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Physiological Responses of the Copepods *Acartia tonsa* and *Eurytemora carolleeae* to Changes in the Nitrogen:Phosphorus Quality of Their Food

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Abstract: Two contrasting estuarine copepods, *Acartia tonsa* and *Eurytemora carolleeae*, the former a broadcast spawner and the latter a brood spawner, were fed a constant carbon-based diatom diet, but which had a variable N:P content, and the elemental composition (C, N, P) of tissue and eggs, as well as changes in the rates of grazing, excretion, egg production and viability were measured. To achieve the varied diet, the diatom *Thalassiosira pseudonana* was grown in continuous culture at a constant growth rate with varying P supply. Both copepods altered their chemical composition in response to the varied prey, but to different degrees. Grazing (clearance) rates increased for *A. tonsa* but not for *E. carolleeae* as prey N:P increased. Variable NH_4^+ excretion rates were observed between copepod species, while excretion of PO_4^{3-} declined as prey N:P increased. Egg production by *E. carolleeae* was highest when eating high N:P prey, while that of *A. tonsa* showed the opposite pattern. Egg viability by *A. tonsa* was always greater than that of *E. carolleeae*. These results suggest that anthropogenically changing nutrient loads may affect the nutritional quality of food for copepods, in turn affecting their elemental stoichiometry and their reproductive success, having implications for food webs.

Keywords: nitrogen; phosphorus; *Acartia tonsa*; *Eurytemora carrolleeae*; fecundity; food quality; ecological stoichiometry

1. 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 (typically measured as carbon, C), with increasing rates related to increased food supply [1–3]. Food quality, the nutritional value of food to a consumer to meet its physiological needs, may also constrain copepod physiological processes [4–7]. Studies on food quality have mainly emphasized nutrient-rich biomolecules in phytoplankton cells such as phosphorus (P)-rich lipids [8,9], and nitrogen (N)-rich proteins [10], or amino acids [8,11]. Effects of food quality as measured by N content have been reported for a few copepods [5,7,12]. There have been comparatively few studies of the effect of variable N:P ratios of prey on estuarine copepods [7], but such studies have recently been undertaken on heterotrophic dinoflagellates, ciliates and rotifers [13–16]. This study was undertaken to determine the effects of altered N:P, as a measure of nutritional quality, on the physiology of two estuarine copepods, *Acartia tonsa* and *Eurytemora carolleeae*.

Over the past several decades, rising inputs of N to estuaries and coastal waters throughout the world have outpaced inputs of P, largely as a result of single-nutrient management strategies that have focused on P reductions while N inputs continue to rise [17,18]. Cultural eutrophication in estuaries and coastal waters, mainly caused by the development of synthetic N fertilizers and their application to land, discharge of human waste, animal and aquaculture production, and the increased combustion of fossil fuels, are largely responsible for these increases [19–24]. This unequal nutrient loading underscores

the relevance of studies of nutrient imbalances on the physiology and growth of not only primary producers [25], but their consumers as well. Nutritional imbalances are generally accompanied by changes in growth, metabolism or reproduction [1,26].

Phytoplankton cellular nutrient stoichiometry (as C, N, or P content) can vary significantly with nutrient concentration and ratio, and is generally thought to reflect ambient nutrient conditions, at least in directionality of stoichiometry, if not in absolute magnitude [27–30]. Consumers, in contrast to phytoplankton, are generally thought to be more homeostatic, with smaller changes in organismal elemental stoichiometry compared to primary producers [29,31,32]. Ecological stoichiometry suggests that consumers can maintain biomass homeostasis through processes such as excretion, egestion, and the allocation of nutrient into eggs to selectively retain or release nutrients [31,33,34]. How consumers vary their physiology in response to food quality has implications for nutrient release to the environment and consumer reproductive success.

Numerous studies of stoichiometric regulation of zooplankton in freshwater have been undertaken, leading to considerable insight into the efficiency of material and energy transfer as it related to stoichiometric imbalances [35–38]. Due to the typical role of P as a limiting nutrient in freshwater, most of these studies have focused on imbalances in C:P in prey. Fewer such studies have been undertaken with marine taxa, and few still consider imbalances in N:P. With rising N:P ratios in many coastal and estuarine systems, questions related to how consumers regulate stoichiometric imbalances in these elements have important food web implications in these systems undergoing nutrient change.

In this study, the small centric diatom *Thalassiosira pseudonana* was grown in continuous culture (turbidostat) at a near-constant growth rate across a gradient of dissolved N:P ratios, and was provided as prey of varying food quality for grazing and egg viability experiments with the calanoid copepod consumers *A. tonsa* and *E. carolleeae* (Figure 1). Previous experiments with *T. pseudonana* have shown that their cellular N:P content varies in relation to available nutrient supply [39]. Furthermore, diatoms have been considered an important food source thought to largely support the traditional pelagic food web [40,41], and even copepods from the Sargasso Sea have been shown to consume diatoms more than any other phytoplankton taxa even when their abundance is comparatively low [42]. The copepods *A. tonsa* and *E. carolleeae*, a sibling species to the cosmopolitan *Eurytemora affinis* [43], were selected because both are important estuarine copepods with widespread distribution [44–46]. These copepods also have different life history strategies: *A. tonsa* is a broadcast spawner while *E. carolleeae* is a brood spawner [47]. Moreover, while *A. tonsa* is considered an opportunistic producer, increasing and decreasing egg production rates in more and less favorable environments, respectively [4,8,48]; *Eurytemora* spp. is thought to be less responsive to environmental change in its egg production rates [46,47].

This study was designed to determine how copepod tissue nutrient stoichiometry, excretion rates, egg stoichiometry, egg production rates, and egg viability respond to food of varying quality—but not quantity—after short-term exposures (4 h on day 1) and after longer-term exposures (7 days). To do this, only one variable, the availability of P, was varied in the growth media, yielding prey of variable N:P content and provided to the zooplankton at a constant level of C. This study thus aimed to test the following hypotheses: (1) copepod stoichiometry and nutrient content should not vary significantly as food N:P stoichiometry varies, (2) excretion rates of P and N should vary in relation to the N:P in the food, (3) egg production rates, egg nutrient content, and egg viability should decline as P content in food decreases, and (4) *A. tonsa*, a broadcast spawner, 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. These results suggest that changing environmental nutrient ratios may affect the nutritional quality of food for copepods, thereby affecting their tissue elemental stoichiometry, excretion, and reproductive success which, in turn, have implications for nutrient release to the environment and food webs in systems undergoing nutrient change.
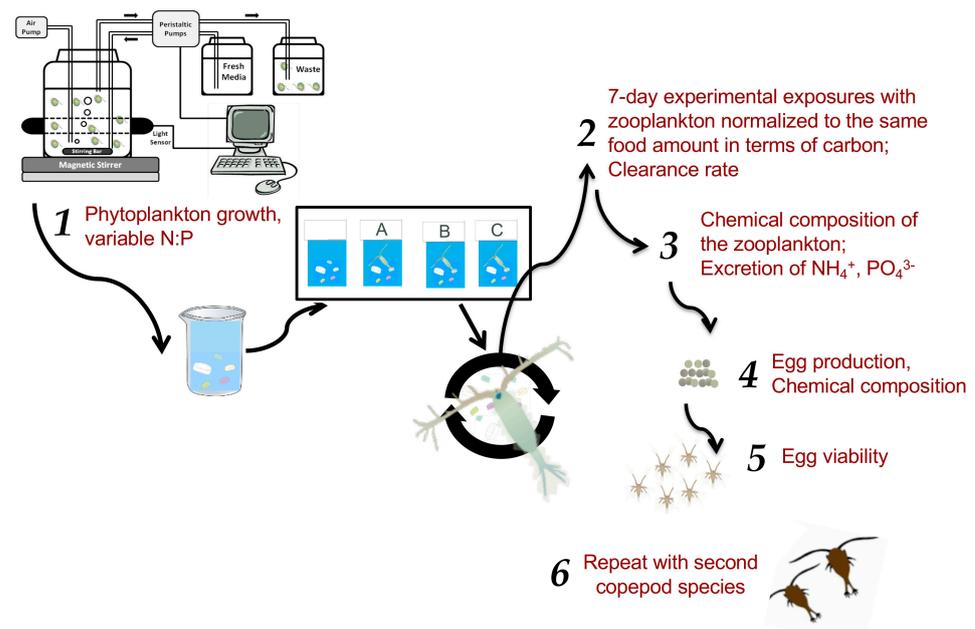
Figure 1. Schematic of the experimental design. Phytoplankton were grown in turbidostat continuous culture, with variable P to achieve differing prey stoichiometry. They were fed to copepods, measured after 4 h (day 1) and again after 7 days. Chemical composition, excretion rates, egg production and egg stoichiometry and egg viability were assessed. Each copepod was tested at 4 prey N:P conditions (but at constant C level), with duplicate treatments at each condition.

2. Results

2.1. Algal Growth and Cell Composition

The diatoms, which were grown in turbidostat continuous cultures, had, by design, relatively constant growth rates for all media N:P conditions. This was evaluated by a proxy for growth rate, the daily average number of media dilutions of the turbidostat chambers (not shown). The cellular molar N:P content of *T. pseudonana* varied from 7.0 ± 2.1 to 15.1 ± 2.5, as media NO_3^-: PO_4^{3-} ranged from 4 to 32 (Figure 2, Table 1; $R^2 = 0.81$, $p < 0.001$). Thus, even though the media supplied ranged from an N:P that was one-fourth to twice Redfield proportions (16:1), the resulting diatom stoichiometry was consistently less than Redfield proportions (except when media N:P was <4).

