STUDIES ON THE STOICHIOMETRY OF SEXUAL DIMORPHISM IN *HYALELLA* AMPHIPODS

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Abstract: Understanding the causes and consequences of variation in across multiple levels of biological organization is a central goal in modern biology. Research integrating key ecological and evolutionary processes necessitates a framework from which phenomena at multiple levels can be tested. Ecological stoichiometry (ES) can serve as a useful integrative framework to both quantify variation at multiple levels and understand the mechanisms that underlie this variation. ES abstracts biological processes down to their constituent atoms of elements to understand ecological and evolutionary patterns. While much work in ES has focused on the causes and consequences of interspecific differences in stoichiometry, little is known about substantial intraspecific variation. Sexual dimorphism is perhaps the most striking example of intraspecific variation in many species, at least at and above the molecular level. Almost nothing is known about dimorphism at the elemental level, and how this dimorphism may drive sex-specific responses to key ecological parameters, such as environmental supply of biogenic elements. In this dissertation, I explore sexual dimorphism on the elemental level, the sex-specific physiological mechanisms organisms use to respond to changing resource availability, and the population-level effects of resource availability on patterns of sexual selection in *Hyalella* amphipods. To that effect, I first quantified sex-specific plasticity in phosphorus (P) content of *Hyalella* amphipods to changes in environmental P supply. I found that the sexes differ in their plasticity of body P content in response to P availability, with male composition behaving plastically and female composition remaining relatively canalized. Second, I used radiotracers to identify age- and sex-specific nutrient processing strategies in response to differences in dietary P. I found both age- and sex-specific patterns of acquisition and assimilation of C and P that are altered by dietary P, as well as preferential allocation to exaggerated male sexual traits relative to nonsexual traits. Third, I used microcosms to quantify population-level responses to environmental P supply and mating trials to explore the interaction between availability of P during development and during mating in influencing mating behavior. No evidence for effects of P on population dynamics were observed, yet mating behavior was influenced by P availability. Finally, understanding that organisms are composed of many elements beyond C, N, and P, I quantified sex-specific and trait-specific composition of 12 mineral and trace elements. Substantial differences between the sexes and traits in elemental composition in multiple dimensions were observed, suggesting potential importance of these elements in sexual dimorphism. Together, these studies highlight the importance of sex-specific responses to variation in the environmental supply of key elements, and the utility of ES in understanding the eco-evolutionary mechanisms that shape sexual dimorphism and selection.

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INTRODUCTION

Biological diversity is a central tenet of both ecology and evolutionary biology. While both of these disciplines place importance on diversity, until the development of evolutionary ecology, integration of these two disciplines was somewhat lacking. This lack of integration between disciplines is likely the result of the both horizontal and vertical fragmentation that is present in most biological disciplines. Specifically, horizontal fragmentation occurs when phenomena are well studied in one taxa, often model organisms, but little effort has been made to assess the generality of those findings. Vertical fragmentation, on the other hand, is the result of a lack of integration of phenomena on one level of organization to either higher or lower levels of organization, often due to the complexities involved in generalizing across levels. Evolutionary biology, since the introduction of the Modern Synthesis (Huxley 1942), has been dominated by a population genetics view of evolution, with variation arising primarily from mutation and recombination. Additionally, evolution is often studied in specific model systems, due to their ease of use within laboratory settings. On the other hand, the role of intraspecific variation within populations has often been overlooked in ecology, especially ecosystem and community ecology. As a result, very little horizontal or vertical integration occurs in either of these disciplines, hindering our ability to fully integrate the two.

Any integration of ecology and evolutionary biology must consider the role of the environment on shaping intraspecific variation, as well as the role of evolutionary change on ecological processes. Recently, many researchers have begun focusing on the importance of the environment in acting not only as a filter of existing phenotypic variation, but as a producer of this variation (West-Eberhard 2003; Pigliucci and Muller 2010). Additionally, with the realization that evolution often occurs on ecological time scales (e.g., Hairston *et al.* 2005), the importance of evolutionary change on ecosystem-level processes has been acknowledged (Post and Palkovacs 2009; Schoener 2011). Although this newfound appreciation for the links between ecology and evolutionary biology has resulted in a wide array of new and exciting studies, a full integration is substantially inhibited by the incredible degree of complexity in phenotypic variation (see, Houle *et al.* 2010) and the environment.

Ecological stoichiometry (ES) provides a useful framework for integrating ecology and evolutionary biology. On a fundamental level, an organism's fitness is the result of its ability to acquire resources from the environment and allocate those resources to fitness-maximizing traits, such as reproduction or growth (van Noordwijk and de Jong 1986; Stearns 1989; de Jong and van Noordwijk 1992). It follows that variation in organismal fitness is, in part, driven by both organismal demand for resources as well as environmental supply of those resources (Kay et al. 2005). At its heart, ES abstracts all biological processes into atoms of elements, traditionally carbon (C), nitrogen (N), and phosphorus (P), and examines the ecological and evolutionary consequences of the mismatch between environmental supply of elements and organismal demand (Sterner and Elser 2002). This abstraction of biological complexity down to the elemental level allows for a reduction of that complexity into a finite number of traits of interest. Additionally, because the stoichiometry of protoplasmic life is somewhat common, ES uses a common currency for which researchers can explore patterns across taxa, as well as fundamental links between ecosystem-level processes and phenotypic evolution (Elser and Hamilton 2007). Given that the supply of elements in the environment is often highly variable both spatially and temporally (Schlesinger 1997), ES is a valuable, ecologically-explicit framework from which to examine phenotypic evolution.

Most studies examining evolution from an elemental perspective have focused on examination of interspecific elemental variation and the life history differences between species that may underlie this variation (e.g., Elser et al. 1996; Quigg et al. 2003; Woods et al. 2004). While these studies have provided a cornucopia of empirical evidence for phylogenetic diversification of organismal stoichiometry, intraspecific variation within populations in stoichiometry is often ignored. Such lack of attention to intraspecific stoichiometric variation makes the paradigm incompatible with evolutionary biology. That is, there is no provision for the potential of organismal stoichiometry to evolve (Jeyasingh et al. 2014). Recent studies have shown that organismal stoichiometry does vary within populations, often as much or greater than the variation between species (Watts et al. 2006; Bertram et al. 2008; González et al. 2011; El-Sabaawi et al. 2012). The sources and mechanisms that underlie this variation, however, have thus far not been explicitly elucidated.

In sexual organisms, sexual dimorphism is a significant source of intraspecific variation. The sexes, despite sharing the same genome and habitat, are often highly divergent in their phenotypic characteristics. Often, sexual dimorphism is manifested as exaggerated male secondary sexual traits (e.g., deer antlers, beetle horns, peacock feathers). These traits are the result of strong directional sexual selection, and males possessing greater trait values are often the most successful at acquiring mates (Andersson 1994). Additionally, theoretical models suggest that these traits are costly to build and maintain, and are tightly coupled with individual variation in quality (i.e., condition dependence; Zahavi 1975; Andersson 1986, 1994; Rowe & Houle 1996). In addition to variation in male quality due to genetic variation, individual condition, and by extension exaggerated trait values, are strongly influenced by the environment (Cotton et al. 2004; Kokko et al. 2006). It follows that sex-specific fitness optima, along with the substantial cost of exaggerated traits, should result in sex-specific nutritional demands and divergent strategies to meet those demands (Morehouse et al. 2010). Because exaggerated traits represent incredible phenotypic divergence between the sexes and are hypersensitive to environmental heterogeneity, a focus on the interaction between ecology and the evolution of these traits can provide useful insights into the mechanisms that underlie sex-specific evolutionary trajectories. Indeed, a complete understanding of population diversification and speciation necessitates exploration into the interactions between sexual selection and environmental heterogeneity (Maan and Seehausen 2011).

Questions

To address, on the elemental level, the eco-evolutionary mechanisms underlying sexual dimorphism and selection, some important questions must be explored. First, do the sexes differ in their elemental demands? Second, are sex-specific elemental demands influenced by environmental heterogeneity in resource supply? Third, are such potential sex-specific differences in demand and response to the environment manifested at the levels of elemental acquisition, assimilation, and/or allocation? Fourth, how do these nutrient processing strategies change as organisms sexually mature? Finally, are there population-level consequences of environmental heterogeneity in elemental supply on various aspects of mating biology?

The above questions are addressed in Chapters 1-3 of this dissertation. In these chapters, I explore the effects of just one elemental parameter, phosphorus (P), on sexual dimorphism and selection in the freshwater amphipod genus *Hyalella*. Amphipods in the genus *Hyalella* are an ideal model for testing aforementioned predictions for two reasons. First, *Hyalella* males possess remarkably exaggerated, condition-dependent claw-like appendages called posterior gnathopods (Cothran and Jeyasingh 2010). Those individuals possessing larger posterior gnathopods relative to body size enjoy higher mating success (Wellborn 1995; Wellborn and Bartholf 2005; Cothran 2008; Cothran et al. 2010). Second, the distribution of *Hyalella* includes the majority of freshwater ecosystems throughout North America (Bousfield 1958), indicating that *Hyalella* amphipods likely experience a wide spectrum of spatiotemporal variation in elemental supply. Male posterior gnathopod expression has been shown to be hypersensitive to environmental phosphorus (P) supply, relative to similar, non-sexual traits (Cothran et al. 2012).

A major reason for the focus on P is that this element is important for many biological structures (Westheimer 1987). Higher P content has been hypothesized to confer to higher growth rates, due to an increased concentration of P-rich rRNA within growing organisms (Elser et al. 1996; Sterner and Elser 2002). Additionally, P supply varies both within and across aquatic ecosystems and is increasing drastically in many parts of the world due to human activities (Smil 2000; Wetzel 2001; Schindler et al. 2008; Hale et al. 2013). Furthermore, changes in P supply invoke gene-by-environment interactions in fitness-relevant traits (Jeyasingh and Weider 2005; Jeyasingh et al. 2009), affect organismal P and C use (Frisch et al. 2014; Roy Chowdhury et al. 2014), result in widespread transcriptional changes (Jeyasingh et al. 2011; Roy Chowdhury et al. 2014; 2015), and are known to influence microevolutionary trajectories (Weider et al. 1997, 2008; Frisch et al. 2014), making P supply an important parameter for determining organismal condition.

The final chapter of this dissertation, Chapter 4, revisits the first question posed above—is there sexual dimorphism in elemental demand?—and explores *Hyalella* sexual dimorphism in elemental demand in multiple elemental dimensions. Although we observed substantial effects of P supply on various aspects of *Hyalella* biology, a focus on just one element potentially masks the importance of trace elements, and the interactions among elements, in the mechanisms underlying sexual dimorphism and sexual trait variation. Considering that there are ~25 elements with known biological functions (Frausto da Silva and Williams 1991), a limited focus on one or a few of these elements precludes a complete understanding of biological processes at the elemental level, and indeed may result in a 'black box-ing' of many important interactions between ecology and phenotypic evolution. Given that the biogeochemical cycles of many elements are rapidly changing due to anthropogenic activities, knowledge gained from a complete stoichiometric view of ecology and evolution is potentially critical for an understanding of biological diversity in the Anthropocene.

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CHAPTER I

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SEX-SPECIFIC PLASTICITY IN BODY PHOSPHORUS CONTENT OF *HYALELLA* AMPHIPODS

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Abstract

Understanding the evolution of sexually dimorphic traits requires knowledge of the genetic and environmental sources of variation. However, we know surprisingly little about how the sexes differ in their responses to environmental nutrient supply. Here, we investigated how phosphorus (P) availability, a key metric of eutrophication, affects body composition in each sex of two *Hyalella* amphipod species. We also examined whether differences in food preference and acquisition are responsible for observed variation in body P. We discovered environmentally-driven changes in body P that were dependent on both species and sex. In both species, males contained less P when raised in low-P laboratory conditions compared to high-P field environments, while females exhibited no significant differences. Importantly, this difference was greater in the species that is known to have larger sexual traits and higher growth rates. Variation in P content was not due to differences in acquisition of P because both sexes preferred high-P food and consumed it at a similar rate. Our study illuminates potentially important sex- and species-specific evolutionary consequences of rapid alterations to P availability due to cultural eutrophication.

Introduction

Human activities have strongly impacted many ecosystems throughout the world. Aquatic ecosystems have been particularly affected by cultural eutrophication caused by agricultural activity. The advent of artificial fertilizers has played a pivotal role in human population growth (Smil, 1999), leading to profound environmental alterations (Smil, 2000). Notably, synthetic phosphorus (P) fertilizer has been used to augment global agriculture for the past 150 years (Brown, 2000). Consequent runoff of P into lakes is a major cause of cultural eutrophication (Schindler et al., 2008). A common effect of excessive P loading is the increase in P content of seston (i.e. planktonic living and non-living matter; Hessen et al. 2002) which can alter the nutrition of aquatic consumers (Sterner & Elser, 2002). These changes in P content of consumer diet alter the development of traits, often with fitness consequences (Jeyasingh and Weider 2005; Bertram et al. 2006; Boersma and Elser 2006; Jeyasingh and Weider 2007; Bertram et al. 2009; Jeyasingh et al. 2009; Cothran et al. 2012).

When examining the fitness consequences of nutrient supply, it is important to examine intraspecific differences in responses to the environment. Arguably the greatest source of intraspecific variation is sexual dimorphism. Sexual dimorphism allows the sexes to use different traits or enhancements of traits to employ different reproductive strategies or, less commonly, to decrease competition by exploiting different resources (Shine 1989; Andersson 1994). Sexual dimorphism implies differences in the material composition of the sexes, and thus they should have distinct nutritional demands (Morehouse et al., 2010). Numerous studies have documented striking differences in the material composition of the sexes (Tarnopolsky and Saris 2001; Raymond and Himmelman 2004) and such compositional differences are often reflected in food choice (Clarke et al. 1998; Ruckstuhl 1998; Beck et al. 2007; Maklakov et al. 2008).

Because P supply is much lower than P demand in most biota (Westheimer, 1987), and P availability is positively related to the expression of sexually dimorphic traits (Bertram et al. 2006, 2009; Cothran et al. 2012), it is likely that P is a limiting resource that underlies honest signaling of male quality (Morehouse et al. 2010). Furthermore, previous studies have shown that P influences female oogenesis (Visanuvimol & Bertram, 2010), resulting in higher P content in females relative to males (Markow et al., 1999). Nevertheless, we know very little about sex-specific responses to P supply. The P content of the sexes could differ constitutively, plastically, or show no difference, and body P content should be driven by the ability of individuals to acquire and process P. Differences in body P content and P processing physiology should have dramatic effects on fitness optima of the sexes, causing one sex to be more dramatically affected by environmental heterogeneity than the other. Finally, while P is important for sexual traits, it also is important for other traits such as growth (Elser et al., 1996) that can be under different patterns of natural selection, especially in aquatic invertebrates (e.g., Wellborn et al. 1996). Thus, sexual differences in P content and responses to P supply should vary depending on the life history differences between closely related species.

Amphipods in the genus *Hyalella* are an ideal system to examine sex differences in P composition and sensitivity to P supply. Because of their vast geographic range, these amphipods inhabit most freshwater ecosystems, and experience a wide variety of nutritional environments (Bousfield, 1958). Laboratory experiments have shown that *Hyalella* obtain most of their nutrition by grazing on periphyton such as diatoms and bacteria, although much is still unknown about their diets in the wild (Hargrave, 1970). *Hyalella* amphipods in North America represent a complex of undescribed species that vary in morphology and life history (Wellborn et al. 2005; Witt et al. 2006; Wellborn and Broughton 2008). The distribution of species in the complex is based primarily on the strength of fish predation (Wellborn, 1994a). Large ecomorph species live in habitats with little or no fish predation. Larval odonates are common predators in these habitats and these predators typically prefer smaller prey and thus select for higher growth rates (Wellborn 1994a, b), which is a P-demanding trait (Elser et al. 2003). In contrast, small ecomorph species are found in habitats with fish, which prefer larger prey, and thus select for lower growth rates.

Large and small ecomorphs also differ in patterns of sexual selection. Although larger males that possess larger posterior gnathopods (PGs; large, claw-like appendages) are generally more successful in obtaining mates, this pattern is much stronger in the large ecomorph than in the small ecomorph (Wellborn, 1995, 2000; Wellborn & Bartholf, 2005). The PG is more sensitive to low P availability than other morphological traits (Cothran et al. 2012). Despite our knowledge of how P availability affects PG expression, it is unclear whether this translates into sex differences in P content.

In this study, we examined how each sex of *Hyalella* amphipods responds to changes in environmental P availability and whether food preferences and differences in acquisition underlie these responses. Specifically, we tested for sex-specific differences in body P content, with the prediction that males would be higher in P content than females because large PGs are likely large resource sinks like other sexual traits (Lincoln, 1992; Andersson, 1994; Emlen & Nijhout, 2000). Second, we tested whether environments with different concentrations of P induced sex-specific plasticity in body P content. Because males were found to be more sensitive in their morphological response to changes in P supply (Cothran et al. 2012), we predicted that such sensitivity would be reflected in P content to a greater extent in males than females. If males are more sensitive to environmental P than females, then we predict that males will show greater preference for P-rich food or acquire P at a faster rate compared to females to optimize their resource intake. Our study tested these predictions in two amphipod species with different life histories, one with rapid growth and another with selection against rapid continual growth (Wellborn, 1994a, 1994b; Wellborn et al., 2005; Wellborn & Broughton, 2008). Because P is important for growth, we further tested the prediction that P content of males from the large ecomorph, which exhibits prolonged, rapid growth should respond more strongly to dietary P supply.

Materials and Methods

Survey of P composition in the bodies of field-collected and lab-raised amphipods

Our first goal was to quantify the body P composition of the sexes for the two ecomorphs collected directly from field environments or collected in field and then raised in laboratory environments. Both of the field environments (Lake Thunderbird, Cleveland County, OK; Lake LeBoeuf, Erie County, PA, USA) are classified as eutrophic lakes with total phosphorus measurements exceeding 40µg/L (OWRB 2005; Butkas and Ostrofsky 2006). Eutrophic lakes have carbon:phosphorus ratios typically lower than 100

(Sterner et al., 2008), indicating that these lakes have high P availability, although we did not directly measure P availability in the lakes. It is well known that the P content of autotrophs tracks inorganic P supply especially in aquatic ecosystems (Elser et al., 2000). Thus, it is likely that field-collected amphipods were experiencing a high P diet.

Large ecomorph amphipods (species OK-L in Wellborn and Broughton 2008) were collected from Lake Thunderbird in October 2011 and raised in the laboratory using 10-L plastic tubs containing water treated with Tetra Aquasafe® (Tetra Werke, Melle, Germany) and spiked with 1000 µM $NaNO_3$ and 5µM KH_2PO_4 to promote periphyton growth. Matala® aquatic filter media were added to each tank to provide cover. Animals were housed in a temperature-controlled room (mean ± SD = 20 ± 1°C) with a 16:8 day:night cycle. The animals were fed twice weekly with a 2:1:1 mix of ground rabbit pellets, Tetramin® fish flakes (Tetra Werke, Melle, Germany), and Spirulina (Nutrex Hawaii Inc., Kailua-Kona HI, USA) that was suspended in Bacto agar. The carbon:phosphorus ratio of periphyton in laboratory tanks (mean ± SD = 611 ± 229), and agar-based pellets (mean ± SD = 351.6 ± 30.0) was high, indicating a P-limited diet for amphipods with body stoichiometry (mean ± SD) of 140.6 ± 31.3 (Frost et al., 2006). Our eutrophic field environments, however, likely exhibit C:P ratios of less than 100 (Sterner et al., 2008). Stock animals were raised in the laboratory for 7 months to ensure that only individuals born and raised in the laboratory were used for elemental analysis. To assess the elemental composition of field animals, additional large ecomorphs were collected from Lake Thunderbird in April 2012.

Small ecomorph amphipods (species C in Wellborn and Cothran 2004) were collected from Lake LeBoeuf (Erie County, PA, USA) in June 2011 and raised in a 90-L outdoor wading pool. These animals were subsequently overwintered in a single 1000-L cattle watering tank filled with 800 L of well water and then placed back into a 90-L wading pool the following spring. Thus, these animals had been exposed to wading pool conditions for several generations and elemental composition was assayed on animals that were born and raised in the wading pool. The pool was filled with approximately 80 L of well water, inoculated with algae from Lake LeBouef, and provided with approximately 13 kg of washed sand. We added 0.968 μM P (as KH_2PO_4), and 53.55 μM N (as $NaNO_3$) to promote algal growth. To assess the elemental composition of field animals, additional small ecomorphs were collected from Lake LeBoeuf in June 2012.

We acknowledge that the two ecomorphs were raised in very different laboratory conditions prior to estimation of body elemental content. Large ecomorph amphipods were housed indoors in multiple containers, while small ecomorphs were reared in a single, outdoor wading pool. Thus, direct comparisons between the ecomorphs in P content should be interpreted with caution because it is likely the P environments vary between the two species. However, our design allows for robust comparisons of P content within ecomorphs (i.e., sex-specific, and environment-specific effects).

To assess body P content, all amphipods were sexed at 4X magnification using a Swift SM90 stereo microscope and then dried individually in a 60°C incubator for 48 hrs. To control for the effect of female reproductive stage on body elemental composition, only females with clearly visible eggs in their ovaries were selected. This ensured that all females used were undergoing the same stage of oogenesis, a particularly P-intensive process for females (Markow et al., 1999, 2001). Twenty-three large ecomorph males, 24 large ecomorph females, and 8 of each sex of small ecomorph amphipods from each environmental group (i.e. lab and field) were analyzed for phosphorus content. A modified sulfuric acid digestion method (APHA, 1992) was used to estimate %P, and verified with a spinach standard (NIST 1570a).

Because direct species comparisons cannot be made, we ran separate two-way ANOVAs of sex and environment for each species. Statistical analyses were conducted using R 2.14.1 (R Foundation for Statistical Computing).

Food-choice experiment

To determine the extent to which species- and sex-specific variation in P acquisition drives differences in body P composition, we gave amphipods a choice between high-P (HP) and low-P (LP) food. All of the food-choice experiment trials were performed at the University of Pittsburgh. Small ecomorph and large ecomorph animals were collected from Lake LeBoeuf and Lake Thunderbird, respectively. Both ecomorphs used in the following experiment were raised in the laboratory under identical conditions, in 14-L tubs filled with carbon-filtered and UV irradiated water. Sand was provided as a substrate while waterweed (*Elodea* sp.) and fake macrophytes made of polypropylene rope served as refugia for amphipods. The tubs were kept in a temperature-controlled (mean ± 1 SD: 22.2 ± 0.2) room with a 16:8 day:night cycle. Amphipods were fed a 3:1 mixture of ground Tetramin® fish flakes and alfalfa (Spring Valley, Bohemia, NY, USA), supplemented with 0.5 grams of Spirulina and 20 mL of high-phosphorus *Scenedesmus* algae suspended in 20 mL Bacto®-agar solution three times a week. Large and small ecomorphs were kept under laboratory conditions for at least 3 and 7 months, respectively, prior to behavioral testing. Given a time to maturity of ~ 21 d, this assured that all adults tested were descendants of wild-collected adults and raised under identical laboratory conditions (Wellborn & Bartholf, 2005).

