ABSTRACT

Effects of Stream Nutrient Enrichment on Aquatic Insect Stoichiometry: Importance of Life-History Traits, Sex, and Ontogeny

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I investigated the effects of stream nutrient enrichment on aquatic insect stoichiometry, particularly in the context of species life-history traits, sex, and ontogeny. The majority of studies investigating nutrient content of organisms preceding this research had assumed that aquatic insect species maintain a homeostatic concentration of carbon, (C) nitrogen (N) and phosphorus (P) content with respect to developmental stage and nutrient enrichment of their food resources. However, P content was shown not to be homeostatic across the ontogeny of 19 species of aquatic insects and C and N were quasihomeostatic. Growth rates for *Caenis* mayflies were higher on P enriched foods, and smaller individuals grew faster than larger individuals. Further, female mayflies were equal to or larger than males and had equal or higher body P content than males, depending upon species. A phylogenetic pattern in body P content was found for baetid and leptophlebiid mayflies. Male and female baetids declined in % P across their ontogeny, whereas leptophlebiid females increased in the 3 species studied. Finally, a study of streams spanning a steep nutrient enrichment gradient revealed that females of *Baetis* sp. and *Neochoroterpes nanita* attained a much larger size than males and were more enriched in P. Body P content of both mayfly species was higher at low P sites, and body size was much larger

at high P sites. In insects, P content is a good indicator of growth rate (higher P, faster growth), and body size is a good predictor of fecundity (bigger body, more fecund). Nutrients not only play a role in determining an organism's reproductive success, growth rate and size. The effects of nutrients are seen in organism nutrient recycling rates, food web organization, and community structure and function.

Effects of Stream Nutrient Enrichment on Aquatic Insect Stoichiometry: Importance of Life-History Traits, Sex and Ontogeny

by

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A Dissertation

Approved by the Department of Biology

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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December 2013

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ACKNOWLEDGEMENTS

I would like to extend thanks to David Lang, Charles Stanley, Becky Shaftel, Justin Grimm, Jason Taylor, Barry Fulton, Ted Valenti, Thad Scott and Jeff Mink for their help with field and laboratory work, as well as their friendship and discussions on ecology and my dissertation project in particular. Additionally, I thank Baylor University and the Center for Reservoir and Aquatic Systems Research for laboratory space and analytical equipment that was essential for the completion of my dissertation research. And to my wife Judy, and children Jesse, Jeffrey, Jordan, and Hannah for their love and support.

CHAPTER ONE

Introduction

Elemental stoichiometry is gaining favor in ecology as a way to study ecosystems, especially the interactions between organisms at all trophic levels and their environment. Stoichiometry examines the abundances and/or ratios of key elements (nutrients), particularly carbon (C), nitrogen (N) and phosphorus (P), in organisms and their food, and how differing elemental abundances and/or ratios affect organisms and ecosystems (Elser et al. 1996). Nutrient ratios are an important aspect of stream ecology because they influence organism growth, reproduction and life history traits and are therefore the basis of trophic interactions. Studies on the stoichiometry of benthic invertebrates and their food sources should improve overall understanding of trophic interactions, as they are an essential part of the food web. However, current knowledge of organism stoichiometry is lacking in terms of species examined, ontogenic changes, and spatial and temporal variation in elemental composition (Frost et al. 2002).

The importance of phosphorus in affecting growth rates has recently been demonstrated (Weider et al. 2005). Phosphorus-rich ribosomal (r)RNA is necessary for protein synthesis which directly influences growth rates. Thus, rapid growth rates are associated with increased P requirements because organisms must disproportionately increase their allocation to P-rich rRNA to meet the protein synthesis demands of rapid growth. This is the basic tenet of the Growth Rate Hypothesis (GRH) which suggests that organisms with rapid growth rates must build P-rich biomass which makes them more susceptible to P-limitation (Elser et al. 1996, 2006, Vrede et al. 2002).

