this index is a measure of the degree of consistency in growth rate across treatments.

As each of the turbidostats was maintained under identical conditions, these continuous culture systems eliminated growth stage and rate as variables within and between experiments and the only variable in prey conditions between experiments was variations in the dissolved nutrients of the media.

To assess the nutrient status of the diatom prey in turbidostat, aliquots of prey culture were filtered onto each of two precombusted (450°C, 2 hr) 25mm GF/F filters that were frozen and later analyzed for particulate C, N, and P, as described below.

Copepod Collection and Growth

The copepods *A. tonsa* and *E. carolleeae* were collected from the Choptank River, a tributary of Chesapeake Bay, using 200 μm plankton nets towed by hand. Single-species copepod cultures were subsequently maintained in an environmental chamber at a salinity of 12, temperature of 14-16°C, and on a 14:10 h light:dark cycle in 2 L glass bottles with bubbling air. Prior to use in the grazing experiments, *A. tonsa* and *E. carolleeae* were maintained on a mono-algal diet of *Rhodomonas sp*. that was grown on f/2 at a salinity of 12.

Prey Quality Experiments

Experiments were conducted on adult stage CVI and some CV copepods that had been starved for 12-24 hs prior to experiments. A total of 16, 7-day experiments using *T. pseudonana* as prey and either *A. tonsa* or *E. carolleeae* as grazers were conducted. Each grazer was used in 8 experiments, representing a day-1 and a day-7 experiment for each of the 4 prey N:P ratios. Copepods were fed prey at a level that was estimated to be twice their daily copepod C demand. Given the average C content of the diatom, which was 8×10^{-6} μg C cell^{-1} (Berges et al. 2002), daily copepod C demand, which was 0.2 μg C adult^{-1} day^{-1} (Heinle and Flemer 1975), and the cell count of the diatom in turbidostat, which was 1.50×10^{6} cells mL^{-1}, a total of 300 μg C L^{-1} was calculated to be needed to meet the daily C demand of the copepod *T. pseudonana*. Thus, to provide twice their daily C demand, prey were added equivalent to 600 μg C L^{-1}. Each day the copepods were transferred to a new bottle with 200 mL of new prey culture. To provide the prey

of a given N:P ratio, equal volumes of the replicate turbidostat cultures were combined and then diluted to achieve the prey concentration needed.

From these copepod cultures with varying prey, aliquots were withdrawn after 4 hours (on day 1) and again after 7 days for measurements of grazing and egg production and for determination of chemical composition. Grazing experiments conducted over 4 hs are considered to be representative of baseline physiological conditions (after 12-24 h starvation) in copepods while the day 7 experiments are considered to reflect a physiological response to eating prey for that number of days at varying N:P ratio.

To initiate grazing experiments, aliquots of each of the diatom cultures were transferred to 5, 600 mL bottles. One of these bottles was a control with no copepods, and to each of the other 4 bottles, 30 female adult copepods and 3- 5 males were added. Three of these bottles were considered replicates for the grazing experiment and included copepods that were subsequently used for egg chemistry measurements, and one bottle was later used for viability and egg production experiments as described below. The bottles for egg viability and production experiments always contained 30 adult stage CVI females. These bottles were incubated on a slowly rotating wheel for 4 h in a 14-16°C environmental chamber during the light cycle.

Samples for dissolved and particulate nutrients, and cell counts were taken at the start and end of the 4 h grazing period, and within 1 h from removal from the grazing wheel. After grazing copepods were removed and 150 mL aliquots of the dilution mixture (start) and particulate material from each grazing incubation bottle (end) were filtered onto pre-combusted 25mm GF/F filters to collect particulate P, C, and N samples to determine changes in particulate nutrients before and after feeding on varying prey. The filtrate from treatment bottles was used to collect samples for analysis of dissolved nutrients (NH_4^+, NO_3^-+NO_2^-, PO_4^{3-}). The differences in

$NH_4^+$and PO_4^{3-} concentrations in the treatment (with copepod grazers and prey) and control (with prey only) grazing bottles over 4 h were compared to estimate excretion rate. Aliquots of 10-20 mL unfiltered material were also collected for enumeration of diatoms and these cell counts were later used for grazing calculations. At the 4 h time point, when copepods were removed, 20 from each replicate treatment bottle were removed for particulate nutrient analysis; about 10 were placed onto each of two pre-combusted 25mm GF/F filters for particulate P, C, and N analysis (Table 1).

Female stage CVI copepods remaining in the 3 treatment bottles (usually about 30 stage C6 females and 3 to 5 males) were gently transferred to 200 µm mesh containers and submerged in low nutrient artificial seawater at a salinity of 12. These copepods were incubated for an additional 24 h in the environmental chamber at 14-16°C on a 14:10 h light:dark cycle. After 24 h, the eggs produced were collected and placed onto two pre-combusted 25mm GF/F filters for subsequent analysis of particulate C, N, and P (Table 2). The contents of remaining (5th) incubation bottle not used for grazing, excretion, and nutrient content analysis were gently transferred to a 200 µm mesh container and submerged in low nutrient artificial seawater and incubated for 48 h in the 14-16 °C environmental chamber and egg production and viability was determined. The contents of this bottle were preserved in 4% formalin and the number of nauplii and unhatched eggs were counted microscopically at a later date.

Analytical

Dissolved nutrient analyses were conducted on a Technicon AutoAnalyzer II. NH_4^+ analysis was based on USEPA Method No. 350.1 and Whitledge et al. (1981). NO_3^-+NO_2^- analysis was based on USEPA Method No. 350.1 and D’Elia et al. (1997). Analysis of PO_4^{3-} was based on methods for soluble reactive PO_4^{3-} analysis including USEPA Method No. 365.1, and

Whitledge et al. (1981). Particulate C and N were determined on an Exeter Analytical, Inc. (EAI) CE-440 Elemental Analyzer. Particulate P was extracted by dry ashing followed by acidification overnight (Aspila et al., 1976, USEPA Method No. 365.1). Cell counts (later used in grazing calculations) were counted under a light microscope at 40x magnification with a hemocytometer counting chamber.

Calculations

Grazing rate calculations and excretion rate calculations were according to Frost (1972) and Miller and Glibert (1998; Table 3; Appendix A). Egg production rates (eggs $female^{-1}$ day^{-1}) and egg viability were calculated for each viability experiment from the preserved samples. Egg production rates were calculated by dividing the total number of eggs produced in the 48 h incubated container by the number of females and dividing by the number of days. Rates of egg viability were calculated as the number of nauplii produced as a percent of the number of free fertilized eggs and nauplii produced.

Statistics

Analysis of variance (ANOVA) tests were calculated to determine differences across the prey N:P gradient. Unpaired t-tests were calculated to compare somatic tissue nutrient content between *A. tonsa* and *E. carolleeae* for each nutrient, C, N, and P, on day 1 and on day 7. Unpaired t-tests were also calculated to compare egg nutrient content between eggs of *A. tonsa* and *E. carolleeae* for each nutrient on each day. Further, unpaired t-tests assuming equal variance were calculated to compare egg nutrient content of C, N, and P on day 1 with the same nutrient content on day 7 in each copepod. Unpaired t-tests were also performed to compare day-1 excretion rates of either NH_4^+ or PO_4^{3-} to day-7 excretion rates of the same nutrient in the same

copepod. Paired t-tests were calculated to compare day-1 to day-7 total egg production and egg viability in a copepod eating prey at the same N:P ratio.

Results

Algal growth and cell composition

Diatom growth rates in turbidostat, evaluated by a proxy for growth rate, the average number of dilutions of turbidostats, were relatively constant (e.g., Figure 2). The cellular N:P content of *T. pseudonana* varied significantly as media NO_3^-:PO_4^{3-} varied (Figure 3, Table 4). *T. pseudonana* molar cellular N:P ranged from 6.95 $\pm$ 2.07 to 15.10 $\pm$ 2.54 as the molar N:P of the media ranged from 4 to 32 (Figure 3; ANOVA p=0.0001).

Grazing Rates

Clearance rates were relatively constant for both copepods across the N:P gradient of their prey during the 4 h experiments and during the day 7 experiment for *E. carolleeae*, while during the day 7 *A. tonsa* experiments clearance rates rose as prey cellular N:P increased (or prey cellular P content declined) (Figure 4). During the 4 h experiments clearance rates ranged from -1.15 to 4.88 mL copepod^{-1} h^{-1} for *A. tonsa* and from -3.16 to 5.20 mL copepod^{-1} h^{-1} for *E. carolleeae*. During the day 7 experiments clearance rates ranged from -5.79 to 3.78 mL copepod^{-1}h^{-1} for *A. tonsa* and from 1.24 to 4.12 mL copepod^{-1} h^{-1} for *E. carolleeae*. Negative clearance rates are likely an effect of the diatom prey growing faster than they were grazed by the copepods.

Nutrient content in copepod somatic tissue

The nutrient content of the copepods varied as a function of prey nutrient content, and differed between the two copepods. The somatic tissue nutrient content (C, N, P) of *A. tonsa* varied significantly with prey cellular N:P on day 1 for C content (Figure 5A, ANOVA p=0.01),

N content (Figure 5B, ANOVA $p=0.031$), and P content (Figure 5C, ANOVA $p=0.04$), while that of *E. carolleeae* varied significantly with prey cellular N:P for C content (Figure 5D, ANOVA $p=0.047$) and P content (Figure 5F, ANOVA $p=0.016$), but not for N content (Figure 5E, ANOVA $p=0.29$) .

E. carolleeae were more C, N, and P rich than *A. tonsa* (Figures 5A-F) across all prey conditions. *E. carolleeae* nutrient contents were on average 12.07 µg C copepod^{-1} (=1.01 µM C copepod^{-1}), 2.00 µg N copepod^{-1} (=0.14 µM N copepod^{-1}), 0.20 µg P copepod^{-1} (=0.007 µM P copepod^{-1}). In contrast, *A. tonsa* nutrient contents were on average 6.64 µg C copepod^{-1} (=0.55 µM C copepod^{-1}), 0.77 µg N copepod^{-1} (=0.06 µM N copepod^{-1}), 0.075 µg P copepod^{-1} (=0.002 µM P copepod^{-1}). By day 7, *E. carolleeae* nutrient contents were on average 9.33 µg C copepod^{-1} (=0.78 µM C copepod^{-1}), 1.35 µg N copepod^{-1} (0.097 µM N copepod^{-1}), 0.279 µg P copepod^{-1} (=0.009 µM P copepod^{-1}) while those of *A. tonsa* nutrient contents were, on average, 5.04 µg C copepod^{-1} (=0.42 µM C copepod^{-1}), 0.59 µg N copepod^{-1} (=0.042 µM N copepod^{-1}), and 0.06 µg P copepod^{-1} (=0.002 µM P copepod^{-1}).When comparing *A. tonsa* and *E. carolleeae* somatic tissue nutrient content for the same nutrient (C, N, or P) on the same day (day 1 or day 7), significant differences (unpaired t-test $p < 0.01$) between the two copepods were found for all nutrients on both days (Figure 5).

Somatic tissue N:P ratios of *A. tonsa* did not vary significantly with prey cellular N:P ratios after either 4 hs (Figure 6A, $p=0.32$), or 7 days of grazing, although day-7 ratios did decline with prey N:P (Figure 6B, $p=0.165$). Somatic tissue N:P ratios of *E. carolleeae* did not vary significantly with prey cellular ratios after 4 h (Figure 6C, $p=0.07$), but did after 7 days of grazing (Figure 6D, $p=0.024$). *A. tonsa* somatic tissue N:P ratios after 4 h of grazing ranged from 18.62 to 27.06 (molar) over the prey cellular N:P range (Figure 6A). Day 7 *A. tonsa* somatic

tissue N:P molar ratios ranged from 15.22 to 41.08 over the prey cellular N:P range (Figure 6B). *E. carolleeae* somatic tissue N:P molar ratios after 4 h of grazing ranged from 12.28 $\pm$ 0.64 to 26.68 $\pm$ 10.56 over the prey cellular N:P range (Figure 6C). Day 7 *E. carolleeae* somatic tissue N:P molar ratios ranged from 5.89 $\pm$ 1.32 to 21.96 $\pm$ 11.46 over the prey cellular N:P range (Figure 6D).

Excretion

A comparison of NH_4^+ and PO_4^{3-} excretion rates on day 1 and day 7 for both copepods showed a different NH_4^+ response in the two copepods but a similar PO_4^{3-} response (Figures 7, 8). *A. tonsa* NH_4^+ excretion rates ranged from -41.62 $\pm$ 15.71 to 9.34 $\pm$ 14.21 ng N copepod^{-1}h^{-1} (= -2.97 $\pm$ 1.12 to 0.68 $\pm$ 1.01 nM N copepod^{-1} h^{-1}) after 4 h, and from -42.05 $\pm$ 18.74 to 33.07 $\pm$ 78.27 ng N copepod^{-1}h^{-1} (= -3.00 $\pm$ 1.33 to 2.36 $\pm$ 5.59 nM N copepod^{-1}h^{-1}) on day 7 (Figure 7A,B). Note that negative excretion rates suggest that the rate of NH_4^+ uptake by the prey was greater than that of excretion by the copepod, resulting in net NH_4^+ depletion over the incubation period. These excretion rates increased as the molar prey cellular N:P increased (Figures 7A,B). *A. tonsa* NH_4^+ excretion rates varied significantly with prey cellular N:P ratios after 4 h (Figure 7A, ANOVA p=0.0097), but did not vary significantly after 7 days (Figure 7B, ANOVA p=0.078). Further, NH_4^+ excretion rates on day 1 and day 7 were not significantly different from each other (unpaired t-test p=0.7).

A. tonsa PO_4^{3-} excretion rates both after 4 h and 7 days were greatest when eating prey with molar cellular N:P ratio of 6.95 $\pm$ 2.07, and declined as prey molar cellular N:P ratio increased to 15.10 $\pm$ 2.54 (Figures 7C,D). PO_4^{3-} excretion rates after 4 h ranged from -8.03 $\pm$ 26.08 to 25.12 $\pm$ 9.23 ng P copepod^{-1}h^{-1} (= -0.26 $\pm$ 0.84 to 0.81 $\pm$ 0.30 nM copepod^{-1}h^{-1}) (Figure 7C). Day 7 PO_4^{3-} excretion rates ranged from -17.30 $\pm$ 9.81 to 35.49 $\pm$ 24.33 ng P copepod^{-1} h^{-1}

(= -0. 56 $\pm$ 0.32 to 1.14 $\pm$ 0.78 µM copepod^{-1} h^{-1}) (Figure 7D). PO_4^{3-} excretion rates varied significantly with prey cellular N:P on day 7 (ANOVA p=0.002) but did not on day 1 (ANOVA p=0.099). Day 1 and Day 7 PO_4^{3-} excretion rates were not significantly different from each other (unpaired t-test p=0.86).