For each sex-by-ecomorph combination we gave groups of five amphipods a choice between HP and LP foraging stations and quantified the proportion of each foraging station consumed by the amphipods. To account for the large variation in size in the large ecomorph and avoid biases due to exploitative intraspecific competition (Wellborn, 1994b), we also tested for size effects on foraging preferences using two size classes for each sex, using head length an indicator of body size [mean ± 1 SD mm]: small males: 0.58 ± 0.07, small females: 0.59 ± 0.06, large males: 0.83 ± 0.09, large females: 0.83 ± 0.063. For the large ecomorph, we performed 17 foraging trials for each sex. For the small ecomorph, we performed 7 and 12 foraging trials for females and males, respectively.

Experimental units were 48-ml plastic cups containing carbon-filtered, UV-irradiated water, and washed play sand. Each cup housed two 1-cm^2 Nitex mesh foraging stations (mesh size = 1 mm) containing green algae (*Scenedesmus* sp.) grown under conditions with either low (5.94 μM P) or high added inorganic phosphorus (59.37 μM P) concentrations (Kilham et al., 1998) and then mixed with 20 mL of Bacto®-agar. These manipulations assured that P availability between our two treatments was much different. To confirm that our algae manipulations were effective, we analyzed carbon using an elemental analyzer (Elementar, Hanau, Germany) and phosphorus via sulfuric acid digestion. Algae reared under different P conditions differed in %P content (mean ± SD; LP, 0.044% ± 0.012%; HP, 0.061% ± 0.008%; t_5=1.989, P=0.05), but not in %C content (LP, 39.6 ± 0.9%; HP, 39.1% ± 0.5%; t_5=0.746, P=0.489).

Amphipods were starved for 4.5 to 6 h in 48-mL plastic cups filled with filtered water to clear their guts (Hargrave, 1970). Each trial was terminated after approximately 40 to 60 percent of the total food available was foraged. Foraging stations were removed and digitized using an Olympus SZX16 microscope fitted with a DP25 digital camera (Olympus America, Center Valley, PA). We quantified the proportion of each food type consumed by counting the number of empty squares on the mesh and dividing by the total number of squares. Although there was some variation among foraging stations in the number of 1 mm mesh squares, this did not differ statistically between food types (large ecomorph: paired $t_{33} = 1.878$, $P = 0.07$; small ecomorph: paired $t_{15} = 1.3$, $P = 0.215$). Eight control foraging arenas, without amphipods, were set up to confirm that absence of food from the squares was due to consumption by amphipods.

Separate experiments were performed for the large ecomorph and small ecomorph; therefore, we analyzed the data for each ecomorph separately. For each ecomorph-by-sex combination (and for size in large ecomorph), we used a Wilcoxon signed-rank for paired samples to test whether amphipods preferred HP algae over LP algae. We then calculated the difference between the proportion of LP and HP food consumed in each replicate and applied a Mann-Whitney U-test to test for sex differences, and for the large ecomorph size differences, in the magnitude of preference for HP food. Finally, for each experimental unit we divided the number of HP squares consumed by the number of days amphipods foraged to assess acquisition rates (number of squares consumed per day) of HP algae. We used a Mann-Whitney U-test to test for sex differences, and for the large ecomorph size differences, in acquisition rates. All statistical analyses for the foraging experiment were conducted using IBM SPSS

Statistics 20.

Results

P content of field-collected and lab-raised amphipods

For the large ecomorph, we found that body P was affected by sex, environment, and their interaction (Table 1). When we compared the two environments, we found that male body P decreased by 26% when raised in the laboratory compared to animals collected from the field, whereas females did not differ (Fig 1a). When we compared the two sexes, we found that females had higher body P when raised in the lab but there was no difference between the sexes in %P when collected from the field.

For the small ecomorph, we found that body P was affected by the environment, but not by sex or the sex-by-environment interaction (Table 1). Across both sexes, %P decreased by 7% when amphipods were reared in the laboratory compared to amphipods collected from the field (Fig. 1b).

We acknowledge that our sample size for the small ecomorph is small (N=32). Therefore we conducted a post-hoc power analysis using a small, medium, and large effect size (0.2, 0.5, and 0.8, respectively). This power analysis yielded powers of 0.194, 0.779, and 0.992 for the small, medium and large effect sizes, respectively. The effect size for the large ecomorph was 0.569, falling between the medium and large effect sizes in the power analysis. Given this information, we had a sufficient small ecomorph sample size (giving us a power > 0.8) to uncover an effect of similar magnitude to that observed in the large ecomorph.

Food-choice experiment

For both ecomorphs, males and females strongly preferred HP algae to LP algae, as did both size classes of the large ecomorph (Table 2; Fig. 2). However, the strength of this preference did not differ between the sexes in either ecomorph (small ecomorph: Z = -0.844 *P* = 0.398; large ecomorph: Z = -0.753, *P* = 0.452) or size class in the large ecomorph (females: Z = -1.092, *P* = 0.275; males: Z = -0.2, *P* = 0.842).

We also found no evidence for sex differences in acquisition rates of HP food (small ecomorph: Z = -1.504 *P* = 0.152; large ecomorph: Z = -0.919, *P* = 0.663). However, large females of the large ecomorph consumed HP food at a higher rate than small females (females: Z = -2.209 *P* = 0.027); a similar pattern was found in males, although it was not significant (Z = -1.66, *P* = 0.109). For both large ecomorph sexes, large amphipods consumed on average four more HP food squares per day than small amphipods (mean ± 1 SD squares consumed per day: large females = 12 ± 3, small females = 8 ± 3, large males = 11 ± 6, and small males = 7 ± 3). Male and female small ecomorph amphipods consumed on average 5 ± 2 and 7 ± 3 HP food squares per day, respectively.

Discussion

We found that two *Hyalella* ecomorphs with different life histories exhibited divergent sex-specific plasticity in P content in response to rearing environment. Specifically, large ecomorph males exhibited plasticity in P content in response to rearing conditions (i.e. lab-reared or field-caught) while no such plasticity was observed in large ecomorph females or either sex of the small ecomorph. In addition, a food choice experiment revealed that such sex-specific environmental responses do not cause different foraging behaviors. Although interspecific variation in body stoichiometry is often explained by broad phylogenetic differences (Fagan et al., 2002; Jaenike & Markow, 2003; Woods et al., 2004), the two ecomorphs used in this study were once considered the same species, *Hyalella azteca*, until recent molecular and life history studies uncovered substantial species diversity within the group (Witt et al. 2006; Wellborn and Broughton 2008). This suggests that even species that are morphologically quite similar can have divergent and environmentally responsive P content.

Phosphorus content of both species of amphipods was dependent on the environment. Amphipods raised in the laboratory fed primarily on periphyton growing in the tanks, supplemented with agar-based food pellets that were low in P, indicating a P-limited environment. On the other hand, both lakes from which animals were sampled are currently recognized as eutrophic (OWRB 2005; Butkas and Ostrofsky 2006), indicating high availability of P, and C:P ratios of autotrophs typically less than 100 (Sterner et al., 2008). Although we did not directly quantify P content of periphyton from these lakes, it is a safe assumption that our lab environment, with C:P ratios between 350-600, was considerably P poor than these eutrophic lakes. Thus, it is likely that variation in P supply, and associated shifts in composition of macromolecules (e.g., classes of carbohydrates, lipids) in the diet of aquatic consumers (Jeyasingh et al., 2011) contributed to differences in elemental composition between laboratory-reared and field-caught amphipods. Furthermore, we acknowledge that other sources of variation (e.g., temperature, sunlight, community structure) in sex specific responses in P content to altered environments should be important, because our laboratory environment for the small species involved outdoor mesocosms. However, the design of this study to explore sex specific variation precludes us from isolating such sources of variation.

Species-level differences in how P content of the sexes responds to changes in dietary supply of P may be due to disparate patterns of sexual selection, yet further study is warranted that directly compares species collected from lakes with known resource C:P and reared under the identical laboratory conditions. In the large ecomorph, larger males have higher mating success, whereas this pattern is weaker in the small ecomorph and countered by positive size-selective predation by fish (Wellborn 1994a; Wellborn 1995; Wellborn and Bartholf 2005). Sexual selection on male body size in amphipods, which have indeterminate growth, is a combination of selection on growth rate and age. Growth rate is strongly associated with P content within organisms as P is a major component of the rRNA necessary to fuel protein synthesis (Elser et al. 1996). Therefore, we might expect higher P demand in large ecomorph males than small ecomorph males, which may explain the sensitivity of large ecomorph males to changes in P availability. Note that counter to predictions (Elser et al., 1996), we found that fast growing large ecomorph amphipods had lower P content. It is likely that the size-dependency of organismal P content drives this effect, because P content decreases with an increase in size as the fraction of rRNA in the body decreases relative to other P-rich molecules (Gillooly et al., 2005).

In addition to differences in growth rates, the importance of exaggerated sexual traits and their demand for P may explain ecomorph differences in sensitivity to the nutrient environment. Large PGs increase mating success in both ecomorphs, however, only large ecomorph males must also continue to invest P to fuel growth after maturation because of selection of smaller individuals by their predators and the resource competition advantages of large body size (Wellborn 1994a, 2002). Large ecomorph PGs also have steeper allometric slope than small ecomorph PGs probably because of the greater returns in mates for investment in PGs at larger size classes in the large ecomorph (Wellborn 1995; Wellborn and Bartholf 2005; Bonduriansky 2007; Cothran and Jeyasingh 2010). Further, a previous study by Cothran et al. (2012) has shown that PG expression is sensitive to low P availability. The greater demand for P to fuel high relative growth rates of PGs combined with competing demands for increasing body size may explain why large ecomorph males are sensitive to changes in the nutrient environment.

While body P analysis revealed sex-specific responses to rearing environment in the large ecomorph amphipods, we found no sex differences in preference or acquisition rate when amphipods were offered foods of different P content in either ecomorph (Fig. 2). Both sexes in each ecomorph preferred high-P to low-P food and these results were consistent across size classes in the large ecomorph. We did find significant differences in acquisition rates between the two ecomorphs, with large ecomorph amphipods acquiring food at a higher rate. These differences are to be expected, however, given the significant size differences between the ecomorphs, and previous work showing that the large ecomorph consumes algal resources at a higher rate (Wellborn, 1994b). These results show that amphipods are able to select food based on relative P content. The ability to choose food based on P content has been observed in *Daphnia* and is believed to be an important in mitigating stoichiometric imbalances between food and consumer (Schatz & McCauley, 2007). However, this ability is not sex-dependent, suggesting that sexual dimorphism is not driven by differential ability to discern food based on P content. Phosphorus availability has been shown to have many effects on the structure and biochemistry of algae (Tillberg & Rowley, 1989; Theodorou et al., 1991; Theodorou & Plaxton, 1993), and it is possible that the amphipods are showing preference for algal characteristics resulting from increased phosphorus. Further study is needed to examine the specific algal characteristics preferred by amphipods.

In summary, we found that closely related, and morphologically similar ecomorphs have divergent body P content. Further, P content was plastic but only in males of the large ecomorph while the P content of small ecomorph males, and females of both ecomorphs were not plastic. However, further studies are warranted in which lineages from multiple species are subjected to identical environmental treatments to examine plasticity in P content across species. Variation in P content is most likely driven by differences in the life history of the ecomorphs and sexes studied. Further, differences in body P content were not explained by differences in food choice or acquisition of P, and are most likely a function of differential processing of P. Elucidating the mechanisms underlying sex-specific responses to changes in the supply of key elements, such as phosphorus, should reveal much about how the environment can affect the evolution of freshwater organisms that inhabit environments experiencing major shifts in key abiotic parameters such as phosphorus loading.

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Tables

Table 1. Two-way ANOVA results showing the effects of environment, sex and their interaction on %P. *F* statistics, degrees of freedom and *P*-values are reported for each ecomorph. Bold values indicate significance at P<0.05

	Large ecomorph			Small ecomorph		
Source	*F*	*df*	*P*	*F*	*df*	*P*
Environment	7.113	1,90	**0.009**	12.788	1,28	**0.001**
Sex	41.503	1,90	<**0.001**	0.994	1,28	0.327
Environment*sex	30.552	1,90	<**0.001**	1.068	1,28	0.310

Table 2. Foraging preference results comparing the proportion of high P and low P algae squares consumed for each sex and also size class for the large ecomorph. In all cases, more high P squares were consumed than low P squares. Wilcoxon sign-rank *Z* and *P*-values are presented. Bold values indicate significance at P<0.05

Ecomorph	Sex	Size class	*Z*	*P*
Large	Female	Small	2.94	**0.003**
		Large	2.032	**0.042**
	Male	Small	2.923	**0.005**
		Large	2.371	**0.018**
Small				
	Female		2.521	**0.012**
	Male		2.366	**0.018**

Figures

Figure 1: Large (a) and small (b) ecomorph responses in male (*filled circles*) and females (*open circles*) in %P. *Markers* represent means and *error bars* ±1 SE

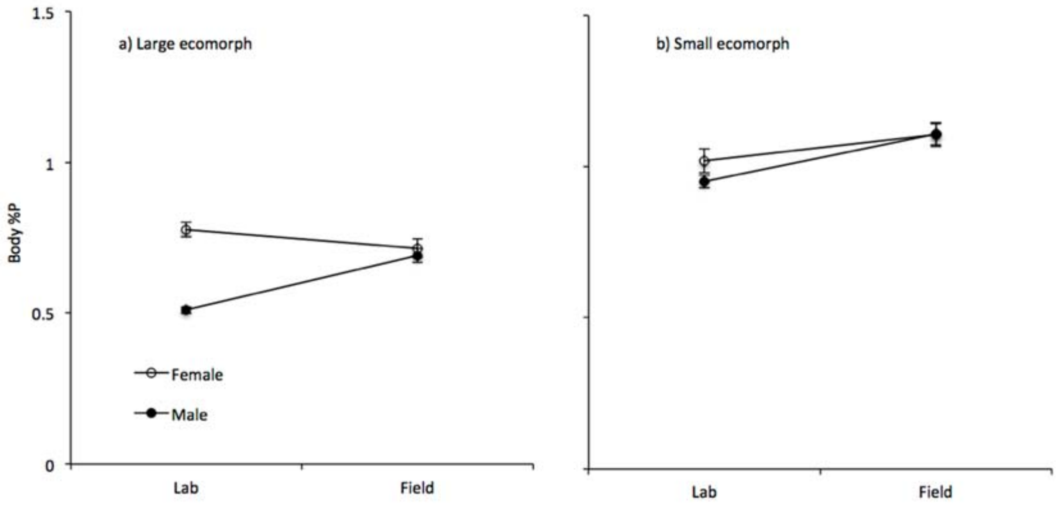
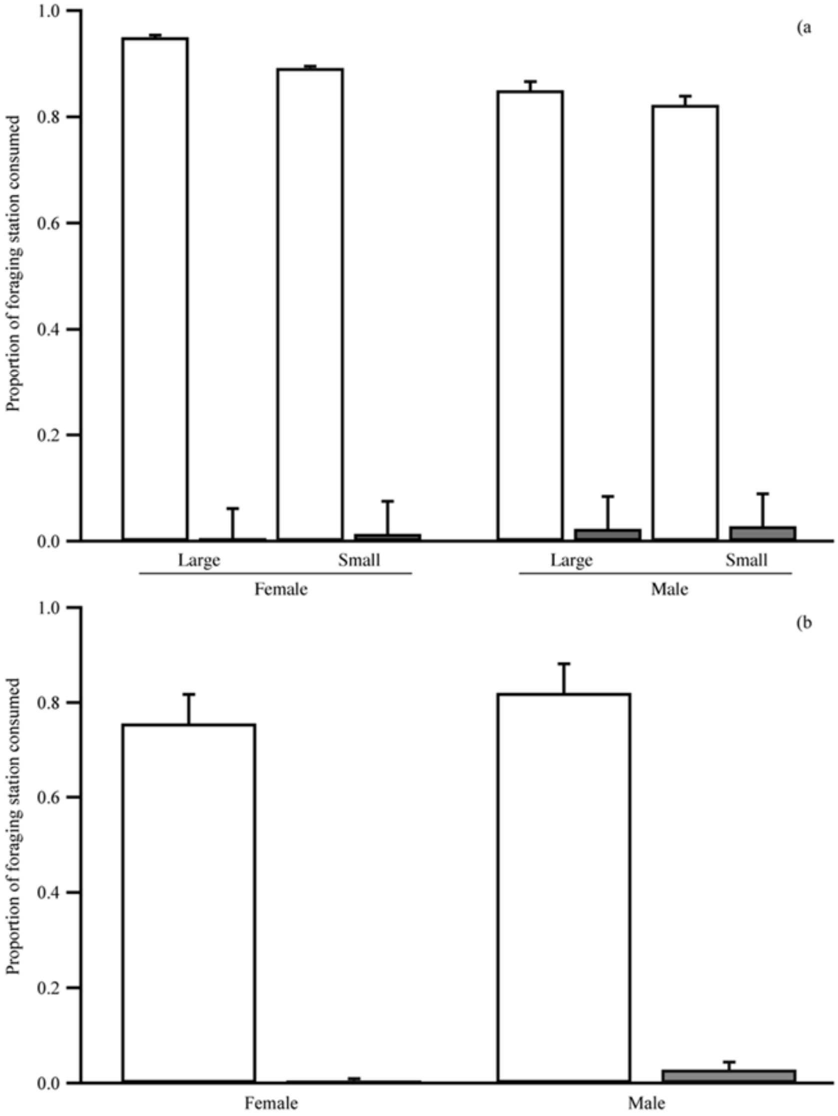


Figure 2: Proportion of high phosphorus (*white bars*) and low phosphorus (*gray bars*) foraging stations consumed by amphipods. Results for both sexes of a) large ecomorph and b) small ecomorph ar presented. The large ecomorph species was further split into small and large size classes. *Bars* represent means ±1 SE.



CHAPTER II

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SEX-SPECIFIC NUTRIENT USE AND PREFERENTIAL ALLOCATION OF RESOURCES TO A SEXUALLY SELECTED TRAIT IN *HYALELLA*

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Abstract

Although sexually dimorphic traits are often well studied, we know little about sex-specific resource use strategies that should underlie such dimorphism. We measured sex-specific responses in acquisition and assimilation of two fundamental resources, carbon (C) and phosphorus (P) in juvenile and mature *Hyalella* amphipods given low and high supplies of inorganic phosphate, analogous to oligotrophic and eutrophic conditions, respectively. Additionally, we quantified allocation of resources to sexual traits in males. Dual radiotracer (^{14}C and ^{33}P) assays revealed substantial age- and sex-specific differences in acquisition and assimilation. Furthermore, a phenotypic manipulation experiment revealed that amphipods fed low-P food allocated more C to all traits than those fed high-P food. Importantly, we found that amphipods preferentially allocated more C to the development of a sexually selected trait (the posterior gnathopod), compared to a serially homologous trait (the fifth pereopod) not under sexual selection. Substantial differences in how the sexes use fundamental resources, and the impact of altered nutrient supply on such differences illuminate sexual dimorphism at the lowest level of biological organization. Such information will be important in understanding how sex- and age-specific life history demands influence nutrient processing in a biosphere characterized by rapidly changing alterations to biogeochemical cycles.

Introduction

Sexual dimorphism accounts for a large portion of the phenotypic variation observed within species. Despite a similar genome, sexes differ extensively in behavior, morphology, and physiology. Often sexual dimorphism is manifested as the exaggeration of traits (e.g., deer antlers or widowbird tails; Andersson 1994). Exaggerated traits have long been thought to be costly, driving many sex-specific behavioral and physiological processes (Andersson, 1994; Emlen, 2001; Lincoln, 1992). Further, the expression of these exaggerated traits is often hypersensitive to organismal condition, defined as the pool of resources allocable to traits (Cotton et al., 2004; Rowe and Houle, 1996). This pool of allocable resources is necessarily influenced by both the environmental supply of those resources and genetic and/or plastic variation in acquiring, assimilating, and allocating resources, resulting in potential tradeoffs between sexual and non-sexual traits. Because sexually dimorphic traits are partially the result of sex-specific selection during development, studies on sexual dimorphism should not preclude potential ontogenetic effects (Badyaev, 2002; Badyaev, 2004). Variation in selective pressures due to sexual dimorphism has been theorized, and empirically shown, to elicit sex- and age-specific strategies for trade offs between life history traits (Bonduriansky et al., 2008; Hunt et al., 2004; Penn and Smith, 2007). With regard to nutrient use, however, little is known about sex-specific responses to changes in the supply of resources in the environment, and whether those responses vary as organisms develop (Morehouse et al., 2010; Snell-Rood et al., 2015).

The sexes can differ markedly in elemental composition, with these differences changing across different life stages (Back and King, 2013; Goos et al., 2014; Gorokhova and Hansson, 2000; Markow et al., 1999). Thus, supply of necessary elements in the environment could invoke sex- and/or age-specific element use strategies, particularly in species exhibiting high degrees of sexual dimorphism. For example, in many deer species, males exhibit age- and sex-specific foraging and nutrient use strategies, particularly in bone minerals (e.g., calcium and phosphorus), linked primarily to the demands of antler production (Atwood and Weeks, 2002; Banks et al., 1968; Cowan et al., 1968; Stephenson and Brown, 1984). The consequences of these sex-specific use strategies (e.g., seasonal osteoporosis during antler development) may be mitigated by the environmental supply of the minerals comprising antlers. On the elemental level, variation in foraging and nutrient use strategies represents changes in the acquisition, assimilation, and allocation of elements. Acquisition, assimilation, and allocation are each sensitive to changes in elemental supply, which is variable in both space and time (Elser, 2003; Sterner and Elser, 2002). Given that human influences on the supply of most biologically important elements have been dramatic (Schlesinger, 1997), examining the age- and sex-specific consequences of such biogeochemical shifts is an important step toward our understanding of the material basis of sexual dimorphism as well as our understanding of microevolutionary shifts in response to global change.

Because exaggerated traits, which are generally condition-dependent resource sinks, are most often found in males, one would expect male acquisition and assimilation strategies to be more sensitive to changes in elemental supply. Additionally, selection on these exaggerated traits should drive greater allocation of important elements to sexually selected traits compared to similar, non-sexual traits. Finally, the patterns observed in elemental processing are expected to change dramatically from juvenility to adulthood, and these shifts should be particularly noticeable late in ontogeny of males as they begin to mature and develop exaggerated, sexually selected traits.