Mayflies (Ephemeroptera) are a widespread and important component of stream ecosystems. Their abundance and the high number of species in central Texas made them an attractive focal species to study. Previous studies have examined the effects of C: N and C: P ratios on mayfly species growth in both field and laboratory settings. Data demonstrate that high quality food (high P and or N content) increases growth and fecundity of several species of mayfly (Soderstrom 1988, Frost & Elser 2002). Mayflies are a good organism for studying possible stoichiometric difference across their ontogeny because development classes have been described (e.g. Taylor & Kennedy 2006) or can be easily determined for most species. Whether or not the growth rate is constant across all development classes is unknown. Moreover, in organisms which undergo metamorphosis (e.g. insects), growth and the loss of juvenile structures and development of adult structures follow a time sequence which may require different nutrient levels. Because adult mayflies do not feed, all the chemical requirements of the adults must be met by the nymphs. Thus nymphal nutrition must play a large role in adult reproductive success. Since egg elemental composition is key to early nymph growth and survivorship (Tessier et al. 1983, DeMott 2003), the availability of nutrients to actively feeding nymphs is of paramount importance. Because all egg production is realized in mayfly nymphs, there should be a direct linkage between nymphal food quality, fecundity, and egg nutrient content in mayflies. This relationship has been shown in *Daphnia* (Sterner 1993, Urabe & Sterner 2001). On the other hand it is possible that this linkage is relaxed because males may contribute phosphorus in their sperm packet that is available for incorporation into eggs. This has been demonstrated in *Drosophila* spp. (Markow et al. 2001).

The CNP content of lotic macroinvertebrates and their food resources has recently received attention (Cross et al. 2003, Bowman et al. 2005, Evans-White et al. 2005, Liess and

Hillebrand 2005, Small and Pringle 2010). Organisms accomplish homeostasis by modifying the quality or quantity of organic matter ingested to maintain adequate supply relative to demand of essential elements required for metabolism, growth, and reproduction (Sterner and Elser 2002). Investigators have implicitly assumed that aquatic macroinvertebrates maintain an approximately constant, or homeostatic, elemental composition across life stages or sizes within species. However, the limited number of studies on CNP content of aquatic and terrestrial invertebrates spanning different developmental stages has revealed nonhomeostatic patterns across the ontogeny of species (zooplankton: Andersen and Hessen 1991, Hessen and Lynch 1991, Main et al. 1997, Villar-Argaiz et al. 2002; *Drosophila*: Elser et al. 2006; mayflies: Frost and Elser 2002, Veldboom and Haro 2011). Specifically for insects, P content of 5 species of *Drosophila* larvae decreased with increasing larval development, and each species had differing P content (Elser et al. 2006). P content decreased across the early developmental stages of *Ephemerella* sp. mayflies (Frost and Elser 2002). P content also increased in late-stage larvae, pupae, and adults of the caddisfly *Brachycentris occidentalis* (Veldboom and Haro 2011). However, these investigators compared, at most, the elemental content of several size classes and did not examine individuals across the continuum of ontogeny.

Food elemental content and species development are intimately linked by changing elemental needs across ontogeny. Because elemental content may change across the ontogeny of a species, the severity of elemental imbalance also may change (increase or decrease) assuming constant C, N, and P content of food. This linkage may affect survivorship of specific life-history stages as demonstrated for the copepod *Diaptomus clavipes* (Villar-Argaiz and Sterner 2002).

Understanding how P content varies across ontogeny is of particular importance because P deficiency limits growth of invertebrates (Urabe et al. 1997, Sterner and Elser 2002).

Furthermore, growth rates are positively correlated with body %P (Elser et al. 1996, 2003, Frost and Elser 2002, Weider et al. 2005) but negatively correlated with adult invertebrate body size (Woods et al. 2004). Phylogeny also may constrain P (Woods et al. 2004) and N (Fagan et al. 2002) content of invertebrates, independent of ontogeny.

