Sex-specific nutrient use and preferential allocation of resources to a sexually selected trait in *Hyalella* amphipods

Jared M. Goos[1]*, Rickey D. Cothran[2], Punidan D. Jeyasingh[1]

[1]Department of Integrative Biology, Oklahoma State University, Stillwater, OK 74078

[2]Department of Biological Sciences, Southwestern Oklahoma State, Weatherford, OK 73096

*Corresponding Author:

501 Life Sciences West

Stillwater, OK 74078

(405) 744-5555

jared.goos@okstate.edu

Summary: Age influences sexual dimorphism in carbon and phosphorus acquisition and assimilation, a pattern potentially driven by the exaggeration of male sexual traits to which resources are preferentially allocated.

Journal of Experimental Biology • Advance article

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.

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 $\pm$ 1 SD; HP: 0.123% $\pm$ 0.033%, LP: 0.016% $\pm$ 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 $\pm$ 1 SD; HP: 48.03% $\pm$ 0.340%, LP: 49.123% $\pm$ 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 $\pm$ 1 SD; HP: 2.50% $\pm$ 1.44%, LP: 1.35% $\pm$ 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.

Competing Interests

The authors declare no competing or financial interests.

Author Contributions

J.M.G, R.D.C., and P.D.J. designed the study. J.M.G. collected the amphipods and performed trait manipulations, radiotracer assays, and statistical analysis. All authors helped draft the manuscript and gave final approval for publication.

Literature Cited

Andersson, M. B. (1994). *Sexual Selection*. 1st ed. Princeton, NJ: Princeton University Press.

APHA (1992). *Standard Methods for the Examination of Water and Wastewater*. 18th ed. Washington, DC: APHA.

Atwood, T. and Weeks, H. (2002). Sex- and Age-specific Patterns of Mineral Lick Use by White-tailed Deer (*Odocoileus virginianus*). *Am. Midl. Nat.* **148**, 289–296.

Back, J. A. and King, R. S. (2013). Sex and size matter: ontogenetic patterns of nutrient content of aquatic insects. *Freshw. Sci.* **32**, 837–848.

Badyaev, A. V. (2002). Growing apart: an ontogenetic perspective on the evolution of sexual size dimorphism. *Trends Ecol. Evol.* **17**, 369–378.

Badyaev, A. V (2004). Developmental perspective on the evolution of sexual ornaments. *Evol. Ecol. Res.* **6**, 1–17.

Banks, W. J., Epling, G. P., Kainer, R. A. and Davis, R. W. (1968). Antler growth and osteoporosis II. Gravimetric and chemical changes in the costal compacta during the antler growth cycle. *Anat. Rec.* **162**, 399–405.

Bearhop, S., Phillips, R. Q., McGill, R., Cherel, Y., Dawson, D. A. and Croxall, J. P. (2006). Stable isotopes indicate sex-specific and long-term individual foraging specialisation in diving seabirds. *Mar. Ecol. Prog. Ser.* **311**, 157–164.

Bertram, S. M., Whattam, E. M., Visanuvimol, L., Bennett, R. and Lauzon, C. (2009). Phosphorus availability influences cricket mate attraction displays. *Anim. Behav.* **77**, 525–530.

Bonduriansky, R., Maklakov, A., Zajitschek, F. and Brooks, R. (2008). Sexual selection, sexual conflict and the evolution of ageing and life span. *Funct. Ecol.* **22**, 443–453.

Boutton, T. W., Tesdahl, K. G. and Slade, N. A. (1983). Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. *Oecologia* **57**, 32–37.

Carman, K. and Guckert, J. (1994). Radiotracer determination of ingestion and

assimilation of periphytic algae , bacteria, and adsorbed amino acids by snails. *J. North Am. Benthol. Soc.* **13**, 80–88.

Cothran, R. D., Kuzmic, A., Wellborn, G. A. and Relyea, R. A. (2010). Phenotypic manipulation provides insights into the function of a sexually selected trait in a freshwater crustacean species complex. *Anim. Behav.* **80**, 543–549.

Cothran, R. D., Stiff, A. R., Jeyasingh, P. D. and Relyea, R. A. (2012). Eutrophication and predation risk interact to affect sexual trait expression and mating success. *Evolution* **66**, 708–719.

Cothran, R. D., Stoler, A. B. and Relyea, R. A. (2014). Leaves and litterbugs: how litter quality affects amphipod life-history and sexually selected traits. *Freshw. Sci.* **33**, 812–819.