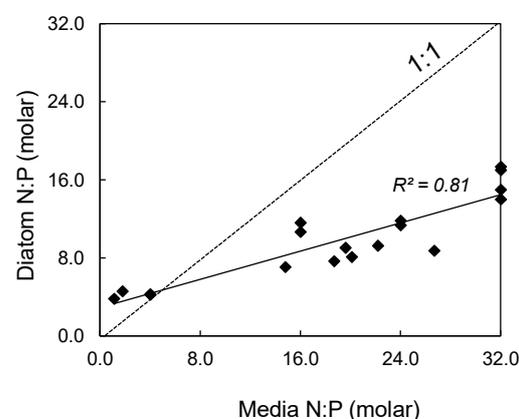
Figure 2. *Thalassiosira pseudonana* cellular N:P as a function of dissolved N:P as $NO_3^-:PO_4^{3-}$ in the growth media of the turbidostat cultures. Dashed line is a 1:1 line. The coefficient of determination was significant at $p < 0.01$.

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

Media N:P (molar)	Diatom N:P (molar)	Diatom C (μM)	Diatom C (μg C L^{-1})	Diatom N (μM)	Diatom N (μg N L^{-1})	Diatom P (μM)	Diatom P (μg P L^{-1})
4	7.0 ± 2.1	393 ± 34	4722 ± 403	67.89 ± 6.32	951 ± 89	11.06 ± 5.18	343 ± 160
16	10.9 ± 8.8	512 ± 62	6154 ± 746	86.67 ± 10.29	1214 ± 144	7.68 ± 0.56	238 ± 17
24	10.5 ± 1.6	486 ± 32	5834 ± 384	84.41 ± 8.33	1182 ± 117	8.32 ± 2.24	258 ± 69
32	15.1 ± 2.5	545 ± 30	6552 ± 357	89.43 ± 5.38	1253 ± 75	6.01 ± 0.74	186 ± 23

2.2. Grazing Rates

Clearance (grazing) rates of both copepods did not vary significantly across the N:P gradient of their prey during either the day-1 or day-7 experiments for *E. carolleeae*, but during the day-7 *A. tonsa* experiments clearance rates rose as prey cellular N:P increased (i.e., as prey cellular P content declined; R^2 = 0.82, $p < 0.05$; Figure 3). These rates ranged from −1.15 to 4.88 mL copepod^{-1} h^{-1} for *A. tonsa* and −3.16 to 5.20 mL copepod^{-1} h^{-1} for *E. carolleeae* on day 1, and from −5.79 to 3.78 mL copepod^{-1} h^{-1} for *A. tonsa* and 1.24 to 4.12 mL copepod^{-1} h^{-1} for *E. carolleeae* on day 7. Given that the prey were provided at twice the concentration required to meet the daily copepod C demand, the removal of prey due to grazing would have been small relative to the total amount of prey available, leading to small differences between treatment (with copepods) and control (without copepods) bottles, thus leading to potential underestimation of grazing.
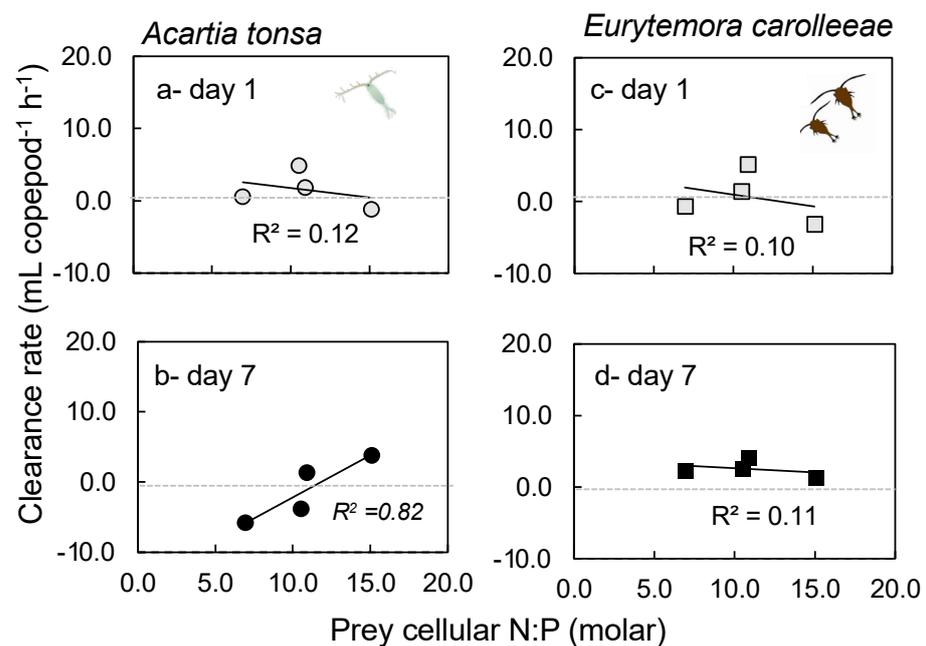

Figure 3. Grazing (clearance) rates of *Acartia tonsa* (panels a,b) and *Eurytemora carolleeae* (panels c,d) measured over 4 h incubation experiments conducted on days 1 (**a,c**) and 7 (**b,d**) of exposure to prey of varying cellular N:P ratios. Coefficients of determination are shown; that for panel b was significant at $p < 0.05$.

2.3. Nutrient Content in Copepod Tissue

The nutrient content of the copepods varied as a function of prey nutrient content, and differed between the two copepods and with the time of exposure to prey. Values of N:P for *A. tonsa* on both measurement days were greater than the N:P of the prey (Figure 4a,b). The initial N:P (day 1) of *A. tonsa* averaged 22.5 and varied little across the N:P spectrum of the prey (Figure 4a). By day 7, the N:P of *A. tonsa* grown on food with the lowest N:P ratio had virtually doubled to 41.1 (Figure 4b). This change represented an increase in N

content (Figure 4e) and not P content (Figure 4f). However, for both days, there was no significant difference in copepod N:P with prey N:P (ANOVA day 1, $F_{(3,8)} = 1.36$, $p = 0.32$; day 7, $F_{(3,7)} = 2.29$, $p = 0.17$). The C:N and C:P contents of *A. tonsa* also increased when fed the low N:P food (Figure 4c,d), reflecting an increase in C content (Figure 4g).
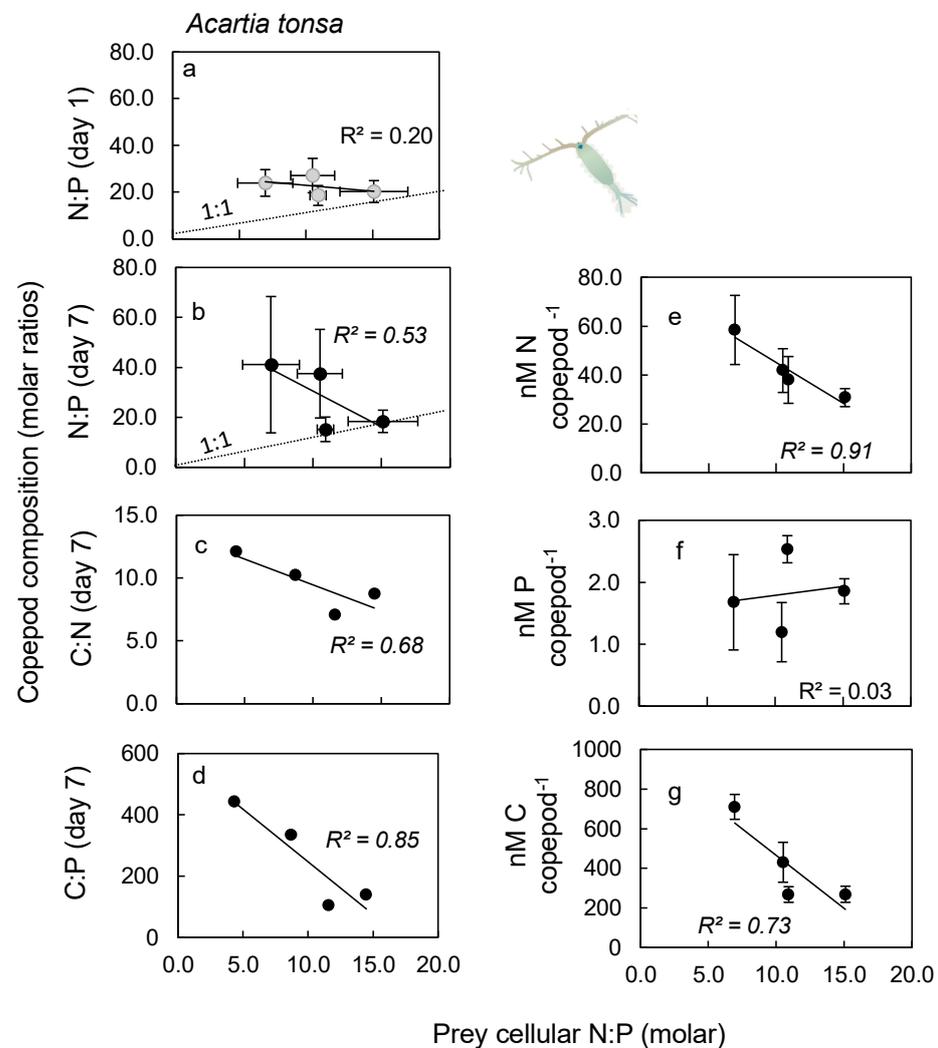

Figure 4. Tissue nutrient elemental ratios (**a–d**) and nutrient content of *Acartia tonsa* (**e–g**) as a function of prey cellular N:P ratio. Panel a is the N:P on day 1; the remaining panels show day 7 results. For panels a and b, the 1:1 line of correspondence between prey cellular N:P and that of copepod tissue N:P is indicated. Values are means for *n* = 3 replicates per treatment. Error bars are standard deviations. Coefficients of determination are shown; those indicated in italics were significant at *p* < 0.05.