Life-history traits, such as reproductive strategy, probably have a strong bearing on P content during ontogeny. P content of invertebrates that produce a single (usually large) batch of eggs (semelparous taxa) may increase as larvae mature. Percent P of somatic tissue is less than that of gametes (especially eggs) in the few taxa examined (Andersen and Hessen 1991, Markow et al. 1999, 2001, but see Færøvig and Hessen 2003). P content of iteroparous taxa (reproduce multiple times, fewer eggs) probably declines as larvae grow because adults can feed and supply nutrients necessary for maintenance and reproduction. Differences in patterns of P content between iteroparous and semelparous taxa may be because semelparous insect taxa do not feed as adults. Therefore, the burden of P (and C, N, and other elements) acquisition rests entirely on larval feeding. Collectively, these previous studies suggest that sex, reproductive strategy, adult feeding status, trophic level, life-cycle completion time, and metamorphosis type may interact to produce varying patterns in P content across invertebrate ontogeny.

Nutrient requirements for growth and reproduction vary widely among and within major groups of animals (Sterner and Elser 2002, Elser et al. 2006). Organisms perceive deficiencies or surpluses in food quantity or quality based on the organisms' energetic and nutritional needs, which reflect its physiological state. Natural energy and material subsidies are common occurrences in many ecosystems (Polis et al 1997). For example, allochthonous inputs of leaf litter from deciduous trees provide a seasonal pulse of organic material and nutrients that can fuel shredder (Cummins et al. 1989) and detritivore food webs (Hall et al 2000, Cross et al. 2003, Danger et al. 2013). Terrestrial insects from riparian habitats can subsidize stream fishes during periods of low aquatic insect emergence (Wipfli 1997, Nakano et al. 1999, Wurtsbaugh 2007). Further, anadromous fishes can subsidize resident stream fishes and invertebrates via eggs and carcasses (Wipfli et al 2003, Moore et al 2008) and provide dissolved nutrients to streams (Levi et al. 2013). All of these subsidies are of short duration, and thus provide a pulse of increased resources.

Nutrients and organic matter from waste-water treatment plant (WWTP) effluent and agricultural runoff are anthropogenic sources of resource subsidies to streams. These subsidies can be virtually continuous in the case of waste water treatment plants (WWTP) effluent. Thus the background concentrations of dissolved nutrients can become elevated far beyond the natural condition and provide an excess of nutrients for primary producers and heterotrophic bacteria. These inputs can change the quality of resources available to consumers (Bowman et al. 2005, Singer and Battin 2007). Resources with increased nutrient content can cause consumers to increase growth rates (Urabe and Sterner 2001, Frost et al. 2006), reproductive output and egg survivorship, (Urabe and Sterner 2001), and the nutrient content of the consumers themselves (Cross et al. 2003, 2005, Small and Pringle 2010).

A species' resource requirements can change across its ontogeny. An ontogenetic approach to nutrient subsidies can provide age specific responses to food quality. This approach is valuable because organism can encounter resource bottlenecks that are life stage specific, and result in decreased survivorship of life stages, as seen in the copepod *Diaptomus clavipes* (Villar-Argaiz and Sterner 2002). Resource demand and nutrient content of consumers can vary with gender also, as shown for several *Drosophila* sp. by Markow et al. (1999, 2001).

Patterns of CNP content of consumers and their food provides a basis for understanding the effects of elemental imbalances on growth rates of consumers (Elser et al. 2003), nutrient recycling (Vanni et al. 2002, Evans-White and Lamberti 2006, Rothlisberger et al. 2008), population dynamics (Andersen et al. 2004, Moe et al. 2005), foodweb organization, (Vrede et al. 2004), and community structure and function (Elser et al. 2000, Cross et al. 2005).

CHAPTER TWO

Ontogenic Differences in Mayfly Stoichiometry Influence Growth Rates in Response to Phosphorus Enrichment

This chapter published as:
Back, J.A., J.M. Taylor, R.S. King, K.L. Fallert, and E.H. Hintzen. 2008. Ontogenic differences in mayfly stoichiometry influence growth rates in response to phosphorus enrichment. *Fundamental and Applied Limnology* 171:233-240.