Cotton, S., Fowler, K. and Pomiankowski, A. (2004). Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proc. Biol. Sci.* **271**, 771–783.

Cowan, R. L., Hartsook, E. W. and Whelan, J. B. (1968). Calcium-strontium metabolism in white tailed deer as related to age and antler growth. *Exp. Biol. Med.* **129**, 733–737.

Darchambeau, F., Faerøvig, P. J. and Hessen, D. O. (2003). How *Daphnia* copes with excess carbon in its food. *Oecologia* **136**, 336–346.

DeMott, W. R., Gulati, R. D. and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* **43**, 1147–1161.

Edwards, T. D. and Cowell, B. C. (1992). Population Dynamics and Secondary Production of *Hyalella azteca* (Amphipoda) in *Typha* Stands of a Subtropical Florida lake. *J. North Am. Benthol. Soc.* **11**, 69–79.

Elser, J. J. (2003). Biological stoichiometry: a theoretical framework connecting ecosystem ecology, evolution, and biochemistry for application in astrobiology. *Int. J. Astrobiol.* **2**, 185–193.

Elser, J. J., Dobberfuhl, D. R. and MacKay, N. A. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* **46**, 674–684.

Elser, J. J., O'Brien, W. J., Dobberfuhl, D. R. and Dowling, T. E. (2000). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* **13**, 845–853.

Emlen, D. J. (2001). Costs and the diversification of exaggerated animal structures. *Science* **291**, 1534–1536.

Fink, P. and Von Elert, E. (2006). Physiological responses to stoichiometric constraints: nutrient limitation and compensatory feeding in a freshwater snail. *Oikos* **115**, 484–494.

García-Berthou, E. and Moreno-Amich, R. (2000). Food of introduced pumpkinseed sunfish: ontogenetic diet shift and seasonal variation. *J. Fish Biol.* **57**, 29–40.

Goos, J. M., French, B. J., Relyea, R. A., Cothran, R. D. and Jeyasingh, P. D. (2014). Sex-specific plasticity in body phosphorus content of *Hyalella* amphipods. *Hydrobiologia* **722**, 93–102.

Gorokhova, E. and Hansson, S. (2000). Elemental composition of *Mysis mixta* (Crustacea, Mysidacea) and energy costs of reproduction and embryogenesis under laboratory conditions. *J. Exp. Mar. Bio. Ecol.* **246**, 103–123.

Hargrave, B. T. (1970). The utilization of benthic microflora by *Hyalella azteca* (Amphipoda). *J. Anim. Ecol.* **39**, 427–437.

He, X. and Wang, W.-X. (2006). Releases of ingested phytoplankton carbon by *Daphnia magna*. *Freshw. Biol.* **51**, 649–665.

He, X. and Wang, W. (2007). Kinetics of phosphorus in *Daphnia* at different food concentrations and carbon : phosphorus ratios. *Limnol. Oceanogr.* **52**, 395–406.

Hessen, D. O. and Anderson, T. R. (2008). Excess carbon in aquatic organisms and ecosystems: Physiological, ecological, and evolutionary implications. *Limnol. Oceanogr.* **53**, 1685–1696.

Hobson, K. A. and Clark, R. G. (1992). Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues. *Condor* **94**, 181–188.

Hunt, J., Brooks, R., Jennions, M. D., Smith, M. J., Bentsen, C. L. and Bussière, L. F. (2004). High-quality male field crickets invest heavily in sexual display but die young.

Nature **432**, 1024–1027.

Jeyasingh, P. D. (2007). Plasticity in metabolic allometry: the role of dietary stoichiometry. *Ecol. Lett.* **10**, 282–289.

Kilham, S., Kreeger, D., Lynn, S. and Goulden, C. (1998). COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* **377**, 147–159.

Kokkotis, A. T. and McLaughlin, J. D. (2002). Instar-specific head and body lengths of *Hyalella* (Amphipoda): criteria for starting and endpoints in experimental studies. *Hydrobiologia* **474**, 223–227.

Lincoln, G. A. (1992). Biology of antlers. *J. Zool.* **226**, 517–528.

Maklakov, A. A., Simpson, S. J., Zajitschek, F., Hall, M. D., Dessmann, J., Clissold, F., Raubenheimer, D., Bonduriansky, R. and Brooks, R. C. (2008). Sex-specific fitness effects of nutrient intake on reproduction and lifespan. *Curr. Biol.* **18**, 1062–1066.