The initial N:P content of *E. carolleeae* averaged 19.6 across all initial N:P prey conditions (Figure 5a); all values were greater than the N:P of the prey. By day 7, the N:P content averaged 14.4 across all prey conditions (Figure 5b), reflecting both an increase in N content (Figure 5e) and a decrease in P content (Figure 5f) as the prey N:P content declined. Tissue N:P ratios of *E. carolleeae* did not vary significantly with prey cellular N:P ratios on day 1 (ANOVA $F_{(3,8)} = 3.49$, $p = 0.07$), while they did vary significantly on day 7 (ANOVA $F_{(3,8)} = 5.55$, $p = 0.02$). The C:N content did not vary with N:P of the prey (Figure 5c), as C content also increased (Figure 5) in parallel with the change in N as N:P of the food decreased. The C:P content of these copepods remained <200 across all prey conditions (Figure 5d). The N and P content for *E. carolleeae* on day 7 (Figure 5e,f) had a greater variation as a function of prey N:P than did that of *A. tonsa* (Figure 4e,f).

Moreover, across all prey conditions, *E. carolleeae* were more C, N, and P rich than *A. tonsa* (Figures 4 and 5). When comparing *A. tonsa* and *E. carolleeae* tissue nutrient content for the same nutrient (C, N, or P), significant differences between the two copepods were found for all nutrients on both days (unpaired *t*-test $p \leq 0.01$ in all cases). The differences in nutrient content between copepods are not likely due to a size difference between the copepods as prosome lengths were similar.
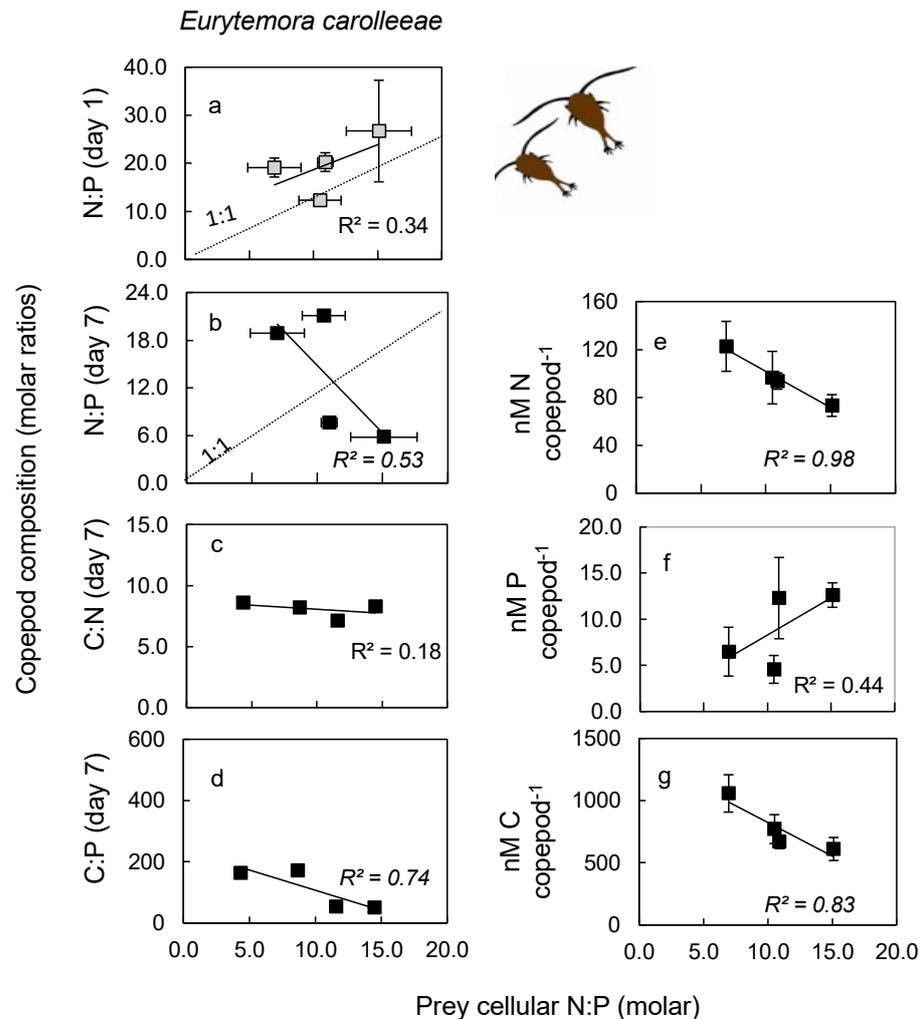

Eurytemora carolleeae

Figure 5. (**a**–**g**) As for Figure 4, except for *Eurytemora carolleeae*.

2.4. Excretion Rates

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 6 and 7). Rates of NH_4^+ excretion by *A. tonsa* increased significantly as prey N:P increased, from -41.6 ± 15.7 to 9.3 ± 14.2 ng N copepod^{-1} h^{-1} ($= -3.0 \pm 1.1$ to 0.7 ± 1.0 nM N copepod^{-1} h^{-1}) on day 1 (ANOVA $F_{(3,10)} = 6.61$, $p = 0.009$), but not significantly, from -42.0 ± 18.7 to 33.1 ± 78.3 ng N copepod^{-1} h^{-1} ($= -3.0 \pm 1.3$ to 2.4 ± 5.6 nM N copepod^{-1} h^{-1}), on day 7 (ANOVA $F_{(3,12)} = 2.90$, $p = 0.08$; Figure 6a,b). Average rates of NH_4^+ excretion on day 1 and day 7 were not significantly different from each other (unpaired *t*-test $p = 0.7$).
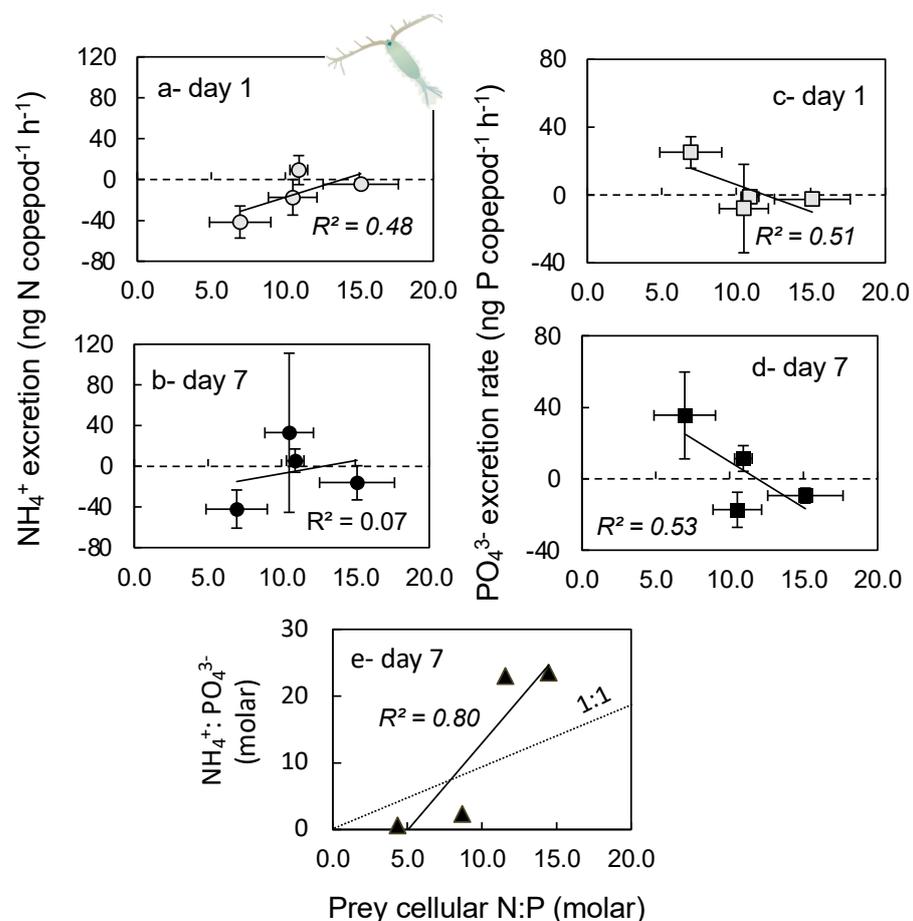
Figure 6. Rates of NH_4^+ (panels a,c) and PO_4^{3-} (c,d) excretion by *Acartia tonsa* measured over 4 h incubation experiments conducted on days 1 (**a,c**) and 7 (**b,d**) of exposure to prey of varying cellular N:P ratios. Values are means ± standard deviation (*n* = 3). (**e**) molar ratio of the means of NH_4^+: PO_4^{3-} excretion rates as a function of prey cellular N:P ratio. Coefficients of determination are shown; those indicated in italics were significant at *p* < 0.05.

The PO_4^{3-} excretion rates of *A. tonsa* on both day 1 and day 7 were highest when the copepods ate prey with nominal molar cellular N:P ratio of ~7, and declined as prey molar cellular N:P ratio increased (Figure 6c,d). Rates of PO_4^{3-} excretion on day 1 ranged from -8.0 ± 26.1 to 25.1 ± 9.2 ng P copepod^{-1} h^{-1} (= -0.3 ± 0.8 to 0.8 ± 0.3 nM P copepod^{-1} h^{-1}; Figure 6c), and on day 7 ranged from -17.3 ± 9.8 to 35.5 ± 24.3 ng P copepod^{-1} h^{-1} (= -0.6 ± 0.3 to 1.1 ± 0.8 nM P copepod^{-1} h^{-1}; Figure 6d). These rates on the two experimental days were not significantly different from each other (unpaired *t*-test *p* = 0.86).

Rates of NH_4^+ excretion by *E. carolleeae* on day 1 ranged from 24.5 ± 11.3 to 36.7 ± 18.8 ng N copepod^{-1} h^{-1} (= 1.7 ± 0.8 to 2.6 ± 1.3 nM N copepod^{-1} h^{-1}; Figure 7a), and from 2.5 ± 23.8 to 38.6 ± 16.5 ng N copepod^{-1} h^{-1} (= 0.2 ± 1.7 to 2.8 ± 1.2 nM N copepod^{-1} h^{-1}) on day 7 as prey N:P decreased (Figure 7b). The rates of NH_4^+ excretion on days 1 and 7 were not significnatly different from each other (unpaired t-test *p* = 0.34).