Abstract. We contrasted the carbon, nitrogen, and phosphorus (C:N:P) stoichiometry of *Caenis* spp. (Ephemeroptera:Caenidae) nymphs from 2 stream reaches differing in P enrichment. We also estimated growth rates of nymphs reared on algae of different P content across four development classes in a laboratory experiment. C:N ratios of field-collected nymphs exhibited variable responses across development classes between sites whereas C:P and N:P ratios showed a clear unimodal response, increasing from classes II through IV but then declining sharply in class V (nymphs nearing eclosion) at both sites. C:P was lower at the highly enriched site for all but the last development class. Growth rates increased in response to P enrichment at the earliest development class, but this growth response diminished in later development classes resulting in a significant interaction between P treatments and development classes. Trends in field data imply that later stages of development have higher P requirements than earlier classes and nutrient enrichment may affect sequestration of P by nymphs. Laboratory data suggest that early development classes are more P limited but in light of field results, nymphs may shift P allocation from somatic growth to reproductive development as organisms mature.

Introduction

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The importance of phosphorus in affecting growth rates has recently been demonstrated (Weider et al. 2005). Phosphorus-rich ribosomal (r)RNA is necessary for protein synthesis which directly influences growth rates. Thus, rapid growth rates are associated with increased P requirements because organisms must disproportionately increase their allocation to P-rich rRNA to meet the protein synthesis demands of rapid growth. This is the basic tenet of the Growth Rate Hypothesis (GRH) which suggests that organisms with rapid growth rates must build P-rich biomass which makes them more susceptible to P-limitation (Elser et al. 1996, 2006, Vrede et al. 2002).

Invertebrates at a given life stage are more homeostatic with respect to their body nutrient content than autotrophs despite variation in the chemical makeup of their food, although the degree of elemental homeostasis varies between species (Sterner & Elser 2002, Peck & Walton

2006). In a field study examining consumer-resource stoichiometry, Cross et al. (2003) found that P content of invertebrates exhibited much greater variability than C or N, and that some taxa might not be stoichiometrically homeostatic across life stages. Although data from this study did not find a significant correlation between body size and %P for the 40 invertebrate taxa examined, the P content of certain functional feeding groups did decrease with increasing body size. Frost & Elser (2002) found a negative linear relationship between P content and body size in *Ephemerella* spp. indicating that P content in this taxon is not necessarily fixed for its lifespan but shifts ontogenetically during development. Similar ontogenetic shifts in P content have been observed in aquatic crustaceans (DeMott 2003, Faerøvig & Hesson 2003).

Mayflies (Ephemeroptera) are a widespread and important component of stream ecosystems. Previous studies have examined the effects of C: N and C: P ratios on mayfly species growth in both field and laboratory settings. Data demonstrate that high quality food (high P and or N content) increases growth and fecundity of several species of mayfly (Soderstrom 1988, Frost & Elser 2002). Mayflies are a good organism for studying possible stoichiometric difference across their ontogeny because development classes have been described (e.g. Taylor & Kennedy 2006) or can be easily determined for most species. Whether or not the growth rate is constant across all development classes is unknown. Moreover, in organisms which undergo metamorphosis (e.g. insects), growth and the loss of juvenile structures and development of adult structures follow a time sequence which may require different nutrient levels. Because adult mayflies do not feed, all the chemical requirements of the adults must be met by the nymphs. Thus nymphal nutrition must play a large role in adult reproductive success. Since egg elemental composition is key to early nymph growth and survivorship (Tessier et al. 1983, DeMott 2003), the availability of nutrients to actively feeding nymphs is of paramount importance. Because all egg production is realized in mayfly nymphs, there should be a direct linkage between nymphal food quality, fecundity, and egg nutrient content in mayflies. This relationship has been shown in *Daphnia* (Sterner 1993, Urabe & Sterner 2001). On the other hand it is possible that this linkage is relaxed because males may contribute phosphorus in their sperm packet that is available for incorporation into eggs. This has been demonstrated in *Drosophila* spp. (Markow et al. 2001).

The objective of this study was to determine growth rates for *Caenis* spp. across a range of development classes spanning several levels of nutrient enrichment. We hypothesize that (1) nutrient stoichiometry will vary through the development cycle of *Caenis* spp, with highest levels of P at early and late development stages due to relatively high levels of somatic and reproductive growth, respectively, (2) that nutrient stoichiometry within a development class from a site will differ depending on nutrient content of food, and (3) growth rates will increase with increasing P content of food resources and decrease with increasing development class.