Markow, T. A., Dobberfuhl, D. R., Breitmeyer, C. M., Elser, J. J. and Pfeiler, E. (1999). Elemental stoichiometry of *Drosophila* and their hosts. *Funct. Ecol.* **13**, 78–84.

Markow, T. A., Coppola, A. and Watts, T. D. (2001). How *Drosophila* males make eggs: it is elemental. *Proc. R. Soc. London B* **268**, 1527–1532.

Morehouse, N. I., Nakazawa, T., Booher, C. M., Jeyasingh, P. D. and Hall, M. D. (2010). Sex in a material world: why the study of sexual reproduction and sex-specific traits should become more nutritionally-explicit. *Oikos* **119**, 766–778.

Navarro, J. M. and Winter, J. E. (1982). Ingestion rate, assimilation efficiency and energy balance in *Mytilus chilensis* in relation to body size and different algal concentrations. *Mar. Biol.* **67**, 255–266.

Neumann, P. T. M., Borgmann, U. and Norwood, W. (1999). Effect of gut clearance on metal body concentrations in *Hyalella azteca*. *Environ. Toxicol. Chem.* **18**, 976–984.

Olijnyk, A. M. and Nelson, W. a. (2013). Positive phenotypic correlations among life-history traits remain in the absence of differential resource ingestion. *Funct. Ecol.* **27**, 165–172.

Penn, D. J. and Smith, K. R. (2007). Differential fitness costs of reproduction between the

sexes. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 553–558.

Plath, K. and Boersma, M. (2001). Mineral limitation of zooplankton: Stoichiometric constraints and optimal foraging. *Ecology* **82**, 1260–1269.

Quinn, G. P. and Keough, M. J. (2002). *Experimental design and data analysis for biologists*. Cambridge, UK: Cambridge University Press.

Robinson, M. R. and Beckerman, A. P. (2013). Quantifying multivariate plasticity: genetic variation in resource acquisition drives plasticity in resource allocation to components of life history. *Ecol. Lett.* **16**, 281–290.

Rowe, L. and Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. Biol. Sci.* **263**, 1415–1421.

Roy Chowdhury, P., Lopez, J. a, Weider, L. J., Colbourne, J. K. and Jeyasingh, P. D. (2014). Functional genomics of intraspecific variation in carbon and phosphorus kinetics in *Daphnia*. *J. Exp. Zool. A. Ecol. Genet. Physiol.* **321**, 387–398.

Ruckstuhl, K. (1998). Foraging behaviour and sexual segregation in bighorn sheep. *Anim. Behav.* **56**, 99–106.

Schlesinger, W. H. (1997). *Biogeochemistry : an analysis of global change*. San Diego, CA: Academic Press.

Skinner, D. M. (1985). Molting and regeneration. In *The Biology of Crustacea*, pp. 43–146.

Snell-Rood, E., Cothran, R., Espeset, A., Jeyasingh, P., Hobbie, S. and Morehouse, N. (2015). Life history evolution in the anthropocene: effects of increasing nutrients on traits and tradeoffs. *Evol. Appl.* **8**, 635–649.

Speakman, J. R. (2008). The physiological costs of reproduction in small mammals. *Philos. Trans. R. Soc. London B* **363**, 375–398.

Stahlschmidt, Z. R., Davis, J. R. and Denardo, D. F. (2011). Sexual variation in assimilation efficiency: its link to phenotype and potential role in sexual dimorphism. *J. Comp. Physiol. B.* **181**, 383–9.

Stephenson, D. C. and Brown, A. D. (1984). Calcium kinetics in male white-tailed deer. *J. Nutr.* **114**, 1014–1024.

Sterner, R. W. and Elser, J. J. (2002). *Ecological stoichiometry: the biology of elements from molecules to the biosphere*. Princeton: Princeton University Press.

Sterner, R. W., Hagemeier, D. D., Smith, R. F. and Smith, W. L. (1992). Lipid-ovary indices in food-limited *Daphnia*. *J. Plankt. Res.* **14** , 1449–1460.

Stockhoff, B. A. (1993). Ontogenetic change in dietary selection for protein and lipid by gypsy moth larvae. *J. Insect Physiol.* **39**, 677–686.