E. carolleeae NH_4^+ excretion rates after 4 hs ranged from 24.53 $\pm$ 11.27 to 36.72 $\pm$ 18.76 ng N copepod^{-1} h^{-1} (equivalent to 1.75 + 0.80 to 2.62 $\pm$ 1.34 nM copepod^{-1}h^{-1}) (Figure 8A), and from 2.51 $\pm$ 23.86 to 38.64 $\pm$ 16.49 ng N copepod^{-1}h^{-1} (equivalent to 0. 18 $\pm$ 1. 70 to 2.76 $\pm$ 1.18 nM copepod^{-1}h^{-1}) on day 7 (Figure 8B). These rates decreased as prey cellular N:P increased (Figures 8A,B); however, *E. carolleeae* NH_4^+ excretion did not vary significantly with prey cellular N:P ratios on day 1 (Figure 8A, ANOVA p=0.624) or day 7 (Figure 8B, ANOVA p=0.086). NH_4^+ excretion rates on day 1 and day 7 were not significantly different from each other (unpaired t-test p=0.34). Rates of PO_4^{3-} excretion after 4 hs for *E. carrollleeae* did not did not vary significantly with prey cellular N:P (ANOVA p=0.06) and ranged from -2.86 $\pm$ 2.52 to 3.93 $\pm$ 4.21 ng P copepod^{-1} h^{-1} (-0.09 $\pm$ 0.08 to 0.13 $\pm$ 0.13 nM copepod^{-1}h^{-1}) (Figure 8C). Rates of PO_4^{3-} excretion for *E. carrollleeae* on day 7 varied significantly with prey cellular N:P (ANOVA p=6.47x10^{-5}), and were greatest when eating prey with molar cellular N:P ratio of 6.95 $\pm$ 2.07, and declined as the molar prey cellular N:P ratio increased to 15.10 $\pm$ 2.54 (Figure 8D). These day 7 excretion rates ranged from -3.32 $\pm$ 2.03 to 50.97 $\pm$ 14.62 ng P copepod^{-1} h^{-1} (equivalent to -0. 107 $\pm$ 0.0654 to 1.64 $\pm$ 0.047 nM copepod^{-1}h^{-1}) (Figure 8D). Rates of PO_4^{3-} excretion measured at both time periods were significantly different from each other both (unpaired t-test p=0.02), and when day 1 and 7 excretion rates of copepods feeding only on prey at prey cellular N:P of 6.95 $\pm$ 2.07 were compared (unpaired t-test p=0.003).

Nutrient content in eggs

On both day 1 and day 7 *A. tonsa* eggs had higher C, N, and P content on average than *E. carolleeae* eggs (Figure 9). Egg nutrient content for the same nutrient (C, N, or P) for both copepods on the same day (day 1 or day 7) were not significantly different (two-sample t-test for Day 1 C p=0.23, for N p=0.21, for P p=0.28, for Day 7 C p=0.15, for N p=0.13, for P p=0.75). It should be noted that egg production was not always sufficient for particulate C, N, and P analysis and thus data for *A. tonsa* egg C and N content on day 1 and *A. tonsa* egg nutrient content on day 7 when eating at media N:P ratios of 4 and 24 (prey N:P ratios of 6.95 $\pm$ 2.07 and 10.51 $\pm$ 1.64 respectively), as well as *E. carolleeae* egg nutrient content on day 7 when eating at media N:P ratios of 16 and 32 (prey N:P ratios of 10.91 $\pm$ 0.60 and 15.10 $\pm$ 2.54), are unavailable (see Table 2, 5, Figure 9). For both *A. tonsa* and *E. carolleeae* egg nutrient contents (C, N, and P) were not significantly higher on day 7 than on day 1 for the same nutrient in the eggs of the same copepod (two-sample t-test, for *A. tonsa* C p=0.15, for N p =0.15, for P p=0.46, for *E. carolleeae* C p=0.17, for N p=0.30, for P p=0.39). *A. tonsa* egg P content after 4 h of feeding declined with increasing prey N:P (or decreasing prey P content) (Figure 9C), while *E. carolleeae* P content, as well as C and N content, was relatively constant across prey N:P ratios (figure 9D-F). On day 7 C, N, and P content of both copepods may have been greater when copepods feed on prey of higher N:P, however since egg nutrient content was determined from a single sample, replicate statistics cannot be performed to determine if the difference is significant or not.

Comparing nutrient content in copepods and eggs

Overall, *A. tonsa* eggs had higher nutrient content than *E. carolleeae* eggs, while the somatic tissue of *E. carolleeae* copepods had higher nutrient content than *A. tonsa* copepods (Figure 10). For *A. tonsa*, copepods richer in P were associated with eggs that were also richer in

P (r^2=0.81; figure 10c). Note that insufficient egg production did not permit such a comparison for C or N for *A. tonsa*. In *E. carolleeae* egg nutrient content did not appear to respond to copepod N or P content on day 1, although C content of eggs was negatively related to C content of the prey (r^2= 0.79) (Figures 10D-F).

Egg production and viability

Both *A. tonsa* and *E. carolleeae* egg production and egg viability responded to declining prey cellular N:P (Figures 11, 12). *A. tonsa* responded to rising prey cellular N:P with decreasing egg production rates (Figure 11A, B) and decreasing egg viability (Figure 12A, B). When *A. tonsa* were fed diatom cells with cellular molar N:P of 15.10 $\pm$ 2.54 and 10.91 $\pm$ 0.60 for 7 days, egg production rates were 0.5 and 3.1 eggs female^{-1} day^{-1}, respectively (Figure 11B). *A. tonsa* eating diatom with molar cellular N:P of 15.10 $\pm$ 2.54 versus 10.91 $\pm$ 0.60 for 4 hs produced fewer eggs per female, 2.9 versus 3.8 eggs female^{-1}day^{-1}, respectively (Figure 11A). When comparing day 7 (Figure 11B) and day 1 (Figure 11A) egg production for *A. tonsa* eating diatom prey with molar cellular N:P of 15.10 $\pm$ 2.54, produced 0.5 eggs female^{-1}day^{-1} versus 2.9 eggs female^{1}day^{-1} (Figure 11A,B). When *A. tonsa* ate diatom prey grown at prey molar cellular N:P of 10.91 $\pm$ 0.60, the copepod produced 3.1 eggs female^{-1}day^{-1} on day 7 compared to 3.8 eggs female^{-1}day^{-1} on day 1 (Figure 11A,B). Differences in egg production on day 1 and day 7, and when the copepod ate prey with molar cellular N:P of 15.10 $\pm$ 2.54 versus 10.91 $\pm$ 0.60 were not significant (unpaired t-test p=0.19, unpaired t-test p=0.14 respectively).

E. carolleeae responded to rising prey cellular N:P with increasing egg production rates although most of these changes were not significant (Figure 11C,D, day 1 ANOVA, p=0.02, day 7 ANOVA, p=0.26), and declining egg viability (Figures 12C,D, day 1 ANOVA, p=0.62, day 7 ANOVA p=0.67). When *E. carolleeae* were fed prey with molar cellular N:P ratios ranging from

6.95 ± 2.07 to 15.10 ± 2.54 for 4 h, egg production rates on day 1 were 3.56 ± 1.33 compared to 18.12 ± 5.66 eggs $female^{-1}$ day^{-1}, respectively (Figure 11C). After 7 days *E. carolleeae* egg production rates ranged from 2.10 ± 0.10 to 11.98 ± 3.59 eggs $female^{-1}$ day^{-1} respectively (Figure 11D). However, when eating prey at molar cellular N:P of 6.95 ± 2.07 on day 7, egg production was 9.49 ± 5.55 eggs $female^{-1}$ day^{-1}, a greater egg production rate than when the copepod ate at prey molar cellular N:P of 10.51 ± 1.64 or 10.91 ± 0.60 on day 7, and nearly as great an egg production rate as when copepods ate at prey cellular N:P of 15.10 ± 2.54 eggs $female^{-1}$ day^{-1} for 7 days (Figures 11C). Further, this day 7 egg production rate, when feeding on prey at 6.95 ± 2.07, is 2.67 times greater than the day 1 egg production rate when feeding on prey of the same cellular N:P (Figures 11C,D).

Egg viability in both copepods on both days declined as prey cellular N:P rose (Figure 12). In the day 1 *A. tonsa* experiments, egg viability was 85.5% and 89.0% when eating prey with cellular molar N:P of 15.10 ± 2.54 and 10.91 ± 0.60, respectively (Figure 12A). In the day 7 *A. tonsa* experiments, egg viability was 38.7% and 69.1 % for prey with cellular molar N:P of 15.10 ± 2.54 and 10.91 ± 0.60, respectively (Figure 12B). Differences in egg viability on day 1 and day 7, and when the copepod ate prey with cellular molar N:P of 10.91 ± 0.60 and 15.10 ± 2.54, were not significant (unpaired t-test p=0.08, unpaired t-test p=0.29 respectively). Egg viability in *E. carolleeae* ranged from 4.4 ± 6.2 to 44.4 ± 52.2 percent on day 1 (Figure 12C), and from 6.2 + 3.1 to 25.7 + 23.5 percent on day 7 (Figure 12D), declining as prey cellular N:P increased. After 7 days the egg viability of both copepods overall was lower than day 1 values.

Discussion

It has been said that "nutrient flux from resources to consumers and then to waste products can be thought of as a chemical reaction wherein mass must balance," (Sterner and

George 2000, p. 127) Herein, we attempted to quantify these fluxes for two contrasting copepods, *A. tonsa* and *E. carolleeae*, one a broadcast spawner and one a brood spawner, when fed food of constant and saturating amounts but of varying nutritional quality as defined by N:P ratio. Maintaining food in a constant physiological state was accomplished by growing the algae in turbidistat. Varying nutritional quality was accomplished by varying the P content in the media of the diatoms, while holding N content constant. These results suggest different responses to food quality in terms of excretion and egg production with implications for their adaptive response and success to changing nutrient regimes.

Algal growth and cell composition

The stoichiometry of phytoplankton reflects their nutritional environment and the physiological state of the cell, for example growth stage and growth rate (Sommer 1989, Sterner and Elser 2002). In order to conduct a food quality study in which the stoichiometry of a cell varies only due to its nutritional environment, growth stage and rate must be held constant to avoid conflicting effects of other changes in physiological state on food quality. In this study, the growth of the *T. pseudonana* in turbidostat was relatively constant (Figure 2). The cellular N:P (molar) of the diatom varied with the N:P (molar) of the media even when growth was maintained at near constant values, as has also been previously shown for this species (Figure 3; Glibert et al. 2013). Diatom cellular nutrient ratios may not match media nutrient ratios exactly since nutrient content in phytoplankton cells is due to both nutrient incorporated in cellular structures and biochemicals and nutrient stored within the cell often due to luxury consumption (Sterner and Elser 2002). Taken together this suggests that our turbidostat cultures successfully maintained cells at similar growth rates and varying media N:P stoichiometric ratios, thereby eliminating the possibility of biochemical differences in nutrient stoichiometry resulting from

physiological variations due to differences in growth rate of the diatoms. These cells represent a gradient of food quality that can be attributed, in terms of mineral nutrient content, to the experimental manipulation of dissolved $NO_3^-:PO_4^{3-}$ in media, which, in these experiments was varied by altering the concentration of PO_4^{3-} while holding NO_3^- constant. Differences in copepod physiological responses to grazing across the diatom prey food quality spectrum can be linked to the experimentally manipulated stoichiometry and P content of the prey.

Grazing

Both copepods ate prey at all cellular N:P ratios after 4 hours (day 1) or 7 days of exposure to the prey (Figure 4). Some grazing rates (reported as clearance rates) were negative but this can be explained by the underestimation of grazing due to the use of twice the saturating concentration of prey (in terms of C) required for the copepods in these experiments. Physiological responses across prey cellular N:P ratios are evident from the data in these experiments (e.g. excretion, egg production, egg viability). Clearance rates were relatively similar between copepods and across prey cellular N:P ratios after 4 hours of exposure (day 1) (Figure 4). However, clearance rates may have been lower when copepods grazed on prey of the highest and lowest cellular N:P (or most P-poor and most P-rich prey) (Figure 4) suggesting that there is a physiological cost to eating unbalanced food, whether P is limiting or saturating.

On day 7 *A. tonsa* clearance rates increased as prey cellular N:P increased (prey cellular P content declined) (Figure 4B). *A. tonsa* ingested more prey cells of low P content than of high P content. They likely increased clearance rates as prey P content declined to obtain a sufficient quota of P. This is similar to results from Houde and Roman (1987) where *A. tonsa* sourced from Chesapeake Bay increased ingestion rates of *T. weissflogii* prey as the prey became more deficient in N or in protein. Similarly, the mixotrophic dinoflagellate *Ceratium furca* was

induced to feed by N or P-deplete conditions and increased feeding as cellular C:N:P ratios deviated farther from the optimum (Smalley et al. 2003). In these experiments *A. tonsa* also increased grazing rates as the diatom prey cellular N:P (prey P content) deviated farther from P-sufficiency. Smalley et al. (2003) suggest that for *C. furca* nutrient ratios are stronger regulators of feeding than absolute nutrient concentrations. *A. tonsa* likely increased grazing rates both to increase intake of P from P-deplete prey, and to balance its stoichiometry.

During the 4 hour experiments clearance rates ranged up to 4.88 mL copepod^{-1}h^{-1} for *A. tonsa* and up to 5.20 mL copepod^{-1}h^{-1} for *E. carolleeae*. These rates compare to those reported by Frost (1972), where *Calanus pacificus* fed *T. fluviatilis* prey at varying cell concentrations had clearance rates of about 1 to 4 mL copepod^{-1} h^{-1}. Further, a prey C concentration of 600 μg C L^{-1} in the *C. pacificus* experiments, the same C concentration of *T. pseudonana* prey used in these experiments, resulted in a clearance rate of about 2 mL copepod^{-1} h^{-1}. Similarly, Saba et al. (2009) reported *A. tonsa* clearance rates of about 1 mL copepod^{-1} h^{-1} when it grazed on *T. weissflogii*.

Nutrient content in copepod somatic tissue

In contrast to phytoplankton with their wide range in cellular nutrient content as a function of nutrients in the media, consumers, including herbivores like these copepods, are generally thought to be homeostatic, maintaining their somatic nutrient stoichiometry in relatively constant proportions within species at a given life stage (Hessen 1992, Urabe 1993, Sterner and Elser 2002, Glibert et al. 2011, Hessen et al. 2013). This is indeed what was seen for both copepods after 4 h of exposure to food of varying stoichiometry on day 1 of the grazing experiments: the nutrient (C, N, and P) content in each copepod as prey cellular N:P varied was similar (Figure 5). However, after 7 days of feeding on food of varying stoichiometry, somatic

tissue C, N and P, and therefore N:P ratios, of the copepods did vary (Figures 5,6). For *A. tonsa,* somatic nutrient contents on day 1 and 7, respectively, were on average 6.64 and 5.04 µg C copepod^{-1}, 0.77 and 0.59 µg N copepod^{-1}, and 0.08 and 0.06 µg P copepod^{-1}. These results are similar to previous studies of *Acartia* species. Thompson et al. (1994) found *A. tonsa* somatic C content ranging from 3.15-5.05 µg C copepod^{-1}, and somatic N content ranging from 0.87-1.43 µg N copepod^{-1} as prosome length ranged from 838 to 987 µm. In addition, from their data Thompson et al. (1994) predicted that *A. tonsa* adult females with a cephalothorax length between 850 and 900 µm (*A. tonsa* prosome length in these experiments was 871 µm) should initially have a somatic C content between about 5 and 10 µg C copepod^{-1} and between 0.7 and 1.0 µg N copepod^{-1}. Similarly, Durbin et al. (1992) reported *A. hudsonica* somatic nutrient content ranging from 3.98-5.32 µg C copepod^{-1}, and 1.08-1.47 µg N copepod^{-1} at temperatures of 15-16°C. Miller and Roman (2008) fed *A. tonsa* 4 different prey types (detritus, a senescent and log-phase stage of a diatom, and ciliates) with naturally varying N:C ratio and reported C content ranging from about 2 to 6 µg C copepod^{-1} and N content ranging from about 0.5 to 1.3 µg N copepod^{-1} as the N:C ratio of the prey types varied. Uye and Matsuda (1988) reported somatic P contents in *A. omorii* of 0.12 µg P copepod^{-1} and in *A. erythraea* of 0.19 µg P copepod^{-1}.