Methods

Study area.

The North Bosque River is a 4th-order (Straehler system, 1:250,000 scale) perennial tributary of the Brazos River located in central Texas, USA. The North Bosque flows predominantly through the Cross Timbers Level III ecoregion (Griffith et al. 2004), an area characterized by semi-arid climate (annual precipitation 40-60 cm/y), shallow alkaline clay soils overlaying heavily fractured limestone bedrock, and flashy stream flow. The longitudinal profile of the North Bosque River exhibits a strong nutrient gradient caused by municipal waste water inputs and runoff associated with concentrated animal feeding operations. These inputs are highest in the upper reaches of the watershed. Consequently, concentrations of dissolved and total phosphorus and nitrogen decrease with downstream direction (Back 2003).

Two sites along the longitudinal P gradient were selected for the field study of *Caenis* spp. to contrast C:N:P ratios among nymphs of differing development classes and from habitats differing in nutrient content of food resources. We chose NBOS-03 (31.97692° N, 98.03974° W), a 4th-order reach near Hico, TX as a relatively high phosphorus site. We selected NBOS-05 (31.63760° N, 97.36640° W), a 4th-order reach near Del Mar Ranch at Valley Mills, TX as a contrasting site of low-to-moderate levels of phosphorus enrichment (Table 2.1). Both stream reaches exhibited similar physical characteristics, typified by short limestone cobble riffles interspersed with long, shallow bedrock glides and pools with moderate deposits of fine sediment. Historical stream flows (United States Geological Survey, http://tx.usgs.gov/basins.html) at each site were also similar, although surface discharge was undetectable at the time of sampling and streams were reduced to a series of long glides and pools interconnected by subsurface flow.

Field study: stoichiometry of Caenis *development classes*

Caenis spp. nymphs were collected from both study reaches between 28 September and 7 October 2006. *Caenis* spp. nymphs were collected using a 250 µm Hess sampler from shallow gravel substrates at pool margins. Samples (15-25) from each site were sieved through soil sieves (2-mm, 1-mm, 0.5-mm, and 0.25-mm). The retained material in the three smallest sieves was stored on ice for transport to the laboratory. We removed in the field all large *Caenis* spp. nymphs retained in the 2 mm sieve. In the laboratory we sorted nymphs into one of five development classes following Taylor & Kennedy (2006) using a Nikon SMZ 1500 dissecting microscope equipped with a Nikon DXM 1200f digital imaging system. Development classes corresponded to external wing pad morphology and pigmentation. Development classes I through V are determined as: I = no wing-pads present, II = clear wing-pads present in thoracic region, III = wing-pads with veins present in thoracic region, IV = wing-pads with veins present in abdominal region or with veins and mottling present in thoracic region, V = wing pads enlarged, with veins and dark mottling reaching abdominal region (Taylor & Kennedy 2006). These external morphological classes are also related to internal changes associated with adult development. The use of development classes is superior to arbitrarily making groups based upon size alone because equal size among individuals does not necessarily mean they are at an equivalent development stage. Because only mature development class V nymphs can be reliably identified to species we could only contrast development classes at the genus level. We dried all development class samples to a constant mass at 50°C, pulverized and homogenized with a mini-beadbeater-8 (Biospec Products) and stored the powder in a desiccator until determination of nutrient content was completed.

Periphyton (defined here as a composite mixture of algae, fine particulate organic matter, and sediment) was collected from each site for determination of C:N:P stoichiometry to evaluate whether food resources at the two sites differed in terms of nutrient content. Periphyton was removed from rocks by scrubbing with hard-bristle brushes. The entire slurry was dried at 50°C for 24 hours and then pulverized and homogenized the dried periphyton with a mini-beadbeater-8 (Biospec Products) and stored the powder in a desiccator until determination of nutrient content was completed.

Surface-water samples were collected from each site for determination of dissolved (0.45 μm filter) and total (unfiltered) N and P. Samples were collected in triplicate for NH_4-N, NO_2-

NO_3-N, TN, PO_4-P, and TP and stored and analyzed samples according to standard methods (APHA 1998). All surface-water nutrients were analyzed on a Lachat Quik-Chem 8500 flow-injection autoanalyzer.