We used a sexually dimorphic amphipod species in the *Hyalella azteca* species complex (the species is undescribed and is in clade OK-L in Wellborn and Broughton 2008) to examine potential sex- and/or age-specific effects of phosphorus (P) supply on acquisition and assimilation of P and carbon (C, representing ~50% of biomass; Sterner and Elser 2002), as well as the allocation of C to male sexually selected, claw-like appendages called gnathopods. This study focuses on the posterior gnathopods which are ~15 times larger in males than females, and can account for up to 10% of male biomass (Wellborn, 2000). Previous studies have shown that the exaggeration of gnathopods occurs late in juvenility (Kokkotis and McLaughlin, 2002). In addition, (Goos et al., 2014) have shown that females of our study species have higher P contents than males. Male P content, however, is more influenced by changes in dietary P supply (Goos et al., 2014), which is likely the result of changes in gnathopod growth under limiting P supply (Cothran et al., 2012; Cothran et al., 2014). As such, we hypothesized that males would exhibit greater plasticity in their acquisition and assimilation of C and P under contrasting P-supply conditions. Second, we hypothesized that age should shift the patterns of both acquisition and assimilation of C and P as elemental demand should vary between juveniles and adults (Villar-Argaiz et al., 2002). Age-driven shifts were predicted to be more pronounced in males as they begin developing exaggerated traits. Third, because exaggerated male sexual traits are presumed to be large resource sinks (Lincoln 1992;

Andersson 1994; Emlen 2001), we hypothesized that biomass allocation to these traits would be greater than to similar non-sexual traits. Additionally, we hypothesized that variation in P availability would induce greater plasticity in biomass allocation toward sexual traits compared to non-sexual traits.

Materials and Methods

To accurately measure sex- and age-specific variation in acquisition and assimilation of elemental resources, we used ^{14}C and ^{33}P radioisotopes. While estimates of acquisition and assimilation are possible using techniques that do not employ radiolabeling (e.g., weighing food before and after feeding, measuring C:P in food and feces, etc.), this technique directly measures atoms of each element that have been consumed. Additionally, because these isotopes become incorporated into biological tissue, we can directly measure isotopic activity after ingestion as a surrogate of assimilation. We assessed biomass allocation to a sexually dimorphic trait by quantifying the amount of assimilated ^{14}C in gnathopod tissue. In all experiments, P availability was manipulated by feeding amphipods either high-P (HP) or low-P (LP) leaf discs, which were radiolabeled with ^{14}C and ^{33}P. We digested all samples using an aqueous tissue solubilizer (Solvable, Perkin Elmer, Waltham, MA, USA), and measured radioactivity using a scintillation counter (LS 600SC, Beckman Coulter, Pasadena, CA, USA).

Study animals and housing conditions

Amphipods were collected from Ten Acre Lake in Oklahoma County, Oklahoma

(35°28’N, 97°15’W), and were housed in 5.7-L plastic tubs containing water treated with Tetra Aquasafe to dechlorinate the water (Tetra Werke, Melle, Germany). Washed pea gravel and aquatic filter media (Matala USA, California, USA) were added to each tub to provide substrate and refugia. Once each week, amphipod stock tanks were fed with one HP leaf.

Manipulation of leaf P content

Oak leaves were conditioned by soaking them in pond water in a 30-L plastic container for one month to allow natural periphyton growth. The container was kept indoors under natural light at 20-23°C, with constant aeration. After conditioning, the leaves were transferred to either HP (50 μM P) or LP (5 μM P) COMBO medium (Kilham et al., 1998). The leaves were left in the medium for two weeks, under the same environment as the conditioning phase, before being used for experiments. Media was changed weekly to ensure a continual supply of elements. To verify that our phosphorus treatments altered P availability in the leaves, we quantified the P content of a sample of leaves that had been dried at 60°C for 48 h with a modified sulfuric acid digestion method (APHA, 1992) that was verified using a spinach standard (National Institute of Standards and Technology 1570a, Gaithersburg, MD, USA). Phosphorus content of the leaves was greater in the HP leaves than in LP leaves (mean ± 1 SD; HP: 0.123% ± 0.033%, LP: 0.016% ± 0.009%; t_4= 6.562, p= 0.003). Additionally, to verify that carbon content of the leaves was not significantly affected by our P treatments, we dried a sample of leaves and quantified %C using an elemental analyzer (varioMicro Cube, Elementar Americas, Mt. Laurel, NJ, USA). There was no difference in C content of the leaves between P treatments (mean ± 1 SD; HP: 48.03% ± 0.340%, LP: 49.123% ±

0.920%; t_4= -0.132, p=0.904). Because it is possible that variation in P supply may influence the content of another important element, nitrogen (N), in the leaves, we also measured %N using the same elemental analyzer. There was no difference in N content of the leaves between P treatments (mean ± 1 SD; HP: 2.50% ± 1.44%, LP: 1.35% ± 0.13%; t_4= 1.132, p= 0.459).

Leaf radiolabeling

Radioisotope assays allowed us to examine element use on a per-atom basis. By introducing radioactive isotopes of both C and P (^{14}C and ^{33}P) to live food, the isotopes are incorporated into biologically available pools. After ingestion by an organism, it is possible to observe both quantity of radioisotopes acquired and precisely to which tissues these resources are allocated. Inorganic radiotracers, such as the ones employed here, are introduced to consumers primarily through ingestion of autotrophic periphyton (Carman and Guckert, 1994). As such, our radioassays provided a robust test of both ingestive and post-ingestive elemental processing strategies. To introduce the radioisotopes into the periphyton on the leaves, we first added HP and LP leaf discs into separate jars filled with 25 ml of COMBO containing no nitrogen or phosphorus. Each jar contained ten 7-mm leaf discs. We then added 0.925 MBq of ^{14}C (as bicarbonate) and 1.3875 MBq of ^{33}P (as orthophosphate) and placed each jar on an orbital shaker for 72 h. After this period, it was assumed that the periphyton on the leaves was radiolabeled uniformly (Hargrave, 1970; He and Wang, 2006). To determine radioactivity in the leaves prior to feeding, we selected five leaf discs and then rinsed and transferred them to scintillation vials for quantification.

Are there age- and sex-specific differences in acquisition and assimilation of elemental resources in response to P supply?

A total of 144 amphipods across two different life stages, late-stage juveniles (male and females both N=36; Fig. 1A) and adults (male and females both N=36, Fig. 1B), from stock populations were used in radiotracer experiments. Age and body size are highly correlated in *Hyalella* amphipods (Kokkotis and McLaughlin, 2002), allowing us to clearly delineate age classes for our radioassays. Specifically, late-stage juveniles were identified as animals with a head length (a reliable indicator of body size; Edwards and Cowell 1992) of greater than 0.275 mm and less than 0.45 mm, and no egg development in the ovaries (immature females) or only slightly enlarged posterior gnathopods (immature males; Fig. 1A). Adult females were identified by egg development in the ovaries or developing embryos in the marsupium, while adult males were identified by fully enlarged posterior gnathopods (Fig. 1B). Since molt and female reproductive cycles are tightly linked in amphipods (Sutcliffe, 1992), we controlled for variation in egg development by selecting only females with embryos in early development (indicated by bright green, oval-shaped embryos) within their marsupium.

Three days prior to radioassays, each amphipod was transferred to a separate 100-ml glass jar filled with COMBO media (Kilham et al., 1998) with no added nitrogen or phosphorus. Individuals were then randomly assigned to treatments (HP or LP) and fed one HP or LP leaf disc (7 mm diameter that did not include any major veins) daily for a two-day period to acclimate the amphipods to the food used in the experiment. Before feeding the amphipods radiolabeled food, all amphipods were starved for 24 h to clear their guts and to maximize foraging activity (Hargrave, 1970). Amphipods were then fed either one HP or LP radiolabeled leaf disc. Because the same animals could not be used to assess acquisition and assimilation of C and P, we used two groups of amphipods to compare age- and sex-specific responses in acquisition and assimilation to P supply. We defined acquisition as the intake of elements in a given period, before those elements have been absorbed through the gut wall. Assimilation is defined as those elements absorbed through the gut wall into body tissue, and allocation as the amount of atoms invested in a trait after assimilation.

To compare acquisition among the experimental groups, amphipods (late-stage juveniles: male and female N=16, and adults: male and female N=16) were allowed to feed on the radiolabeled leaves for 2 h (less than average published estimates of gut passage time in amphipods [~3.5 hours, on average]; Hargrave 1970; Neumann et al. 1999; Willoughby and Earnshaw 1982). Once the feeding period was over, amphipods were immediately rinsed and transferred to scintillation vials. Because amphipod foraging behavior can displace the periphyton on the leaves, accurate acquisition measurements cannot be obtained by measuring radioactivity in the leaves before and after feeding. As such, within the context of our experiment, this method of estimating acquisition is the most accurate, as it directly measures all radioactive material that has been ingested by the amphipod in the 2-h period.

To compare assimilation among the experimental groups (late-stage juveniles: male and female N=20, and adults: male and female N=20), amphipods were fed for 2 h, as in the acquisition experiment, but were then rinsed and transferred into fresh beakers, and fed one non-radiolabeled 70-mm leaf disc of the same P treatment as that of the acquisition experiment. At 1, 2, 4, 8, and 12 h after removal of the amphipods,

amphipods were transferred to new media and given a new non-radiolabeled leaf disc to minimize recycling of the radioisotopes. At 12 h, amphipods were rinsed and transferred to scintillation vials. Because the upper limit of observed gut passage times is 6 h (Neumann et al. 1999), we assumed that any radioactivity left in the body after 12 h were assimilated from the gut into body tissue.

Prior to statistical analyses, we converted the activity of the radioisotopes, in disintegrations per minute (DPM), to μg of radioactive C or P acquired or assimilated. Low phosphorus leaves were ~2.5X more radioactive than HP leaves, which would confound results of the amphipod acquisition and assimilation assays. We accounted for differences in the radioactivity available in the leaves by multiplying the HP amphipod radioactivity by the ratio of mean LP leaf radioactivity to mean HP leaf radioactivity. This correction allows us to compare the two treatments after accounting for differences in initial leaf radioactivity. All C and P acquisition and assimilation values were corrected for body size by dividing these measurements by body mass, calculated from a head length vs. mass regression equation. This size adjustment is more appropriate for our experimental design than including body size as a covariate in our statistical models because amphipod life stage is highly correlated with body size. As a result, our groups have dissimilar covariate values with little overlap, violating a key component of covariate analyses (Quinn and Keough, 2002). We obtained head length vs. mass regression equation by randomly selecting 32 amphipods, ranging in head length from 350-750 μm, from our stock populations. Each amphipod was analyzed for head length using ImageJ and immediately dried in a 60°C drying oven and then weighed. To determine whether males and females differ in their head length:body size relationships, we ran an analysis of covariance (ANCOVA), with log-transformed mass as our dependent variable, sex as our independent factor, and log-transformed head length as our covariate. The results of this ANCOVA indicated that neither the slopes ($F_{1,28}$= 0.692; p= 0.412) nor the intercepts ($F_{1,28}$= 0.656; p= 0424) of the head length:body size relationship differed between the sexes. As such, we then ran a linear regression of log-transformed mass to log-transformed head length for all amphipods together. This regression was highly significant (p<0.001), with 91.6% of the total variation explained by our regression equation. Using this regression equation, we then calculated body mass for all individuals used in the radiotracer experiments. To satisfy assumptions of normality, our size-adjusted values for C and P acquisition and assimilation were log-transformed.

Amphipods that died during the radiotracer experiments were not included in the statistical analyses (<3% late juvenile mortality, 0% adult mortality). Additionally, samples that resulted in error in activity quantification, identified as those readings that were orders of magnitude higher or lower than those in the same treatment, were also removed from analyses. These errors were likely due to no feeding activity or radioactive contamination of scintillation vials for the low and high outliers, respectively. In total, for the acquisition assay we removed four late stage juveniles and adults from analysis due to quantification error. Additionally, we removed five late stage juveniles and four adults from analysis in the assimilation assay. The final sample sizes in the acquisition assay were 27 and 28 for late-stage juveniles and adults, respectively. For the assimilation assay, sample sizes were 34 and 38 for late-stage juveniles and adults, respectively.

To examine how sex-specific responses to P availability in the C and P acquisition and assimilation experiments may change, we ran general linear models

(GLMs) separately for acquisition and assimilation with log-transformed ^{14}C or ^{33}P (ug) per mg of dry mass as our dependent variable and life stage, P-availability, and sex as fixed factors.

Is there preferential allocation of carbon to an exaggerated, sexually selected trait?

Hyalella amphipods, like many crustaceans, have the capacity to regenerate their limbs within only a couple of molts (Skinner, 1985). As such, by allowing amphipods with amputated traits to begin regenerating their limbs, we can isolate patterns of allocation to re-development of specific traits. From the stock population, we randomly selected 30 adult male amphipods and divided them into three appendage amputation groups (N=10 for each group). Each male was anesthetized prior to amputation using a clove oil solution (Venarsky and Wilhelm 2006). All amputations were performed under a stereo microscope (Swift SM90, Schertz, TX, USA) using fine surgical forceps (#5, Dumont SA, Montignez, Switzerland). In the first group of males (hereafter, 'amputated gnathopod' males), we removed the carpus, propodus, and dactyl of both posterior gnathopods (Fig. 2). In the second group of males (hereafter, 'amputated leg' males), we removed the carpus, propodus, and dactyl of both 5th pereopods (i.e. walking legs; Fig. 2). The 5th pereopod is serially homologous to the posterior gnathopod but is much smaller and not used in mate acquisition (Cothran et al., 2010). Finally, the third group of males was assigned to an amputation control group (hereafter, 'unamputated' males). These males were anesthetized and sham operated on, but no appendages were amputated. By comparing unamputated males to our two amputation groups, we can observe allocation differences between trait regeneration and strictly trait maintenance.

After surgery, all males were transferred individually to 200-ml glass jars filled with treated water and a square of filter media (Matala USA, Laguna Hills, CA, USA) for substrate. We then fed each male one 14-mm diameter HP leaf disc every three days for two weeks. This two-week period served as a recovery period that was sufficient for all amphipods to begin regeneration of traits. We included this recovery period to isolate allocation to regrowth from that of acute wound repair. Amphipods were then randomly assigned to either HP or LP treatments, transferred individually to new jars, and fed either one HP or LP leaf disc, radiolabeled with ^{14}C, each day for six days, with daily media changes. After feeding on radiolabeled food for six days, each amphipod was again individually transferred to another 200-ml glass jar and fed non-radiolabeled HP or LP food for two more weeks (i.e. diet treatments continued during this period), to allow the radiolabeled C time to be allocated to tissue. At the conclusion of the two-week feeding period, we measured body size, the size of the walking legs and the size of the gnathopods to determine size-adjusted allocation of ^{14}C for each trait. We photographed each male on both sides and analyzed each photograph with ImageJ (version 1.46r). We measured the width of the gnathopod at the widest part of the propodus and the total length of the carpus, propodus, and dactyl of the 5th pereopod. We then dissected the carpus, propodus and dactyl of the gnathopods and 5th pereopod from each male and quantified radioactivity in these two traits and the rest of the body.

Prior to statistical analysis, radioactivity within each trait was corrected for initial radioactivity within the leaves using the same method as in the acquisition and assimilation assays. Trait-specific radioactivity was then converted to µg of ^{14}C allocation per mg of trait tissue. Because we were unable to measure the dry mass of each trait used in the radiotracer experiment (per radiation safety protocol), we performed mass conversions using trait size-mass regressions. These conversions were performed on amphipods that had undergone the same trait manipulations as those used in the study, but were not exposed to radiation. First, we randomly selected 60 males from our stock tanks and divided them into three groups of 20, representing our three manipulation groups. Then, for each group, we amputated the appropriate trait (i.e. amputated gnathopods, amputated legs, no amputations). The groups that were manipulated were then transferred into individual jars filled with no nitrogen or phosphorus COMBO and each fed one HP leaf disc daily for a period of two weeks to allow regrowth to occur. After the two-week period, both the gnathopods and the legs were amputated from each individual. Trait sizes, along with total body size, were then measured using ImageJ. The gnathopods, legs, and body of each individual were then dried at 60°C for 48h and weighed to the nearest 0.1 μg (Mettler Toledo XP2U, Columbus, OH, USA). The weights of the legs and gnathopods were divided by two to determine the average weight for just one limb. We performed separate linear regressions for each manipulation group and trait with trait size as our independent variable and mass as our dependent variable. All regressions were highly significant ($p<0.001$), with trait size explaining 70-90% of the variation in trait mass. The mass of each trait used in the radiotracer experiment was determined by converting the trait size measured to trait mass using the regression equation for each group.

Our goal in this experiment was to examine the effects of P availability and amputee group on carbon allocation. Thus, we ran a GLM that included log-transformed ^{14}C activity in target traits as the dependent variable and P availability, trait, and amputee group as fixed factors. This initial model revealed a trait-by-amputee group interaction (see Results). Therefore, to examine trait-specific manipulation or P availability effects, we ran separate GLMs for each trait (whole body, gnathopod, and leg), including only amputee group and P availability as fixed factors. For significant results, we then ran Tukey HSD post hoc analyses to determine differences within factors.

Results

Are there age- and sex-specific differences in acquisition and assimilation of elemental resources in response to P supply?

For acquisition, we found a significant interaction between sex and life stage for both C and P acquisition (C: $F_{1,47}$= 13.945, p= 0.001; P: $F_{1,47}$= 13.183, p= 0.001). Late-stage juvenile males and adult males did not significantly differ in their acquisition of C and P (Fig. 3A, B). However, late-stage females acquired 494% more C and 392% more P than adult females (Fig. 3A, B). Additionally, irrespective of sex or age, we observed a smaller, but significant, effect of P availability on the acquisition of C, but not P, with acquisition increasing in the LP treatment by 32% ($F_{1,47}$= 4.185, p= 0.046).

Assimilation of C and P was dependent on the three-way interaction of sex, life stage, and P availability (C: $F_{1,64}$= 10.048, p<0.001; P: $F_{1,64}$= 10.459, p<0.001). This interaction indicates that sex-specific assimilatory responses to P availability change with age. In each life stage, female assimilation was largely unchanged by P availability (Fig. 4). However, in each life stage, there was a significant interaction between sex and P availability that was driven by a plastic male response. Specifically, late-stage juvenile males exhibited a 63% and 65% decrease in assimilation of C and P when fed LP food

(Fig. 4A, B). In contrast, adult males significantly increased assimilation of C and P by 243% and 152%, respectively, when fed LP food (Fig. 4C, D). These plastic male responses resulted in convergence of the sexes in assimilation under contrasting food quality. Late-stage juveniles converged in the LP environment, while adults converged in the HP environment (Fig. 4).

Is there preferential allocation of carbon to an exaggerated, sexually selected trait?

We observed a significant interaction between amputee group and trait, indicating trait-specific responses to amputation ($F_{4,69}$= 5.75, p<0.001). There was greater ^{14}C activity (~404%) in all traits under LP environments than HP environments ($F_{1,69}$= 350.65, p<0.001; Fig. 5). The interaction observed between trait and amputee group was largely driven by the response of the gnathopod to amputation. Post-hoc analyses revealed ^{14}C activity within the gnathopod in the amputated gnathopod group was significantly higher than in the amputated leg, or the unamputated groups (Fig. 5B), while the other traits (leg and whole body) did not differ in ^{14}C activity across amputation groups (Fig. 5A, C). Amphipods within the amputated gnathopod group had, on average, 115% higher ^{14}C activity in their gnathopods than those within the other two groups.

Discussion

The results of our study show that P supply invoked differing degrees of plasticity in acquisition and assimilation of two key elemental resources, C and P. Further, our results clearly show age and sex have an interactive effect on the acquisition and assimilation of both C and P. Finally, our study revealed preferential allocation of biomass to an exaggerated trait in males. Note that our measures of radioactivity in our samples only represented the amount of radiolabeled C or P found in the sample, and does not represent an estimate of the *total* C or P acquired or assimilated. As such, these measures are just a fraction of the total C or P acquired, assimilated, or allocated by the amphipod. Nevertheless, radiolabeling has been shown to be reliable indicator of the physiological kinetics of both C and P (e.g., DeMott et al. 1998; He and Wang 2007; Roy Chowdhury et al. 2014).

Age-specific sexual dimorphism in C and P acquisition in response to P supply

We found strong, interactive effects of age and sex on the acquisition of C and P (Fig. 3), as well as a significant effect of P supply on the acquisition of C. It is possible that the increase in C acquisition under LP conditions is due to compensatory feeding, which has been proposed, and observed in a few taxa, as a potential mechanism that organisms use to meet their elemental demand under low supply conditions (e.g., Plath and Boersma 2001; Fink and Von Elert 2006). Additionally, acquisition of C and P appear to vary together, which suggests an observed increase in P acquisition is due to greater overall feeding effort. Given the temporal nature of sexual divergence, it is not surprising that we observed substantial sex-specific differences in the acquisition of both C and P that are driven by age. Because the sexes have differing elemental demands, differences in foraging behaviors likely play a central role in meeting those demands. Indeed, previous studies in a wide array of taxa from crickets to birds and mammals, have observed sex-specific differences in foraging strategies (e.g., intake rates, food selection, and foraging behavior) on the molecular level (e.g., Bearhop et al., 2006; Maklakov et al., 2008; Ruckstuhl, 1998), but, to our knowledge, there are no rigorous explorations of such sex-specific differences on the elemental level.

Previous studies in a variety of taxa (e.g., García-Berthou and Moreno-Amich, 2000; Stockhoff, 1993) have found that specific life history demands drive foraging strategies with juveniles ingesting different, or different quantities of, resources than adults. While exaggerated traits are thought to be costly to build and maintain, the results of our acquisition experiments suggest that the development of the exaggerated gnathopod in *Hyalella* males does not drive an increase in C or P acquisition (Fig. 3). In fact, the age-by-sex interaction observed is driven primarily by changes in female, not male, acquisition from late juvenility to adulthood. Late-stage juveniles differ from adults in that they must allocate resources to both a high overall growth rate and the development of reproductive traits, both processes that are highly C- and P-intensive (Bertram et al., 2009; Cothran et al., 2012; Elser et al., 1996; Elser et al., 2000; Markow et al., 2001; Speakman, 2008; Visanuvimol and Bertram, 2010). One possible explanation for the observed age-by-sex interaction is that males are foraging at their maximum rate in both late juvenility and adulthood, because selection on these traits may be consistent across these two life stages. Alternatively, females in late juvenility may be acquiring resources at an increased rate due to the demands of somatic growth along with ovary and egg development. While adult females bear the demands of oogenesis, a P intensive process (Back and King, 2013), the selection for somatic growth in adulthood is much lower in adults than juveniles because adults reach a size refuge from their main predator—dragonfly naiads (Wellborn, 1994; Wellborn et al., 2005). Given the sex differences in P content within amphipods (Goos et al., 2014), it is somewhat surprising that no sexual dimorphism in acquisition exists in adulthood. A possible explanation for this result could be that adult females assimilate more of their acquired P. Alternatively, adult females may have a greater ability to store acquired P than males. Studies examining foraging behaviors, the effects of egg development, and other post-ingestive processes, on sex-specific acquisition will provide important insights into the mechanisms underlying the observed patterns in acquisition.