The copepods did differ in C, N, and P content between the two species when both ate the same prey and were exposed otherwise similar environmental conditions. Grazers are known to have variable interspecific stoichiometry but relatively constant intraspecific stoichiometry (Andersen and Hessen 1991, Sterner and Elser 2002). *E. carolleeae* have greater C, N, and P in their tissue relative to *A. tonsa* (Figure 5). A body C content calculation based on prosome length (794 µm) predicts 3.25 µg C copepod^{-1} for *E. carolleeae* females (Kankaala and Johansson 1986, Mauchline 1998). Similarly, Kimmerer (2014) reported C content of *E. affinis* ranging from 0.8

to 4 μg C copepod^{-1}. For the data herein, *E. carolleeae* had an average somatic C content higher than these estimates, ranging from 12.07 μg C copepod^{-1} on day 1 to 9.33 μg C copepod^{-1} on day 7. The C weights here may be higher due to copepod stage, CVI females, and the presence of eggs within the copepod body or extruded into sacs, both of which have been shown to weigh more than younger stages of females and/or egg-free females in *E. herdmanni* (McLaren and Corkett 1981). *E. carolleeae* P content was, on average, 0.20 μg P copepod^{-1} on day 1, and 0.28 μg P copepod^{-1} on day 7. A calculation of somatic P content based on prosome length of 11 copepod species predicts P content for a copepod the size of *E. carrolleae* (794 μm) at 15°C to be 0.12 μg P copepod^{-1} (Uye and Matsuda 1988, Mauchline 1998). This is within the range of our P content measurements.

The difference between copepods in their nutrient content is not likely due to a size difference between the copepods as prosome lengths were similar, and, in fact, temperature-based prosome length calculations predict a longer prosome length of the less nutrient-rich *A. tonsa;* 871 μm (Heinle 1969, Mauchline 1998) compared to 794 μm in *E. carolleeae* (Mauchline 1998, Lloyd et al. 2013). This *A. tonsa* prosome length is comparable to prosome lengths measured and predicted in other studies (Heinle 1969, Cataletto and Umani 1994). This prosome length calculation for *E. carolleeae* agrees with data from the Chesapeake Bay that predicts a prosome length of about 800 μm by relating measurements of prosome length in field-collected *E. carolleeae* to the ambient temperature they were collected at (Lloyd et al. 2013). Further, ingestion rates of both copepods were relatively similar on day 1 (Table 4), so this difference in nutrient content cannot likely be explained as a consequence of eating more or less of the nutrient. Sterner and Elser (2002) suggest that closely related animals can have varying elemental chemistry as a result of different life-history strategies that require the animal to

allocate nutrient resources differentially. In this study, *A. tonsa*, the broadcast spawning copepod, had lower nutrient content overall than the brooding copepod *E. carolleeae*.

Excretion

Excretion is one mechanism by which consumers maintain homeostasis. As copepods eat food of higher N:P, food that is poor in P and thus N rich relative to P content, excretion stoichiometric theory predicts the relative proportions of N:P in excreta should vary in response (Elser et al 1988, Sterner 1990, Urabe 1993, Elser and Hasset 1994, Elser and Urabe 1999, Sterner and Elser 2002 and references therein). Even though N was maintained at constant supply in the food as the N:P of the food varied, *A. tonsa* excreted more NH_4^+ in response to increasing N:P in food (Figure 7), while *E. carolleeae* appeared to excrete less NH_4^+ as prey cellular N:P increased (Figure 8). Excretion rates were negative for *A. tonsa* NH_4^+ at lower prey cellular N:P ratios because copepods excreted less N, relative to P but what was excreted was subsequently taken up by the diatom prey. Miller and Glibert (1998) also reported low NH_4^+ excretion rates of *A. tonsa* ranging from non-detectable up to 2 ng N $copepod^{1}h^{-1}$ with an average of less than 1 ng N $copepod^{-1}h^{-1}$. Similarly, Miller and Roman (2008) reported low NH_4^+ excretion rates for *A. tonsa*, ranging from about 2.08 to 6.67 ng N $copepod^{-1}h^{-1}$, as the N:C ratio of the experimental prey types on which they grazed varied. The difference between these results and the more variable results of this study can likely be explained by the qualities of the varying prey used and the varying nutrient ratios. Miller and Glibert (1998) maintained the *A. tonsa* in mesocosms where they fed on a natural plankton assemblage from the Choptank River, a sub-estuary of the Chesapeake Bay in summer, while Miller and Roman (2008) provided monocultures of 4 prey types of naturally varying N:C ratio (detritus, a senescent stage and log-phase stage of a diatom, and ciliates). This study provided only one prey type, a log-phase

diatom, that was experimentally manipulated to vary in N:P ratio. Further, in this study the N and C content of prey was held constant; only P was allowed to vary. Nutrient content of prey was not closely controlled in either of these studies. *A. tonsa* would likely have been responding to several features of the uncontrolled prey including size, type, and nutritional quality while the only variable in this study was the N:P ratio, specifically the P content, in the prey. This could explain our higher and more variable rates of NH_4^+excretion. Similar data on the excretion rates of *E. carolleeae* was not found.

When eating P-rich food, both copepods responded by increasing PO_4^{3-} excretion as predicted by the hypothesis that when a grazer eats a prey of unbalanced N:P it will excrete the nutrient in excess (Urable 1993, Sterner and Elser 2002). In *A. tonsa* this pattern can be seen on day 1 and day 7, although the trend across the prey N:P gradient is greater on day 7. In *E. carolleeae* this pattern was only seen on day 7. Similarly, the day 7 P excretion in *A. tonsa* was greater than that on day 1. Even though *E. carolleeae* is the more P-rich copepod (see Figure 5), it excreted P at a higher rate than *A. tonsa* when both are eating the same food. Similarly, when *Daphnia magna* fed on prey of decreasing C:P (increasing P content), excretion of P increased (He and Wang 2007). When feeding on food of increasing C:P, the rates of P release by *D. magna* declined to maintain P homeostasis (He and Wang 2007). DeMott et al. (1998) also reported that *D. magna* grazing on P-deficient *Scenedesmus acutus* prey excreted P at a lower rate than when it grazed on the P-sufficient prey.

Morales (1987) suggested that copepods can vary the C and N content of fecal pellets in response to food quality. The lack of sufficient production of fecal pellets for analysis in this study precluded any comparisons of fecal pellet stoichiometry.

Egg nutrient stoichiometry

The stoichiometry and production of eggs is another pathway by which copepods rebalance C, N and P. In general, it is thought that proportionately more P should be allocated to eggs because of their high growth demand (Færøvig and Hessen 2003; Laspoumaderes et al. 2010). Here, differences were found in nutrient allocation to eggs between the two copepods. When comparing the egg nutrient content of *E. carolleeae* and *A. tonsa* (Figure 9), it was seen that *E. carolleeae*, the more nutrient rich copepod, had less nutrient rich eggs in terms of N, P, and C. *E. carolleeae* also had constant C, N, and P egg content across prey cellular N:P ratios on day 1 (Figure 9 D-F). While *E. carolleeae* P content did not vary as P content in food varied, *A. tonsa* appeared to vary its egg P content in relation to variable food P content (Figure 9C) increasing egg P content as prey cellular N:P declined (prey P content increased). Further support for this response is apparent when egg P content was compared to copepod somatic P content (Figure 10C). As the *A. tonsa* copepod P content increases slightly, egg P content increases dramatically (Figure 10C). DeMott et al. (1998) similarly found that *D. magna* had lower P content when they grazed on P-deficient prey.

Egg production and viability

It was hypothesized that due to the need for P in eggs, food P content should affect both egg production and viability. P is required for egg production, naupliar hatching (egg viability), and growth because P is a major component of ribosomes, the machinery of growth (Sterner and Elser 2002). As prey cellular N:P increased, (P content declined) *A. tonsa* egg production declined (Figures 11A,B). This pattern was clear when *A.tonsa* copepods had been grazing for 4 h (day 1) but was greater after 7 days.

A. tonsa egg production rates reported in the literature have a wide range. For example, Durbin et al. (1983) measured egg production rates of *A. tonsa* incubated in ambient Narragansett Bay, Rhode Island, water and found they ranged from 1.6 to 51.6 eggs female^{-1} day^{-1}, and egg production rates incubated in ambient bay water enriched with a mixture of laboratory-cultured algae ranged from 11.8 to 64.2 eggs female^{-1} day^{-1}. Dagg (1977) reported *A. tonsa* egg production that ranged from 20 to 40 eggs female^{-1} day^{-1}. Kimmerer et al. (2005) reported egg production rates of less than 10 and up to 20 eggs female^{-1} day^{-1} at temperatures of 15°C or less and attributed these rates to food quantity limitation. Castro-Longoria (2003) measured *A. tonsa* egg production rates over 6 days and reported rates between 5 and 15 eggs female^{-1} day^{-1} at a salinity of 32 and temperature of 15°C, while a salinity of 15 and temperature of 20.5°C resulted in egg production rates < 5 up to 10 eggs female^{-1} day^{-1}. At 28°C *A. tonsa* eating a food concentration of 150 µg C L^{-1} (which was considered to be saturating for copepod ingestion) of laboratory-cultured *T. weissflogii* for 48 h produced about 3 eggs female^{-1} day^{-1} (Kleppel and Burkart 1995). In a laboratory study of *A. hudsonica,* fed *T. weisflogii,* at varying food quantity, *A. hudsonica* produced from 2.1 ± 0.4 to 11.4 ± 0.7 eggs female^{-1} day^{-1} on average (Durbin et al. 1992). Further, when copepods were maintained on *T. weissflogii* for 7 days, egg production ranged from 0 to about 4 eggs female^{-1} day^{-1} on low food concentrations and from about 8 to 13 eggs female^{-1} day^{-1} on high food concentrations (food quality was constant) (Durbin et al. 1992).

A. tonsa egg production is influenced by food quantity, food quality, food type, ambient temperature, salinity, and female size (e.g. Mauchline 1998 and references therein). In this study food quantity, food type, temperature, salinity, and female stage (related to female size) were held constant throughout experiments of varying food quality (food quantity was also saturating

for copepod daily carbon demand). Variation in one or more of these factors likely explains the wide ranging egg production rates in the literature. While our measured egg production rates are within the range of these reports, they are on the low end, ranging from 0.52 to 3.80 eggs $female^{-1} day^{-1}$ overall. These overall lower rates of egg production compared to literature values could be a result of temperature differences, a strong controller of egg production, differences in salinity, shown to have an effect on egg production rates (e.g. Castro-Longoria 2003), or the use of a single diatom food source or lack of a mixed diet; diatoms are thought to depress copepod reproductive success (i.e. Ban 1997, Ianora 2003), though this is controversial (i.e. Dutz 2008) and species specific (i.e. Ban 1997), while there is some suggestion that a mixed diet is necessary for high levels of egg production (Kleppel 1993). Nevertheless, for this study, it is not the overall egg production rates that are important, but the differences between the rates when the copepods ate prey of lower and higher P content (higher and lower N:P, respectively) since these experiments manipulated only prey quality and did so via N:P ratio which was controlled by P content. *A. tonsa* are opportunistic egg producers, and tend to produce more eggs when environmental conditions provide nutrient-sufficient prey (of a high enough concentration) and decrease egg production in unfavorable conditions (Dagg 1977, Jónasdóttir 1994). In these experiments high prey N:P ratios (or low prey P content) represent unfavorable conditions, food of poor quality. This study found that egg production rates declined under constant food quantity, when prey (food) quality declined. This result mirrors food quantity studies in which egg production rates were lower when *A. tonsa* ate at a low food quantity and higher when it ate at a high food quantity (food quality held constant) (eg. Durbin et al. 1992, Kimmerer et al. 2005), suggesting that food quality may be as important as food quantity (when other environmental and physiological factors do not vary) to egg production rates in *A. tonsa*.

E. carolleeae, in contrast to *A. tonsa*, are brood spawners and are not thought to respond opportunistically to environmental conditions (Mauchline 1998). *E. carolleeae* in this experiment, however, increased rates of egg production as prey cellular N:P ratios increased (P content declined) (Figures 11 C, D). Further, when comparing day 1 to day 7 egg production rates at the lowest prey cellular N:P ratio (greatest prey cellular P content), day 7 egg production rates were higher than those for day 1. This is the only ratio in either copepod species where egg production is higher on day 7. Although not significant herein, the patterns suggest that day 7 *E. carolleeae* egg production rates may have been elevated when P was both limiting (prey cellular N:P of 15.10) and saturating (prey cellular N:P of 6.95) in prey. Further, *E. carolleeae* egg production rates were always higher than *A. tonsa* rates when the copepods ate at the same N:P ratio (Figure 11).

Egg production rates measured here correspond to the literature when rates are considered in terms of lower and higher prey cellular N:P ratios. Hirche (1992) showed that *E. affinis* incubated at 10°C produced 5.5 eggs female^{-1} day^{-1}, while at 15°C it produced 5.85 eggs female^{-1} day^{-1}. Bunker and Hirst (2004), in a global synthesis of copepod reproduction, suggest that sac-spawners such as *E. affinis or E. carolleeae* produce an average of 5 eggs female^{-1}day^{-1}. This data is similar to the egg production rates measured at lower prey cellular N:P ratios (higher prey P content) in this study with the exception of the egg production at the lowest prey cellular N:P ratio on day 7 (Figure 11 C, D). At lower prey cellular N:P ratios copepods would have been grazing on high quality prey (high P content) at saturating levels of food quantity in terms of C since N and C were held constant throughout experiments. Lloyd et al. (2013) reported that Chesapeake Bay *E. affinis,* found in estuarine turbidity maximums where they were food saturated and ate a natural plankton assemblage, produced 15-20 eggs female^{-1} day^{-1}. This result

is similar to our egg production rates for *E. carolleeae* at higher prey cellular N:P ratios (P-poor prey) (Figure 11C,D). At higher prey cellular N:P ratios (lower P content) in these experiments copepods would have been grazing on prey of poor food quality but at saturating levels of food quantity in terms of C. *E. carolleeae* did not produce more eggs at these higher N:P prey ratios as a result of increased grazing because clearance rates did not change substantially across prey cellular N:P ratios after either 4 hs or 7 days of grazing (Figure 4). It is possible that *E. carolleeae* produced more eggs when eating poor quality food as a response to a P-poor environment in which more eggs may have a greater chance of survival than fewer eggs.

The variable *E. carolleeae* egg production at both the lowest and highest prey N:P where prey P content was saturating and limiting, respectively, can be explained by different mechanisms. When prey P content was saturating high egg production was likely a result of more P available in the maternal diet, similar to the *A. tonsa* egg production pattern. After 7 days of grazing on P-sufficient food, copepods may have acclimated to a P-sufficient environment and as a result produced more eggs after 7 days on a P-sufficient diet than after 4 h on the same diet. When prey P content was low it is possible that increased egg production in *E. carolleeae* was a response to a P-poor environment, where a larger number of eggs may have a greater chance of survival. However, clearance rates did not increase substantially as prey cellular N:P varied, so it is unclear how the copepods would have been able to produce increasing numbers of eggs on an increasingly P-poor diet, unless egg production in this copepod is more strongly controlled by N or C. Maternal reproductive responses to nutritionally poor environments have been documented (Frost et al. 2010, Helland et al. 2003). For example the offspring of P-stressed *D. magna* mothers are smaller and contain less P than their peers produced by P-sufficient mothers but, they can recover from P-stress by growing faster than their peers if released to a P-rich

environment. In contrast, if released to a P-poor environment they will grow more slowly than their peers and delay their reproduction. If a P-stressed mother will be forced to release P-stressed offspring to a P-poor environment, then, it may be more beneficial to release a larger number of offspring to increase the chance of that some will survive to adulthood to reproduce, albeit at a later age than their P-sufficient peers.