Sutcliffe, D. W. (1992). Reproduction in *Gammarus* (Crustacea, Amphipoda): basic processes. *Freshw. Forum* **2**, 102–128.

Venarsky, M. P. and Wilhelm, F. M. (2006). Use of clove oil to anaesthetize freshwater amphipods. *Hydrobiologia* **568**, 425–432.

Villar-Argaiz, M., Medina-Sánchez, J. M. and Carrillo, P. (2002). Linking life history strategies and ontogeny in crustacean zooplankton: Implications for homeostasis. *Ecology* **83**, 1899–1914.

Visanuvimol, L. and Bertram, S. M. (2010). Dietary phosphorus availability influences female cricket lifetime reproductive effort. *Ecol. Entomol.* **35**, 386–395.

Wellborn, G. A. (1994). Size-biased predation and prey life histories: a comparitive study of freshwater amphipod populations. *Ecology* **75**, 2104–2117.

Wellborn, G. A. (2000). Selection on a sexually dimorphic trait in ecotypes within the *Hyalella azteca* species complex (Amphipoda: Hyalellidae). *Am. Midl. Nat.* **143**, 212–225.

Wellborn, G. A. and Bartholf, S. E. (2005). Ecological context and the importance of body and gnathopod size for pairing success in two amphipod ecomorphs. *Oecologia* **143**, 308–316.

Wellborn, G. A. and Broughton, R. E. (2008). Diversification on an ecologically constrained adaptive landscape. *Mol. Ecol.* **17**, 2927–2936.

Wellborn, G. A., Cothran, R. and Bartholf, S. (2005). Life history and allozyme diversification in regional ecomorphs of the *Hyalella azteca* (Crustacea: Amphipoda) species complex. *Biol. J. Linn. Soc.* **84**, 161–175.

Willoughby, L. G. and Earnshaw, R. (1982). Gut passage times in *Gammarus pulex* (Crustacea, Amphipoda) and aspects of summer feeding in a stony stream. *Hydrobiologia* **97**, 105–117.
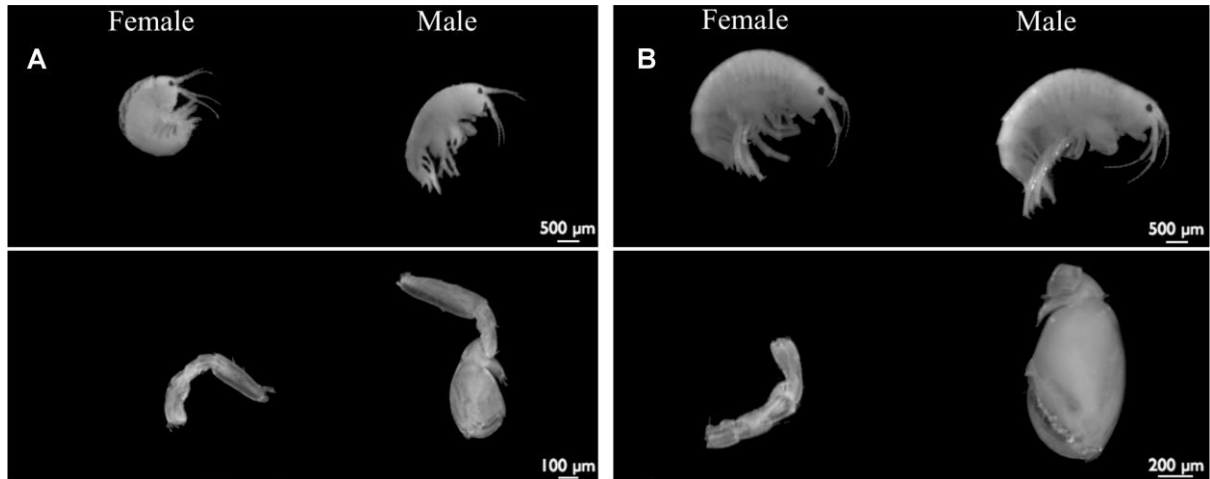
Figures