Age-specific sexual dimorphism in C and P assimilation in response to P supply

Our results clearly show that age- and sex-specific assimilation strategies are not independent from the effects of dietary P supply (Fig. 4). Male, not female, assimilation was significantly influenced by P availability, but the direction of this response differed between late-stage juveniles and adults. These sex-specific patterns are likely due to divergent life history demands in *Hyalella* amphipods. Specifically, males and females within this genus exhibit substantial sexual size dimorphism and have different life histories, with males being larger than females in the OK-L clade (Cothran et al., 2012; Wellborn and Bartholf, 2005; Wellborn et al., 2005). Additionally, developing exaggerated gnathopods has been shown to be a P-intensive process, with P availability influencing both male growth rate and gnathopod growth (Cothran et al., 2012). Late-stage juvenile males assimilated comparatively more C and P under HP than LP conditions, while adults exhibited the opposite pattern (Fig. 4). Adult males grown under differing P environments have been shown to have differing P contents, with males raised under LP conditions containing less P than those raised in HP conditions (Goos et al., 2014). Within the current study, all amphipods were raised under HP conditions, likely increasing their somatic P contents. The pattern observed in adult males, with HP males assimilating comparatively less C and P than LP males seems to suggest that adult males are ramping up assimilation in LP environments in order to meet their P demands in a comparatively resource-poor environment. The pattern observed in late-stage juvenile males may be due to the interaction between acquisition and assimilation. Particularly, the increased acquisition observed under LP may induce a decrease in assimilation, resulting from a decrease in gut passage time caused by constant feeding (Navarro and Winter, 1982; Stahlschmidt et al., 2011). Given that juveniles have a shorter gut length than adults, it is possible that increases in acquisition may influence juvenile assimilation to a different degree than it does adults. Alternatively, the pattern we observe between these two sexes, with juveniles and adults exhibiting contrasting responses to P availability, may be due to differences in metabolic plasticity. In a study examining the effects of low P food on metabolic activity in *Daphnia*, Jeyasingh (2007) found that low P food both increases feeding effort and metabolic activity. Additionally, metabolic scaling was affected by low P food, driven primarily by the greater metabolic response of smaller *Daphnia* species. While the age classes in our study are not as variable in size as interspecific differences in *Daphnia*, it is possible that juvenile males exhibit much greater metabolic activity under LP food than adult males, resulting in an overall decrease in assimilated C and P. It is important to note that our measure of assimilation is not interchangeable with measures of retention or assimilation efficiency. Because we could not measure acquisition and assimilation within the same individual, we were unable to calculate assimilation efficiency at the individual level. As such, our estimate of assimilation is a gross estimate, not accounting for the effects of acquisition, and more research is needed to determine whether assimilation efficiency is indeed decreased under LP due to higher feeding rates. Regardless, the patterns of assimilation that we observed clearly indicate that assimilatory responses to P availability are dependent upon the age and sex of an organism. Together, these results point to the potentially important role of physiological processes in driving allocation of resources to traits (Olijnyk and Nelson, 2013; Stahlschmidt et al., 2011), which is generally thought to be controlled by acquisition (Robinson and Beckerman, 2013).

Phosphorus supply alters carbon allocation to a sexually selected trait

In addition to sexual dimorphism in acquisition and assimilation of elements, our results revealed the importance of P availability in allocation to all traits, with greater overall biomass allocation when P was in limited supply (Fig. 5). This increase in allocation of C under LP food is likely due to the higher acquisition and assimilation of C in adult males when feeding on LP food. While it is known that consumers feeding on carbon-rich, nutrient-poor diets can deal with such imbalances by respiring or egesting excessive C (Darchambeau et al., 2003; Hessen and Anderson, 2008; Jeyasingh, 2007), they are also known to store excess C as fats (Sterner et al., 1992). Although we did not measure respiration or fat content, higher ^{14}C activity under LP conditions indicate that storing excessive dietary C as fats may be a more prevalent mechanism used by amphipods to deal with stoichiometric imbalances.

Using our method of trait manipulations, we were able to isolate resource allocation to both the development and maintenance of sexual traits and serially homologous non-sexual traits. Specifically, we found that regrowth of the gnathopod, but not the walking leg, induced an increase in ^{14}C allocation, suggesting that a greater fraction of recently-ingested C was allocated to gnathopod regrowth compared to leg regrowth. While our data seems to suggest a pattern of preferential allocation of ingested

C to gnathopod regeneration, the precise physiological mechanisms that underlie this preferential allocation require further investigation. Additionally, we observed that ^{14}C activity was always the lowest in body tissue, compared to the other two traits. This pattern may be due to the relative turnover rates of each body tissue. For example, provided the overall C turnover rate in amphipod tissue is slower than the two-week period between radiolabeling and ^{14}C quantification, it possible that the walking leg or gnathopod possesses a significantly faster C turnover rate due to the high abundance of metabolically active muscle tissue (Boutton et al., 1983; Hobson and Clark, 1992). Our results indicated that the demands of developing a sexual trait (i.e. the male gnathopod), as opposed to possessing an already developed trait, play an important role in determining carbon allocation in male amphipods, suggesting that sexually selected, exaggerated traits are significant resource sinks, particularly later in ontogeny when sexual differentiation occurs.

Conclusion

We found sex- and age-specific variation in the acquisition and assimilation of two key elemental resources that make up approximately 50% of amphipod biomass. Further, the environmental supply of phosphorus, at levels similar to those found in oligotrophic and eutrophic conditions had a significant effect on how individuals use key elemental resources. Moreover, we found that changes in P supply affected biomass allocation to all traits, and that biomass allocation to the development of the sexual trait was prioritized. Our results highlight the importance of both pre- and post-ingestive processes that influence how sexes respond to rapid changes in nutrient availability. It is likely that such alterations will affect the sexes differently, and alter the expression of sexually selected traits, perhaps with important demographic and evolutionary consequences.

Acknowledgments

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Figures

Figure 1: Life stages of *Hyalella* amphipods used in acquisition and assimilation radiotracer experiments. A) late-stage juveniles B) adults. The whole organism is represented on the top row and the posterior gnathopod on the bottom row. For both life stages, females are on the left, males on the right.

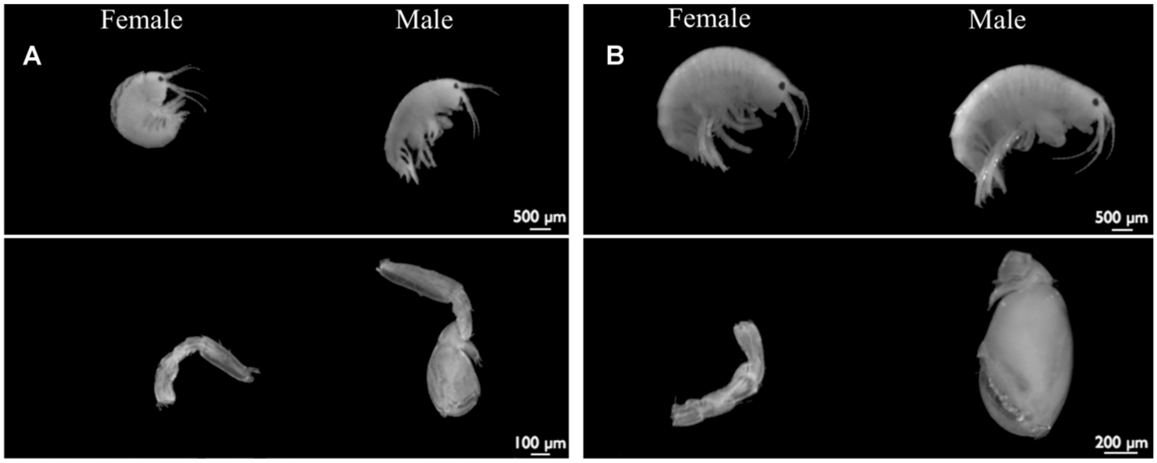


Figure 2: Male *Hyalella* amphipod showing points of dissection for appendage amputation groups. The black lines indicate the point where we ablated the carpus, propodus, and dactyl of the gnathopods (PG) and fifth pereopods (5P).

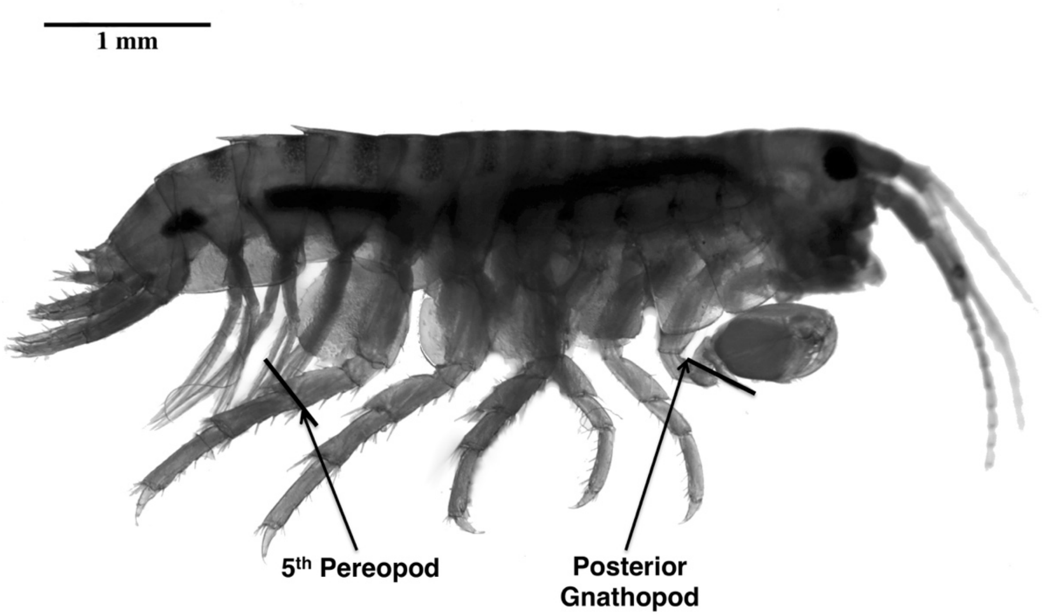


Figure 3: Age-specific patterns of sexual dimorphism in acquisition of phosphorus and carbon over two hours during late ontogeny.. Symbols represent means of log-transformed, size-adjusted ^{33}P (A) and ^{14}C (B) present in the body, error bars 95% confidence intervals.

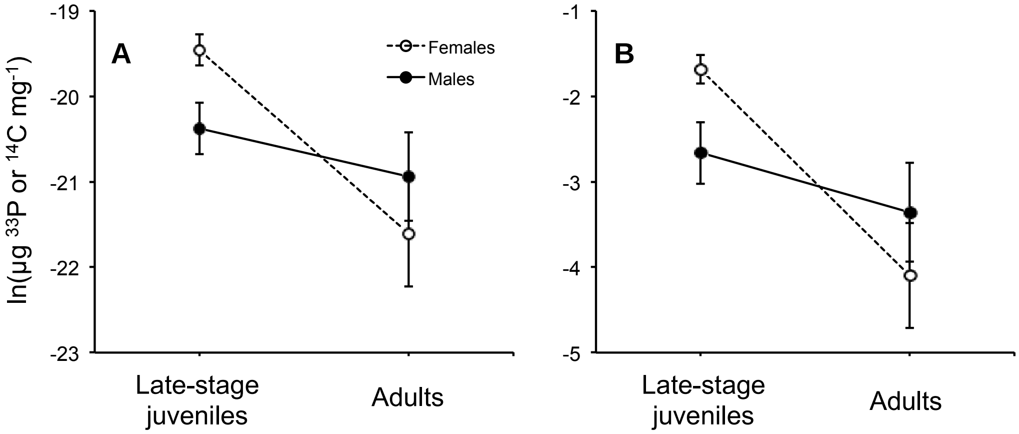


Figure 4: Sex-specific patterns of assimilation of C and P in late-stage juveniles and adults in response to P availability. (A,B) Late-stage juveniles and (C,D) adults. Symbols represent means of log-transformed, size-adjusted^{33}P (A,C) and ^{14}C (B, D) present in the body, error bars 95% confidence intervals.

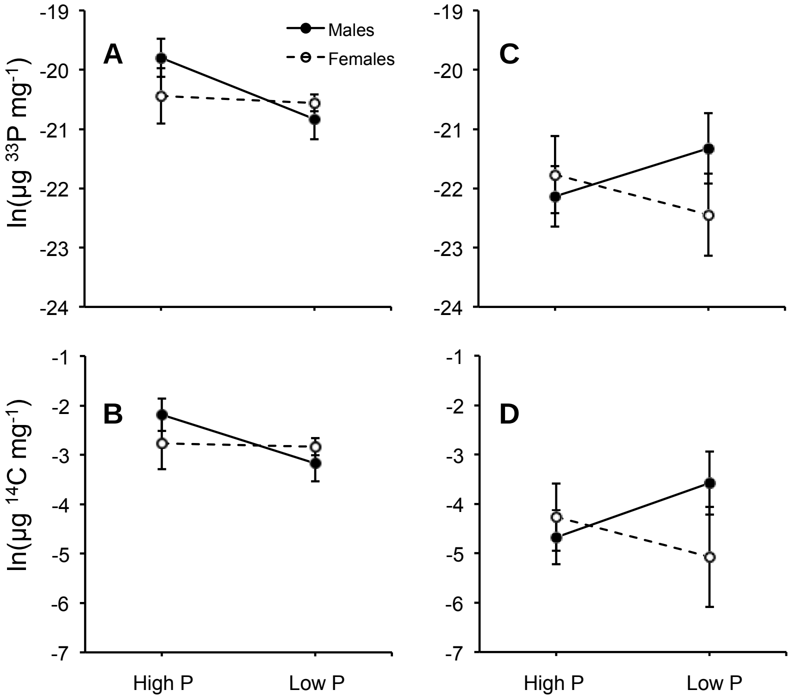
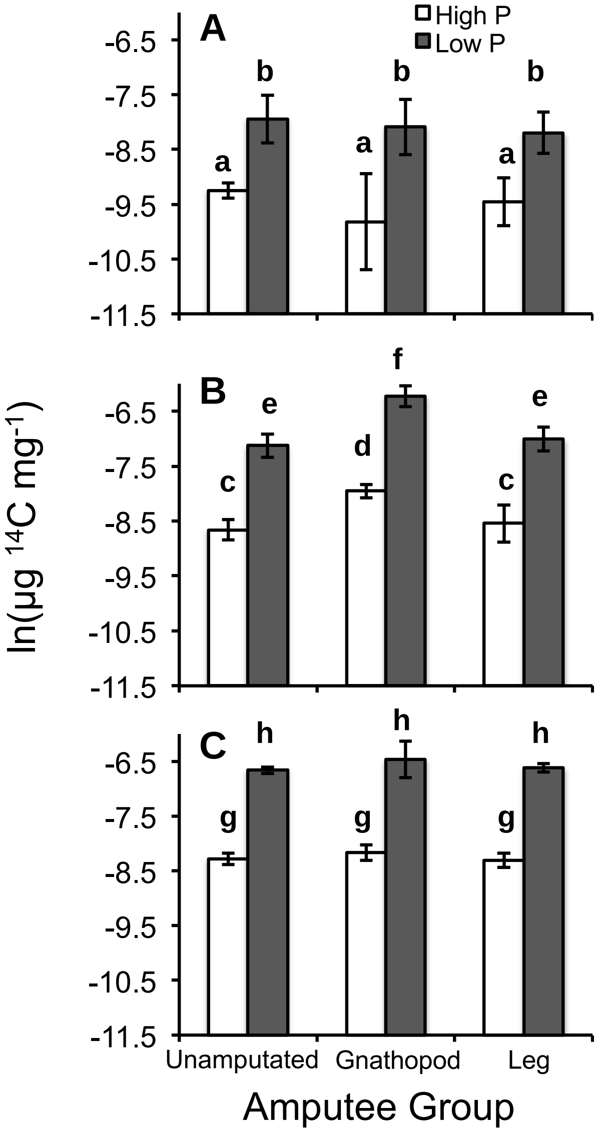


Figure 5: Carbon allocation in the male A) body, B) gnathopod, and C) leg across amputation groups. Values are means of log-transformed carbon allocation (µg C/mg). Error bars are 95% confidence intervals. Different lower case letters indicate significantly different carbon allocation, as indicated by Tukey tests.



CHAPTER III

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SUBTLE VARIATION IN PHOSPHORUS AVAILABILITY INFLUENCES MATING BIOLOGY IN *HYALELLA* (AMPHIPODA: HYALELLIDAE) AMPHIPODS

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Abstract

Eutrophication caused by anthropogenic activity has had tremendous impacts on aquatic ecosystems across the world. A primary cause of this eutrophication is increased phosphorus (P) runoff into water bodies, resulting in alterations to the quantity and quality of food available for consumers. Previous studies have shown that large differences in P availability affect expression of traits and mating biology at the individual level. Here, we manipulated the supply of phosphate, which altered the quantity and quality of food (e.g., periphyton) available to consumers, and documented the population-level responses of *Hyalella* amphipods. We also performed a mating experiment to examine the effects of P availability during development on the expression of sexual traits, and P availability during mating on mating behavior. Our results indicate that subtle variation in P availability has no effect on population density or sex ratio. The amount of P available during development and P available during mating, however, did affect mating behavior. Further, the patterns in mating behavior observed were not attributable to P-induced changes in sexually selected traits. Our study indicates that subtle changes in P availability can affect mating biology, possibly due to physiological or behavioral changes, but such effects may not necessarily be mediated by differential expression of sexually selected traits.

Introduction

Human activity has fundamentally altered ecosystems at an unprecedented rate and scale resulting in evolutionary changes in many organisms (Palumbi, 2001). Altered biogeochemical cycles are perhaps the most studied aspect of global environmental change (Schlesinger, 1997). In the last few decades, human activities such as fertilizer (e.g., phosphate) mining and application have caused the eutrophication of many ecosystems (Hale *et al.*, 2013), and phosphorus (P) runoff into aquatic ecosystems is the primary cause of eutrophication (Schindler *et al.*, 2008). Further, increased P supply has been shown to increase the P content of seston (i.e. planktonic living and nonliving matter) and periphtypon (i.e. attached algae and microbial communities) in aquatic ecosystems (Hillebrand & Kahlert, 2001; Hessen, 2006). Therefore, the addition of inorganic P to aquatic ecosystems can dramatically change the quantity and quality of food available to consumers. The ecological consequences of eutrophication have been widely studied (e.g., Smith, Tilman, & Nekola, 1999; Smith, 2003; Khan & Ansari, 2005; Kemp *et al.*, 2005), however, we have only begun to uncover the evolutionary implications of eutrophication.

Despite its importance in determining fitness, however, we know relatively little about how changes in P supply affect the mating biology of organisms. While there have been a few studies on the effects of eutrophication on mating biology, most of these studies have focused on how eutrophication directly influences the evolution of sensory systems (Van der Meer *et al.*, 2012) and affects sensory reception of mating signals. For example, eutrophication increases phytoplankton abundance, resulting in greater turbidity that interferes with visual mating cues (Seehausen, van Alphen, & Witte, 1997; Sundin, Berglund, & Rosenqvist, 2010; Lane, Forrest, & Willis, 2011; Fischer & Frommen, 2013). In addition to altering the visibility of signals, eutrophication will also affect the quality of food available for heterotrophic organisms (Sterner & Hessen, 1994). Because reproduction is a resource-intensive activity (Speakman, 2008; Morehouse *et al.*, 2010), P supply has the potential to influence the mating biology of organisms.

Our understanding of how changes in P affect organismal performance largely arises from studies that employ highly contrasting conditions of P availability. For example, studies documenting the effects of P supply on aspects of mating biology in crickets have used highly contrasting diets differing roughly by an order of magnitude in P content (e.g., 0.2% and 1%; Bertram *et al.*, 2009; Visanuvimol & Bertram, 2010). To our knowledge, the response of mating biology to more subtle changes in P content of food that may be representative of seasonal or spatial changes in P availability (e.g., Hessen, Van Donk, & Gulati, 2005) has not been studied. Further, these previous studies have generally focused on the effects of dietary P on the expression of male sexual traits. However, mating is inherently an interactive process, and we know little about how dietary changes in P availability affect females and consequently sexual interactions (Morehouse *et al.*, 2010).

When examining the effects of P supply on mating biology, most studies have focused on changes occurring at the individual level with little attention to demographic patterns (e.g., density and sex ratio). This is surprising because much of mating biology is driven by population dynamics, as it is both density and sex ratio dependent (Kokko & Rankin, 2006).In fact, changes in density and sex ratio have been found to affect several aspects of mating biology such as female resistance and choosiness (Rowe *et al.*, 1994;

Bleu, Bessa-Gomes, & Laloi, 2012), male weapons and ornaments (Buzatto, Tomkins, & Simmons, 2012; Vergara *et al.*, 2012), male mate preferences (Gosden & Svensson, 2009), mate guarding (Jormalainen, 1998) and alternative mating strategies (Tomkins & Brown, 2004; Dennenmoser & Thiel, 2008; Leary, Garcia, & Knapp, 2008). Although it is well known that changes in P supply often increase population size, and drive population dynamics in a variety of taxa (Andersen, Elser, & Hessen, 2004; Moe *et al.*, 2005), very little is known about the concomitant impacts of such dynamics on the mating biology of species.

We performed two experiments to assess how modest changes in P supply affect demographics, sexual trait development, and mating behavior. First, using a mesocosm study, we examined whether P supply results in changes in population density and sex ratio. Second, using animals from the mesocosm study above, we conducted a mating behavior experiment to examine how changes in P availability affected mating interactions. By selecting animals from the population study for this experiment we were able to address whether differences in P availability affect sexual trait development and consequently mating interactions. We also manipulated the quality of food (i.e. food was collected from either high P or low P tanks in the population study) available to animals *during* the behavior experiment to address whether short-term access to P rich food affects mating interactions (perhaps through changes in behavior, as opposed to developmental shifts).

Materials and Methods

Study species and housing of animals

To examine the effects of P supply on population dynamics and mating biology, we used amphipods in the genus *Hyalella*. *Hyalella* amphipods are found in most freshwater ecosystems throughout North America and are thus exposed to a variety of nutritional environments (Bousfield, 1958). This genus is composed of a complex of undescribed species and many of these species can be divided into either small ecomorph or large ecomorph types that differ in morphology, behavior and life history (Wellborn, Cothran, & Bartholf, 2005; Witt, Threloff, & Hebert, 2006; Wellborn & Broughton, 2008).