Rates of PO_4^{3-} excretion on day 1 for *E. carolleeae* did not vary significantly with prey cellular N:P (ANOVA F(3,8) = 3.61, *p* = 0.06) and ranged from -2.9 ± 2.5 to 3.9 ± 4.2 ng P copepod^{-1} h^{-1} (= -0.1 ± 0.1 to 0.1 ± 0.1 nM P copepod^{-1} h^{-1}; Figure 7c). However, rates of PO_4^{3-} excretion for these copepods on day 7 decreased significantly, ranging from 51.0 ± 14.6 to -3.3 ± 2.0 ng P copepod^{-1} h^{-1} (= 1.6 ± 0.1 to -0.1 ± 0.1 nM P copepod^{-1} h^{-1}; ANOVA F(3,9) = 28.32, *p* ≤ 0.001), as the molar prey cellular N:P ratio increased to 15.1 (Figure 7d). Rates of PO_4^{3-} excretion measured at both time periods were significantly different from each other (unpaired *t*-test *p* = 0.02).

For both copepods, the directionality of the trend in $NH_4^+:PO_4^{3-}$ excretion rates increased as N:P in their diet increased (Figures 6 and 7e). Yet, for *A. tonsa*, $NH_4^+:PO_4^{3-}$ excretion rates more closely matched that of the diet (Figure 6e), while the excretion ratio for *E. carolleeae* reflected a more N-rich excretion across the dietary spectrum and increased significantly as N:P of the diet increased (Figure 7e). The latter trend is in keeping with the trend in the N:P of tissues, as N of tissue decreased and P of tissues increased as N:P in the diet increased (Figure 5e,f).
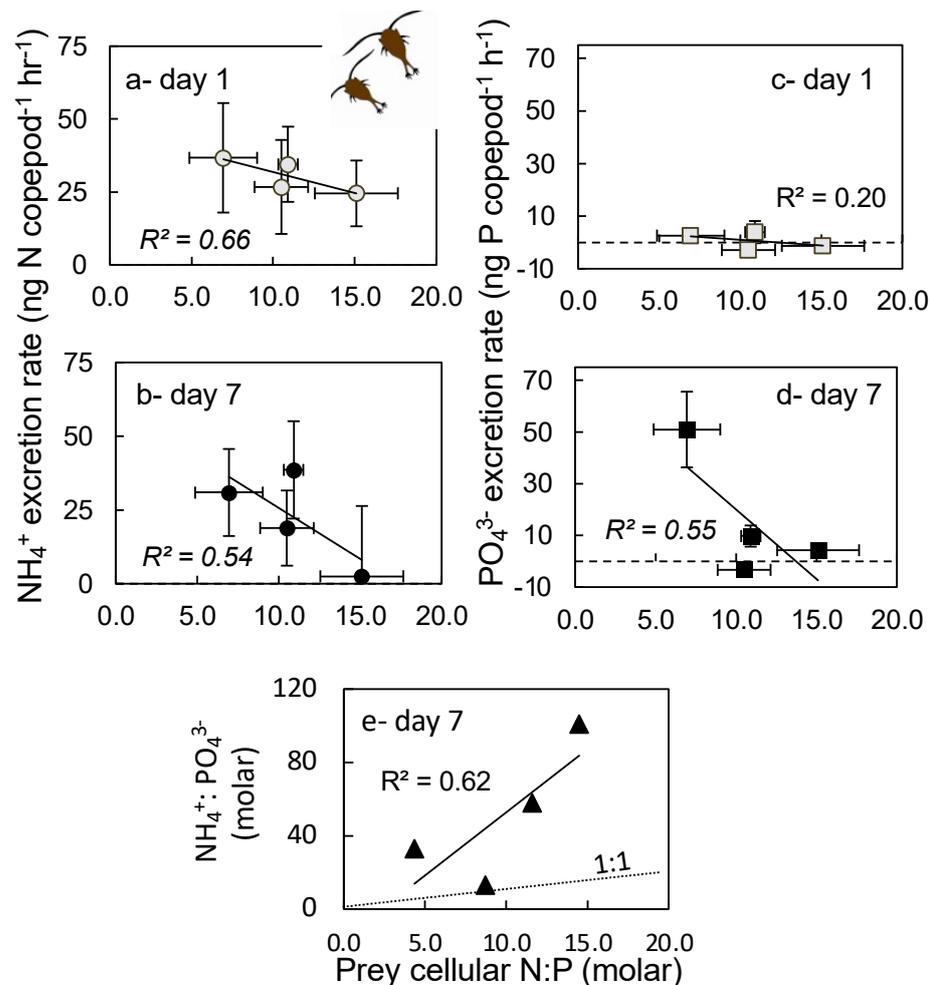

Figure 7. (**a**–**e**) As for Figure 6, except for *Eurytemora carolleeae*.

2.5. Nutrient Content in Eggs

During some of the experiments egg production was not sufficient for particulate C, N, and P analysis and thus comparisons across the prey spectrum do not carry high significance. For *A. tonsa*, eggs collected on both days 1 and 7 had N:P content well in excess of Redfield proportions, and the trend suggested higher N:P and C:P content with lower N:P in prey (Figure 8a,c; note, no data available for the treatments of prey N:P < 10). The C:N content of eggs of *A. tonsa* were similar for the different prey conditions tested on both days (Figure 8b). In contrast, the N:P of eggs of *E. carolleeae* were <30 for all prey conditions tested on both experimental days (Figure 9a), and there was no trend in C:P (Figure 9c), while C:N fluctuated with N:P in prey (Figure 9b). On both days 1 and 7, *A. tonsa* eggs had higher C, N, and P content, on average, than *E. carolleeae* eggs (Figures 8 and 9d–f), though egg nutrient content for the same nutrient for both copepods on the same day were not significantly different (unpaired *t*-test $p > 0.10$ in all cases).
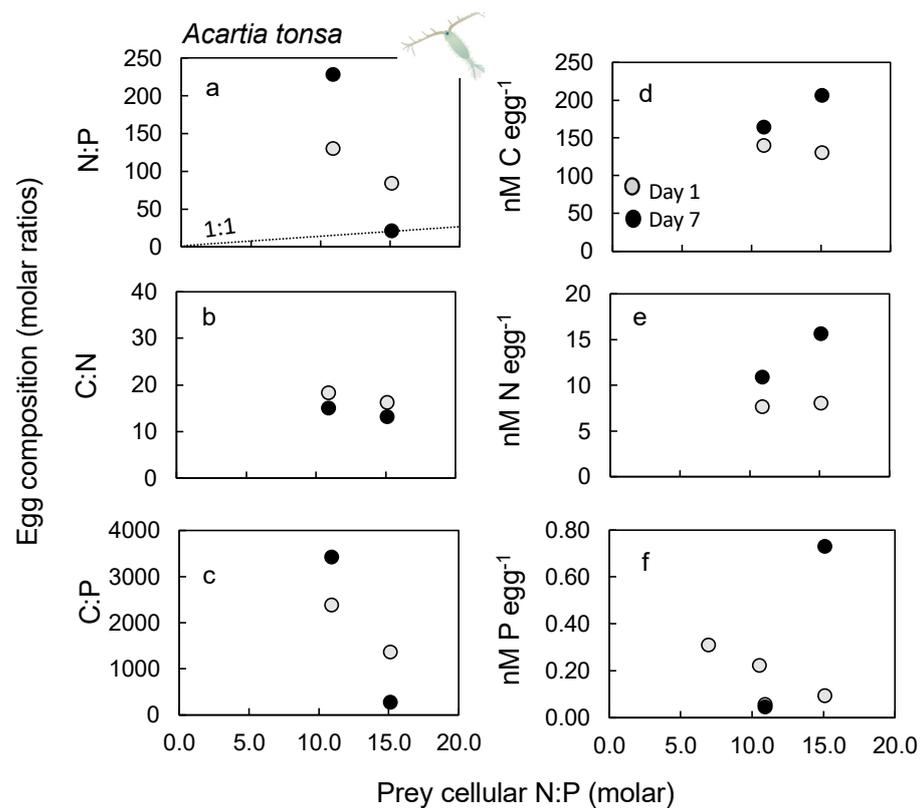
Figure 8. Egg nutrient elemental ratios (**a**–**c**) and nutrient content of *Acartia tonsa* (**d**–**f**) as a function of prey cellular N:P ratio. Gray points are from day 1 measurements, black points from day 7. The 1:1 line of correspondence between prey cellular N:P and that of copepod egg N:P is indicated in (**a**). Please note that no data are available for *A. tonsa* at prey < 10 for N or C.

2.6. Egg Production and Viability

For *A. tonsa*, egg production was not measurable at the lowest N:P prey conditions (N:P < 10). Across all measurable conditions (excluding the day 7 N:P prey = 10.9 measurement), *E. carolleeae* produced about an order of magnitude more eggs per female than did *A. tonsa* (Figure 10). On day 1, egg production by *E. carolleeae* increased significantly with N:P of prey provided, but this was not the case on day 7 (Figure 10c,d; day 1 ANOVA $F(3,4) = 10.02$, $p = 0.02$; day 7 ANOVA $F(3,4) = 1.97$, $p = 0.26$).