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.
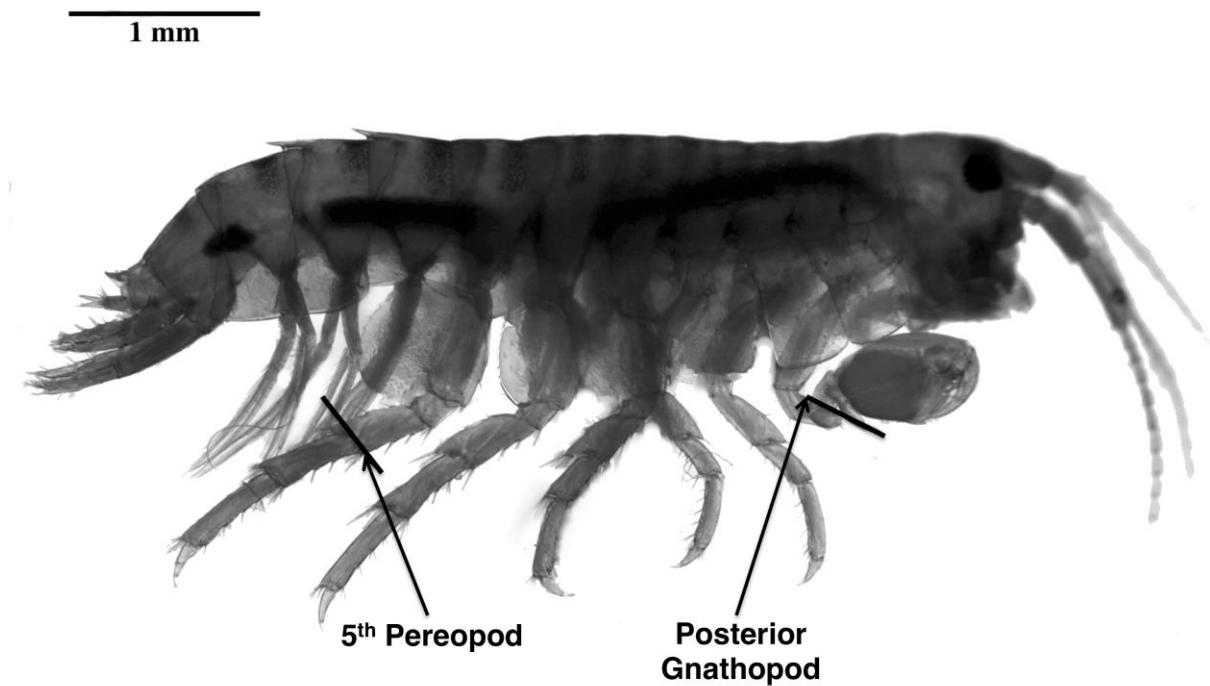
Figure 2: Male *Hyalella* amphipod showing points of dissection for appendage amputation groups. The black lines indicate the point where we ablated the carpus, propodus, and dactyl of the gnathopods (PG) and fifth pereopods (5P).
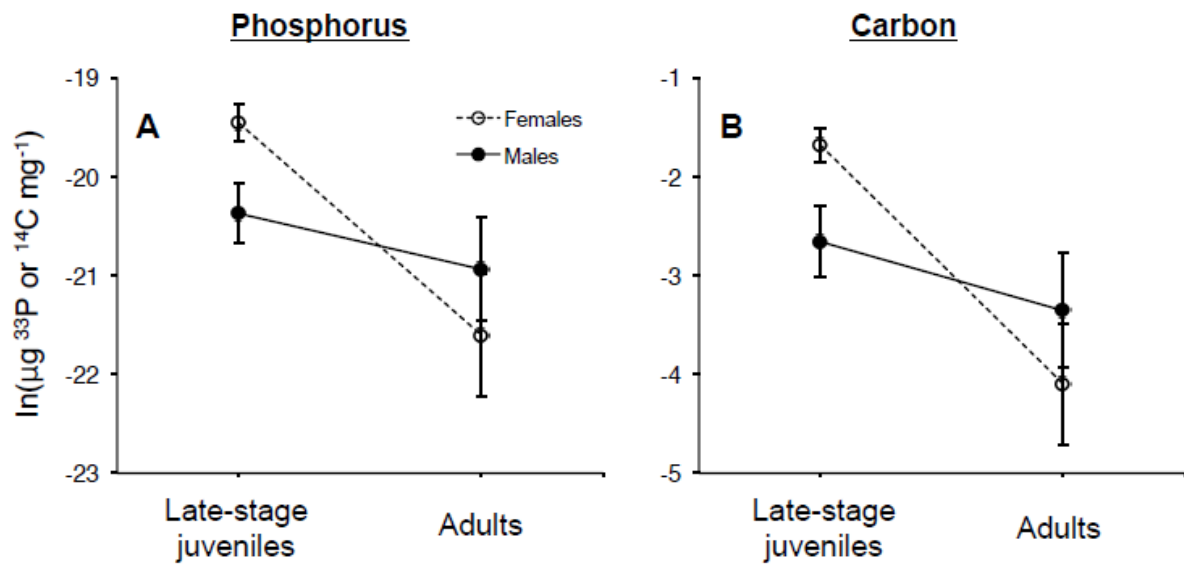
Figure 3: Age-specific patterns of sexual dimorphism in acquisition