When either food quantity or food quality is not controlled varying egg production rates can sometimes be attributed to either, assuming other environmental factors are held constant. Kimmerer (2014) reported egg production rates of *E. affinis* in the field ranging from 0 up to 10-15 eggs female^{-1} day^{-1} in 2006, while in 2007 egg production rates overall were lower and always remained below 10 eggs female^{-1} day^{-1} with most measurements below 5 eggs female^{-1} day $^{-1}$. Based on literature values, the authors suggested that rates for food-saturated *E. affinis* range from about 15 to 40 eggs female^{-1} day^{-1} and attribute the lower rates reported in their study to food quantity limitation (Kimmerer et al. 2014). Our rates when copepods fed at high N:P (and saturating levels of food quantity), poorer quality food, are more similar to the literature ranges reported in Kimmerer et al. (2014) than to the results reported in Kimmerer et al. (2014). Our rates when copepods fed at low N:P (and saturating levels of food quantity), higher food quality, are more similar to the results reported in Kimmerer et al. (2014). In the case of *E. carolleeae*, according to these experiments, the patterns of egg production in response to poor food quality (increasing egg production) do not agree with the response to low food quantity (decreasing egg production). This would suggest that food quantity and quality may affect egg production in opposite directions for *E. carolleeae*, but not in *A. tonsa*. In *A. tonsa* declining food quantity and/or declining food quality will lead to lower rates of egg production. *E. carolleeae*, on the other hand, grazing on saturating levels of lower quality food may produce many eggs, but when

grazing on low concentrations of higher quality food they may produce fewer eggs. This contrast begs the question of how food quantity and quality interact to influence the reproductive success of *E. carolleeae*.

The second feature of reproductive success in these studies, egg viability, also varied with food quality. In both copepods, grazing on increasingly P-poor food led to reduced egg viability (Figure 12). This effect was visible on day 1 (Figure 12A), but was enhanced on day 7 (Figure 12B) for *A. tonsa*, and present on both days for *E. carolleeae* (Figure 12C,D). This fits the hypothesis that P-poor food should lead to less viable eggs. Ambler (1985) also found initially high egg viability when field-collected *A. tonsa* were fed *T. weissflogii* at saturating levels in a laboratory experiment. The viability in that study ranged from 81 $\pm$ 11 to 98 $\pm$ 2% on this diet and fell to 70% or lower when compared to field collected copepods that had been taken from a low quality food environment. Other studies have also measured declines in reproductive success when copepods are fed a poor quality diet (e.g., Jónasdóttir and Kiørboe, 1996; Kleppel et al., 1998; Nobili et al., 2013). The low P content in the prey may have prevented copepods from obtaining sufficient P to produce viable eggs.

Copepod egg viability is often related to egg content and composition of P-rich fatty acids (Kleppel et al. 1998; Ianora et al. 2003; Arendt et al. 2005; Koski et al. 2012), which can be related to the nutritional quality of the maternal diet (Frost et al. 2010, Kleppel 1993, Kleppel et al. 1998, Helland et al. 2003). Since *A. tonsa* eating a mono-algal diet of *T. pseudonana* grown at N:P 32, a P-poor food, showed very low egg viability relative to a diet of the diatom grown at N:P 16 (Fig. 12a,b), and *E. carolleeae* egg viability declined as prey cellular N:P increased, these results support the hypothesis that poor food quality, here food with very low P content, can have detrimental effects on copepod reproduction, and thus potentially on population dynamics

Comparing the egg production and viability of both copepods it appears that *A. tonsa* responded to P-poor prey by decreasing both egg production and viability relative to when that copepod ate P-sufficient food. Further, *A. tonsa* was shown to allocate P to eggs as P became available to the copepod. Under a P-poor diet *A. tonsa* produced fewer, less P-rich, less viable, eggs than when it ate a P-sufficient diet, yet the viability of these eggs was still greater than the viability of *E. carolleeae* eggs produced when that copepod ate food of the same P content. Under a P-poor diet *E. carolleeae*, in contrast to *A. tonsa*, produced more eggs, of equivalent P content, that had lower viability than eggs it produced when it ate a P-sufficient diet, yet the number of eggs produced was always greater than the number of eggs *A. tonsa* produced for a given diet. The data suggests that under a P-poor diet *A. tonsa* produced fewer, more P-rich and more viable eggs than *E. carolleeae* eating the same diet. *E. carolleeae*, on the other hand, produced a greater number of eggs having lower viability and equivalent P content than *A. tonsa* eating the same diet. *A. tonsa*, the broadcast spawner, had been hypothesized to respond to nutritionally poor prey via egg production, but not via egg nutrient content. The brood spawner, *E. carolleeae*, had been hypothesized to respond to nutritionally poor prey via viability and egg nutrient content where the mother was expected to maintain constant nutrient content in eggs which should have led to high viability. Instead, *A. tonsa* responded with reduced egg production and viability overall, but allocation of P to eggs that likely created greater egg viability in its eggs compared to those of *E. carolleeae* eating the same diet, while *E. carolleeae* responded with increased egg production and decreased viability.

It is not likely that these results can be explained as a function of egg development time since this experiment allowed eggs 48 h to hatch (develop) and the egg development time of *E. affinis* at 14-16°C was reported to center around 2 days (Sullivan and Kimmerer 2013), while

Ambler et al. (1985) reported that 30 h was sufficient to allow the eggs of *A. tonsa* to develop. Further, some studies suggest that eating a monoalgal diatom diet can lead to reduced egg viability and production (i.e. Ban et al. 1997, Ianora et al. 2003), although this is controversial (i.e. Dutz et al. 2008).

Conclusions and conceptual model

Results of these experiments suggest that both *A. tonsa* and *E. carolleeae* respond physiologically to food of varying quality when it is given at saturating concentrations. However, the pathways, and the relative importance of those pathways, that the copepods use to maintain their homeostasis for N and P vary (Figure 13). *A. tonsa* are broadcast spawners and are reproductively opportunistic, producing more eggs when environmental conditions provide nutrient-sufficient prey and fewer eggs in unfavorable conditions (Dagg 1977, Jónasdóttir 1994). *E. carolleeae*, however, a brood spawner, did not vary egg nutrient content as food nutrient content varies. Instead, *E. carolleeae* eggs had a similar nutrient input despite alterations in prey cellular P content or in response to somatic tissue P content.

Both copepods excreted N and P, but the magnitude and direction, and trend with prey P content, was different (Figure 13). P was released partially to excretion of PO_4^{3-} and partially to egg nutrient content in *A. tonsa* while in *E. carolleeae* excess P in prey was only released to excretion. As N in prey increased, *A. tonsa* responded by increasing NH_4^+ excretion, while *E. carolleeae* did not (Figure 13). It is possible that this excess N in food that is not excreted, and not found in copepod somatic tissue or eggs, is released through fecal pellets, which were not measured in this study.

These copepods thus appear to have different methods of regulating their homeostasis. Our first hypothesis, that copepods would maintain relatively constant somatic nutrient

stoichiometry, was demonstrated. We also hypothesized that excretion rates of P when eating a P-rich food, or N when eating and N-rich food, would be higher than when copepods ate foods that were more balanced. This has been demonstrated for PO_4^{3-} excretion in both copepods. Excretion of N, as its content in food increased is not as clear. However, in this experiment only P content was varied in the media of the prey, and thus responses would only be expected in P. Our third hypothesis stated that decreasing prey P content should decrease P content in eggs, egg production rates and viability. *E. carolleeae* egg nutrient content showed no trend with prey or copepod P content. *A. tonsa* egg nutrient content, on the other hand, increased as prey and copepod P content did. Egg production rates declined for *A. tonsa* eating P poor food, but not for *E. carolleeae*. Egg viability declined for both copepods when eating P poor food.

Implications

In this study we altered food quality by altering the N:P ratio of media diatom prey were grown on, which altered the N:P of the prey. Given the anthropogenic changes to N and P in eutrophic and eutrophying estuaries throughout the world (Howarth et al. 2000), copepods can be expected to be exposed to prey of varying food quality, and increasingly higher N:P (lower P) as N loads are increasing faster than those of P (Boesch 2002, Howarth and Marino 2006, Bricker 2008, Glibert et al. 2013, 2014). Although the environmental factors selecting for varying copepods and their population success are many, these results suggest that nutrient content of the prey may be important.

The recruitment of copepod populations depends on reproductive success, which this study has shown to be reduced by food of poor quality. The decline of copepod populations due to poor quality food may lead to the collapse of higher trophic levels that depend on these copepods as a food source (Laspoumaderes et al. 2010, Malzahn et al. 2010, Winder and Jassby

2011, Hessen et al. 2013). For example, in the San Francisco Bay Estuary, long-term trends in increasing N:P ratios have been correlated with declines over the same time period in the abundance of the copepods *A. clausii* and *E. affinis* (Glibert et al. 2011). This correlation may be a result of shifts away from diatoms as dominant phytoplankton, thought to be a major food source for these copepods, to phytoplankton that are of poorer food quality for these copepods (Glibert et al. 2011). This correlation may also be due to shifting nutritional quality of the phytoplankton even without changing dominants (Glibert et al. 2011). These experiments support the consideration of food quality in an explanation of declining abundances of *A. clausii* and *E. affinis* in the San Francisco Bay Estuary.

These experiments varied the P content of prey. How copepod physiology will respond under experimental conditions in which N content rather than P content varies is not clear. Further, these experiments were done using a single food type, a centric diatom, although in the field copepods likely eat a mixed diet. There is much not yet known about how other phytoplankton groups vary their N:P content in response to varying available nutrient. Finally, these food quality experiments were conducted at saturating C prey content. How effects of food quality would vary as food quantity also varies is also not clear and needs to be considered.

Tables

Table 1. Number of copepods per filter per treatment for particulate carbon and nitrogen (CHN) and particulate phosphorus (PP) analysis.

Species	media N:P ratio	Experimental Day	CHN analysis replicate 1	CHN analysis replicate 2	CHN analysis replicate 3	PP analysis replicate 1	PP analysis replicate 2	PP analysis replicate 3
Acartia tonsa	4	1	10	10	10	9	9	10
Acartia tonsa	16	1	14	13	12	12	12	13
Acartia tonsa	24	1	10	10	10	10	10	10
Acartia tonsa	32	1	13	11	13	11	13	12
Acartia tonsa	4	7	5	5		6	5	
Acartia tonsa	16	7	9	8	11	9	9	9
Acartia tonsa	24	7	6	8	12	6	7	11
Acartia tonsa	32	7	10	10	10	9	10	10
Eurytemora carolleeae	4	1	10	10	10	10	10	10
Eurytemora carolleeae	16	1	9	9	8	11	9	11
Eurytemora carolleeae	24	1	10	10	10	10	10	10
Eurytemora carolleeae	32	1	11	8	10	11	16	12
Eurytemora carolleeae	4	7	12	10	8	12	12	8
Eurytemora carolleeae	16	7	14	11	15	16	13	15
Eurytemora carolleeae	24	7	15	15	15	11	15	23
Eurytemora carolleeae	32	7	14	19	10	18	15	12

Table 2. Number of eggs per filter for particulate carbon and nitrogen (CHN) and particulate phosphorus (PP) analysis.

Species	media N:P ratio	Experimental day	CHN number of eggs	PP number of eggs
Acartia tonsa	4	1		92
Acartia tonsa	16	1	71	73
Acartia tonsa	24	1		140
Acartia tonsa	32	1	40	43
Acartia tonsa	16	7	52	73
Acartia tonsa	32	7	40	34
Eurytemora carolleeae	4	1	109	143
Eurytemora carolleeae	16	1	113	117
Eurytemora carolleeae	24	1	129	189
Eurytemora carolleeae	32	1	188	203
Eurytemora carolleeae	4	7	179	201
Eurytemora carolleeae	24	7	103	314

Table 3. Calculations of grazing and excretion.

To calculate	Symbol	Equation:	Variables:	Source
Growth constant for algal growth	k	$C_2=C_1e^{k(t_2-t_1)}$	C_1 and C_2 are cell concentrations (cells mL^{-1}) at time 1 (t_1) and time 2 (t_2) in the control bottle	Frost 1972
Grazing coefficient	g	$C_2*=C_1*e^{(k-g)(t_2-t_1)}$	C_1* and C_2* are cell concentrations in grazing bottles at times t_1 and t_2	Frost 1972
Average cell concentration	⟨C⟩	$\langle C\rangle=\frac{C_1*\left[e^{(k-g)(t_2-t_1)}-1\right]}{(t_2-t_1)(k-g)}$	defined above	Frost 1972
Volume swept clear (also called grazing rate)	F (mL $copepod^{-1}$ h^{-1})	F=Vg/N	V= volume (mL) in bottle N=number of copepods in bottle g defined above	Frost 1972
Ingestion rate	I (cells eaten $copepod^{-1}$ h^{-1})	$I=\langle C\rangle*F$	defined above	Frost 1972
Excretion rate	Excretion rate (ER)	$ER=\frac{(\Delta C_t-\Delta C_c)\times V}{T \times N}$	ΔC_t and ΔC_c are the change in concentration of the nutrient (NH_4^+ or $PO4_3^-$) in the treatment and control bottles, respectively V=volume (mL), T=length of incubation (h), N=number of grazers	Miller and Glibert 1998

Table 4. Diatom prey cellular N:P ratio in molar (M) and nutrient (C, N, P) content in micromolar (µM) and weight (µg nutrient L^{-1}) units. Values are means ± standard deviation (n=4 at N:P 16, 32, n=6 at N:P 4, 24).

media N:P (M)	prey N:P (M)	prey C (µM)	prey C (µg C L^{-1})	prey N (µM)	prey N (µg N L^{-1})	prey P (µM)	prey P (µg P L^{-1})
4	6.95 ± 2.07	393.20 ± 33.52	4722.74 ± 402.60	67.89 ± 6.32	950.96 ± 88.51	11.06 ± 5.18	342.53 ± 160.37
16	10.91 ± 8.82	512.40 ± 62.11	6154.45 ± 746.06	86.67 ± 10.29	1214.04 ± 144.12	7.68 ± 0.56	237.98 ± 17.36
24	10.51 ± 1.64	485.76 ± 31.95	5834.45 ± 383.72	84.41 ± 8.33	1182.26 ± 116.64	8.32 ± 2.24	257.71 ± 69.47
32	15.10 ± 2.54	545.48 ± 29.69	6551.74 ± 356.65	89.43 ± 5.38	1252.59 ± 75.32	6.01 ± 0.74	186.25 ± 23.04