By day 7, egg viability in both copepods trended downward as prey cellular N:P rose, but these trends were not significant (Figure 11). On day 1, *A. tonsa* egg viability was 85.5% and 89.0% when eating prey with cellular molar N:P of 15.1 and 10.9, respectively (Figure 11a), but by day 7, *A. tonsa* egg viability declined to 38.7% and 69.1% for prey with cellular molar N:P of 15.1 and 10.9, respectively (Figure 11b). Egg viability in *E. carolleeae* decreased from $44.4 \pm 52.2\%$ to $4.4 \pm 6.1\%$ on day 1 (Figure 11c), and from to $25.7 \pm 23.5\%$ to $6.2 \pm 3.1\%$ on day 7 (Figure 11d), as prey cellular N:P increased.
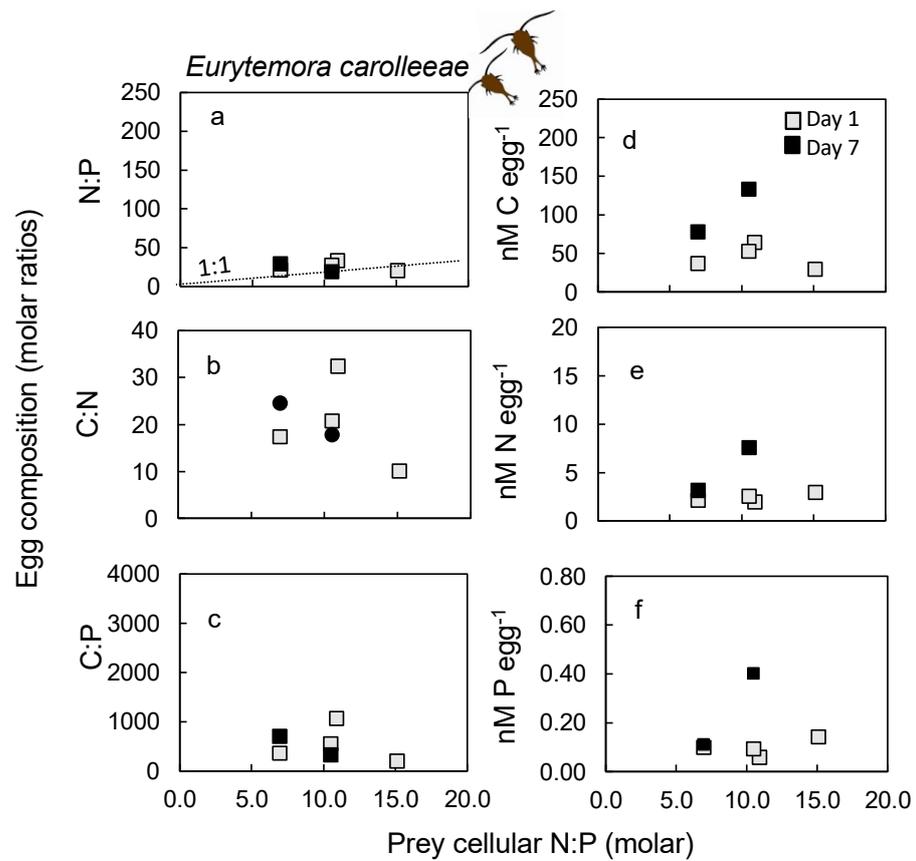
Figure 9. (**a–f**) As for Figure 8, except for *Eurytemora carolleeae*.
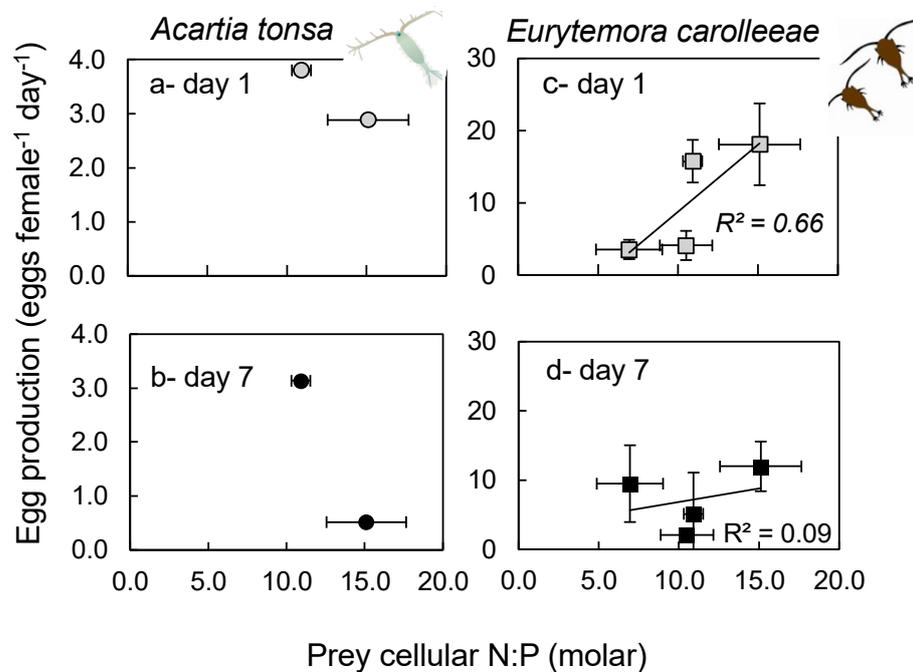
Figure 10. Egg production of *Acartia tonsa* (**a,b**) and *Eurytemora carolleeae* (**c,d**) measured over 24 h following days 1 (**a,c**) and 7 (**b,d**) of exposure to prey of varying cellular N:P ratios. Values are means ± standard deviation (*n* = 2 for *E. carolleeae*, *n* = 1 for *A. tonsa*). Please note that no data are available for *A. tonsa* at prey < 10. Coefficients of determination are shown for *E. carolleeae*; those indicated in italics were significant at *p* < 0.05.

Figure 11. Egg viability of *Acartia tonsa* (**a**,**b**) and *Eurytemora carolleeae* (**c**,**d**) measured over 48 h following days 1 (**a**,**c**) and 7 (**b**,**d**) of exposure to prey of varying cellular N:P ratios. Values are means ± standard deviation ($n = 2$ for *E. carolleeae*, $n = 1$ for *A. tonsa*.). Please note that no data are available for *A. tonsa* at prey < 10. Coefficients of determination are shown for *E. carolleeae*; those indicated in italics were significant at $p < 0.05$.
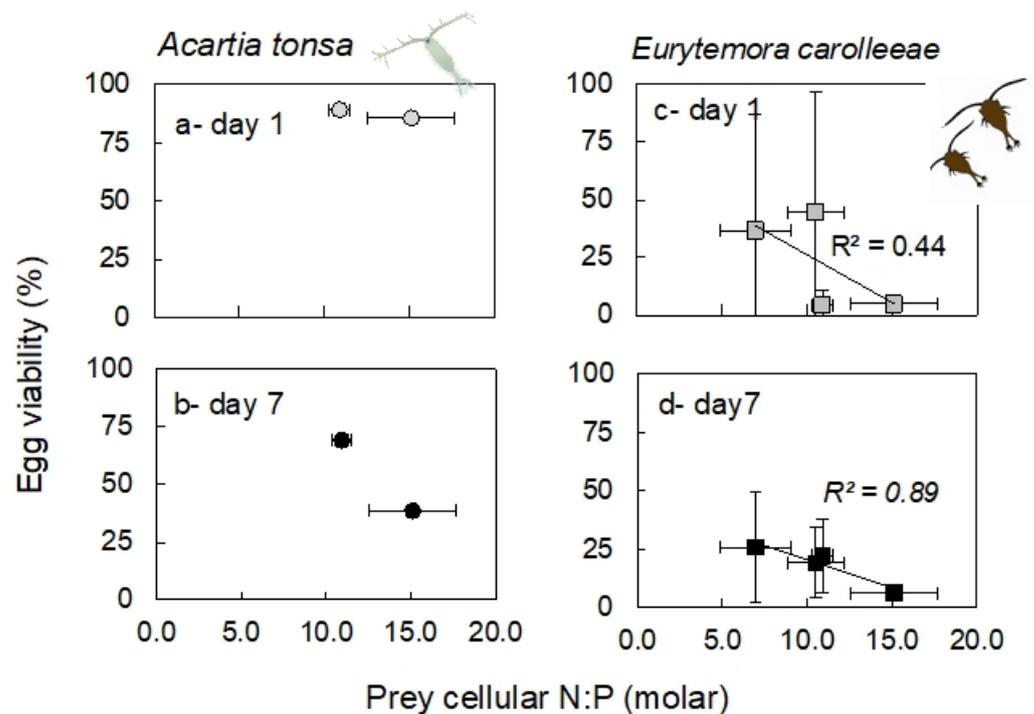
3. Discussion

Given the anthropogenic changes to N and P in estuaries and coastal waters throughout the world, copepods can be expected to be exposed to prey of varying food quality even under conditions of food sufficiency. In particular, higher N:P conditions are emerging in many systems as N loads are increasing faster than those of P [17,18,39]. Sterner and George [49] stated that "nutrient flux from resources to consumers and then to waste products can be thought of as a chemical reaction wherein mass must balance." Although the environmental factors selecting for varying copepods and their population success are many, how grazers do, in fact, respond metabolically and reproductively as nutrient conditions change has become an important question with important implications for food webs.

The work of Malzahn and Boersma [6] convincingly demonstrated that *A. tonsa* reared on P-poor diets had lasting effects on copepod growth that were non-reversible even when the animals were subsequently exposed to more nutritious food. They also showed that the effect on growth was related to the duration of exposure to the low-quality food. Furthermore, Shoo et al. [32] showed that poor quality food for copepods has effects higher in the food web as the resulting nutritionally poor copepods themselves are grazed. The decline of copepod populations due to poor quality food have far-reaching effects and may lead to the collapse of higher trophic levels that depend on these copepods as a food source [34,50,51]. As evidence, Nobili et al. [7] showed that changes in the quality and quantity of phytoplankton resulting from changing nutrient ratios in the North Pacific Subtropical Gyre have resulted in changes in zooplankton structure; as larger zooplankton declined, the biomass of smaller zooplankton increased, in turn affecting energy transfer and productivity at higher tropic levels, and Beaugrand et al. [52] showed that declines in abundance of *Calanus* zooplankton in the North Sea have resulted in long-term declines in cod.