We estimated %C and N content of periphyton and *Caenis* spp. nymphs from the field sites using a ThermoQuest Flash EA™ 1112 elemental analyzer. Percent P content was estimated using a Lachat QuikChem 8500 flow-injection autoanalyzer using the molybdate colorimetric method following digestion in 5-mL 32M sulfuric acid at 350 °C on a digestion block for 2-h. Soil (Thermo Finnigan 1.99 %C) and peach leaf (SRM 1547, 0.137 %P, 2.98 %N) standards were used for QA/QC to determine C, N, and P recoveries, which were all quite high (89-107%) and consistent among replicates (n=5 per standard).

Because of the small size of *Caenis* nymphs (development classes I-V mean mass per individual were 0.002, 0.01, 0.071, 0.289, and 0.461 mg, respectively) relative to the mass required to achieve detectable concentrations of P in our digests (10-20 mg), we analyzed composite samples of several to hundreds of nymphs from each site (Table 2.2). In those cases that we had less than the required mass, we decreased the acid volume and dilution volume proportionately (3-9 mg material would be digested in 2.5 ml H_2SO_4 and then diluted to 37.5 mls). We were unable to collect sufficient biomass of development class I nymphs for C:N:P determination.

Polynomial least-squares regression was used to fit continuous relationships between increasing development stages and C:P and N:P ratios of nymph composites. Regression equations were fitted to data for each site and patterns were qualitatively contrasted between sites to assess whether the higher-P site (NBOS-03) tended to have lower C:P and N:P ratios than the lower-P site (NBOS-05).

Laboratory growth experiment

Green algae (*Cladophora* and *Spirogyra* spp.) mats were collected from Neils Creek, a low-nutrient tributary of the North Bosque River, near Valley Mills, TX, to culture for food treatments in the growth experiment. Algal mats were collected on 24 September 2006 and split this material into four equal masses of approximately 20 g wet mass. We placed split fractions of algae in 1 L beakers of filtered site water enriched with Na_2HPO_4 ranging from +0 (no enrichment, background TP 10 μg/L), +30, +90 and +270 μg/L P respectively. Algal cultures were held in control and enriched stream water at 20ºC with continuous light for 48 hours to allow uptake of P into algal tissues. We did not determine the ingestion rates of *Caenis* spp. across its development classes and potentially could have not provided enough food at higher development classes. However, our lowest supply (0.15 mg C/cm^2) was 3-times higher than that used by Frost & Elser (2002) to represent low food quantity for similarly sized larvae (our development class I). Algal samples were removed from beakers and allowed to air dry at 20ºC for several days. Samples were ground to a fine powder using a mortar and pestle and stored in airtight containers. Nine subsamples were analyzed from each algal enrichment treatment for C, N and P content using laboratory methods previously described in the field study.

We collected *Caenis* spp. nymphs for the growth study from a perennial pool in shallow gravel/sand substrates from the lower-P site (NBOS-05) on the North Bosque River. Twenty-five Hess samples (250 μm mesh) were collected as described in the field study in order to obtain enough nymphs for the experiment. Live nymphs were separated into the five development classes and measured each individual for head capsule width (HCW) using a Nikon SMZ 1500 stereomicroscope equipped with a Nikon DXM 1200f digital imaging system. We initially considered all five development classes, but preliminary results suggested that development class

V nymphs were too close to maturation and would likely emerge during the experiment. Thus, we focused only on nymphs of development classes I-IV for the growth experiment.

Nymphs were placed individually into 20 mL glass scintillation vials filled with 15 mL of filtered stream water. Ten nymphs were reared from each of the four development classes, on each of the four algal treatments in incubators set at 20ºC with 12 hour light cycles for ten days. Nymphs were fed in order of increasing development class 0.15, 0.3, 0.6, and 1.2 mg C cm^{-2}, respectively. We replaced filtered stream water and algae every 3-4 days. Post incubation HCW measurements were made for each individual and used these measurements to calculate growth rate. Growth rate was calculated as:

$$\mu = [\ln(B_2) - \ln(B_1)] \ / \ time$$

where μ = growth rate, B_1= estimated initial mass and B_2= estimated final mass. We estimated initial and final masses (mg) by inserting HCW measurements (µm) into a published HCW-weight regression (dry mass = 23.09548 $e^{\mathrm{HCW} + 3.19737}$, $r^2 = 0.97$) developed for *Caenis latipennis* by Taylor & Kennedy (2006).