Figures

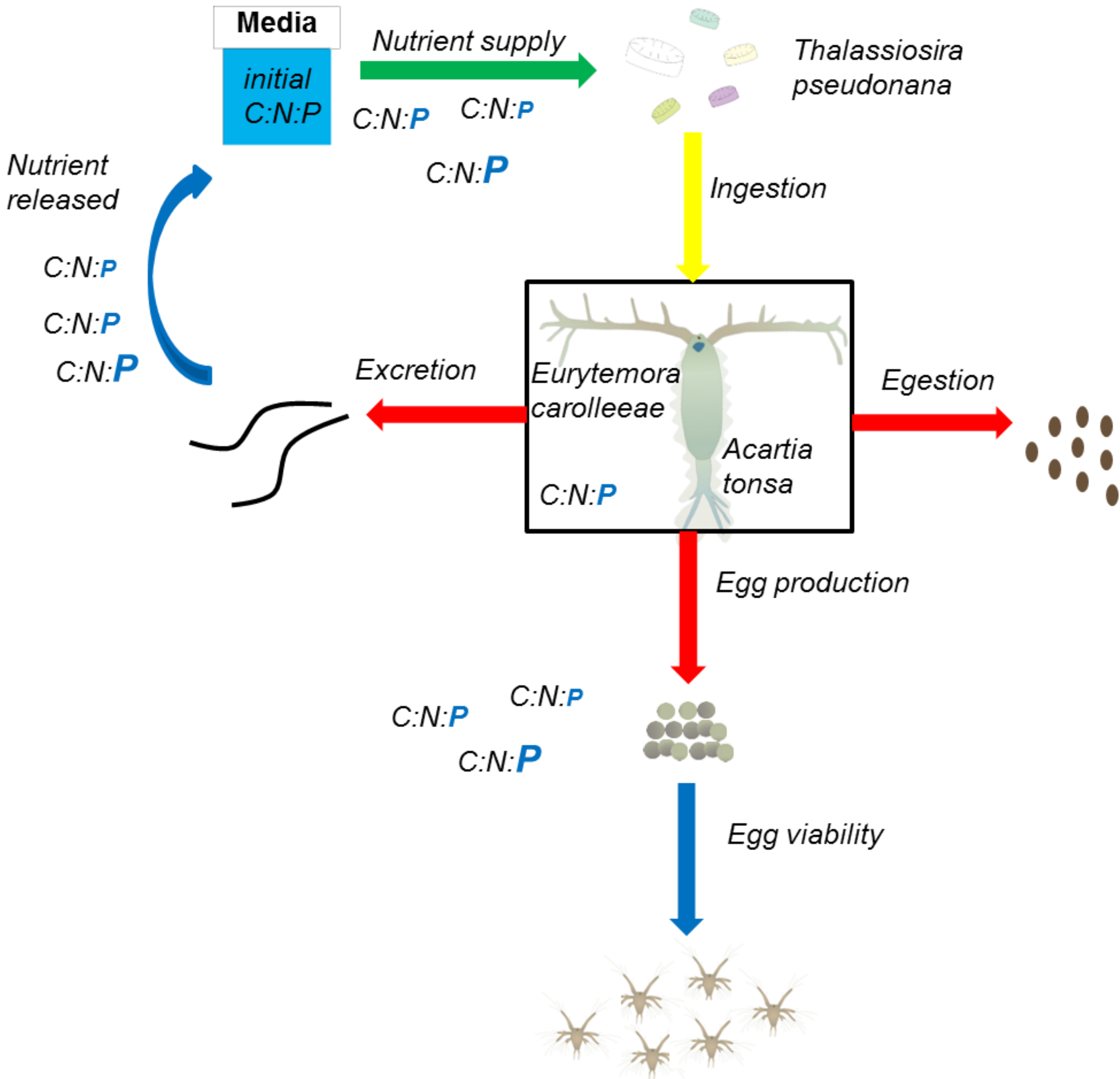

Figure 1. Conceptual model of copepod physiological mechanisms in response to food (prey) of varying N:P ratio. The only input is ingestion of prey (yellow arrow). Prey are the diatom *Thalassiosira pseudonana* and the copepod is either *Eurytemora carolleeae* or *Acartia tonsa*. Mechanisms (red arrows) are pathways of nutrient output and include excretion, egestion, and egg production. The stoichiometry of these released products can vary in response to the stoichiometry of the prey represented as C:N:P ratios with varying P content. This stoichiometry can have implications for egg viability and nutrient release to the environment (blue arrows). All pathways except egestion were included in this study.

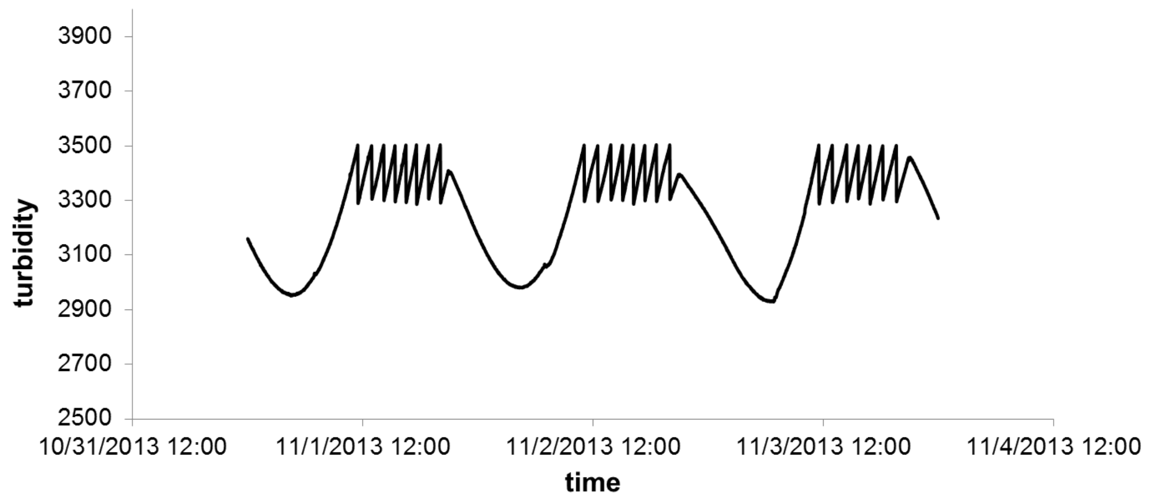

Figure 2. *Thalassiosira pseudonana* turbidostat culture turbidity readings recorded over three consecutive days showing the number of dilutions in the turbidostat each day, a proxy for growth rate. A dilution is marked by a spike in turbidity reaching the threshold of 3500 followed by a rapid decline in turbidity.

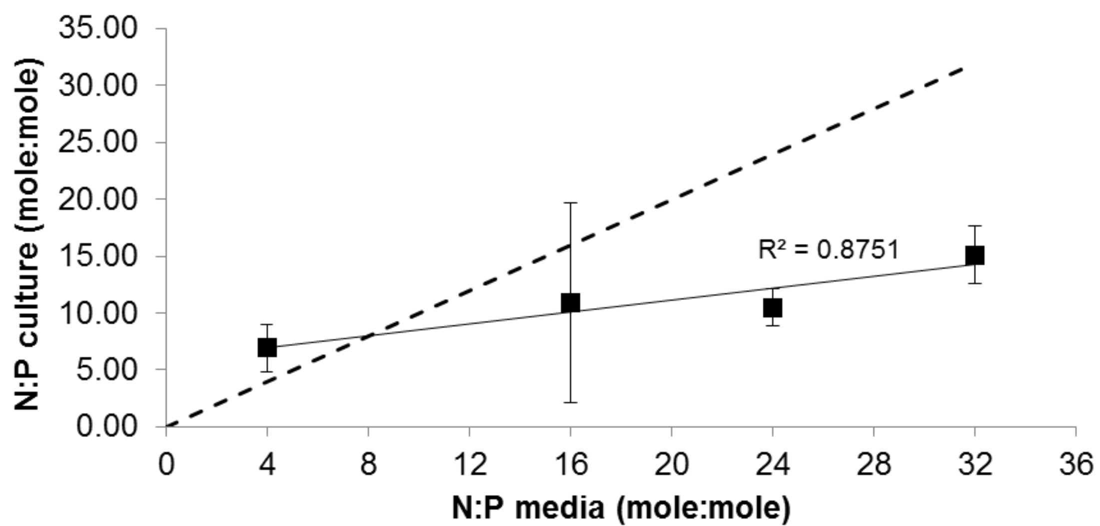

Figure 3. *Thalassiosira pseudonana* cellular N:P in turbidostat as a function of NO_3^-:PO_4^{3-} in media. Values are means $\pm$ standard deviation (n=4 at N:P 16, 32, n=6 at N:P 4, 24). Dashed line is a 1:1 line.

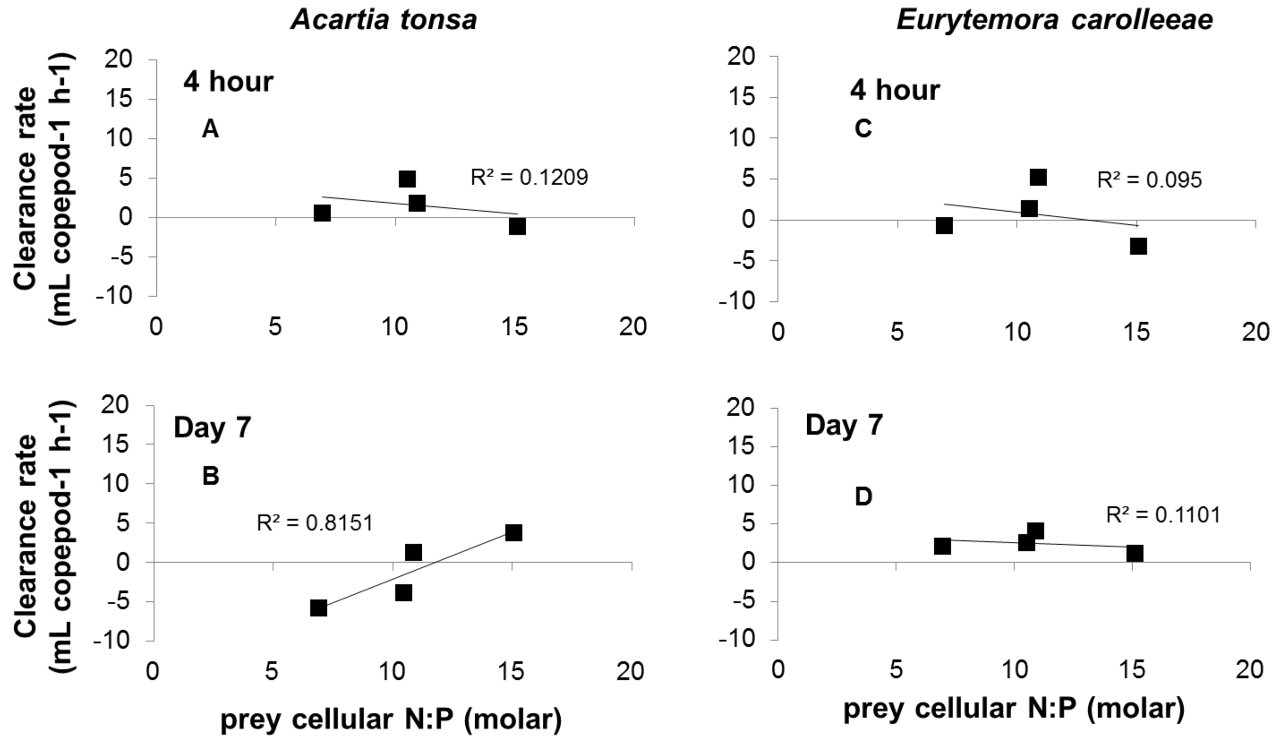

Figure 4. Clearance rates for *A. tonsa* and *E. carolleeae* on each experimental day across prey cellular N:P ratios. Clearance rates were calculated from the equation for volume swept clear (F) in Frost (1972) where k is held to 0 to eliminate effects of diatom prey growth on clearance rates.

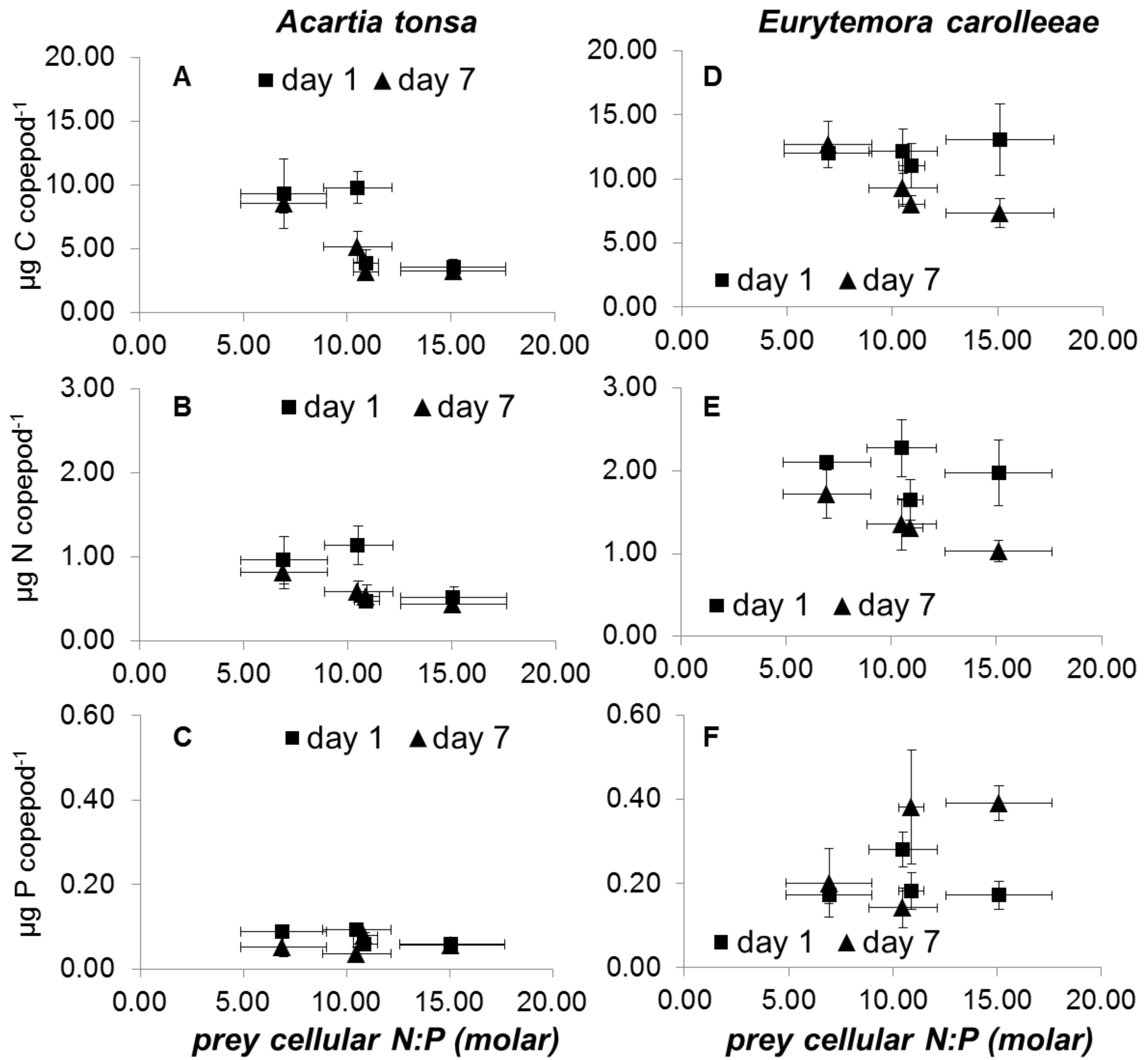

Figure 5. Copepod somatic nutrient (C, N, P) content as a function of prey cellular N:P after 4 hours (on day 1) and 7 days of exposure to food of experimentally set food quality. Values are means ± standard deviation (n=3).