Like many crustaceans, the mating biology of *Hyalella* is tightly linked to the female molt cycle, because the opportunity to fertilize eggs is limited to the period just after the female molt (Sutcliffe, 1992). Males are the mate searching sex, and *Hyalella* populations have female-biased sex ratios where males are typically 20% to 40% of the population (Wellborn & Bartholf, 2005). However, because female molt cycles are asynchronous the operational sex ratio is highly male biased – only about 9% of females are receptive at a given time (Wellborn, 1995). As a result of this time-limited female receptivity to fertilization, males exhibit mate guarding as a time investment strategy (Jormalainen, 1998; Wellborn & Cothran, 2007). Females exhibit resistance behavior to pairing by thrashing and curling their body, which affects pairing duration (Wellborn, 1995; Cothran, 2008). Males use their enlarged, claw-like appendages, the posterior gnathopods (hereafter gnathopods) to initiate pairing (Cothran *et al*., 2010). Prior studies have shown that larger size-specific gnathopods provide a mating advantage and this trait likely serves as an important male offense trait in sexual conflict over pairing duration

(Wellborn, 1995, 2000; Cothran *et al.*, 2010). For the current study, we used a large ecomorph species (referred to as OK-L in Wellborn & Broughton, 2008) from Cowen Creek, a spring-fed creek located in Marshall County, OK, USA (33°55’N, 96°51’W). The OK-L amphipod species is ideal for this study because it exhibits a continuous large gnathopod advantage as body size increases (Wellborn & Bartholf, 2005). Additionally, gnathopod size and antenna size, a trait that is sexually dimorphic and under sexual selection in an isopod species with a similar mating biology (Bertin & Cézilly, 2003), have both been found to increase in size in response to increased P availability in individual-level studies performed on this species (Cothran *et al.*, 2012). The experiment was performed in a greenhouse at the University of Oklahoma Biological Station at ~26°C.

Set up of amphipod population tanks

We set up fourteen 30-L tanks (67.95cm X 40cm X 18.10cm) divided equally into two P supply treatments (HP = 60 µM P [K_2HPO_4] and LP = 10 µM P [K_2HPO_4]; Supplementary Info Figure S1a). Each tank was filled with treated well water, and washed pea-sized gravel substratum was added to a depth of approximately 2 cm. Algae and amphipods were added after two weeks to allow for dechlorination of the water. The tanks were then covered with shade cloth to reduce colonization by terrestrial organisms. All tanks received 500 ml of a periphyton slurry collected from Cowen Creek and were fertilized with a source of nitrogen (1 mM N [$NaNO_3$]) and the amount of phosphorus corresponding to their treatment. Because periphyton growth was minimal in the tanks after two weeks, we added an additional 500 ml of periphyton collected from nearby Lake Texoma. Microscope slides that had been scoured with steel wool were added to each tank to collect periphyton periodically for elemental analysis. Twenty-five randomly chosen gravid females from Cowen Creek were added to twelve of the tanks (six HP, six LP), and populations were allowed to grow for three weeks before sampling began. In addition to the tanks with amphipods, four amphipod-free tanks (two HP and two LP) were set up as controls: periphyton used in the mating trials was collected from these tanks (Supplementary Info Figure S1a). Water was added to the tanks once a week to keep the tank water level relatively constant throughout the experiment.

Population-level responses to P supply

To examine whether population density and sex ratio were affected by P supply, each tank was sampled every two weeks, beginning after three weeks of initial population growth. We used a 10.16-cm diameter pipe to subsample each tank. On each sampling date, we sampled from one randomly selected quadrant (Supplementary Info Figure S1b). We lowered the pipe into the chosen quadrant, making sure to push the pipe through the substrate to the bottom of the tank. We then collected all amphipods within the pipe using a brine shrimp net. Immediately after collection, we quantified the number of males, females, and juveniles by examining all amphipods under a stereo microscope (Leica S8APO, Leica Microsystems, IL). Amphipods were characterized as juveniles if they did not have any eggs visible in ovaries or embryos in the marsupium and enlarged gnathopods. Using these data, we compiled a life table and calculated population density and sex ratio (male:female). Population density was calculated by summing all adults and juveniles collected per sample and dividing by the area of the 10.16-cm collection pipe; total density is reported in individuals/m^2. Samples were collected seven times over 17 weeks, representing between four and five generations of amphipods.

To quantify the P content of periphyton, we collected one microscope slide with attached periphyton from each tank before amphipods were added and after completion of the study. Microscope slides were frozen immediately until P analysis was performed. Periphyton from each sample was brushed from the microscope slide into distilled water and filtered onto glass filters (0.7 μM; Whatman International Ltd, Maidstone, England). The filters were dried at 60°C for 48 h and P content was quantified in duplicate using a modified sulfuric acid digestion method (APHA, 1992) and verified with a spinach standard (NIST 1570a). This method involves digesting the sample with sulfuric acid and reacting the digestion with ammonium molybdate and antimony potassium tartrate. The resulting complex is then reduced to form a blue color that is then analyzed using spectrophotometry.

To determine the effect of sampling date and P treatment on the population density and sex ratio of the tanks, we performed two separate repeated-measures (RM) ANOVAs with the population density and sex ratio as dependent variables, sampling date as a within-subjects variable, and P supply treatment as the between-subjects variable. Additionally, we performed a RM-ANOVA to determine the effect of sampling date and treatment on the P content of periphyton in the tanks, using arcsine-transformed %P as our dependent variable.

Effects of P supply on mating

To examine the effect of P supply on mating success, we set up a 2-by-2 completely randomized, factorial design with female rearing environment and mating environment as treatments (HP or LP female rearing environment and HP or LP mating environment). Each treatment was replicated 30 times for a total of 120 experimental units. Females were selected randomly from the tanks and transferred to 150-ml plastic cups. Each cup was filled with water and periphyton was added (amphipods were fed *ad libitum* during the experiment) from either the HP or LP control tanks according to the mating environment assigned to the cups. Chemical analysis confirmed that the HP periphyton was higher in P content than the LP periphyton in these control tanks ($F_{1,7}$=58.75, $p<0.001$) All of the females were acclimated in the cups for 24 h. During this time, two mature males, one from an HP tank and one from an LP tank were randomly selected from the experimental tanks and added to each cup (Supplementary Info Figure S1c), with randomization at both the within- and between-tank levels. To differentiate between the two males, we anesthetized the amphipods with a clove oil solution (Venarsky & Wilhelm, 2006) and clipped the right or left fifth walking leg removing two to three of the most distal segments of the leg. The side of the leg clipped was alternated within treatments between cups. The males were isolated in cups filled with water from their rearing environment (either HP or LP) for 24 h to recuperate from surgery. After the 24-hour recuperation period, males were added to the experimental cups housing the females.

Daily observations of each cup were made at 0800, 1200, 1600, and 2000 hours. If a pair was observed, we removed the unpaired male and recorded the side of his leg clip to determine the identity of the paired male. A trial was ended when the pair separated and eggs were deposited into the external brood pouch. The successful male was defined as the male last observed paired with the female before oviposition. Because females do not store sperm, the last paired male is likely the successful male (Wellborn & Cothran, 2007). Replicates were discarded if either the female died or pairing occurred after the death of a competing male. During the mating behavior experiment, 13 of the 120 experimental units were discarded due to death, resulting in a total of 107 experimental units used for the analyses (HP female/HP environment: N=26, HP female/LP environment: N=27, LP female/HP environment: N=27, LP female/LP environment: N=27). After a trial ended, all amphipods from each cup were preserved in 70% alcohol for further morphometric analysis.

Because we know that morphology can determine the outcome of mating (Wellborn, 1995), we performed morphometric analysis in which we measured 4 traits for all males. Head length was measured as an indicator of body size (Edwards & Cowell, 1992). We also measured the maximum width of the propodus of the posterior gnathopod and length of the 3rd peduncle segment of the second antenna (hereafter antenna) because both traits are known sexually selected traits in amphipods and isopods that share a similar mating biology (Wellborn, 1995; Bertin & Cézilly, 2003; Wellborn & Bartholf, 2005). Additionally, we measured a control trait, the width of the basis of the fifth pereopod, which is comparable in size to the gnathopod but has no known sexual function (Cothran *et al.*, 2012). All trait measurements were log-transformed before statistical analyses.

Because most traits are correlated with body size, we adjusted all traits for body size using a multivariate analysis of covariance (MANCOVA) to determine the unique trait effects on mating success. We used the treatment of the male as a fixed factor and head length as a covariate and saved the residuals of the analysis. For each trait, these residuals were added to the estimated marginal mean for each treatment to calculate size-adjusted trait size (Cothran *et al.*, 2012). A major assumption of this size-correction method is that the allometric slopes of the traits are equal between treatments (McCoy *et al.*, 2006), and this assumption held true for all traits in our study (See Supplementary Info Table S1).

For each size-adjusted trait, we ran paired t-tests to determine if traits differed in size between HP-reared and LP-reared males within each competing pair of males. Additionally, to determine whether the female's rearing environment and the current mating environment affected which male was successful, we ran a backwards elimination logistic regression with the rearing treatment of the successful male as the dependent variable and female rearing environment, mating environment, and the interaction between the two as independent variables. All statistical procedures were performed using SPSS version 20 (IBM, 2011)

Results

Population-level responses to P supply

Of the 12 experimental tanks, 3 (two HP and one LP) experienced significant population crashes about halfway through the experiment and, as a result, these tanks were discarded from further analyses. We found an effect of sampling date (beginning versus the end of the experiment; $F_{1,16}$=98.75, p<0.001) and P treatment ($F_{1,16}$=50.75, p<0.001) on the P content of periphyton, but no significant sampling date-by-treatment interaction ($F_{1,16}$=0.583, p=0.456; Figure 1). The P content of the HP tanks was on average 1.69 times greater than the P content of the LP tanks. Further, the P content at the beginning of the experiment was on average 2.31 times greater than the P content at the end of the experiment for both treatments.

RM-ANOVA on population density indicated an effect of sampling date ($F_{6,42}$= 8.737, p<0.001), but not P treatment ($F_{1,7}$=0.035, p=0.857) or the sampling date-by-P treatment interaction ($F_{6,42}$=1.12, p=0.367). Population density generally increased early in the experiment before reaching a plateau (Figure 2).

For sex ratio, the RM-ANOVA showed an effect of date ($F_{6,42}$=3.76, p=0.004) and no effect of P treatment ($F_{1,7}$=1.258, p=0.299) or the sampling date-by-P treatment interaction ($F_{6,42}$=0.670, p=0.674). The populations showed no strong temporal trends in sex ratio across time, although sex ratios tended to be more female biased early in the experiment (Figure 3).

Effects of P supply on mating

Males from the two rearing environments did not differ in size (measured as head length: paired-t_{106}=1.276, p=0.205), gnathopod size (paired-t_{106}=1.734, p=0.086; Figure 4 or antenna size (paired-t_{106}=0.322, p=0.748; Figure 4). However, pereopods of HP males were significantly larger than those of LP males (paired-t_{106}=2.706, p=0.008; Figure 4).

The success of LP males against HP males was affected by an interaction between female rearing environment (i.e. whether females were raised on LP or HP algae) and mating environment (i.e. whether amphipods were fed LP or HP algae during the mating experiment; Table 1, Figure 5). Females reared on LP algae were equally likely to pair with a LP or HP male regardless of the type of algae the animals were being fed during the mating experiment. Females reared on HP algae were more likely to pair with HP males when being fed LP algae (19/27 or 70% of the trials) and LP males when being fed HP algae during the mating experiment (17/26 or 65% of the trials).

Discussion

We found that relatively small differences in P supply have no appreciable effect on population size and sex ratio. Nevertheless, our results indicate that P supply influenced mating patterns. The differences in pairing patterns were not due to differences in crowding or male-biased sex ratios experienced in rearing tanks, both of which may affect sexual selection and sexual conflict (Kokko & Rankin, 2006). It is also unlikely that differences in pairing patterns were due to changes in male sexual traits because the size of male gnathopods and antennae did not differ between P supply treatments. The pairing patterns observed are likely due to differences in behavioral responses to a combination of current P-availability in food and the environments in which the amphipods were raised. P availability in the developmental environments may have caused physiological shifts that resulted in different behavioral responses to the quality of food available in the mating environment. However, the effects of physiological shifts caused by the developmental environment on responses to food quality have yet to be studied in depth, and our results suggest this should be examined in future studies.

Previous studies have shown that eutrophication increases growth rates in amphipods and isopods (Hemmi & Jormalainen, 2002; Kraufvelin *et al.*, 2006; Cothran *et al.*, 2012). Faster growth should result in shorter time to sexual maturation, leading to shorter generation times and steeper population growth trajectories. Further, increases in female body size should lead to increased population growth as fecundity increases with body size in many animals (Blueweiss *et al.*, 1978; Shine, 1988; Honek, 1993; Preziosi *et al.*, 1996; including *Hyalella* amphipods in Strong, 1972). Indeed, evidence exists that eutrophication promotes population growth in marine amphipods, although abundance differences exist only between environments that differ dramatically in nutrient availability (i.e. nutrient enriched vs. no enrichment; Pardal *et al.*, 2000; Kraufvelin *et al.*, 2006). Analysis of the periphyton in the tanks indicated that the HP and LP tanks were different in P content (Fig. 1). However, such small differences in P supply (only 1.69 times greater in HP than LP) do not accelerate population growth (Fig. 2). In contrast, Kraufvelin *et al*. (2006) found 2- to 3-fold increases in amphipod (*Gammarus locusta*) abundance in nutrient-enriched mesocosms that contained algae with P contents 3-6 times higher than ambient levels. It should be noted that because our tanks were standing stocks with no P addition throughout the experiment, it is possible that P-turnover may be higher in the LP tanks. In turn, this might actually have resulted in HP amphipods being more P-limited than LP amphipods, further inhibiting population growth.

High within-treatment variation in population density may explain why relatively small increases in P supply did not significantly affect population dynamics. This variation is likely due to either environmental stochasticity or demographic stochasticity in our tanks. Because our tanks were in a controlled environment, environmental stochasticity is unlikely, yet possible due to accidental colonization of competing microinvertebrates or other unmeasured environmental factors. Indeed, the early population crash in one of the tanks eventually discarded from the dataset was likely due to intense competition from ostracods and copepods. Further, our results indicate that P content decreased significantly in all tanks temporally, suggesting that much of the P in the tanks was sequestered into consumer biomass as the experiment progressed, possibly resulting in resource stress as the amount of P per algal cell decreased temporally (Gulati, Martinez, & Siewertsen, 1995). Demographic stochasticity, either due to a skew in sex ratio or random variation in survival may be a large driver of variation in population dynamics as some tanks may experience drift and higher inbreeding (Nei, Maruyama, & Chakraborty, 1975; Lande, 1988; Simberloff, 2009). Colonies of *Hyalella* established by a single gravid female have been shown to be extremely impacted by demographic stochasticity (Wellborn & Capps, 2013). It is difficult, however, to determine how important demographic stochasticity was in influencing population dynamics in the 12 experimental populations, each established from 25 wild-caught gravid females.

Our results indicated that P supply influences the mating behavior of *Hyalella* amphipods. However, these changes in mating behavior seem unlikely to be directly related to population dynamics. The traits known to be important determinants of mating success (gnathopod size and antenna size; Wellborn, 1995; Bertin & Cézilly, 2003; Wellborn & Bartholf, 2005) were not sensitive to the subtle differences in P availability. The strength of natural selection within our populations, due to high competition and resource stress, could have been greater than sexual selection. Natural selection is very likely a strong constraint on sexual traits, especially when sexual traits are density-dependent (Andersson, 1994; Arnqvist, 1994). Both treatments contained population densities much greater (~20,000-50,000 amphipods/m^2) than observed in the wild (~700-8,000 amphipods/m^2; Wellborn, 1994). In fact, the mean gnathopod size in our study compares to gnathopod size in food-stressed (based on density) gnathopods in a previous study (Cothran & Jeyasingh, 2010). However, males in our study possessed larger gnathopods than those in the natural populations, suggesting further selective forces beyond just density (e.g. predation) may be constraining gnathopod size in the wild (Wellborn *et al.*, 2005). Further, while HP males were predicted to have higher mating success, this was only true in one of the four treatment groups.

The male rearing environment was a determinant of mating success only when HP females were available. It is possible that HP females are more selective in choosing mates, due to changes in female condition that affect resistance behavior. Females raised in LP environments may be less likely to resist mating attempts by males due to a greater overall cost of resistance in the low P environment. This decrease in female resistance should result in relatively random mating. On the other hand, HP females may have been in better condition allowing them to invest more in resistance behaviors. Future studies should address whether females fed high P food are more effective at resisting male mating attempts than females fed low P food.

The patterns of mating success observed in our study may also be due to differences in male competitive or coercive ability that are only evident in certain environments. Given the lack of an effect of P availability on male sexual traits, our results suggest that the small differences in P applied in this study, when placed in a population context in which animals had to compete for access to P (unlike in Cothran *et al*. 2012), have no effect on sexual selection. Instead, the patterns in male mating success may be due to physiological or behavioral changes induced by the differences in P available in food. For example, HP males may be of greater overall condition allowing them to exploit resources at a higher rate. Alternatively, LP males may be more efficient at retaining P (Goos, Cothran, & Jeyasingh, unpublished data), possibly providing an advantage in a new food environment. Studies examining the physiological consequences of changes in P availability and their effect on mating behavior may shed more light on the patterns observed in our study. Of particular interest, is the fact that HP males possessed significantly larger pereopods than LP males. Although we know nothing about the utility of the fifth pereopod in mating, our results suggest that this trait deserves further attention in future studies as it may play a role in foraging or competitive ability.

Collectively, we found that, subtle changes in P availability do not affect population growth or sex ratio. Nevertheless, we observed effects of P availability on mating success, suggesting that P supply has the potential to affect individual level processes that ultimately drive mating biology. Although recent studies demonstrate that sexual traits are extremely sensitive to resource supply (Cotton, Fowler, & Pomiankowski, 2004; Hunt *et al.*, 2004; Cothran & Jeyasingh, 2010; Lewis *et al.*, 2012; Cothran *et al.*, 2012), our results indicate that at the population level, this sensitivity may be dampened by natural selection. Given the high densities observed in our study, it is possible that resource competition had an effect on the development of male sexual traits. Additionally, while most studies of amphipod mating biology focus on morphology in determining male mating success (Wellborn, 1995, 2000; Cothran *et al.*, 2010, 2012), the results of our study indicate that mating success may also be shaped by the effect of both the nutritional history and the current nutrition of food on an individual's behavior. Studies focusing on the effects of nutrient dynamics and other ecosystem-level processes on mating biology should consider population-level issues to garner a more complete understanding of the effects of global change on sexual selection.

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Tables

Table 1: Results of backward elimination procedure examining the effect of female treatment and mating environment on whether an HP or LP male paired

Variable	ΔG	*df*	p-value
Female treatment[3]	2.593	1	0.107
Mating environment[2]	0.000	1	1.000
Female treatment*Mating environment	**4.364**	**1**	**0.037**

ΔG= change in log likelihood statistic when a term was removed. Superscripts refer to the step in the backward elimination procedure the variable was removed from the model. Significant effects are in bold.

Figures

Figure 1: Phosphorus content of periphyton collected from experimental tanks at the beginning and the end of the experiment. Closed circles represent HP tanks and open circles represent LP tanks. Markers represent means ±1 SE. HP = 60 μM phosphorus (K_2HPO_4); LP = 10 μM phosphorus (K_2HPO_4).

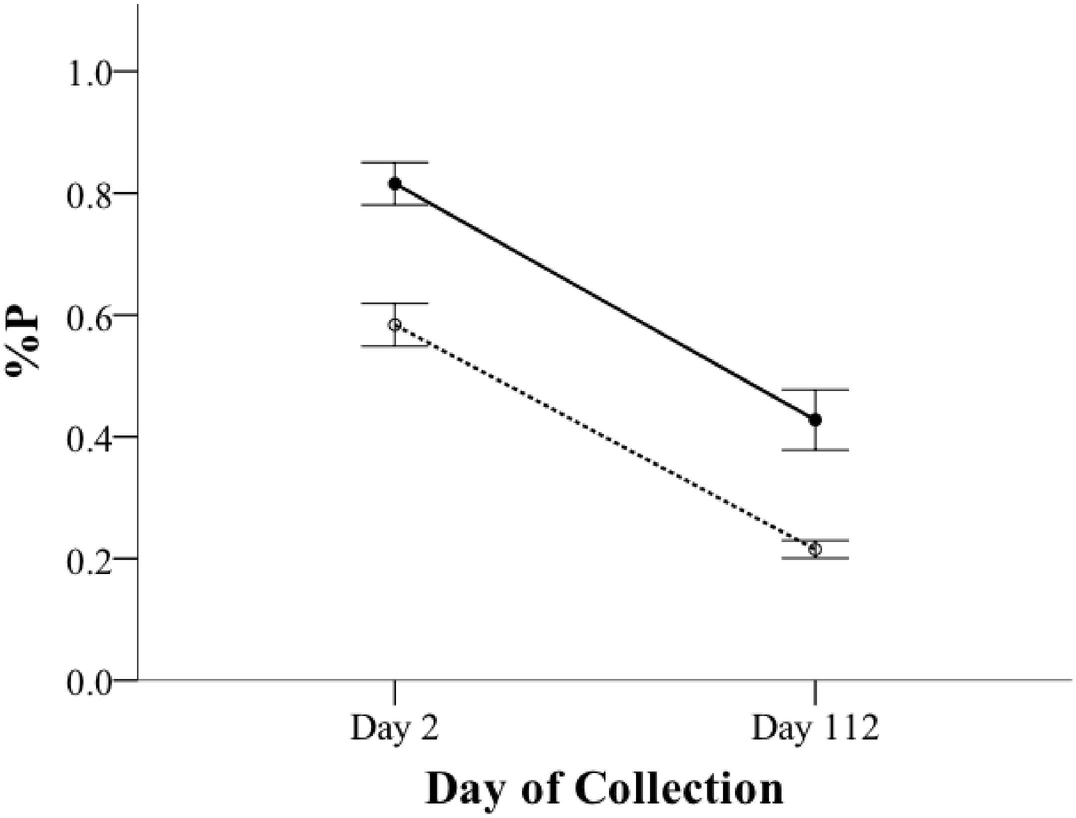


Figure 2: Population densities during the experiment. Markers represent means ±1 SE. HP = 60 μM phosphorus (K_2HPO_4); LP = 10 μM phosphorus (K_2HPO_4).