Two-way ANOVA was performed on growth-rate data using development class (I-III) and food quality (four C:P treatments) as main effects and a development class x food quality interaction term. High mortality of nymphs in class IV treatments reduced our degrees of freedom below an acceptable level for detecting statistical interactions so we only analyzed growth-experiment data from development classes I-III . One-way ANOVA was run *a posteriori* to test the influence of food quality on growth rate within each development class, including development class IV. Tukey's studentized range test was used to assess the relationships of means between groups for ANOVA results. Because not all data was distributed normally, all analyses were conducted on ranked data. Effects were considered significant when $p \leq 0.05$. ANOVA was performed in SAS 9.1 (SAS Institute, Cary, NC, USA).

Results

Field study: stoichiometry of Caenis *development classes*

Surface-water and periphyton had higher N and P levels at NBOS-03 when compared to the downstream site (NBOS-05). This suggests that nutrient availability to periphyton indeed was higher at NBOS-03 and nutrient content of an important food resource to *Caenis* reflected these differences in its elemental composition (Table 2.2).

C:P ratio of *Caenis* spp. nymphs exhibited a unimodal relationship with increasing development class ($r^2=0.993$, $F_{2,1}=74.7$, $p= 0.081$ and $r^2=0.995$, $F_{2,1}= 107.8$, $p = 0.048$ for NBOS-03 and NBOS-05, respectively; Figure 2.1a). C:P ratio increased from development class II to III, but decreased slightly (NBOS-03) to markedly (NBOS-05) from class III to V. C:P ratio at NBOS-05 was greater than the more P-enriched NBOS-03 for all development classes except class V (Figure 1a). We observed a similar hump-shaped N:P relationship across development classes and between sites ($r^2=0.971$, $F_{2,1}=17.32$, $p=0.168$ and $r^2=0.998$, $F_{2,1}=207.8$, $p=0.041$ for NBOS-03 and NBOS-05, respectively). This pattern in N:P ratio was largely due to less dramatic shifts in % N content across development classes than those observed for %P (Table 2.2, Figure 2.1b). Neither C:N nor % C showed a consistent trend with increasing development class between sites, increasing from II-V at NBOS-03, but no clear pattern at NBOS-05 (Table 2.2).

Laboratory growth experiment

Artificially enriched algal samples produced a strong P gradient across the four algal treatments with P increasing substantially while little change was observed in C and N. This resulted in a shift in molar C:P ratios from 960 for the control to 62 for the highest P treatment (Table 2.3).

Caenis spp. growth rates increased in response to P enrichment in the earliest development class but this growth response diminished in later development classes (Fig 2). Growth rate of *Caenis* spp. was significantly influenced by development class ($F_{2, 67} = 3.41$, $p = 0.0388$) and the interaction between development class and P treatment ($F_{6, 67} = 2.36$, $p = 0.0397$); however, P treatment ($F_{3, 67} = 2.51$, $p = 0.0661$) had no effect on growth rate (two-way ANOVA on ranked data). Tukey's studentized range test ($\alpha = 0.05$) could not separate the rank sums of the three development classes into statistically different groups. A post hoc analysis on data analyzed separately for development classes I thru IV showed that P treatment had a highly significant effect on growth rate in development class I but not development classes II, III and IV (One-way ANOVA on ranked data; Table 2.4). Only the rank sums of P treatments 62 and 960 were significantly different for development class I (Tukey's studentized range test on ranked data, $\alpha = 0.05$; Fig. 2.2).