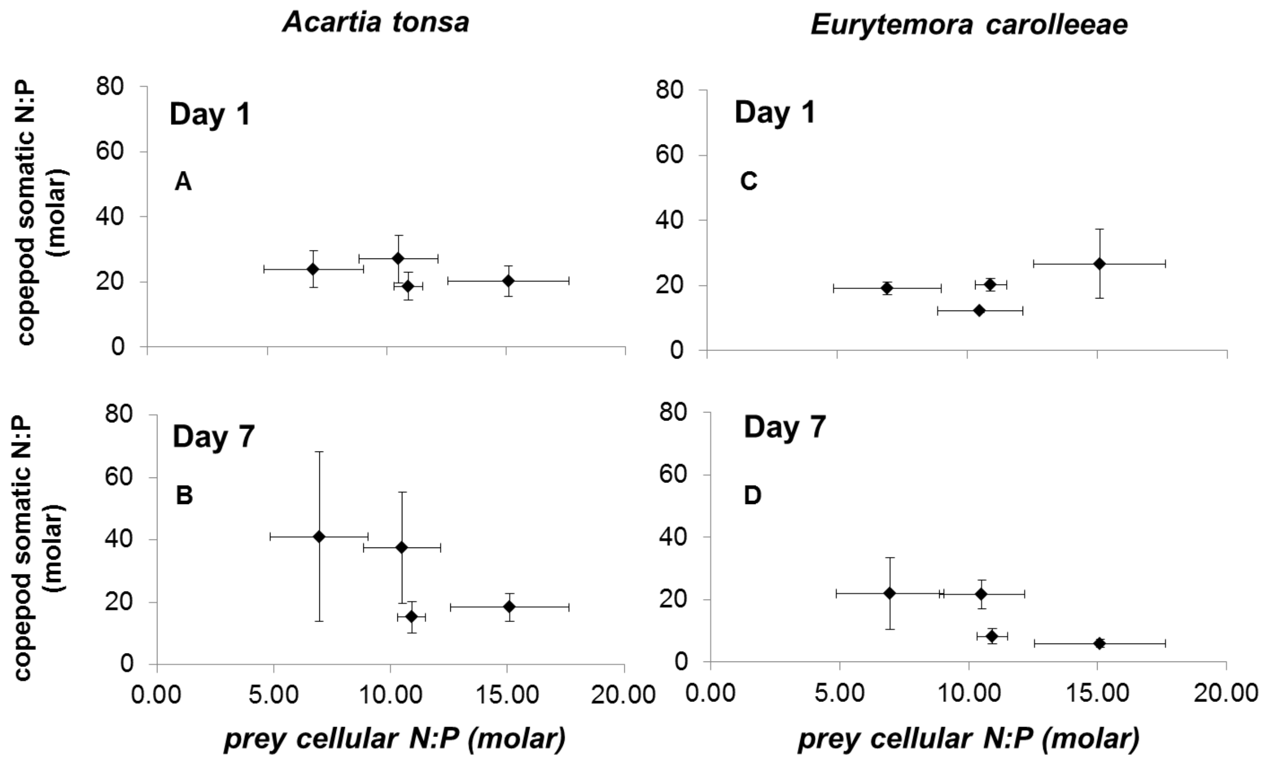

Figure 6. Copepod somatic tissue N:P as a function of prey cellular N:P. Values are means ± standard deviation (n=3).

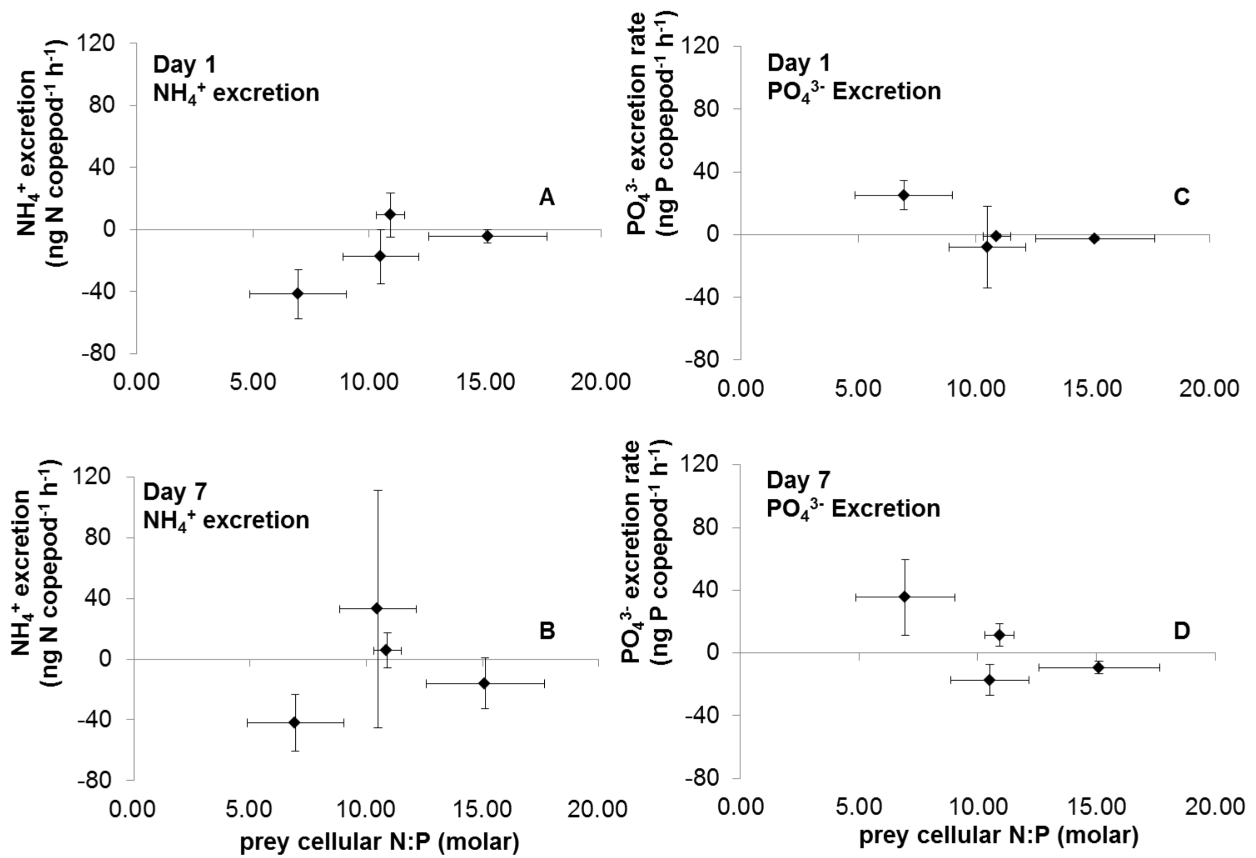

Figure 7. *Acartia tonsa* NH_4^+ and PO_4^{3-} excretion rates after 4 hours (on day 1) and 7 days of exposure to food of experimentally set N:P ratio. Values are means $\pm$ standard deviation (n=3).

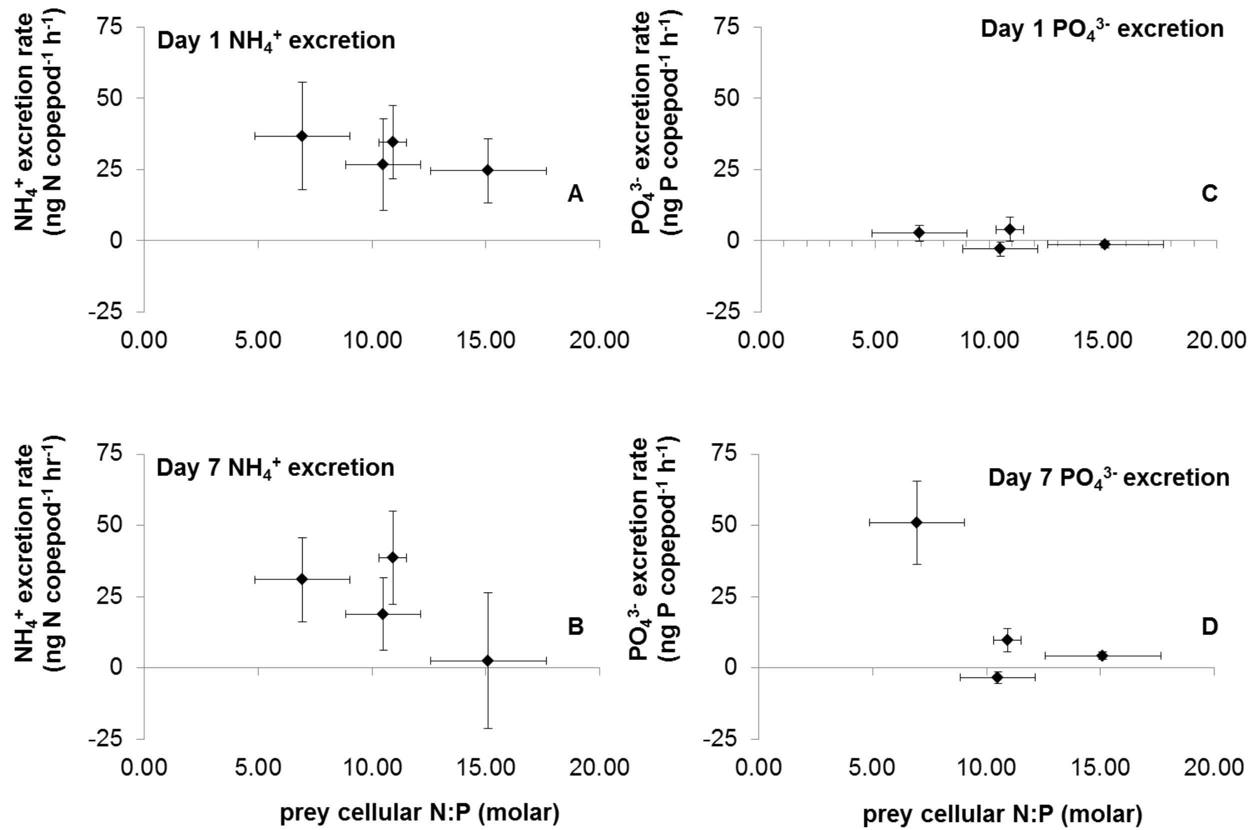

Figure 8. *Eurytemora carolleeae* NH_4^+ and PO_4^{3-} excretion rates after 4 hours (on day 1) and 7 days of exposure to food of experimentally set N:P ratio. All values are means ± standard deviation (n=3).

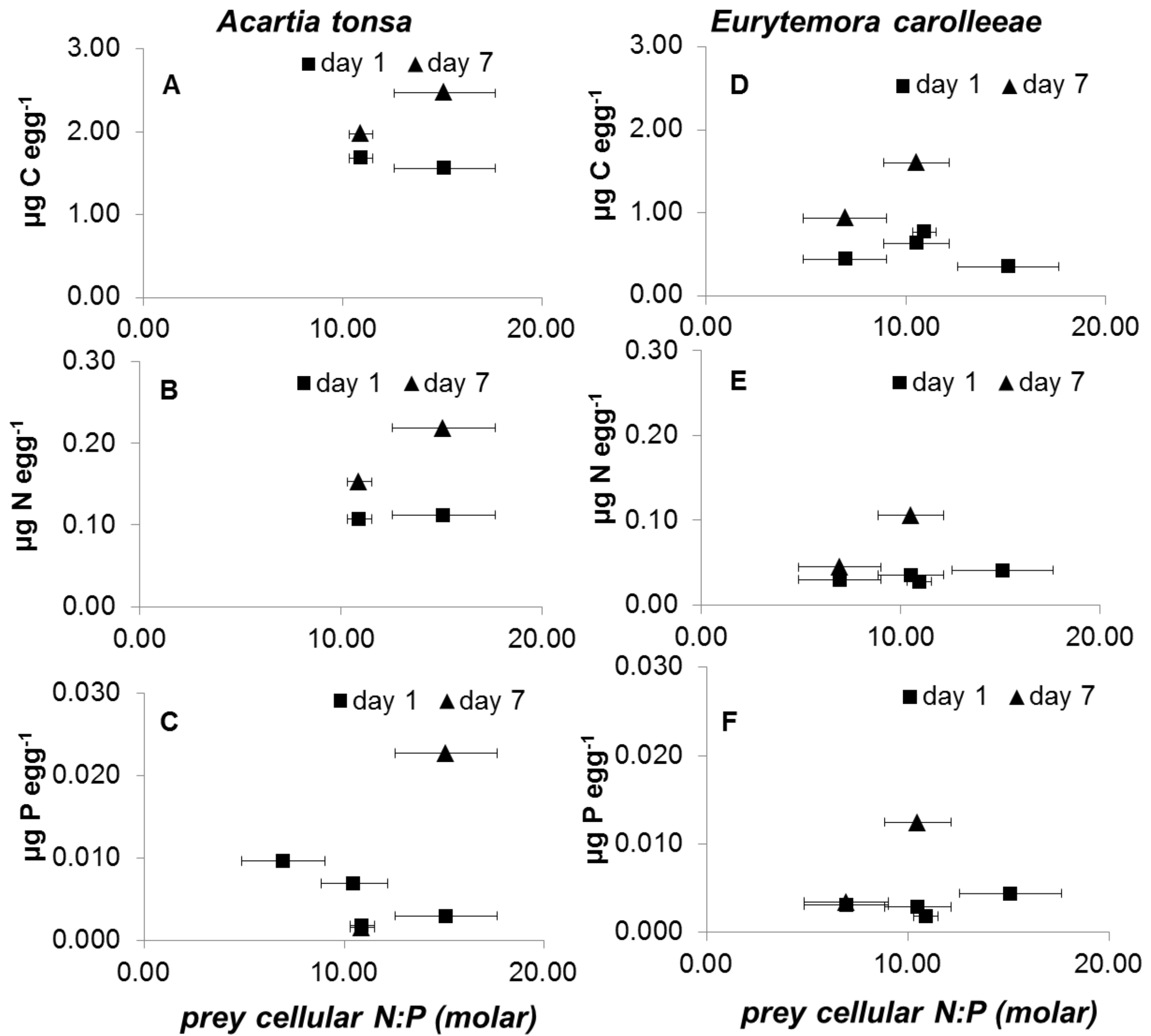

Figure 9. Egg nutrient (C, N, P) content as a function of prey cellular N:P after 4 hours (on day 1) and 7 days of exposure to food of experimentally set food quality. Values of egg nutrient content are multiple eggs combined into one sample (see Table 2). Values of prey cellular N:P are means ± standard deviation (n=4).