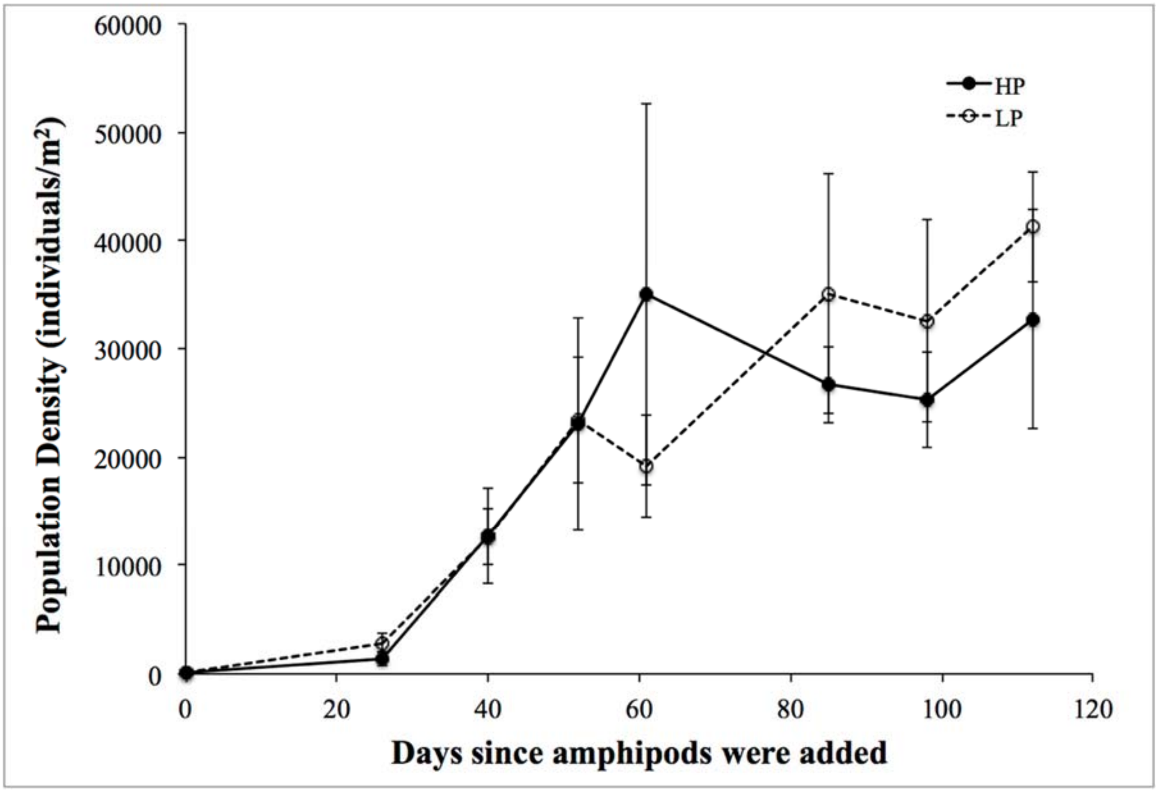


Figure 3: Sex-ratios during the experiment. Markers represent means ±1 SE. Because density within the tanks varies significantly within treatments, sample sizes for sex-ratio estimates also vary considerably (See Supplementary Info Table S2 for sample size ranges). HP = 60 μM phosphorus (K_2HPO_4); LP = 10 μM phosphorus (K_2HPO_4). M, male; F, female.

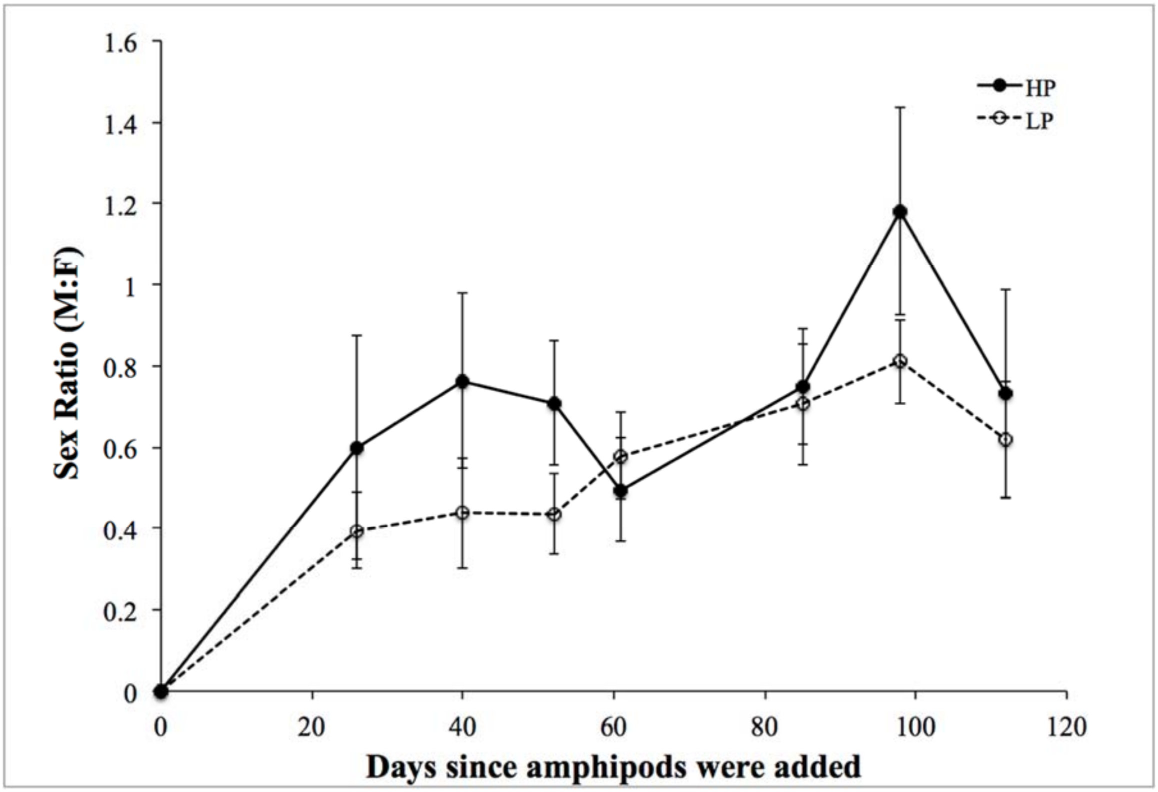


Figure 4: Size-adjusted trait sizes of males in mating experiment. Markers represent means ±1 SE. HP = 60 µM phosphorus (K_2HPO_4); LP = 10 µM phosphorus (K_2HPO_4).

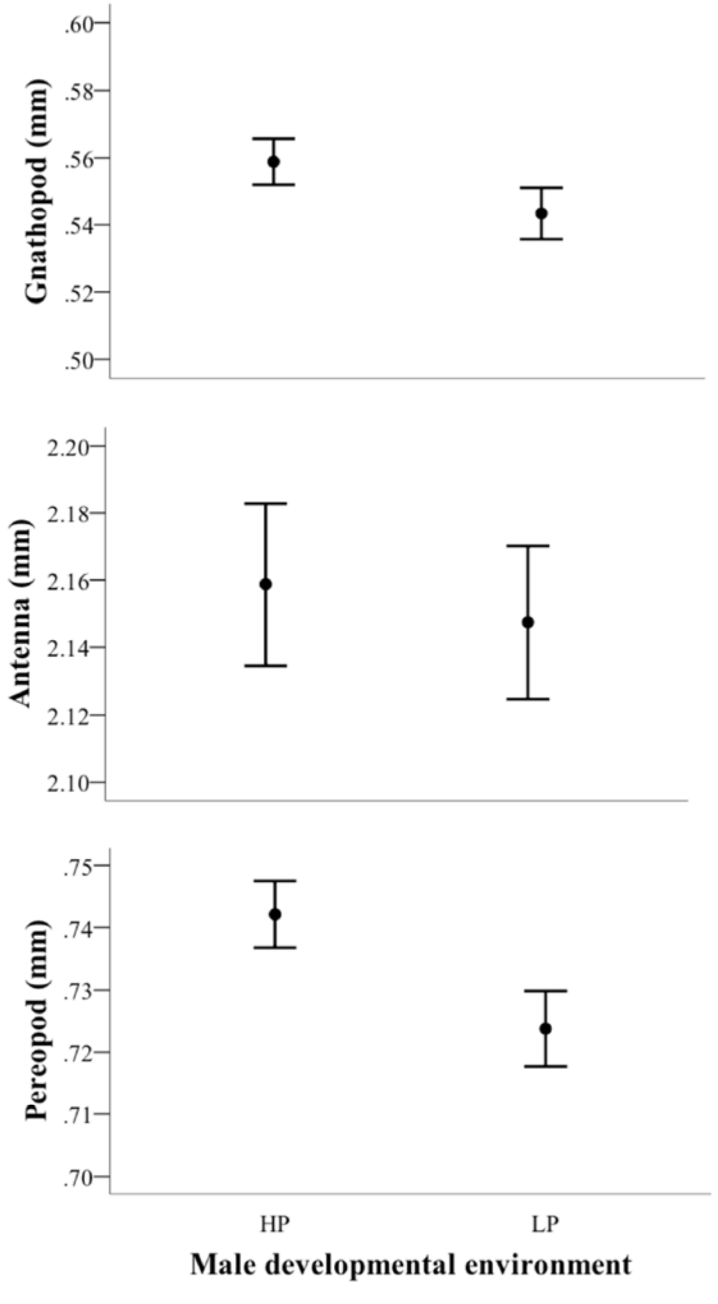
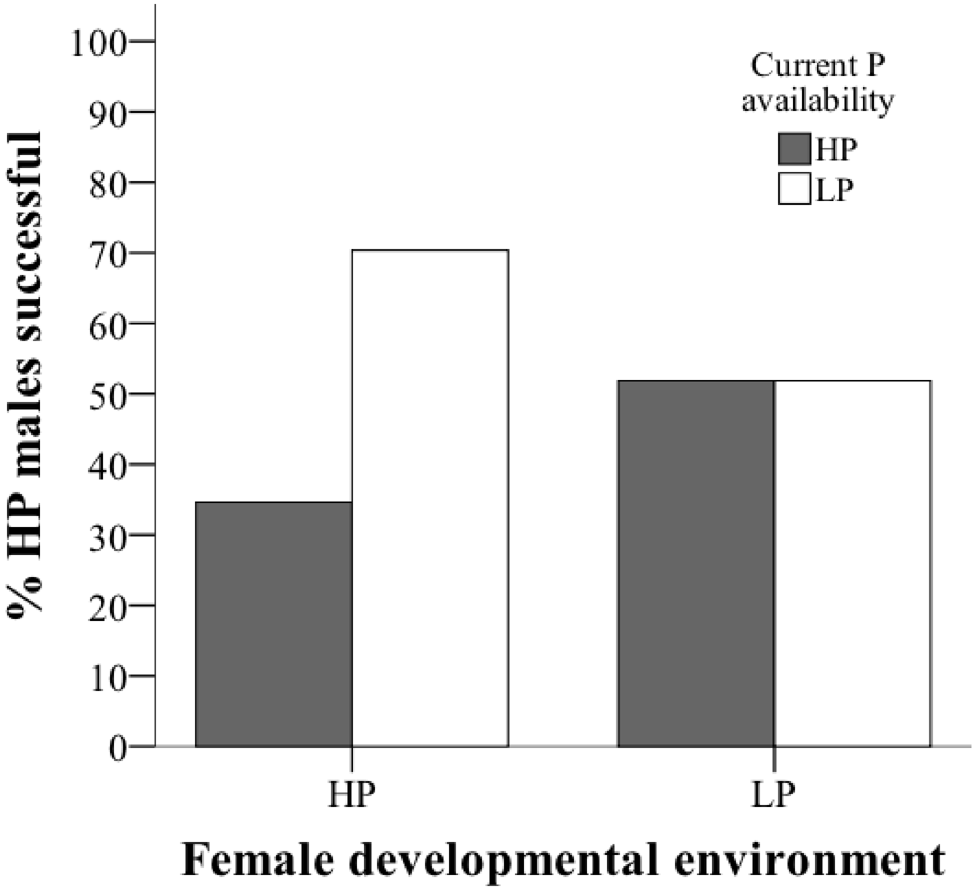


Figure 5: Percentage of HP males successful in mating in each female-by-food group. HP = 60 µM phosphorus (K_2HPO_4); LP = 10 µM phosphorus (K_2HPO_4).



Supplementary Information

Figure S1: a) Spatial distribution of tanks used in the population dynamics experiment. b) Sampling method showing the sampling quadrants in each tank. c) Mating experiment design.

Table S1: MANCOVA results for interaction between Male Developmental Environment and Head Length. Results indicate no difference in slope between treatments

Table S2: Sample size ranges for estimates of population density and sex ratio in 4 HP and 5 LP experimental tanks.

Figure S1

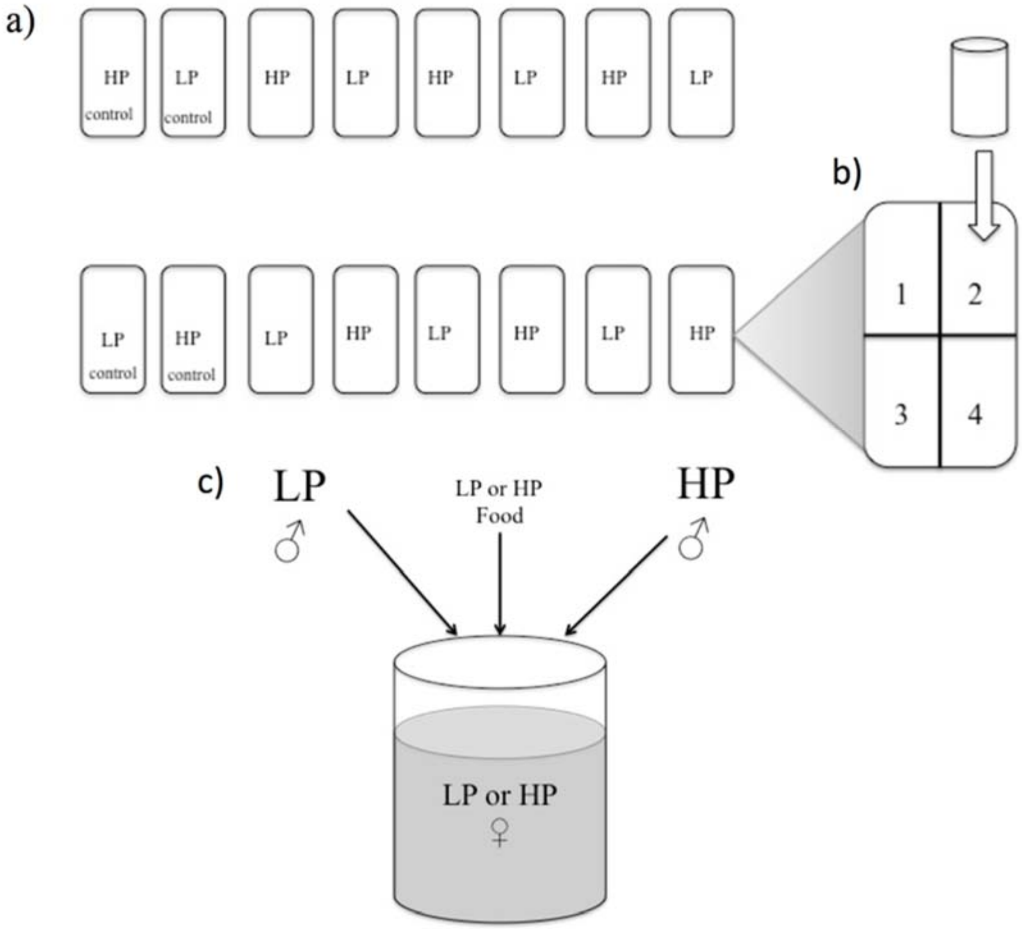

a)
HP control
LP control
HP
LP
HP
LP
HP
LP
LP control
HP control
LP
HP
LP
HP
LP
HP
b)
1
2
3
4
c)
LP
♂
LP or HP Food
HP
♂
LP or HP
♀

Table S1

Source	Dependent Variable	F	p
Male Dev. Environment*ln(Head Length)	ln(Gnath. Width)	0.582	0.446
	ln(Antenna)	1.237	0.267
	Ln(Pereopod)	1.555	0.214

Table S2

Sample	Population Density		Sex Ratio	
	HP	**LP**	**HP**	**LP**
Sample1	3-56	12-105	3-25	9-59
Sample 2	13-174	43-149	2-26	9-24
Sample 3	40-354	58-295	4-119	12-147
Sample 4	43-690	63-281	32-375	41-140
Sample 5	143-271	128-606	63-213	77-357
Sample 6	125-289	118-464	58-149	94-279
Sample 7	116-434	199-434	52-203	87-225

CHAPTER IV

THE STOICHIOMETRY OF SEXUAL DIMORPHISM IN MULTIPLE DIMENSIONS

Jared M. Goos, Rickey D. Cothran, Punidan D. Jeyasingh

Abstract

Sexual dimorphism represents the most striking phenotypic differences observed within a population. Although difficult to quantify, exaggerated trait expression is hypothesized to affect other traits through tradeoffs. Advances in spectrometry enable rapid quantification of the elements that make up traits, which can be used to compare the elemental signatures of traits. We measured dimorphism in the stoichiometry of *Hyalella* amphipods, and examined the extent to which exaggerated traits contributed to dimorphism. Multivariate elemental sexual dimorphism was apparent. Calcium, strontium, phosphorus, and sulfur exhibited the greatest degree of sexual dimorphism, as indicated by comparison of effect sizes. Sexual and nonsexual traits differed significantly in mass-specific stoichiometry. These results indicate dimorphism in the acquisition or processing physiology of these elements, and sex-specific selection on the underlying loci. High throughput, multidimensional data on the stoichiometric composition of males and females will provide a powerful diagnostic tool in understanding the ecology of sexual dimorphism.

Introduction

Sexual dimorphism is arguably the greatest source of intraspecific variation in sexually reproducing organisms. Despite sharing a common genome (Ellegren & Parsch 2007), males and females often exhibit staggering divergence in traits. Such divergence is driven by natural and/or sexual selection on traits that maximize fitness in each sex. Sex-specific selection on certain traits may result in sex-specific life history tradeoffs and resource allocation (Bonduriansky *et al.* 2008). Often, these selective pressures result in the exaggeration of sexually dimorphic traits (e.g., beetle horns or peacock feathers; Andersson 1994). Developing and maintaining these exaggerated traits is thought to be costly resulting in differences in male and female behavior and physiology (Zahavi 1975; Andersson 1994; Emlen 2001; Kotiaho 2001). Additionally, these exaggerated traits are highly dependent on organismal condition, itself a product of environmental supply of resources and genetic variation in the acquisition and utilization of resources (Rowe & Houle 1996; Cotton *et al.* 2004; Hedrick 2005).

While much work has been done to elucidate the ultimate, evolutionary mechanisms that drive sexual dimorphism (e.g., Lande 1980; Hedrick & Temeles 1989; Punzalan & Rowe 2015), most of these studies have focused exclusively on the role of exaggerated traits in driving sexual dimorphism within species. Research focused on these exaggerated traits, as well as other sexually dimorphic traits such as body size and composition (see Badyaev 2002; Wells 2007), has provided diverse avenues of research toward a greater understanding of sexual dimorphism. Regardless, the focus on only a few traits of interest potentially masks the complex interactions between traits, and may lead to misleading mechanistic conclusions for the evolution of sexually dimorphic traits, and traits in general (Cotton *et al.* 2004; Houle *et al.* 2010). Clearly, measuring all traits within an organism is incredibly difficult, if not impossible, but emerging technology and analytical methods have opened the door for high-throughput phenotyping on the whole organism level (Houle *et al.* 2010). Because all traits require elemental resources, the elemental composition of an individual can serve as a composite trait representing the whole individual (Sterner & Elser 2002). Due to the finite number of elements underlying all biological processes, high dimensional analyses of organismal elemental composition, or 'ionomics', has been proposed as a powerful approach to addressing the complexity of characterizing whole organism genotype-phenotype correlations (Baxter 2010; Jeyasingh *et al.* 2014).

Ecological stoichiometry (ES) abstracts complex biological interactions into atoms of biologically relevant elements, particularly carbon (C), nitrogen (N), and phosphorus (P). A central principle of ES is that the relatively fixed nature of organismal demand (i.e. homeostasis) can result in mismatches between demand and environmental supply, potentially driving trade-offs that influence the expression of fitness-maximizing traits (Elser *et al.* 2000). Variation in organismal composition is, in part, a function of evolutionary history and the current selective pressures imposed on traits. Indeed, broad patterns in organismal composition have been observed across a wide array of species as the result of life history differences, trophic position, and phylogeny (Elser *et al.* 1996; Fagan *et al.* 2002; Woods *et al.* 2004; González *et al.* 2011). In addition to the widely studied interspecific patterns of organismal composition, some recent studies have begun to show extensive intraspecific variation in organismal composition (Bertram *et al.* 2008;

González *et al.* 2011; El-Sabaawi *et al.* 2012; Morehouse *et al.* 2012; Goos *et al.* 2014; Tobler *et al.* 2016). Previously, intraspecific variation in organismal composition was thought to be of much smaller magnitude than interspecific variation due to regulation of elemental composition, or homeostasis, and has been largely ignored (Karimi & Folt 2006; Persson *et al.* 2010). However, intraspecific variability in organismal composition has been shown to vary substantially due to genetics as well as the environment (Schade *et al.* 2003; Small & Pringle 2010; El-Sabaawi *et al.* 2012; Goos *et al.* 2014). Additionally, organismal composition has been shown to vary substantially due to variation in life history demands, such as ontogenetic or sex effects (Nakazawa 2011; Back & King 2013; Goos *et al.* 2014).

Most studies examining sexual dimorphism in the context of ecological stoichiometry have focused on the importance of just one element, P, in affecting sex-specific fitness-maximizing traits. Specifically, these studies have found that sexual dimorphism manifests at the elemental level (Goos *et al.* 2014), and that the development of sexually dimorphic traits (Bertram *et al.* 2006, 2009; Cothran *et al.* 2012) and reproductive traits, such as egg production (Markow *et al.* 2001; Visanuvimol & Bertram 2010) are sensitive to dietary P supply. Further, we have also found that the sexes use divergent strategies to meet their P demands in response to variation in dietary P supply (Goos *et al.* 2016). These studies have illuminated some of the myriad ways in which supply of an important elemental resource may influence variation in fitness-conferring traits. Roughly 25 elements are important for the fundamental biochemistry of life, including metabolic processes and the expression of morphological traits (Frausto da Silva & Williams 1991). While P clearly plays a role in many organismal processes, a focus on a limited subset of biologically important elements precludes a complete understanding of the sources of intraspecific variation in organismal stoichiometry, especially considering the documented importance of less abundant elements and interactions between elements in biological processes (Cullen & Sherrell 2005; Karimi & Folt 2006; Wright *et al.* 2011; Dudley *et al.* 2012). A single- or few-element focus may result in failure to recognize important ecological and evolutionary patterns due to correlated evolution in other traits, which is a central issue in understanding the causal mechanisms underlying complex traits (Houle *et al.* 2010).