of A) phosphorus and B) carbon over two hours during late ontogeny.. Markers represent means of log-transformed, size-adjusted ^{14}C and ^{33}P present in the body, error bars 95% confidence intervals.
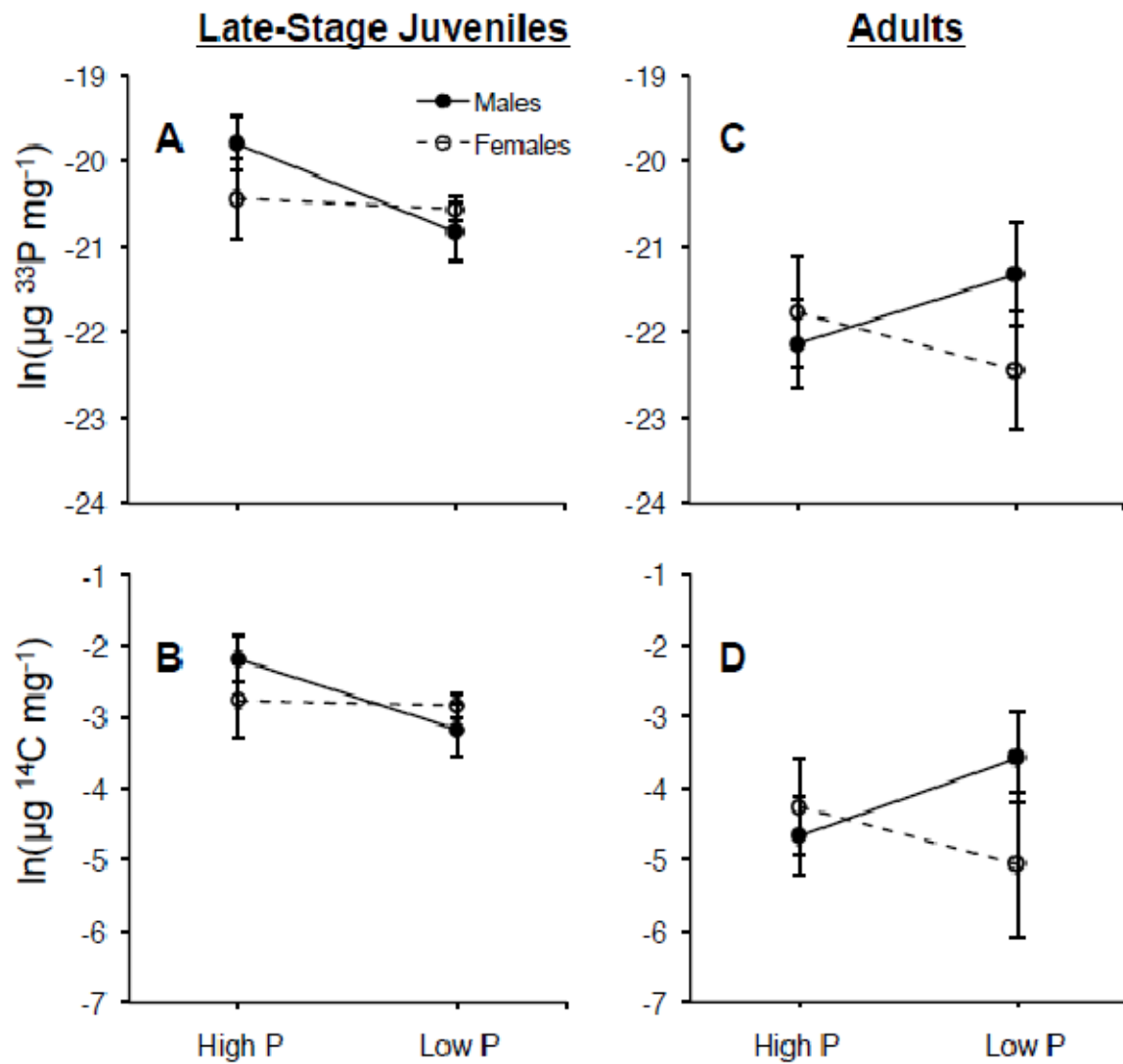
Figure 4: Sex-specific patterns of assimilation of C and P in A) late-stage juveniles and B) adults in response to P availability. Markers represent means of log-transformed, size-adjusted ^{14}C and ^{33}P present in the body, error bars 95% confidence intervals.
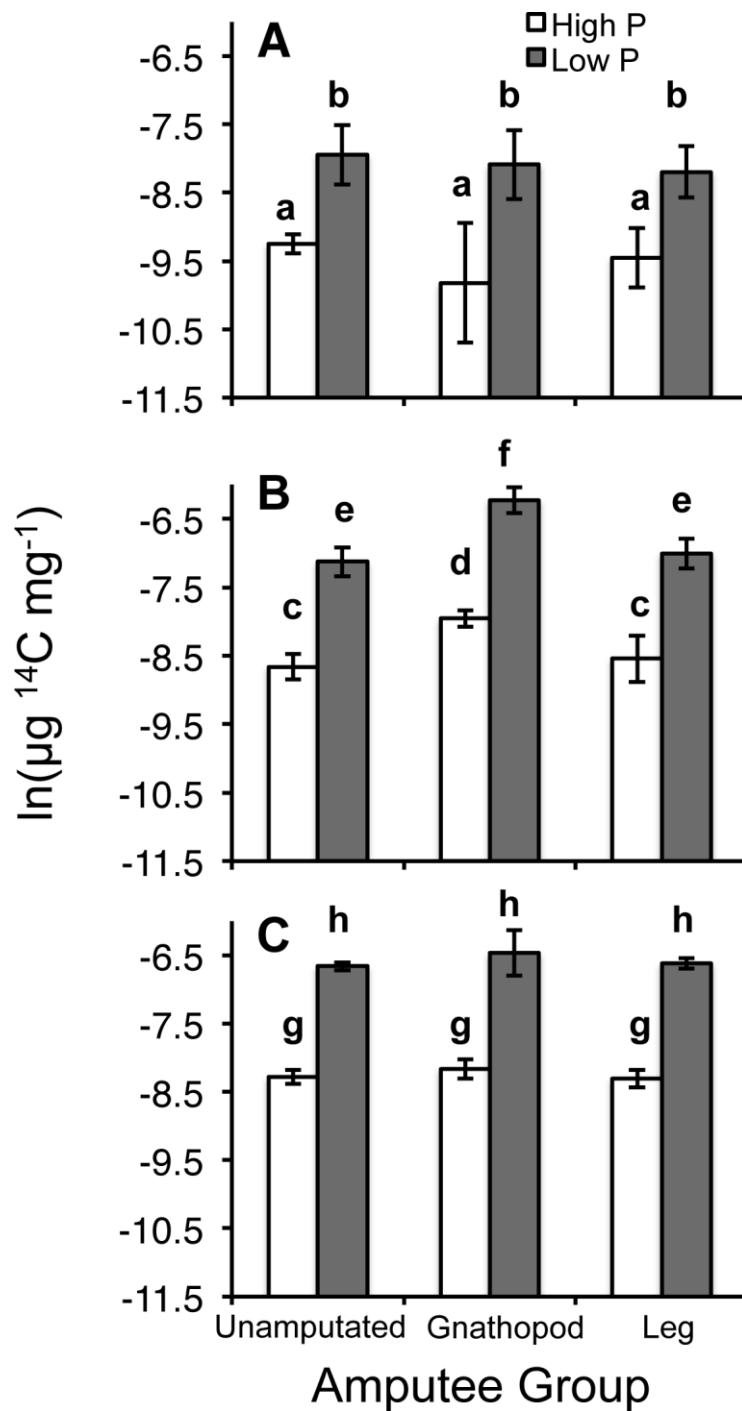
Figure 5: Carbon allocation in the male A) body, B) gnathopod, and C) leg across amputation groups. Values are means of log-transformed carbon allocation (μg C/mg). Error bars are 95% confidence intervals. Different lower case letters indicate significantly different carbon allocation, as indicated by Tukey tests.