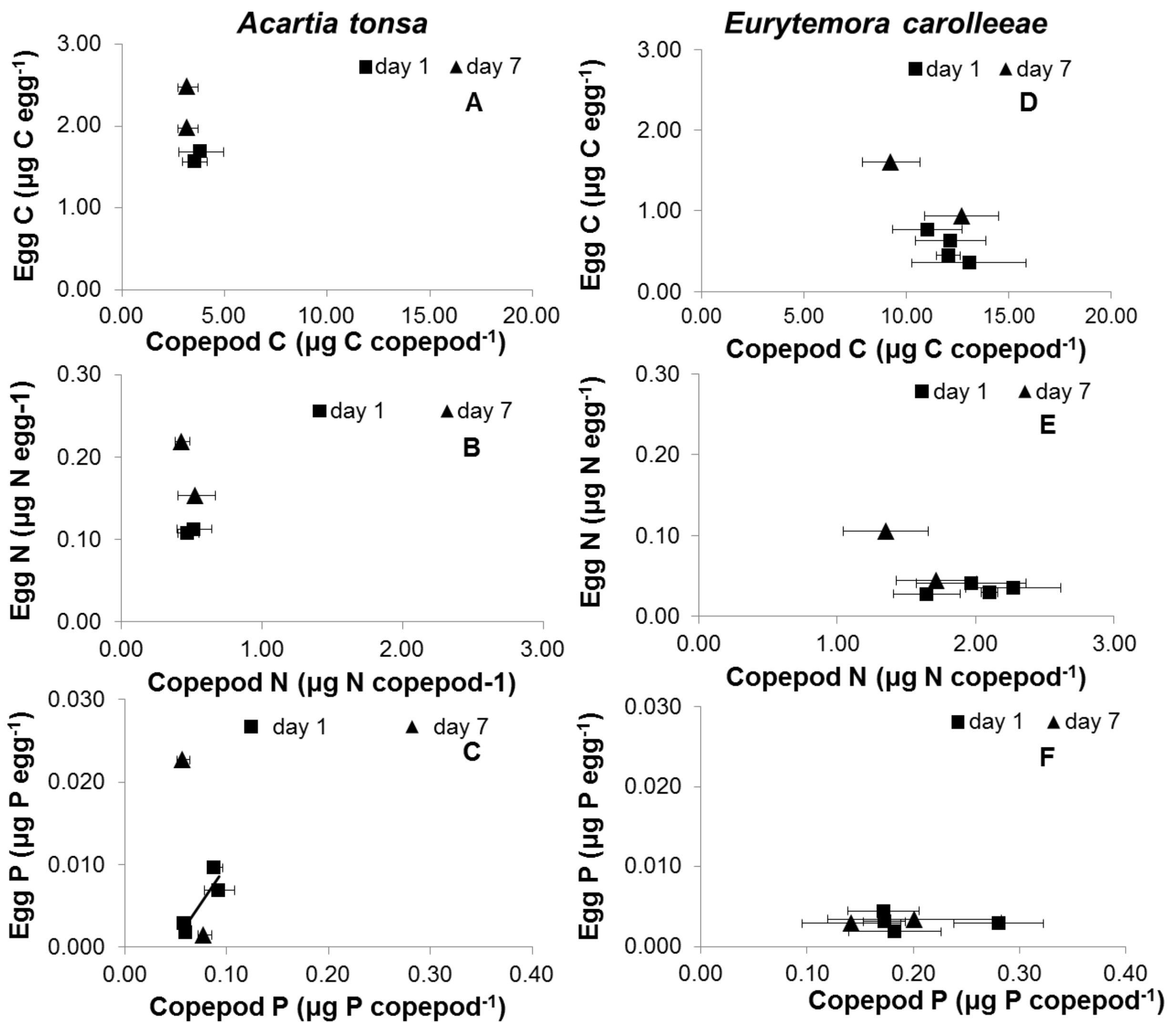

Figure 10. Egg nutrient (C, N, P) content as a function of copepod nutrient content after 4 hours (on day 1) and 7 days of exposure to food of experimentally set food quality. Values of egg nutrient content are multiple eggs combined into one sample (see Table 2). Values of prey cellular N:P are means ± standard deviation (n=4).

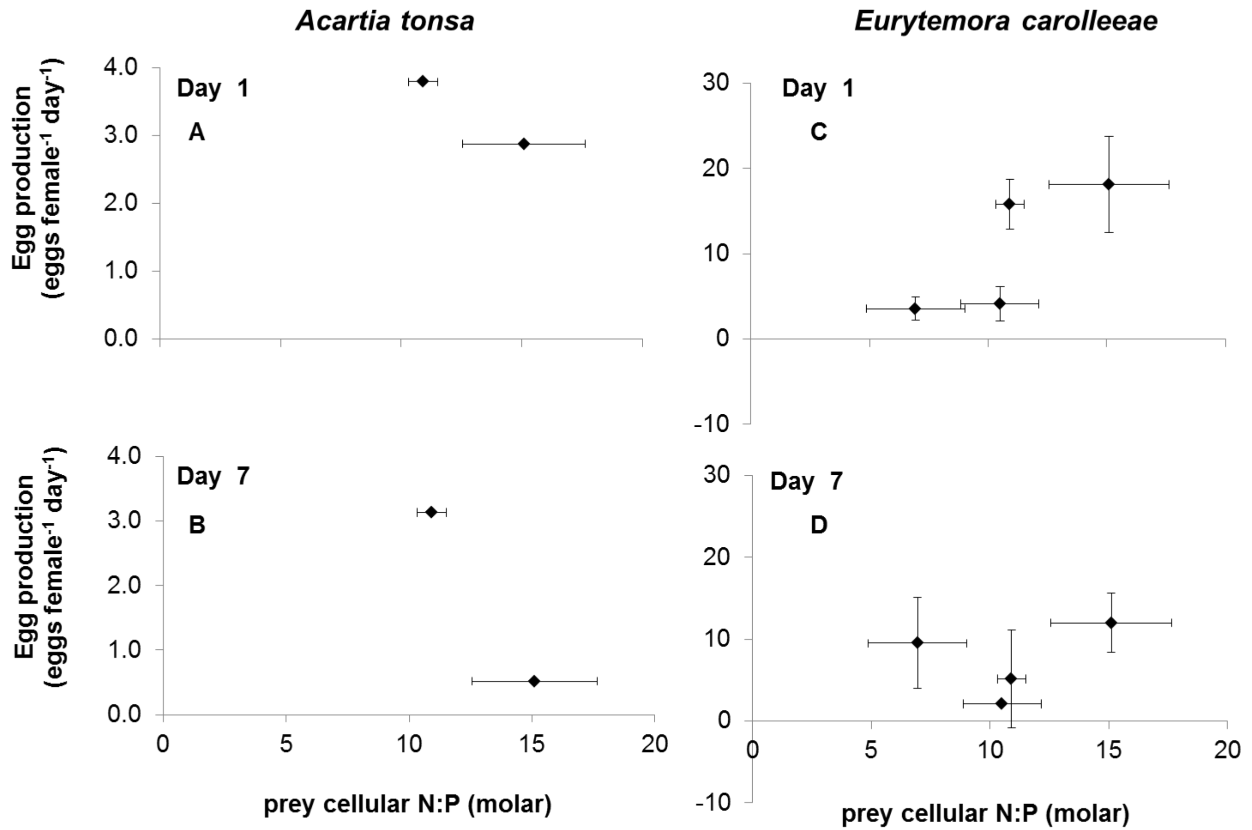

Figure 11. Egg production after 4 hours (on day 1) and day 7 for *Acartia tonsa* and *Eurytemora carolleeae*. Values are means ± standard deviation (n=2 for *Eurytemora carolleeae* n=1 for *Acartia tonsa.*).

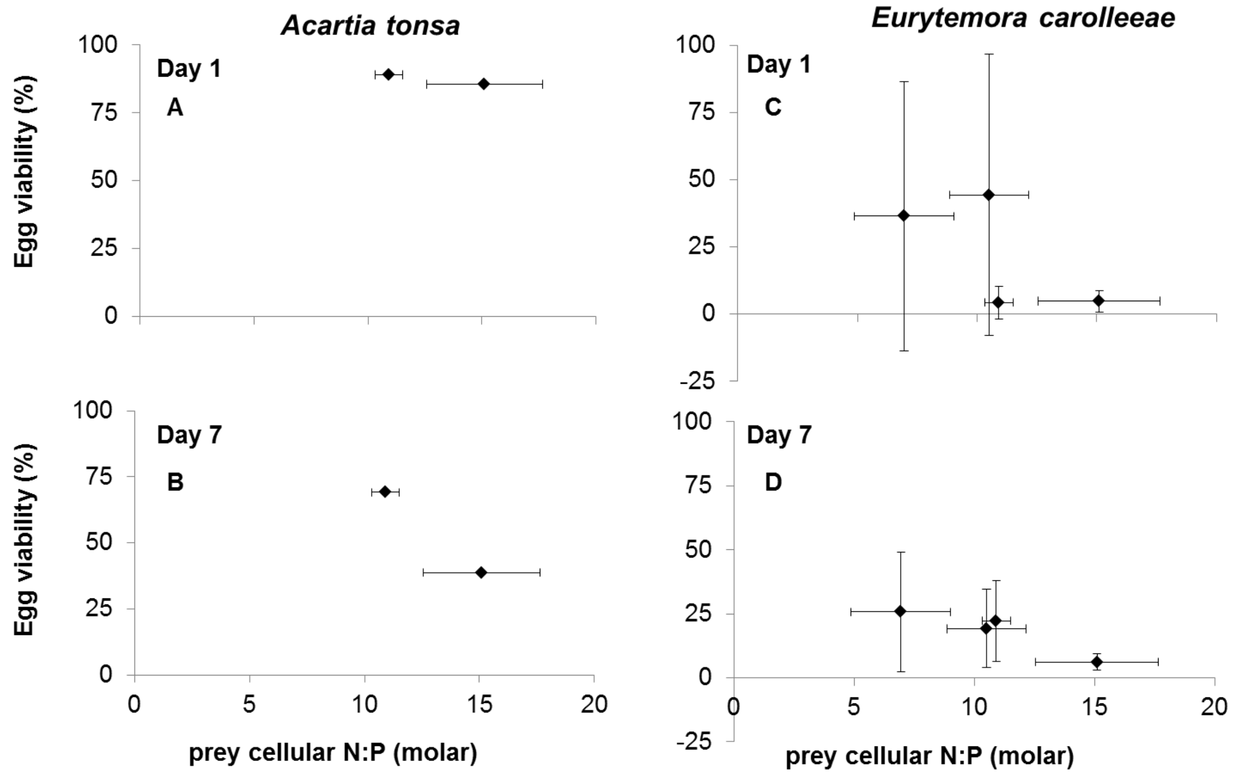

Figure 12. Egg viability after 4 hours (on day 1) and day 7 for *Acartia tonsa* and *Eurytemora carolleeae*. Values are means ± standard deviation (n=2 for *Eurytemora carolleeae*, n=1 for *Acartia tonsa*.)

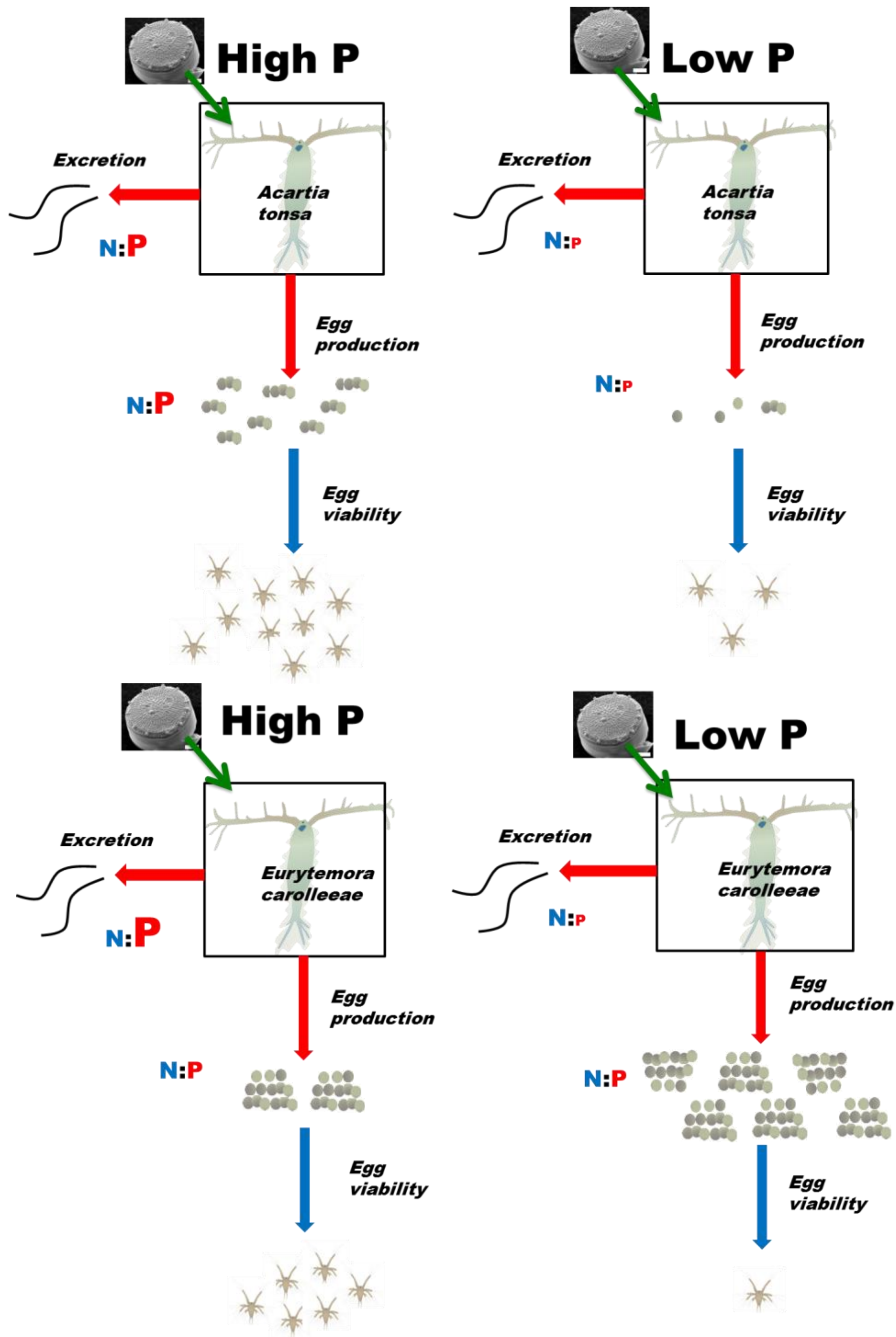

Figure 13. Conceptual diagram of *A. tonsa* and *E. carolleeae* response to eating prey of high and low P content. When eating a high P prey *A. tonsa* excreted P at a greater rate, and produced more eggs with higher P content and greater viability compared to when it ate a low P prey. When eating a high P prey *E. carolleeae* excreted P at a greater rate, and produced fewer eggs with similar P content and greater viability compared to when it ate a low P prey. *A. tonsa* egg viability was always greater than *E. carolleeae* egg viability, while *E. carolleeae* egg production rates were always greater than *A. tonsa* egg production rates when the copepods ate the same prey.

Appendix A

Grazing calculations

Grazing calculations were variable and did not show a consistent trend across prey cellular N:P ratios or within copepods (Table A1). Most of the variation was due to variation in the algal growth constant between experiments and across prey cellular N:P ratios (Table A2, Figure A1). For example, clearance rates (Figure A2), calculated from the equation for volume swept clear (F) in Frost (1972), more strongly reflect algal growth rates (Figure A1), than clearance rates that have been corrected for algal growth by holding the constant for algal growth (k) equal to zero (Figure A3). It is reasonable to correct the clearance rates for algal growth by holding k equal to 0 because the diatom prey was grown in turbidostat where it was maintained at a constant growth rate and in the same physiological state across all N:P ratio experiments before the prey was added to grazing incubations. While algal growth did vary during 4 hour grazing incubations (Table A1, Figure A1), eliminating its effect on grazing calculations more clearly demonstrates the clearance rates of both copepods on both experimental days across prey cellular N:P ratios (compare Figures A1, A2, and A3).

Negative clearance rates where k is calculated according to Frost (1972) in these experiments can be explained by 3 factors. Negative clearance rates where k is set to zero can be explained by the first two factors. First, in grazing incubations copepods were provided with twice the amount of food (in terms of C) that they would need to saturate their daily C demand (600 μg C L^{-1}, see discussion in methods) with the goal of maintaining prey quantity in excess and at constant saturating levels over the entire 4 h incubation period. Since grazing rate calculations are based on changes in the number of cells in treatment and control bottles over the grazing incubation, a removal of cells due to grazing from a large proportion of prey will be less

detectable than a removal of cells due to grazing from a smaller proportion of prey. This could have led to an underestimation of grazing contributing to negative grazing calculations.