In this study, we assessed whether sexual dimorphism in a freshwater amphipod species manifests along multiple elemental axes, and whether there are sex-specific tradeoffs between elements beyond the traditional stoichiometric variables of C, N, and P. Additionally, we examined whether the trait-specific elemental demand of exaggerated, sexually selected traits differs from that of similar, non-sexual traits and whether that demand is correlated with variation in trait morphology. Because exaggerated traits are generally considered resource sinks, we expected to observe significant divergence in trait-specific demand. Trait-specific demand is expected to result from the disproportionate allocation of elemental resources for the development and maintenance of exaggerated traits compared to smaller morphological traits.

Materials and Methods

Study system

To address the above questions, we used a freshwater amphipod species in the *Hyalella azteca* species complex (the species is undescribed and is in the clade OK-L in

Wellborn & Broughton 2008). Amphipods within this genus exhibit significant morphological sexual dimorphism, with males possessing enlarged, claw-like appendages called posterior gnathopods (hereafter, just gnathopods) that are ~15 times larger in males than females and constitute up to 10% of male biomass (Wellborn 2000). These enlarged gnathopods facilitate pairing between the sexes, as larger gnathopods relative to body size are important in determining pairing success, which is a prerequisite for mating (Wellborn 1995, 2000; Cothran *et al.* 2010). Previous studies have shown that posterior gnathopods in males are much more sensitive to ecological stressors such as food availability, environmental P supply, and predation, than the same trait in females and similar, nonsexual traits (Cothran & Jeyasingh 2010; Cothran *et al.* 2012). Additionally, amphipods within the OK-L clade of *Hyalella* exhibit significant sexual dimorphism in P content, as well as sex-specific patterns of compositional plasticity in response to resource availability (Goos *et al.* 2014).

Study organisms

Amphipods were collected from Dripping Spring in Custer County, OK (35°34’32.1816” N, 98°54’2.2998” W). In the lab, amphipods were housed indoors under natural light at 20-23°C in 5.7-L plastic tubs filled with no nitrogen or phosphorus COMBO media (Kilham *et al.* 1998), with Nitex mesh (mesh size = 1 mm) provided as refugia and constant bubbling. An artificial diet mixture consisting of 4 g of pure Spirulina and 1g of Spirulina fish flakes was suspended in 50 mL of Bacto-agar solution and cooled in a petri dish. We then fed the lab stock populations five 4-mm pellets of the artificial diet once weekly. Media within our stock populations was changed fortnightly.

To ensure that all amphipods used in our study were raised in identical conditions, we randomly selected 75 adult males and 75 adult females from our stock populations and paired each male with one female in a 100-mL jar filled with no N/P COMBO. We allowed all pairs to mate and, once the neonates were released from the mother's marsupium, we removed the adults. Juvenile amphipods were then allowed to mature in each jar, with one food pellet being fed to them every three days, and media changes occurring every ten days. Once mature, we selected one male and one female from each jar for elemental analysis. Each individual was then washed, in triplicate, in ultrapure (Type 1) water. To control for the effects of female egg production on the variation in body composition, females were selected only when eggs were clearly visible in their ovaries, ensuring all females were undergoing the same stage of oogenesis. After selection, females were immediately transferred to individual tin boats to be dried and subsequently weighed, while males were dissected (see below) before being transferred to tin boats. In total, 56 females and 48 males were analyzed for their elemental composition. Eight jars contained only females, while 19 jars were discarded due to complete neonate mortality (10), death of the mother (6), or no mating observed (3).

To quantify elemental demand in male sexual traits in comparison to homologous nonsexual traits, we dissected the sexually selected posterior gnathopods and the nonsexual fifth pereopod (hereafter walking leg) from each male. After removal from their respective jars, each male was immediately dissected under a stereo microscope (Swift SM90, Schertz, TX, USA) using fine surgical forceps (#5, Dumont SA, Montignez, Switzerland). Both gnathopods and walking legs for each male were dissected at the basis (Fig. 1). After dissections, we transferred both gnathopods, walking leg, and the rest of the body of each male to separate tin weigh boats to be dried and weighed.

Elemental analysis

All samples were analyzed for 29 elements using an inductively coupled plasma optical emission spectrometer (ICP-OES; Thermo Scientific iCAP 7400, Waltham, MA, USA). In 15 mL centrifuge tubes, we digested each sample with 200 µL of trace metal grade 100% HNO_3 and 100 µL of trace metal grade 100% H_2O_2 overnight. We then diluted all samples to 5 mL with ultrapure (Type 1) water. Aqueous multi-element standard reference solutions (CCV Standard 1A &B, CPI International, Santa Rosa, CA) were used to validate the ICP-OES and calibrate our analysis. Of the 29 elements analyzed, we excluded 17 from further analysis because the measured values of the samples were within the range of the standard deviation of our blank controls, indicating that the concentration of these elements within our samples are close to or below the detection limit of the machine. The remaining 12 elements (Ca, Cu, Fe, K, Li, Mg, Mn, Na, P, S, Sr, and Zn) were all above the limits of detection and the concentrations of these elements (in $\mu g\ g^{-1}$) were $\log_{10}$-transformed before statistical analyses to satisfy assumptions of normality.

Statistical analysis

Because elemental composition within an organism is inherently multivariate, with correlations occurring between the elements, we first ran a multivariate analysis of variance (MANOVA) on all elemental concentrations for each individual, with sex as a fixed factor. Then, to determine which elements exhibit sexual dimorphism, we ran separate univariate analyses of variance (ANOVAs), with sex as a factor. While a MANOVA is a frequently used multivariate analysis, this method only determines the multivariate differences between groups along one axis (the first discriminant function). To examine organismal elemental composition in multivariate space more completely, we ran a principal components analysis (PCA) on all individuals, ignoring sex. A PCA is a multivariate analysis aimed at transforming many variables of interest into fewer, uncorrelated variables that adequately summarize the original data structure. We performed our PCA using a correlation matrix, as this method standardizes all variables to a mean of zero and a unit variance, thus eliminating the possibility of certain variables that exhibit high variance from disproportionately influencing the analysis (Quinn & Keough 2002). To improve interpretability of the components extracted by the PCA, we applied an orthogonal rotation (varimax) to the components. Four PC axes exhibited eigenvalues >1 and the individual PC scores from these axes were extracted for further analyses. We then ran separate analyses of variance (ANOVAs) on the principal component scores of the four PC axes, with sex as the only predictor variable. This method is similar to a MANOVA, but allows for multivariate analyses along many, uncorrelated axes (Quinn & Keough 2002). To test for potential trait-specific differences in elemental composition, we again ran a MANOVA and individual ANOVAs on all elemental variables, with trait as a fixed factor. Additionally, we ran a varimax-rotated PCA on all male traits based on the correlation matrix. This analysis resulted in five PC axes with eigenvalues>1. We then ran separate ANOVAs on the individual PC scores for all five axes, with trait as our lone predictor variable. Effect sizes for all models were calculated as eta squared (η^2), defined as the proportion of the total variance explained by the group variable. All statistical analyses were performed using SPSS (version 20, IBM), unless otherwise stated.

Results

Sexual dimorphism in multielement stoichiometry

The results of our MANOVA indicated that the sexes differed in their elemental compositions along the first discriminant axis (Wilks' $\lambda = 0.303$, $F_{12,91} = 17.42$, $p<0.001$). Individual ANOVAs indicated the largest effect of sex on concentrations of Ca (Table 1; Fig. 2a), P (Table 1; Fig. 2a), S (Table 1; Fig. 2a), and Sr (Table 1; Fig. 2a). Smaller, significant effects of sex were observed on concentrations of Li (Table 1; Fig. 2a), Na (Table 1; Fig. 2a), and Zn (Table 1; Fig. 2a). Additionally, there were weak, but significant effects of sex on Fe (Table 1; Fig. 2a) and K (Table 1; Fig. 2a) concentrations. No significant differences were observed between the sexes in Cu (Table 1; Fig. 2a), Mg (Table 1; Fig. 2a), and Mn (Table 1; Fig. 2a). Of the nine elements exhibiting sexual dimorphism, only Ca, Li, and Sr were greater in males than females (Fig. 2a). Principal components analysis resulted in four PCs that explained 74.06% of the total variation in elemental composition in amphipods (Table S1). PC1 primarily explained the variance in Ca, Li, S, and Sr, PC2 primarily explained the variance in Cu, K, Na, and Zn, PC3 primarily explained the variance in Mg and P, and PC4 primarily explained the variance in Fe and Mn (Table S1). The PCA plots indicate that the sexes differ in their elemental compositions in multidimensional space, with most of the separation between the sexes occurring along PC1 (Fig. 2b; Fig. S1). Males are clustered toward more positive values along PC1, while females tend to be more negative. Additionally, females tend to vary more along this axis than males. The results of our individual ANOVAs for each PC indicated strong, significant differences between the sexes in elemental composition observed along PC1 ($F_{1,102}$= 49.696, p<0.001, η^2= 0.328 :Fig. 2b). Weak, but significant differences between the sexes were also observed along PC2 ($F_{1,102}$= 6.730, p= 0.011, η^2= 0.062; Fig. 2b) and PC3 ($F_{1,102}$= 8.771, p= 0.004, η^2= 0.079; Fig. S1a). No sexual dimorphism was observed along PC4 ($F_{1,102}$= 3.819, p= 0.053; Fig. S1b).

Trait specific multielement stoichiometry and its correlations with trait value

Using a MANOVA, we observed strong differences between gnathopods and legs in elemental composition (Wilks' λ= 0.136, $F_{12,91}$= 43.924, p<0.001). Additionally, individual ANOVAs indicated that there was a strong effect of trait on Ca (Table 2; Fig. 3a), K (Table 2; Fig. 3a), S (Table 2; Fig. 3a), and Sr (Table 2; Fig. 3a) concentrations. Additionally, we observed weaker, but still significant, effects of trait on Mg (Table 2; Fig. 3a) and P (Table 2; Fig. 3a) concentrations. We observed no significant differences between the traits in Cu (Table 2; Fig. 3a), Fe (Table 2; Fig. 4), Li (Table 2; Fig. 3a), Mn (Table 2; Fig. 3a), Na (Table 2; Fig. 3a), and Zn (Table 2; Fig. 3a). Calcium and Sr were the only elements of the six exhibiting significant trait differences that were greater in walking legs than gnathopods (Fig. 3a). Principal components analysis resulted in five PCs that explained 74.83% of the total variation in male amphipod trait elemental composition (Table S2). PC1 primarily explained the variance in Ca and Sr, PC2 primarily explained the variance in Na and S, PC 3 primarily explained the variance in K, Mg, and P, PC4 primarily explained the variance in Fe, Li, and Mn, and PC5 primarily explained the variance in Cu and Zn (Table S2). The PCA plots indicate that traits differ in their elemental stoichiometry along multiple elemental axes (Fig. 3b; Fig. S2). Again, this separation primarily occurs along PC1, with gnathopods having much lower values of PC1 than walking legs, in general (Fig. 3b). Further, the variation in trait stoichiometry within each trait tends to be greater in PC2-4 than in PC1. The results of the ANOVAs for each PC axis indicated a strong effect of trait on elemental concentrations along PC1 ($F_{1,94}$= 199.393; p<0.001, η^2= 0.680; Fig. 3b). Weak, but significant effects of trait were also observed on PC2 ($F_{1,94}$= 4.288; p= 0.041, η^2= 0.044; Fig. 3b), and PC3 ($F_{1,94}$= 9.652; p= 0.003, η^2= 0.093; Fig. S2a). We did not observe significant differences between the traits in PC4 ($F_{1,94}$= 0.385; p= 0.536; Fig. S2b) or PC5 ($F_{1,94}$= 0.008; p= 0.928; Fig. S2c).

Discussion

Our results show intersexual differences in elemental composition across multiple elemental axes beyond C, N and P, despite all organisms being raised in a common garden. Additionally, our results show that elemental concentrations within organisms exhibit complex correlative relationships that point toward important sex-specific stoichiometric constraints. Patterns of sexual dimorphism in stoichiometry were observed in the concentrations of individual elements, as well as in multivariate space, taking into account the various interactions between elements within amphipod tissue. In addition to the sexual dimorphism we observed in multielement stoichiometry, we also found differences between the stoichiometry of sexual and nonsexual traits both individually and in multivariate space. These findings suggest that the elemental demands of different traits are unique, and may drive important resource tradeoffs that influence sex-specific fitness.

In multivariate space, the axis explaining the greatest proportion of total elemental variation in amphipods, PC1, was primarily a combination of Ca, Sr, Li, and S. The effect sizes calculated in the ANOVA of this axis indicated that sex explains five times more variation on this axis than the other axes exhibiting sexual dimorphism, and the PCA plots show separation of the sexes along this axis (Fig. 2b). Of the elements exhibiting sexual dimorphism, Ca, Sr, and Li were the only elements that were more concentrated in males than females. For all other elements exhibiting sexual dimorphism, concentrations were higher in females than males (Fig. 2a). Additionally, Ca, Sr, P, and S exhibit the greatest degree of sexual dimorphism, as indicated by the effect sizes (Table 1).

The sexual difference in Ca content is particularly interesting, as Ca is abundant in the crustacean body and is involved in key processes. In crustaceans, Ca is most abundant in the exoskeleton as $CaCO_3$ and, to a large extent, Ca composition of crustaceans is tightly linked to the crustacean molt cycle, as Ca is periodically lost and replaced throughout this cycle (Ahearn *et al.* 2004). Regression analysis revealed a weak, but significant, positive allometric relationship between body size and Ca content (Table S3). The positive allometric relationship we observed seems to contradict expectations based on surface area to volume, however, further examination into these data suggest that this positive correlation is driven primarily by the heteroscedastic nature of Ca content, driven primarily by sex. Variation in female Ca content was much higher than male Ca variation. Thus, the sexual dimorphism observed in Ca content is more likely a product of sexual dimorphism in form, rather than just size. Because male *Hyalella* have higher Ca demands than females, low ambient Ca levels, along with related environmental parameters such as pH and conductivity, can impose sex-specific selective pressures, with substantial fitness costs (Connallon *et al.* 2010).

In addition to the observed sexual differences in Ca content, our multivariate analysis indicated positive correlations between Li and Sr content and Ca content, as well as a strong negative correlation between S and Ca/Sr/Li (Table 1; Fig. 2b). Strontium is an alkaline earth metal that resembles many chemical characteristics of its fellow alkaline earth metal, Ca. Often, Sr is a chemical substitute for Ca in various cellular processes, as well as in calcified tissues, particularly when environmental supply of Ca is low and organismal demand is high (Cowan *et al.* 1968; Gunatilaka 1981; Matsumura 1981). Considering crustaceans have high Ca demands (e.g., Prater *et al.* 2015), it is possible that the positive correlation between Sr and Ca is due to organismal demand outpacing the supply of Ca within our media, resulting in Sr substitution to meet these demands.

Our observation of sexual dimorphism in P content is consistent with other studies examining P differences between the sexes in arthropods, and is thought to be a result of the substantial demand for P-rich RNA during oogenesis (Markow *et al.* 1999, 2001; Gorokhova & Kyle 2002; Goos *et al.* 2014). A regression analysis revealed a significant, moderately negative allometric relationship between P content and body size (Table S3), a relationship predicted by the 'growth-rate hypothesis' (Elser *et al.* 1996). While we could not quantify C and N content in the current study because there is not enough biomass in individual amphipods for CN as well as ICP-OES analysis, prior work observed dimorphism in these elements, with males containing more N (Goos *et al.* 2014; Goos *et al.*, unpublished data).

Although its physiological role is largely unexplored (Schou 1957; Schrauzer *et al.* 1992), our results suggest that Li, and its interactions with S, Ca, and Sr, may also be important for some aspect of sexual divergence. In contrast to the positive relationships between Ca, Sr, and Li, the strong negative correlation between S and Ca/Sr/Li suggests that uptake of these elements may be antagonistic. This antagonism between S and Ca/Sr/Li may be a direct antagonism where Ca/Sr/Li inhibit the uptake of S, although we are unaware of any studies examining this. Alternatively, this antagonism may be the result of indirect processes, such as the upregulation of proteins rich in methionine and cysteine, S-rich amino acids (Brosnan & Brosnan 2006), in organisms with lower Ca demands.

In addition to the differences between the sexes in elemental composition, we also observed differences in elemental composition between sexual and nonsexual male traits. Univariate analyses revealed elemental differences between gnathopods and walking legs primarily in the bulk elements and not in trace elements (Fig. 3a). As in our intersexual comparison of elemental compositions, Ca and Sr exhibited the strongest differences between the two traits. The differences between walking legs and gnathopods in these elements may be due to differences in exoskeleton investment as a result of disparate trait shape. Specifically, the walking legs are laterally compressed compared to the exaggerated gnathopods, and thus will have higher surface area-to-volume and consequently exoskeleton will contribute toward a higher proportion of leg mass. We also observed strong trait differences in K and S content, but these differences are not to the same degree as the differences observed in Ca and Sr. Finally, there were small differences between Mg and P content of the traits. Multivariate analyses also indicate that the traits largely separate on the axis explaining variation in Ca and Sr (PC1, Fig. 3b). Along this axis, K is weakly negatively correlated with Ca/Sr, indicating that there may be weak antagonism between these elements. Variation within each trait is generally greater in PCs 2-5 than in PC1. This pattern suggests that differences between traits are generally linked to Ca and Sr, whereas trait variation is a function of the other elements.

Our results show the power of including multidimensional elemental data in the application ES principles to elucidate evolutionary questions. It is clear that a focus on a single or few elements is bound to miss important variation that underlies striking sexual dimorphisms and intraspecific trait diversity. While the precise mechanisms underlying the observed patterns of sexual dimorphism along these elemental axes require further study, these patterns have to be driven by sex-specific acquisition, assimilation, allocation, and excretion strategies. Additionally, knowledge of organismal demand in multivariate space can elucidate the complex interactions between an organism and its environment that shape important fitness-conferring life history traits. Analyzing high throughput, multi dimensional elemental data using the theoretical framework of ecological stoichiometry, as demonstrated here, is a powerful way to illuminate biologically relevant variation in a world characterized by rapidly changing biogeochemical cycles.

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Tables

Table 1: ANOVA results comparing elemental concentrations of male and female *Hyalella* amphipods. Bold values indicate significance at $p<0.05$.

Variable	$F_{1,102}$	Effect size (η^2)	P
Ca	47.850	0.319	**<0.001**
Cu	1.044	0.010	0.309
Fe	5.073	0.047	**0.026**
K	5.164	0.048	**0.025**
Li	19.939	0.164	**<0.001**
Mg	0.000	0.000	0.988
Mn	3.647	0.035	0.059
Na	19.164	0.158	**<0.001**
P	64.701	0.388	**<0.001**
S	57.267	0.360	**<0.001**
Sr	42.435	0.294	**<0.001**
Zn	14.844	0.127	**<0.001**

Table 2: ANOVA results comparing elemental concentrations of male gnathopods and walking legs. Bold values indicate significance at $p<0.05$.

Variable	$F_{1,94}$	Effect size (η^2)	P
Ca	194.796	0.675	**<0.001**
Cu	0.164	0.002	0.687
Fe	0.269	0.003	0.605
K	38.698	0.292	**<0.001**
Li	0.105	0.001	0.747
Mg	6.029	0.060	**0.016**
Mn	1.084	0.011	0.300
Na	0.223	0.002	0.638
P	4.868	0.049	**0.030**
S	19.704	0.173	**<0.001**
Sr	182.247	0.660	**<0.001**
Zn	2.268	0.024	0.135

Figures

Figure 1: Male *Hyalella* amphipod showing points of dissection for trait-specific elemental composition comparisons. The black lines indicate the point where the dissection occurred on the gnathopod and fifth pereopod (a.k.a. walking leg).

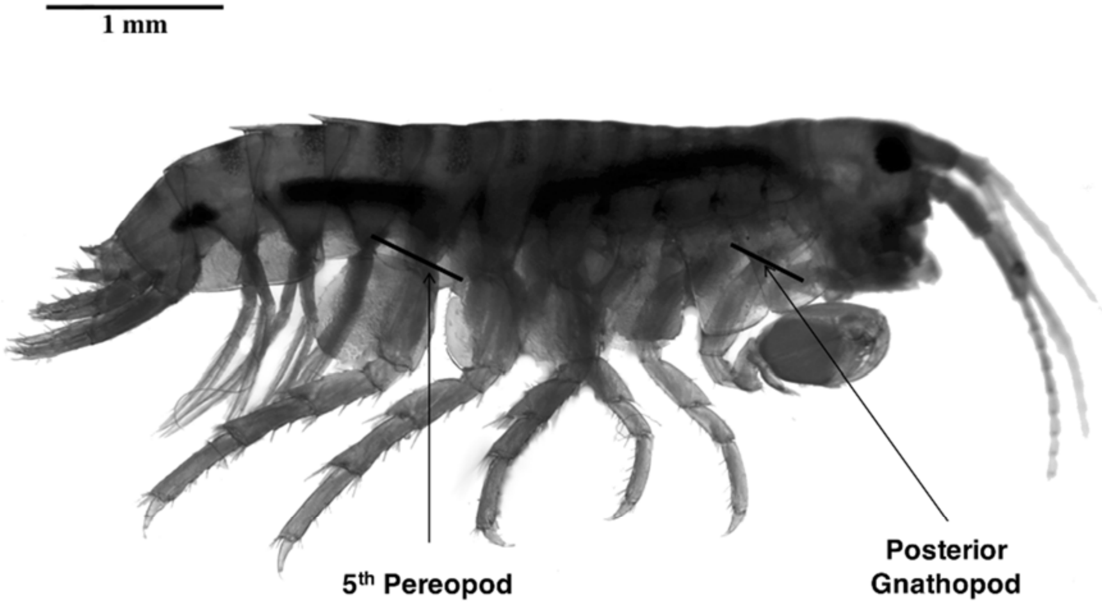


Figure 2: Elemental composition of male and female *Hyalella* amphipods (a) for each individual elemental and (b) in multidimensional space. For individual element comparisons, error bars represent 95% confidence intervals and asterisks indicate significant differences between the sexes ($p<0.05$). In multidimensional space, the first two PCs are shown, with the percentage of total elemental variation explained by these axes. The markers represent individual amphipods and vectors linear component loadings for each element.