Herein, we attempted to quantify changes in copepod tissue nutrient content, excretion, egg nutrient content, and egg production and viability as a function of N:P in food for two contrasting copepods, *A. tonsa* and *E. carolleeae*, one a broadcast spawner and one a brood spawner. Maintaining prey in a constant physiological state was accomplished by growing the diatom prey in a turbidostat. Varying nutritional quality was accomplished by varying the P content in the media of the diatom prey, holding N content in the media constant, and by providing food at a constant level based on C content. Turbidostat growth also allowed the phytoplankton to have variable nutrient content without being nutrient starved, a condition more likely experienced in natural eutrophic conditions such as estuaries. The variability in the diatom N:P was less than that in the media (Figure 2), a finding in keeping with the analysis of Hillebrand et al. [30] showing that fast-growing phytoplankton (here, non-nutrient-limited) have a more constrained stoichiometry than would slow-growing, nutrient-limited cells. The following discussion evaluates the initial hypotheses and places the findings in the context of previous findings for these and related copepods.

3.1. Evaluating Hypotheses

The first hypothesis, that copepods would maintain relatively constant somatic nutrient stoichiometry, was not supported by the data, but changes with food quality in terms of N, P and C copepod content varied by copepod species (Figure 12a). The tissue N:P of *A. tonsa* was consistently higher than that of *E. carolleeae* across all prey conditions and the slope of change was greater for this copepod as food N:P changed. The second hypothesis, that excretion rates of P when eating a P-rich food (low N:P) would be higher than when copepods ate foods that were more balanced, was demonstrated for PO_4^{3-} excretion in both copepods. Yet, excretion of NH_4^+ with N:P food quality differed by copepod species. For both copepods the ratio of NH_4^+:PO_4^{3-} excretion rose as the N:P of the food rose, driven by a decline in P excretion with increasing N:P (declining P content) of the prey (Figure 12b).

The third hypothesis, that decreasing prey P content should decrease P content in eggs, and that egg production rates and viability would be negatively affected, was variably supported. Egg nutrient content of *E. carolleeae* showed no trend with prey or copepod P content (Figure 12c). On the other hand, the N:P of eggs of *A. tonsa* decreased as food N:P increased, reflecting a P-poor diet (Figure 12c). 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 (Figure 12d). Under a P-poor diet *E. carolleeae*, in contrast to *A. tonsa*, produced more eggs, of relatively consistent 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 (Figure 12d).

Finally, *A. tonsa*, the broadcast spawner, had been hypothesized to respond to nutritionally poor prey through declining egg production and viability, but not through egg nutrient content, which is thought to be related to egg viability. Instead, when grazing on nutritionally poor prey, *A. tonsa* responded with the hypothesized reduced egg production and viability overall, and the unanticipated increased egg viability when *A. tonsa* fed at all prey nutritional conditions compared to the viability of *E. carolleeae* eggs when that copepod ate the same diet. The brood spawner, *E. carolleeae*, had been hypothesized to respond to nutritionally poor prey through declining egg production, but egg viability and egg nutrient content were hypothesized to remain constant because the mother was expected to maintain constant nutrient content in eggs which should have led to constant viability. However, *E. carolleeae* responded to nutritionally poor prey with increased egg production, decreased egg viability, and relatively consistent egg nutrient content.
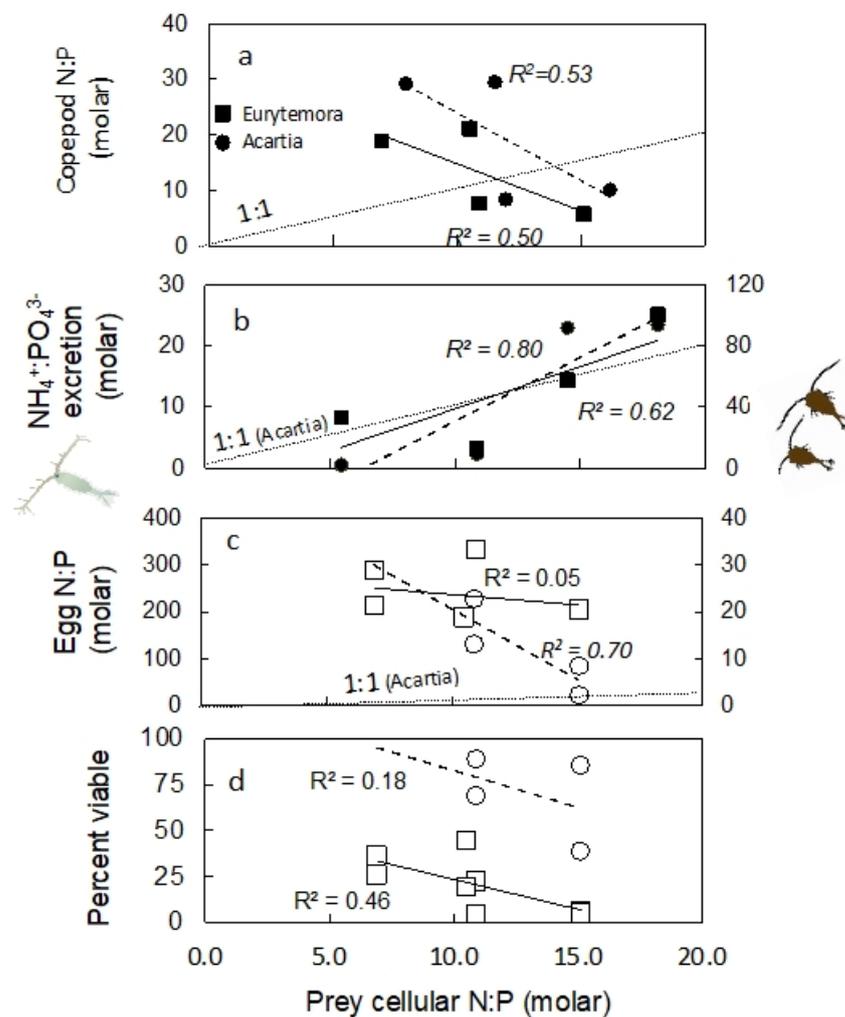
Figure 12. Summary comparison of the two experimental copepods and their tissue N:P (**a**), $NH_4^+:PO_4^{3-}$ excretion ratios (**b**), egg N:P (**c**) and egg viability percentages (**d**) in relation to prey N:P. Where the scale of the copepod responses differ, the Y axis on the left corresponds to *Acartia tonsa*, and the Y axis on the right corresponds to *Eurytemora carolleeae*. For (**a–c**), the 1:1 correspondence of the response parameter to prey cellular N:P ratio is indicated; for (**b,c**) this line is shown for *A. tonsa* only. (**a,b**) Results of day 7 only, (**c,d**) composite of day 1 and 7 results. Coefficients of determination are shown; those indicated in italics were significant at $p < 0.05$.

3.2. Grazing

Both copepods ate prey at all cellular N:P ratios during both days of experiments and clearance rates were relatively similar between copepods and across prey cellular N:P ratios on day 1 (Figure 3). After 7 days of exposure, *A. tonsa* ingested more prey cells of low P content than of high P content, likely increasing clearance rates as prey P content declined to obtain a sufficient quota of P. This is similar to results from Houde and Roman [53] in which *A. tonsa*, sourced from Chesapeake Bay, increased ingestion rates of *Thalassiosira weissflogii* prey as the prey became more deficient in N or in protein. Similarly, Malzhahn and Boersma [6] found *A. tonsa* to have ingestion rates 5 times higher when fed P-deficient food (*Rhodomonas* sp.) compared to P-rich food. Richman et al. [54] found *A. tonsa* filtering (clearance) rates on a natural Chesapeake Bay plankton assemblage ranged from 0–1.50 mL copepod^{-1} h^{-1}, with most values falling below 0.80 mL copepod^{-1} h^{-1}. Saba et al. [55] reported average *A. tonsa* clearance rates between 0.5 and 1.0 mL copepod^{-1} h^{-1} when it grazed on *T. weissflogii* at an initial food concentration of 160 μg C L^{-1}. Please note that in this study the food concentration was 3-4 times greater (600 μg C L^{-1}) than in the

Saba et al. [55] study and nutritional food quality was varied here, while Saba et al. [55] varied the type of food.

Similarly, for *E. carolleeae* clearance rates reported here (average of 0.68 mL copepod^{-1} h^{-1}) compared favorably with values previously reported. Richman et al. [54] reported *E. affinis* filtering (clearance) rates on a natural Chesapeake Bay plankton assemblage that ranged from 0 to 1.50 mL copepod^{-1} h^{-1} with most values falling <0.60 mL copepod^{-1} h^{-1}. The higher clearance rates measured in this study may be due to the differences in diet between the two studies; a mono-algal diatom diet maintained at high food concentrations in this study, and a natural plankton assemblage in the Richman et al. [54] study.

3.3. Nutrient Content in Copepod Tissue

The somatic nutrient contents for *A. tonsa* reported here are similar to previous studies of *Acartia* spp. This study reported C, N, and P content of *A. tonsa* ranging from 269.3–710.8 nM C copepod^{-1} (3.23–8.53 µg C copepod^{-1}), 30.7–58.4 nM N copepod^{-1} (0.43–0.82 µg N copepod^{-1}), and 1.2–2.5 nM P copepod^{-1} (0.04–0.08 µg P copepod^{-1}) (after a 7–day exposure to food). Thompson et al. [56] found *A. tonsa* C content ranged from 3.15–5.05 µg C copepod^{-1}, and N content ranged from 0.87–1.43 µg N copepod^{-1} as prosome length ranged from 838–987 µm. In addition, Thompson et al. [56] 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. [57] reported *Acartia 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 [58] 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. However, Shoo et al. [32] reported *A. tonsa* copepodites fed P-limited food contained 0.36 µg C copepod^{-1}. Uye and Matsuda [59] reported somatic P contents in *Acartia omorii* of 0.12 µg P copepod^{-1} and in *Acartia erythraea* of 0.19 µg P copepod^{-1}. Meunier et al. [60] showed that *A. tonsa* selected P-rich algae as nauplii, while copepodids favored N-rich prey.