Second, the grazing equations of Frost (1972) rely on a calculation of algal growth constant (k) based on changes in diatom prey cells over the grazing incubation in the control bottle, which contained prey but no copepod grazers. This leads to an underestimation of algal growth in the treatment bottles, which contained both prey and copepod grazers. This depressed k value propagates through the other grazing calculations causing, for example, negative calculations of grazing coefficients (g), clearance or grazing rates (called volume swept clear, F), which are based on the grazing coefficient, and ingestion rates (I), which are based on the algal growth constant, the grazing coefficient, and the volume swept clear (see Table 3). Each calculation depends on the one that precedes it. The grazing coefficient calculation is calculated as the change in the number of cells over a grazing incubation in a treatment bottle subtracted from k .When k is depressed, or negative, a negative g can result, which may lead to the calculation of a negative F, and a calculation of a negative I.

Third, this assumption that k calculated for a control bottle with no grazers is equivalent to k in a treatment bottle with grazers is incorrect because grazers release nutrient that may then be taken up by prey and used for growth. In these experiments algal growth in response to copepod nutrient release is further complicated by the interaction of copepod nutrient remineralization with prey cellular N:P and the interaction of diatom prey nutrient uptake with copepod nutrient remineralization based on prey nutrient sufficiency or deficiency. In these experiments the P content of prey varied but N content was held constant. Copepods released more P when eating prey of low cellular N:P (P-rich) than when eating prey of high cellular N:P (P-poor). Copepods released some N at all prey cellular N:P ratios. Diatom prey that were of low

cellular N:P (P-rich) were sufficient for P but took up N to balance their cellular stoichiometry, and could then use this nutrient to grow. Diatom prey that were of high cellular N:P (P-poor) were insufficient for P, and copepods eating these prey did not release P at high rates, though any P that was released would have been taken up rapidly by the P insufficient diatom prey.

While negative grazing calculations are not ideal, in these experiments the comparison between copepods, and changes in grazing across prey cellular N:P ratios are more important than the values of grazing calculations. In this experiment the negative excretion rates could not be eliminated by using either a smaller bottle with the same number of grazers due to the large volume required for dissolved and particulate nutrient samples, or by including more grazers in the grazing bottle because this would have led to bottle effects, where measurements such as of grazing, excretion, or algal growth may have been responses to being confined in a bottle rather than responses to the experimental manipulation.

Appendix table A1. Grazing calculations for this data based on Frost (1972) where k is growth constant for algal growth, g is grazing coefficient, F is volume swept clear, and I is ingestion rate.

			Acartia tonsa				*Eurytemora carolleeae*			
Day	N:P media	N:P prey	k (hr^{-1})	g (hr^{-1})	F (mL $copepod^{-1} hr^{-1}$)	I (cells $copepod^{-1} hr^{-1}$)	k (hr^{-1})	g (hr^{-1})	F (mL $copepod^{-1} hr^{-1}$)	I (cells $copepod^{-1} hr^{-1}$)
1	4	6.95 ± 2.07	0.09	0.12	2.33	32976.57	-0.26	-0.30	-5.95	-159590.66
1	16	10.91 ± 0.6	-0.21	-0.08	-1.19	-18689.09	-0.32	-0.07	-1.51	-21470.16
1	24	10.51 ± 1.64	-0.04	0.20	4.08	87936.91	-0.11	-0.04	-0.87	-31400.12
1	32	15.1 ± 2.54	0.03	-0.05	-0.75	-13855.47	0.16	-0.01	-0.18	-2896.21
7	4	6.95 ± 2.07	-0.04	-0.14	-7.99	-86261.40	0.22	0.33	6.31	63323.09
7	16	10.91 ± 0.6	-0.35	-0.28	-5.29	-139001.15	-0.62	-0.35	-5.20	-47319.73
7	24	10.51 ± 1.64	-0.07	-0.18	-6.34	-247909.50	0.07	0.24	3.81	101009.16
7	32	15.1 ± 2.54	0.00	0.22	3.78	53328.61	-0.26	-0.18	-2.68	-88475.68

Appendix table A2. Grazing calculations for this data based on Frost (1972) when k=0. Where k is growth constant for algal growth, g is grazing coefficient, F is volume swept clear, and I is ingestion rate.

			Acartia tonsa				*Eurytemora carolleeae*			
Day	N:P media	N:P prey	k (hr^{-1})	g (hr^{-1})	F (mL $copepod^{-1}$ hr^{-1})	I (cells $copepod^{-1}$ hr^{-1})	k (hr^{-1})	g (hr^{-1})	F (mL $copepod^{-1}$ hr^{-1})	I (cells $copepod^{-1}$ hr^{-1})
1	4	6.95 ± 2.07	0.00	0.03	0.59	8333.33	0.00	-0.03	-0.70	-18750.00
1	16	10.91 ± 0.6	0.00	0.13	1.86	29176.90	0.00	0.25	5.20	74127.91
1	24	10.51 ± 1.64	0.00	0.24	4.88	105208.33	0.00	0.07	1.39	50000.00
1	32	15.1 ± 2.54	0.00	-0.08	-1.15	-21311.48	0.00	-0.17	-3.16	-50781.25
7	4	6.95 ± 2.07	0.00	-0.10	-5.79	-62500.00	0.00	0.11	2.16	21710.53
7	16	10.91 ± 0.6	0.00	0.07	1.27	33517.35	0.00	0.27	4.12	37500.00
7	24	10.51 ± 1.64	0.00	-0.11	-3.88	-151571.86	0.00	0.16	2.63	69690.27
7	32	15.1 ± 2.54	0.00	0.22	3.78	53328.61	0.00	0.08	1.24	40966.39

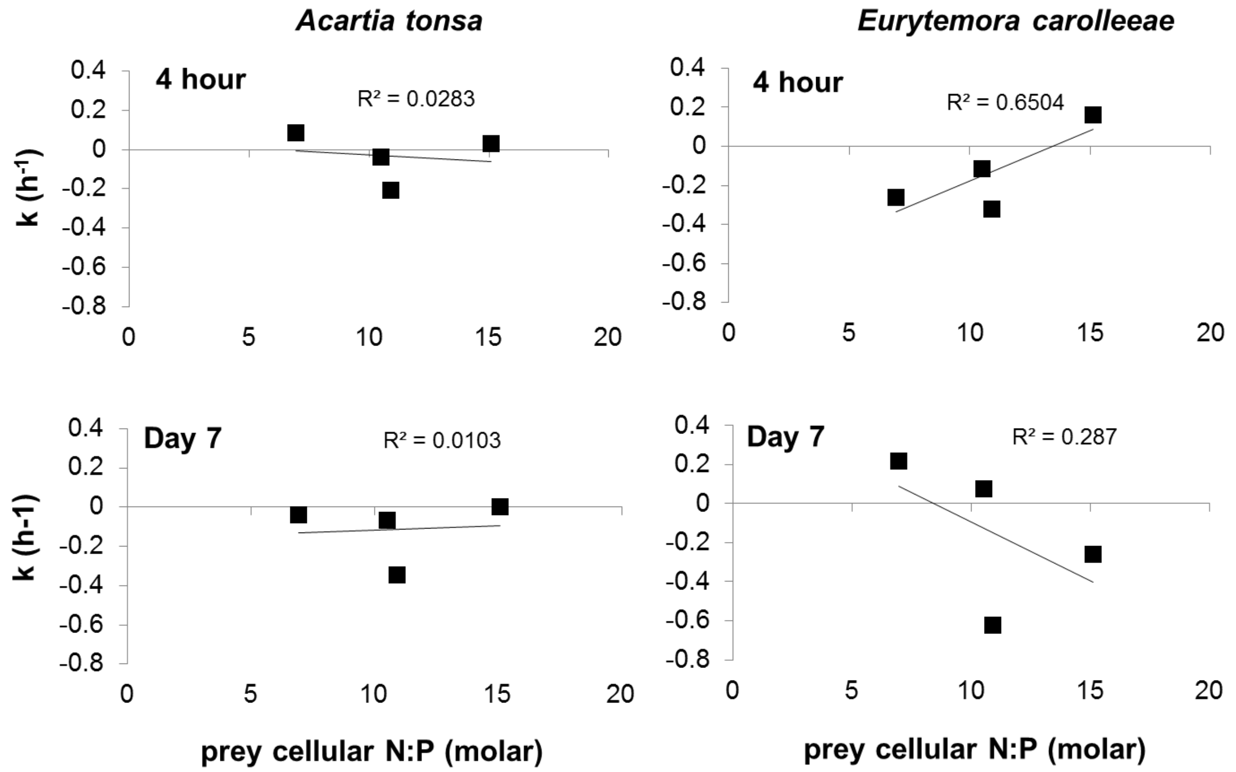

Figure A1. Growth constant for algal growth (k) calculated for data in these experiments and based on the equation in Frost (1972).

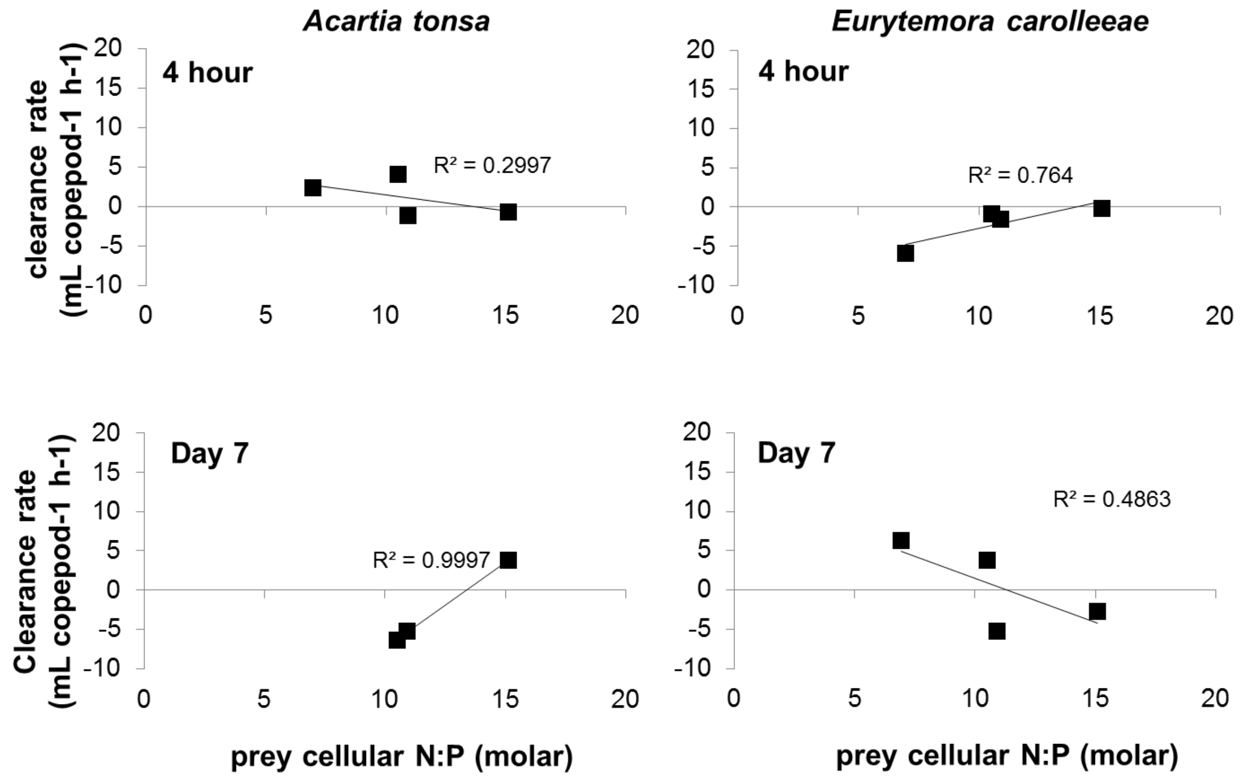

Figure A2. Clearance rate calculated for data in these experiments based on the equation for volume swept clear (F) in Frost (1972).

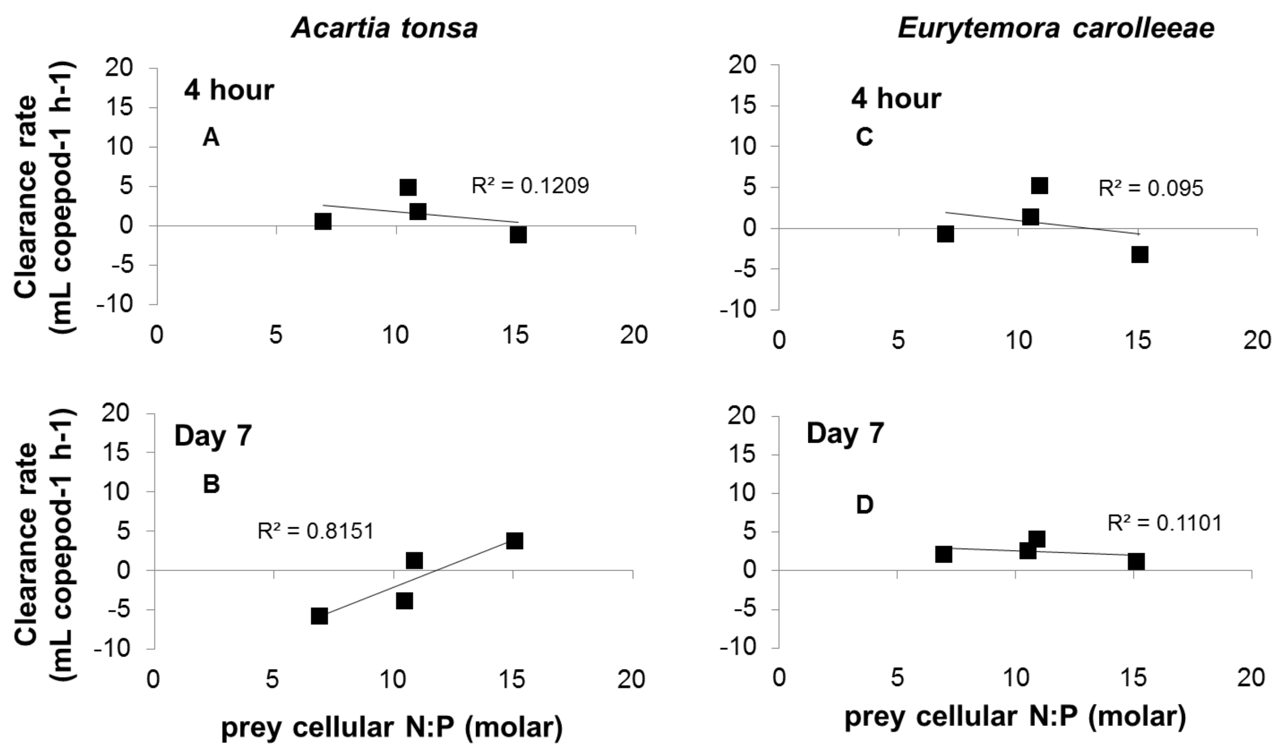

Figure A3. Clearance rates calculated for *A. tonsa* and *E. carolleeae* on each experimental day across prey cellular N:P ratios. Rates are calculated from the equation for volume swept clear (F) in Frost (1972) where k is held to 0 to eliminate effects of diatom prey growth on clearance rates. Same as figure 4 in text and provided here for comparison.

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