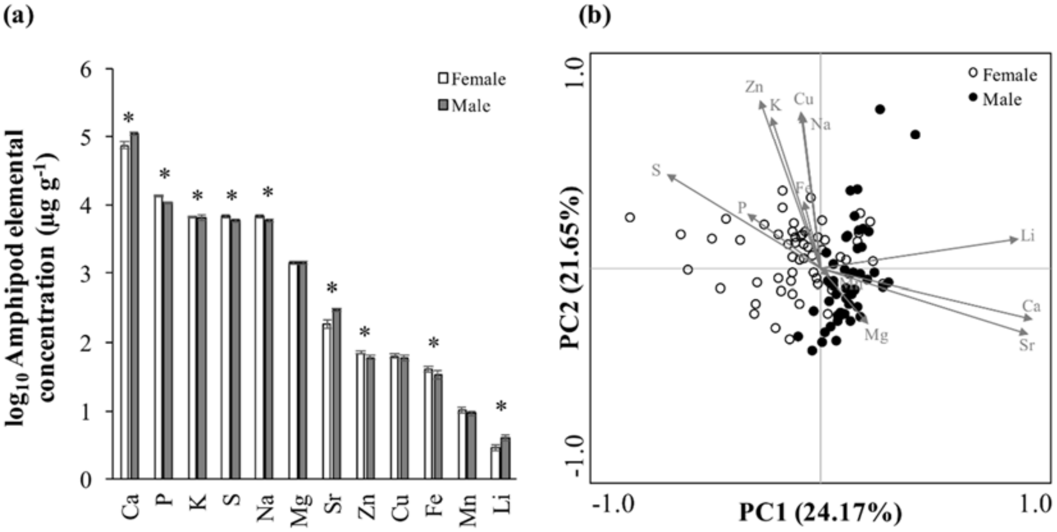
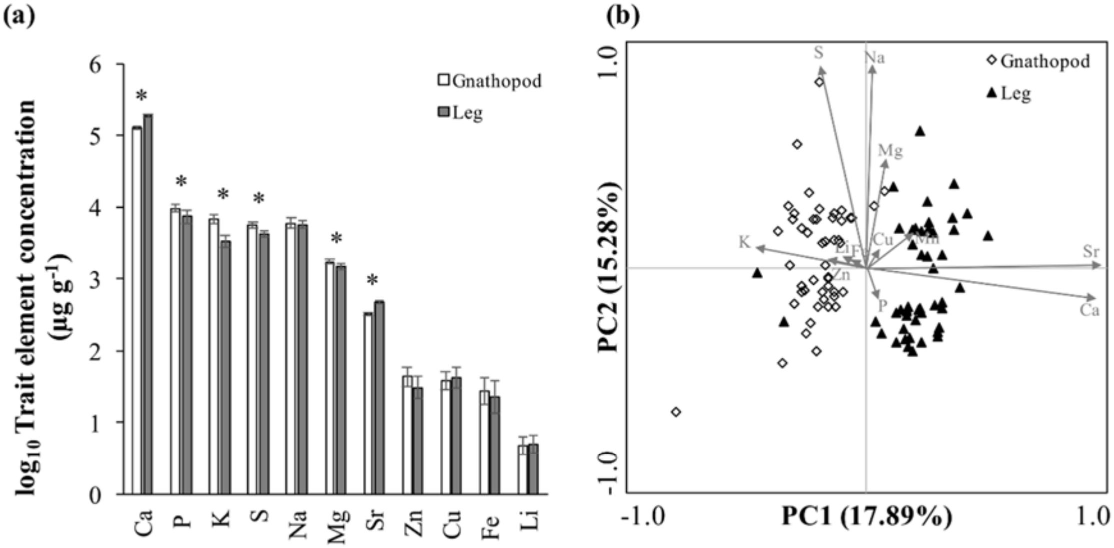


Figure 3: Elemental composition of male *Hyalella* gnathopods and walking legs (a) for each individual element and (b) in multidimensional space. For individual element comparisons, error bars represent 95% confidence intervals and asterisks indicate significant differences between traits ($p<0.05$). In multidimensional space, the first two PCs are shown, with the percentage of total elemental variation explained by these axes. The markers represent individual traits and vectors linear component loadings for each element.



Supplementary Information

Table S1: PC loadings for whole body ionomic analyses

Table S2: PC loadings for gnathopod vs. leg ionomic analyses

Table S3: Correlations of individual elements with log-transformed mass (mg). Bold values indicate significant correlations at $p<0.05$.

Figure S1: Elemental compositions of male and female *Hyalella* amphipods along the PCs (a) PC3 vs. PC1 and (b) PC4 vs. PC1. Markers represent individual amphipods and vectors represent linear component loadings for each element.

Figure S2: Elemental compositions of *Hyalella* gnathopods and walking legs along the PCs (a) PC3 vs. PC1, (b) PC4 vs. PC1, and (c) PC5 vs. PC1. Markers represent individual amphipods and vectors represent linear component loadings for each element.

Table S1

	PC1	PC2	PC3	PC4
Eigenvalue	2.900	2.598	1.696	1.693
% variance	24.17	21.65	14.14	14.11
Cumulative %	24.17	45.82	59.95	74.06
Loadings				
Ca	0.886	-0.227	0.049	-0.184
Cu	-0.082	0.708	-0.241	-0.162
Fe	-0.073	0.305	-0.007	0.709
K	-0.210	0.686	0.269	0.107
Li	0.827	0.131	-0.163	0.180
Mg	0.199	-0.250	0.863	-0.022
Mn	0.039	-0.037	0.119	0.889
Na	-0.08	0.699	-0.067	0.226
P	-0.311	0.246	0.851	0.173
S	-0.642	0.422	0.135	-0.070
Sr	0.868	-0.295	0.176	-0.180
Zn	-0.254	0.765	0.021	0.421

Table S2

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	2.147	1.834	1.811	1.639	1.549
% variance	17.89	15.28	15.09	13.66	12.91
Cumulative %	17.89	33.17	48.26	61.92	74.83
Loadings					
Ca	0.938	-0.130	-0.054	0.036	0.006
Cu	0.050	0.085	-0.107	-0.006	0.793
Fe	-0.060	0.041	0.029	0.850	0.106
K	-0.447	0.091	0.644	0.180	0.369
Li	-0.096	0.052	-0.090	0.563	-0.188
Mg	0.080	0.474	0.624	-0.103	-0.382
Mn	0.186	0.153	0.111	0.699	0.214
Na	0.024	0.874	-0.154	0.249	0.033
P	0.043	-0.136	0.937	-0.016	0.079
S	-0.185	0.875	0.132	0.031	0.146
Sr	0.975	0.004	0.022	-0.036	-0.088
Zn	-0.157	0.037	0.227	0.060	0.713

Table S3

Variable	Pearson Correlation (r)	P
Ca	0.273	**0.005**
Cu	-0.002	0.983
Fe	-0.192	0.051
K	-0.104	0.294
Li	0.169	0.087
Mg	-0.168	0.088
Mn	-0.197	**0.045**
Na	-0.439	**<0.001**
P	-0.572	**<0.001**
S	-0.362	**<0.001**
Sr	0.289	**0.003**
Zn	-0.218	**0.026**

Figure S1

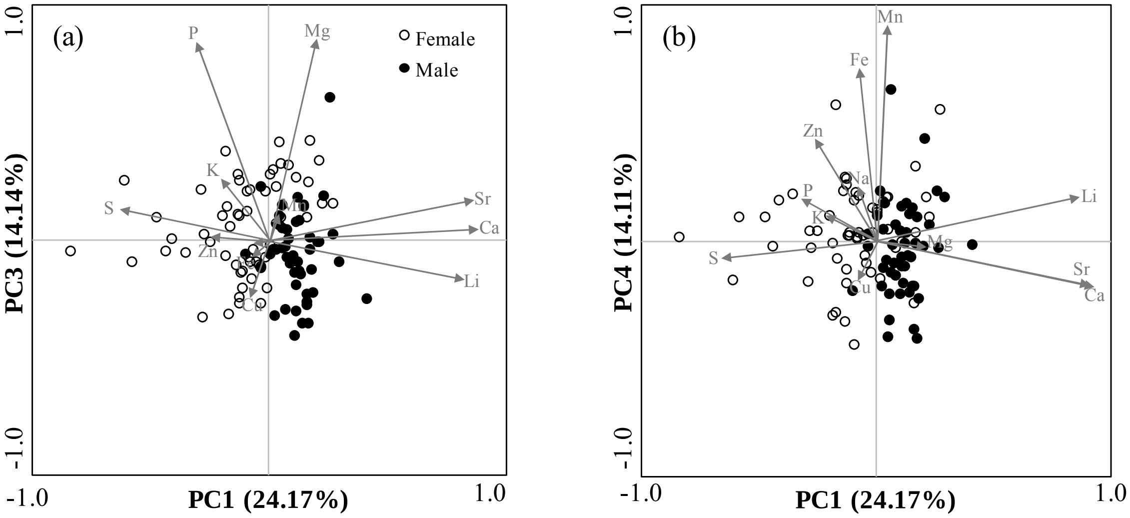
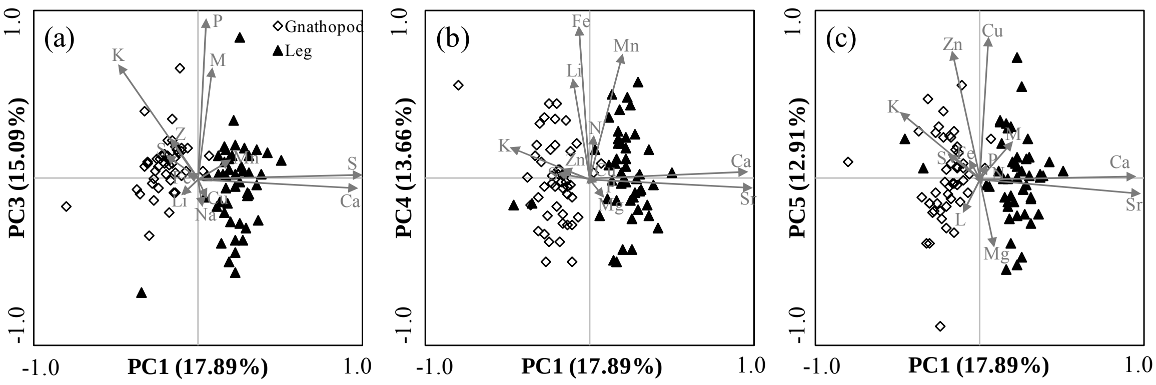

(a)
P
Mg
○ Female
● Male
K
S
Mn
Sr
Ca
Zn
Cu
Li
PC3 (14.14%)
1.0
-1.0
-1.0
PC1 (24.17%)
1.0
(b)
Mn
Fe
Zn
Na
P
K
S
Mg
Li
Sr
Ca
Cu
PC4 (14.11%)
1.0
-1.0
-1.0
PC1 (24.17%)
1.0

Figure S2


(a)
◇Gnathopod
▲Leg
P
M
K
Z
S
Mn
S
Fe
Li
Cu
Na
Ca
PC3 (15.09%)
PC1 (17.89%)
1.0
-1.0
-1.0
1.0
(b)
Fe
Mn
Li
N
K
Zn
Cu
P
Mg
Ca
Sr
PC4 (13.66%)
PC1 (17.89%)
1.0
-1.0
-1.0
1.0
(c)
Cu
Zn
K
M
S
Fe
P
N
L
Mg
Ca
Sr
PC5 (12.91%)
PC1 (17.89%)
1.0
-1.0
-1.0
1.0

SYNTHESIS

An elemental view of ecology and evolution can help in both horizontal and vertical integration of biological research. In recent years, great strides have been made towards a mechanistic understanding of eco-evolutionary interactions on the elemental level, with the elucidation of phylogenetic (e.g., Woods et al. 2004), community-level (e.g., Elser and Urabe 1999), life history (e.g., Jeyasingh and Weider 2005), transcriptomic (Jeyasingh et al. 2011; Roy Chowdhury et al. 2015), and microevolutionary (Frisch et al. 2014) patterns counted among those discoveries. While the use of the elemental perspective is growing as a tool for integrating ecology and evolution, largely unexplored is the role of sexual selection in shaping these patterns. Considering many of these studies are examining these patterns at the interspecific level, or utilize clonal model organisms, it is no surprise that sexual dimorphism and selection has been underrepresented in stoichiometric research. As arguably the greatest source of variation within sexual populations, sex cannot be ignored when attempting to understand the important intrinsic (e.g., genetically determined ability to acquire, assimilate, and allocate resources) and extrinsic (e.g., environmental resource supply) mechanisms that shape intraspecific evolution.

In this dissertation, I have studied how the environmental supply of phosphorus (P) affects: i) sex-specific patterns of plasticity in stoichiometric demand (Chapter One), ii) sex- and age-specific patterns of nutrient acquisition, assimilation, and allocation to exaggerated sexual traits (Chapter Two), and iii) population-level demographics, trait variation, and mating behavior (Chapter Three). Revisiting the patterns of sexual dimorphism on the elemental level observed in Chapter One, I also studied multi-dimensional elemental sex- and trait-specific stoichiometric demand in a common garden environment (Chapter Four). Examining the effects of this relevant environmental parameter on various aspects of *Hyalella* biology on multiple organizational levels has illuminated potential ultimate and proximate mechanisms that may be responsible for shaping sexual dimorphism rapidly changing environments.

Sex-specific plasticity in P content

In Chapter One, I examined whether sexual dimorphism manifests at the elemental level and how P availability influences patterns of stoichiometric dimorphism. Because of the differences in life history, morphology, and physiology, the sexes should also differ in the constituent elements comprising these higher-level phenotypes. A characterization of these sex-specific demands for resources is needed in order to derive second-order hypotheses on the proximate and ultimate causes and consequences of differential demand in the face of ecological change. I found substantial sexual dimorphism in body P content only in two amphipod species raised in a low-P laboratory environment. This dimorphism was much greater in the species that exhibits a greater degree of both sexual size dimorphism and larger exaggerated sexual traits relative to the body. Contrary to predictions based on the growth rate hypothesis (Elser et al. 1996), this faster growing species has a lower P content than the slower growing species. The sexual dimorphism in P content observed in the lab was not present in wild caught amphipods of both species from high-P water bodies. In both species, only males exhibited plasticity in their P contents driven by the environmental conditions in which they were raised, while female P content seems relatively canalized. All of the patterns I observed in this study were not found to be due to differences between the sexes or species in their foraging behavior, as all amphipods overwhelmingly preferred feeding on high P food, suggesting that the variation in body P due to sex or species are driven primarily by variation in nutrient processing strategies. Although the environments differed in their P supplies, the specific causes of this plasticity from lab to field still must be elucidated, as it is difficult to separate the effects of P and other environments in this study. For example, it is possible that selective forces present in the wild (e.g., predation, competition, etc.) can result in little variation in body P content between all individuals in a population. Regardless, it is clear that males and females differ in both their elemental demands and their potentials for plasticity in these demands.

Effects of P on nutrient processing

In the second chapter of this dissertation, I found significant interactive effects of P availability, age, and sex on the acquisition and assimilation of both C and P. Fundamentally, organisms must meet their resource demands utilizing the nutrient processing strategies of acquisition, assimilation, and allocation. Based on first principles, it follows that variation in demand should be tightly linked to variation in these strategies. In this study, I observed a significant effect of P availability on the acquisition of resources, as organisms likely compensated for limited resource availability (Plath and

Boersma 2001). Additionally, I found an interactive effect of age and sex on the acquisition of C and P, as female decreased resource acquisition as they matured. Curiously, I did not observe sexual dimorphism in adult acquisition activity, as predicted by the observed dimorphism in P demand from Chapter One. This suggests that P demands are not necessarily met by acquisition activity alone. In an examination of assimilation activity, I found that assimilatory responses to P availability are dependent upon both the age and sex of an organism. Importantly, the patterns of assimilation we observed do not follow the patterns of acquisition, suggesting that assimilation and acquisition are somewhat independent processes that can be used by organisms to meet their resource demands. This finding is especially important as it suggests that the ultimate fate of resources within organisms (i.e., allocation) is not necessarily controlled by resource acquisition, as has been previously theorized (Robinson and Beckerman 2013).

Sexual dimorphism in *Hyalella* amphipods is largely driven by the expression of the exaggerated posterior gnathopods in males. Intense directional sexual selection for exaggerated gnathopods should drive preferential allocation to these traits. The development of gnathopods has previously been shown to be dependent on environmental resource supply (Cothran and Jeyasingh 2010; Cothran et al. 2012). The mechanisms for resource allocation to these exaggerated traits in the face of environmental P supply are, however, much more elusive. Using the same techniques in radioisotope analysis I used to examine acquisition and assimilation, I studied the allocation of C to both the sexual gnathopods and other nonsexual traits. This analysis found that allocation to all traits was substantially increased when amphipods were fed low P food, suggesting that amphipods hold onto more of their acquired C under these conditions, a finding supported by our measures of assimilation. Additionally, I found evidence for preferential allocation to the exaggerated gnathopod only during gnathopod development. This evidence suggests that the maintenance of the exaggerated gnathopod is not considerably more costly than that of similar nonsexual traits, but that the cost of developing these traits is high and may impose a substantial burden to amphipods in late ontogeny.

Together, the results of this chapter point toward the need to view variation in organismal demand through the lens of the specific mechanisms that organisms use to meet their demands. Specifically, studies that examine sex differences in resource demand should pay particular attention to the role post-ingestive processes play in helping organisms meet their resource demands. Further, these results highlight the importance of examining resource demands and organismal responses to resource supply in the context of ontogeny, as resource demands and thus selection, shift as organisms age.

Effects of P supply on population dynamics and mating biology

Mating biology is highly dependent on population-level parameters, as selection for behaviors and traits are often linked to population density or sex ratios (Kokko and Rankin 2006). The effects of resource supply on various aspects of mating biology have often only been studied at the individual level, with little evidence that these observed patterns apply to higher levels of biological organization. Additionally, studies of P supply effects on mating biology often use highly contrasting P environments, while exploration into the more subtle changes that are potentially more representative of seasonal or spatial variation in P (Hessen et al. 2005) remains lacking. In Chapter Three, I tested the effects of subtle changes in P supply on population dynamics and trait variation. Additionally, using binary mating trials, I tested the interactive effects of P supply during development (long term) and P supply during mating (short term) on female choice. My results indicated that subtle changes in P supply has no effect on population density or sex ratios. Further, gnathopod development was also not affected by subtle changes in P supply, suggesting that the effect previously observed on the individual level is dampened in populations, possibly due to constraints imposed by competition for food. When examining the influences of P supply on mating behavior, I found evidence that organismal nutritional history (i.e., the supply of nutrients during development) and current P supply interact to determine the mating success of males. Together, the results from this chapter highlight the importance of considering population-level dynamics in studies on environmental effects on mating biology, especially considering that effects observed on the individual level may be dampened by selective forces present in higher-level contexts. Moreover, further attention must be paid to ensuring that experimental manipulations in nutrient supply encompass ecologically-relevant ranges that represent the subtle spatial and temporal variation often observed in nature.

Sexual dimorphism in multiple elemental dimensions

The focus on C, N, and P in most stoichiometric studies has provided for a wealth of information on the importance of these elements in key ecological and evolutionary processes on multiple levels (see, Hessen et al. 2013). Organisms, however, are composed of many more elements than just these three. Focusing on a limited subset of elemental variables only captures part of the overall stoichiometric picture, and ignores the documented importance of trace elements in ecological and organismal processes (e.g., Cullen and Sherrell 2005; Karimi and Folt 2006; Wright et al. 2011; Dudley et al. 2012). As such, any framework of eco-evolutionary dynamics at the elemental level must incorporate the entire suite of elements present in biological systems (i.e., the ionome; *sensu* Salt et al. 2008) or risk black-boxing important interactions among elements composing biomass and the ecological consequences of these interactions. Chapter Four of this dissertation revisits the central question of the first chapter in determining whether sexual dimorphism manifests at the elemental level, this time along multiple elemental axes. Additionally, I assessed what, if any, differences there are between sexually selected traits and similar nonsexual traits in their relative elemental demands in multiple dimensions. I found significant dimorphism and trait differences in multidimensional elemental space. The driver of the differences between the sexes and traits was calcium, an important element for the formation of the amphipod exoskeleton. I also found evidence for positive and negative correlations between elements, emphasizing the potential for elemental co-limitation, in which the ability to acquire one nutrient is dependent on the supply of another (Saito et al. 2008). This examination of sexual dimorphism along multiple elemental axes has illuminated the complexity of elemental interactions that may underlie many of the phenotypic differences observed between the sexes. While the sources of this dimorphism and the mechanisms responsible for the interactions between elements may prove to be challenging to uncover, such bioinformatic tools may provide for an ecologically-explicit understanding of the causes and consequences of sexual dimorphism and sexual trait variation.

Conclusions and future directions

Studying the responses of males and females to variation in P supply has enabled me to study how nutrition and sexual selection interact to shape sexual dimorphism, sexual trait development, and mating biology. Such information is critical in furthering our understanding of the condition-dependent nature of sexual traits, particularly in light of global environmental change. It should be acknowledged that this dissertation is limited by its lack of direct quantification of the fitness consequences of the patterns observed within. For example, while I observed sex-specific effects of P supply on the composition, acquisition, assimilation, and allocation of resources (Chapters One and Two), whether these effects result in appreciable sex-specific fitness consequences has yet to be fully explored. Further, our understanding of the genetic sources of stoichiometric variation is lacking. Without an integration of quantitative genetics approaches into studies on stoichiometric variation, the evolutionary implications of studies showing organism-level consequences of variation in elemental supply cannot be fully deciphered. To that end, future studies examining the evolution of condition-dependent sexual traits can explore how variation in environmental supply of elements affects organismal stoichiometry, and whether sexual trait variation is related to organismal stoichiometry. Quantitative genetics techniques can then be used to compare these effects across genotypes, to determine the genetic architecture of condition-dependent trait evolution on the elemental level. In addition, high-throughput "-omics" tools can be incorporated into the framework of ecological stoichiometry to gain a better understanding of the mechanisms responsible for both inter- and intrasexual phenotypic variation and its relationship to ecosystem processes.

Most studies exploring sexual dimorphism and sexual trait variation do so purely on the organismal level, including most of the studies within this dissertation. The utility of an elemental perspective, however, is the ability to use a common currency of elements to address questions across both taxa (i.e., horizontal integration) and levels of biological organization (i.e., vertical integration). To this end, future studies must explore the role of ecosystem-level parameters in shaping sexual dimorphism and trait evolution in natural settings. By using an elemental approach to reduce complexity at both the ecosystem and organismal levels, we can begin to understand the complex, multifarious selective forces that shape broad spatial diversification within species. Knowledge of the variation within species in responses to rapid environmental alterations is critical to our understanding of both the evolution of species and the ecological consequences of these evolutionary changes.

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VITA

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