The C, N, and P content of *A. tonsa* and *E. carolleeae* were different, even when both ate the same prey and were exposed to otherwise similar environmental conditions. The differences in nutrient content between copepods are not likely due to a size difference between the copepods as prosome lengths were similar. Furthermore, clearance rates of both copepods were relatively similar on day 1. Sterner and Elser [31] suggest that closely related animals can have varying elemental chemistry (as mineral nutrient content or mineral nutrient ratios) as a result of different life-history strategies that require the animal to allocate nutrient resources differentially. For example, Gaudy and Boucher [61] summarized molar N:P ratios of over 20 species of copepods collected from the Indian Ocean (none were *Acartia* or *Eurytemora* genera) and they ranged from 2.27 to 44.46. Labuce et al. [62] reported the elemental composition of *E. affinis* from the Gulf of Riga, Baltic Sea, and molar C:N values ranged from 3.9 to 8.6, with males having the higher value. The higher N demand of females could relate to the N demand of egg production [63].

Kimmerer et al. [3] reported C content of *E. affinis* ranging from 0.8–4 µg C copepod^{-1}. In our study, *E. carolleeae* had an average C content higher than these estimates, ranging from 12.07 µg C copepod^{-1} (1005.8 nM C copepod^{-1}) on day 1 to 9.33 µg C copepod^{-1} (777.5 nM C copepod^{-1}) on day 7. The C weights here may be higher due to copepod stage. Adults, together with the presence of eggs–within the copepod body or extruded into sacs–were included in the weights reported herein, both of which have been shown to weigh more than younger stages of females and/or egg-free females in *Eurytemora herdmanni* [64]. A calculation of 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} [47,59]. These values approximate the P content measurements herein, 0.20 μg P copepod^{-1} on day 1, and 0.28 μg P copepod^{-1} on day 7.

3.4. Excretion

As copepods eat food of variable N:P, stoichiometric theory predicts the relative proportions of N:P in excreta should vary in response ([31], and references therein). Even though N was maintained at constant supply in prey, *A. tonsa* excreted more NH_4^+ (Figure 6), while *E. carolleeae* appeared to excrete less NH_4^+, as prey cellular N:P increased (Figure 7). Excretion rates of NH_4^+ were negative for *A. tonsa* at lower prey cellular N:P ratios because copepods excreted less N relative to P, but what was excreted was apparently taken up by the diatom prey. Miller and Glibert [65] also reported low NH_4^+ excretion rates of *A. tonsa* ranging from 0–2 ng N copepod^{-1} h^{-1}. Similarly, Miller and Roman [58] reported low NH_4^+ excretion rates for *A. tonsa*, ranging from about 2.08–6.67 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 results of this study can likely be explained by the qualities of the varying prey used and the varying nutrient ratios. Miller and Glibert [65] maintained the *A. tonsa* in mesocosms where they fed on a natural plankton assemblage from a sub-estuary of the Chesapeake Bay in summer, while Miller and Roman [58] provided monocultures of four prey types of naturally varying N:C ratio (detritus, a senescent stage, and a log-phase stage of a diatom, and ciliates).

When eating P-rich food (low N:P), both copepods responded by increasing PO_4^{3-} excretion as predicted by the hypothesis that a grazer will excrete the nutrient in excess in its food [31,66]. For both copepods, this pattern can be seen by day 7. Even though *E. carolleeae* is the more P-rich copepod (Figures 4f and 5f), it excreted P at a higher rate than *A. tonsa* when both were eating the same food. Similarly, when *Daphnia magna* fed on prey of decreasing C:P (increasing P content), excretion of P increased [67]. DeMott et al. [68] 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 [69] 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.

3.5. Egg Nutrient Stoichiometry

The stoichiometry and production of eggs is another pathway by which copepods may rebalance C, N, and P content. In general, it is thought that proportionately more P should be allocated to eggs because of their high growth demand [70,71]. Here, differences were found in nutrient allocation to eggs between the two copepods. The more nutrient-rich copepod, *E. carolleeae*, had comparatively nutrient-poor eggs (Figure 9). The egg P content of *E. carolleeae* after a 4 h exposure (day 1) was also relatively consistent (Figure 9), while that of *A. tonsa* declined as prey N:P increased, reflecting a relatively more P-poor food (Figure 8). On day 7, though there are limited data, both copepods appear to increase P, N, and C allocation to eggs. Both DeMott et al. [68] and He and Wang [67] similarly found that *D. magna* mothers that grazed on P-deficient prey were more P-deficient themselves and produced daughters with lower P content, and Frost et al. [72] also found effects of poor-quality food for *D. magna* to be transgenerational. The species-specific differences found here may reflect the different reproductive strategies, brood spawning vs. broadcast spawning, implying different approaches to allocation of resources for reproduction.

3.6. Egg Production and Viability

Due to the need for P in eggs, food P content should affect both egg production and viability. Egg production rates of *A. tonsa* reported in the literature have a wide range. For example, Durbin et al. [73] found that egg production ranged from 1.6–51.6 eggs female^{-1} day^{-1} after incubating *A. tonsa* in water from Narragansett Bay, Rhode Island, but that egg production rates incubated in ambient bay water enriched with a mixture of laboratory-

cultured algae ranged from 11.8–64.2 eggs female^{-1} day^{-1}. Dagg [48] reported that *A. tonsa* egg production ranged from 20–40 eggs female^{-1} day^{-1}. Kimmerer et al. [44] 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 [74] 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 at a salinity of 15 and temperature of 20.5 °C, rates were lower, from <5–10 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} [75]. Furthermore, when copepods were maintained on *T. weissflogii* for 7 days, egg production ranged from 0–4 eggs female^{-1} day^{-1} on low food concentrations, and from about 8–13 eggs female^{-1} day^{-1} on high food concentrations when food quality was constant [57]. While the egg production rates measured in this study, 0.5–3.8 eggs female^{-1} day^{-1}, are within the range of the cited reports, they are near the reported minima. These lower rates of egg production, compared to literature values, could result from temperature or salinity differences, both of which have been shown to have strong effects on egg production rates [47,74], or the use of a single diatom food source rather than a mixed diet [76].

Diatoms have been shown to depress copepod reproductive success [77,78], although this is controversial [79] and species specific [77]. Some diatom taxa have been shown to increase production of the cytotoxic polyunsaturated aldehydes (PUA) under nutrient stress, in particular with Si and P limitation [80,81], which may have led to reductions in reproductive output or success if it occurred in our experiments. There is some evidence that a mixed diet is necessary for high levels of egg production [82,83]. In these experiments high prey N:P ratios (or low prey P content) represent unfavorable conditions or food of poor quality, and egg production rates declined, under constant food quantity, when prey quality declined. This result mirrors other food quantity studies in which egg production rates were lower when copepods ate at a low food quantity and higher when they ate at a high food quantity (food quality held constant) [7,8,44,57,84], 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*.

In contrast to *A. tonsa*, *E. carolleeae* are brood spawners and are not thought to respond as opportunistically to environmental conditions [47]. Egg production rates of *E. carolleeae* were comparable to previously reported rates for *Eurytemora* spp. Hirche [85] showed that *E. affinis* incubated at 10 °C produced 5.5 eggs female^{-1} day^{-1}, while at 15 °C they produced 5.8 eggs female^{-1} day^{-1}. Bunker and Hirst [86], 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 value 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 9c,d). Lloyd et al. [46] reported that *E. affinis,* found in estuarine turbidity maximum of Chesapeake Bay, where they were food saturated, produced 15–20 eggs female^{-1} day^{-1}. This result is similar to the egg production rates reported here for *E. carolleeae* at higher prey cellular N:P ratios.

When either food quantity or food quality is not controlled, varying egg production rates can be attributed to either, assuming other environmental factors are held constant. Kimmerer et al. [3] reported egg production rates of *E. affinis* in the San Francisco Bay Delta 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 <5 eggs female^{-1} day^{-1}. Based on literature values, the authors suggested that rates for food-saturated *E. affinis* range from about 15–40 eggs female^{-1} day^{-1} and attributed the lower rates reported in their study to food quantity limitation [3]. In this study, egg production rates, when copepods were fed on prey of high N:P and at saturating levels of food quantity, are more similar to the literature ranges than the experimental

results reported in Kimmerer et al. [3]. 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 may lead to comparatively 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 but more viable 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 (Figures 11 and 12d). Ambler [87] 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 81–98% on this diet and fell to <70% 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 [7,8]. The low P content in the prey may have prevented copepods from obtaining sufficient P to produce viable eggs, or may have stimulated production of cytotoxic compounds in the diatoms, suggesting the potential for both direct and indirect impacts of changing nutrient ratios on consumers. These results clearly show that food quality impacts reproductive success.

4. Methods

4.1. Overview

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

4.2. 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 [88] with a concentration of PO_4^{3-} adjusted to achieve a range of inorganic N:P ratios bracketing the N:P = 16:1 Redfield proportions (treatment molar N:P = 4, 16, 24, 32; [89]). Media was made in artificial seawater (Crystal Sea Marine Mix and ultra-pure water) with a salinity of 12.

The diatoms were grown in a turbidostat continuous culture system [90] in which growth was exponential and near constant for all nutrient conditions (Figure 1). Note that in turbidostat growth, nutrients are not limiting, and thus the prey had variable but non-limiting nutrient supply. Four 2 L turbidostat chambers were set up in an environmental chamber at temperatures of 14–16 °C and a 14:10 light:dark cycle for all experiments. For each experiment, two nutrient ratios were tested simultaneously, with replicate turbidostats set to the lower N:P ratios (4 and 16) or the higher N:P ratios (24 and 32).

In the turbidostat systems, cells were grown exponentially, and the rate was maintained by dilution of the culture with fresh media. Turbidity readings of the cultures were made with an optical turbidity sensor and recorded every minute, and used as a proxy for growth rate. As cells grew, turbidity increased, and once turbidity reached a preset threshold value, new media was automatically delivered, resulting in a 5% reduction in

optical turbidity signal, corresponding to an approximate 10% volume dilution [90]. The rate of dilution, or number of dilutions over time, was proportional to growth rate. As each of the turbidostats was maintained under identical conditions, these continuous culture systems eliminated differences in growth stage and rate for the prey within and between experiments, and the only variable in prey conditions between experiments was the variation in the dissolved nutrients of the media on which the prey were raised. More specifically, the variation was in the P content, as N was held constant. 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 h) 25 mm GF/F filters at the beginning of experiments. Filters and filtrates were frozen and later analyzed for particulate C, N, and P, and dissolved N and P, as described below.

4.3. 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 bubbled with 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, and fed to the copepods at saturating concentrations.

4.4. Prey Quality Experiments

Experiments were conducted on adult-stage copepods that had been starved for 12–24 h prior to each experiment. A total of 16 experiments using *T. pseudonana* as prey and either *A. tonsa* or *E. carolleeae* as grazers were conducted; each copepod taxa was used in 8 experiments, on day 1 and again on day 7 for each of the 4 prey N:P treatments. Copepods were fed prey at a level that was estimated to be twice their daily C demand. The average C content of the diatom was estimated to be 8×10^{-6} µg C cell^{-1} (=0.67 × 10^{-6} µM C cell^{-1} [91]), daily copepod C demand was estimated to be 0.2 µg C adult^{-1} day^{-1} (=0.02 µM C adult^{-1} day^{-1}; [92]), and the cell count of the diatoms in turbidostat was ~1.50×10^6 cells mL^{-1}, and thus 300 µg C L^{-1} (=25 µM C L^{-1}) was estimated to meet the daily C demand of the copepods. Twice their daily C demand was provided as prey each day during the 7 days of exposure for each N:P treatment. To do this, the copepods were transferred daily to a new bottle containing prey culture prepared at the concentration needed by subsampling and mixing aliquots from the respective replicate turbidostats. Thus, with N held constant in the diatom media, and diatom prey added to copepod treatments at constant amounts, the C:N ratio of the food was constant, while C:P and N:P varied.

The grazing measurements, which were conducted on days 1 and 7, were initiated by transferring the appropriate concentration of diatom prey to five, 600 mL bottles. Bottle 1 was a control with no copepods, and to the remaining 4 bottles, 30 female adult copepods and 3–5 males were added (Figure 1). For the day 7 experiments, these animals were retrieved from their respective prey quality condition. Bottles 2–4 were considered replicates for the grazing experiment and included copepods that were subsequently used for copepod nutrient content and egg chemistry measurements. The 5th bottle, which always contained 30 adult females and 5 males, was used for both grazing experiments and subsequently for egg production and viability measurements as described below. All bottles were incubated on a slowly rotating wheel (1–2 rpm) in a 14–16 °C environmental chamber on the same light cycle as the turbidostat cultures. The duration of incubations for the grazing experiments was 4 h.

Aliquots were withdrawn from all bottles before and after grazing experiments conducted on both days 1 and 7. Samples for dissolved (N, P) and particulate (C, N and P) nutrients, as well as for cell counts of the algae, were taken at the start and end of the 4 h grazing period. At the beginning of experiments, 150 mL aliquots from each bottle were

collected and filtered onto pre-combusted 25 mm GF/F filters to collect particulate P, C, and N samples and dissolved nutrient samples (NH_4^+, $NO_3^- + NO_2^-$, PO_4^{3-}). After the 4 h incubation period, copepods were removed from the bottles by collecting them on a 64 μm mesh sieve, and the sample was refiltered, and filters and filtrates collected and frozen for later analysis of dissolved and particulate nutrients. The differences in NH_4^+ and PO_4^{3-} concentrations in the treatment (with copepod grazers and prey) and control (with prey only) were used to estimate excretion rate (Figure 1). Aliquots of 10–20 mL of unfiltered material were also collected at the start and end of each experiment and from each bottle for enumeration of diatoms, and these cell counts were later used for grazing calculations. At 4 h, when copepods were removed, 20 copepods from each bottle were removed for particulate nutrient analysis; about 10 were placed onto each of two pre-combusted 25 mm GF/F filters for particulate C, N, and P analysis.

Adult copepods remaining in bottles 2–5 after 4 h were each gently transferred to a container with an insert that had a 200 μm mesh bottom and submerged in low-nutrient artificial seawater at a salinity of 12 and incubated for either an additional 24 h (bottles 2–4) or 48 h (bottle 5) in the environmental chamber at 14–16 °C on a 14:10 h light:dark cycle. The insert allowed the separation of the adults from the nauplii and any released, unhatched eggs that may have been produced during this period. After 24 h, the eggs were collected and placed onto two pre-combusted 25mm GF/F filters for subsequent analysis of particulate C, N, and P. Between 40 and >200 eggs were collected on a filter for each treatment to obtain sufficient material for analysis of particulate C, N, and P (Figure 1). Eggs produced by copepods that had grazed in the 5th bottle and incubated for 48 h were preserved in 4% formalin and the number of nauplii and unhatched eggs were counted microscopically to determine egg viability.

4.5. Analytical

Dissolved nutrient analyses were conducted on a Technicon AutoAnalyzer II according to Lane et al. [93]. Particulate C and N were determined on an Exeter Analytical, Inc. (EAI) CE-440 Elemental Analyzer (North Chelmsford, MA, USA). Particulate P was extracted by dry ashing followed by acidification overnight [94]. Samples of diatom cells for grazing calculations were counted under a light microscope at $40\times$ magnification with a hemocytometer counting chamber.

4.6. Data Analysis

Grazing rate calculations were performed according to Frost [95] (Table 2), for which it was assumed that k = 0 (prey growth rate) due to the short time frame (4 h) of the experiments. Excretion rate calculations were according to Miller and Glibert [65] (Table 2). Egg production rates (eggs female^{-1} day^{-1}) were calculated from the preserved samples by dividing the total number of eggs produced after 48 h by the number of females and the number of days. Rates of egg viability (%) were calculated as the number of nauplii produced as a percent of the total number of eggs and nauplii produced (*A. tonsa*) or as a percent of the number of fertilized eggs and nauplii produced (*E. carolleeae*).

One-way analysis of variance (ANOVA) tests were performed to determine differences in diatom cellular N:P, copepod nutrient content and ratios (C, N, and P, N:P), NH_4^+ and PO_4^{3-} excretion rates, and *E. carolleeae* egg production rates and egg viability across the prey N:P gradient. Unpaired t-tests were used to compare the following: tissue nutrient content between *A. tonsa* and *E. carolleeae* for the same nutrient (C, N, P) on the same experimental day (day 1 or 7); NH_4^+ and PO_4^{3-} excretion rates on day 1 to day 7; egg nutrient (C, N, P) content between the two copepods for the same nutrient on the same experimental day; egg nutrient (C, N, P) content between day 1 and day 7 for the same nutrient in the same copepod; and day-1 and day-7 egg production rates and egg viability. Regression analysis was performed to compare various copepod response parameters to the diatom nutrient ratios. Statistics were performed using Excel.

Table 2. Equations used for calculations of grazing and excretion rates.

Parameter	Equation	Detail and Definitions	Source
Algal growth rate, k	$C_2 = C_1 e^{k(t_2 - t_1)}$	C_2, C_1 = algal cell concentrations in control treatments at times t_2 and t_1	[95]
Grazing coefficient, g	$C_2* = C_1 * e^{(k - g)(t_2 - t_1)}$	C_2*, C_1* = algal cell concentrations in experimental treatments at times t_2 and t_1	[95]
Average cell concentration, <C>	$\langle C \rangle = \frac{C_1 * \left[e^{(k - g)(t_2 - t_1)} - 1 \right]}{(t_2 - t_1)(k - g)}$	as above	[95]
Clearance rate, F	$F = Vg/N$	V = volume (mL) in experimental bottle N = number of copepods g as above	[95]
Ingestion rate, I	$I = \langle C \rangle * F$	as above	[95]
Excretion rate, ER	$ER = \frac{(\Delta Ct - \Delta Cc) * V}{T * N}$	ΔCt, ΔCc = change in concentration of the nutrient (NH_4^+ or PO_4^{3-}) in the treatment and control bottles V = volume of the bottle T = duration of incubation N = numbers of grazers	[65]

Responses are reported relative to the N:P of the media for the diatom metrics or N:P of the prey for the zooplankton metrics.

5. Conclusions

In all, both copepods altered their somatic chemical composition, excretion rates, egg production, and egg viability in response to prey of variable chemical composition, but to different degrees. Notably, *A. tonsa* responded to feeding on P-poor prey (prey with high N:P ratio) by excreting more N, and producing fewer eggs with decreased viability compared to feeding on P-rich prey. However, the viability of *A. tonsa* eggs was always greater than the viability of *E. carolleeae* eggs when feeding at the same prey N:P ratio. *E. carolleeae* responded to feeding on P-poor prey (prey with high N:P ratio) by excreting less N, and producing more eggs with decreased viability compared to feeding on P-rich prey. However, *E. carolleeae* always produced more eggs than *A. tonsa* feeding at the same prey N:P ratio.

These results suggest that changing nutrient ratios may affect the nutritional quality of food for copepods, thereby affecting their tissue elemental stoichiometry, excretion, and reproductive success which has implications for nutrient release to the environment and food webs in systems undergoing nutrient change. Further study on the impacts of shifting nutrient supply on consumers including evaluation of mixed diets, the interaction of food quality and quantity, and impacts on food webs will be valuable. 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. Furthermore, 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 and microzooplankton vary their N:P content in response to varying available nutrient (but see for example [14–16]). Finally, these food quality experiments were conducted at saturating C prey content. The synergistic effects of poor quality and quantity on copepod growth has been shown (e.g., [32]), but much is yet to be understood in

terms of the trophic consequences of these factors in systems undergoing rapidly changing nutrient regimes.

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