Discussion

Ontogenetic differences in Caenis *stoichiometry*

The C:P ratios for *Caenis* were all in the lower range of values reported 100-800 (mean 263 ± 113) for mayflies in Evans-White et al. (2005). Although we have no measurement of the amount of variation within each development class, C:P and N:P ratios showed a clear pattern of increasing and then decreasing values across development classes at both sites. Admittedly, having no measure of variation in C:P and N:P ratios decreases the certainty of a unimodal pattern. However Frost & Elser (2002) showed that the smallest mayflies had the highest P content. Extending this trend to Fig. 2.1 would further support a unimodal pattern. If individual species could be identified for all development classes, their stoichiometry could result in patterns different than those we observed. However, *Caenis* are collector-gathering detritivores and variability in food type and quality among species is probably low. Therefore, the level of elemental imbalance between different *Caenis* species and their food should be similar. This could lead to species with unique elemental content but not necessarily differing patterns in stoichiometry across their ontogeny.

Why would *Caenis* spp. nymphs differ in their C:N:P stoichiometry across development classes? We believe that it is a result of differing N and P demands needed for the development of somatic and reproductive structures across the ontogeny of *Caenis* spp. When eggs hatch and nymphs grow and metamorphosis progresses through development classes, the early development classes first produce somatic tissue, and later (development classes IV and especially V) produce reproductive structures and gametes. Thus the decline in C:P ratios in development class IV and V represents a high P investment in gametes, which is probably most evident in females. This idea is supported by Markow et al. (1999, 2001) who demonstrated that adult *Drosophila melanogaster* and *D. nigrospiracula* females are 3-times more phosphorus rich than males and that eggs and male ejaculate are P rich. Furthermore, males of *D. nigrospiracula*, which feed on P-poor cacti, had a longer time lag in mating after eclosion than those of *D. melanogaster*, which feed on P-rich fruit. This time lag for *D. nigrospiracula* was thought to represent a longer P acquisition time for males because of the low P content of its food (Markow et al. 2001). Follicular development in *D. nigrospiracula* was much slower than that in *D. melanogaster* at eclosion. Markow et al. (2001) suggest that *D. nigrospiracula* may have to allocate a higher proportion of P to somatic growth in the larval stages due to low P food. Thus more egg maturation takes place in the adult stage in this species. Elser et al. (2006) showed that P content of larvae in five *Drosophila* spp. decreased as larvae grew. This is not the pattern we observed for *Caenis* spp. However, mayflies have a major difference in their life history: adults do not feed. Thus, larvae must acquire all materials necessary for adult survival and reproduction. Based on the high P content of adult *Drosophila* reproductive structures (Markow et al. 2001), it makes sense that organisms with non-feeding reproductive stages would necessarily have late development stage larvae that are richer in P than earlier stages because gametes develop and mature in the larvae. The onset of morphological sexual differentiation and development of reproductive structures coincides with decreasing C:P ratios in *Caenis* spp. nymphs in our study. Examinations of mature female mayfly larvae (i.e. darkened wing pads) of *Caenis* spp. and two other mayflies at our sites, *Neochoroterpes nanita* and *Stenonema femoratum*, revealed their entire abdomens were full of eggs and no digestive tract was evident. Frost & Elser (2002) showed a steady decline in %P content in *Ephemerella* sp. mayflies. Although they did not report development classes in their study, mature larvae of *Ephemerella* sp. are 9-15 mm in total length and they complete their life cycle in 9-12 months (Edmunds et al. 1976). Since Frost & Elser (2002) collected newly hatched larvae and their experiment duration was six days, they could not have included late development class nymphs in their experiment. Thus the declining body %P demonstrated in their study supports what we demonstrated in early development class larvae of *Caenis* spp.

Trends in *Caenis* spp. nutrient stoichiometry between sites of differing nutrient status are consistent with those shown for aquatic insects by Cross et al. (2003). They showed insects from enriched sites had higher nutrient content than those from less enriched sites. Mayfly development classes showed higher percent nutrient content at the enriched (NBOS-03) site relative to the less enriched site (NBOS-05). Percent C increased across development classes. This probably represents an increase in exoskeleton and other C rich structures associated with growth (i.e. larger body size). The % N and P at NBOS-03 was equivalent or higher than NBOS-05 in every case except development class V. Again, this probably reflects the nutrient content of the dominant food source which was higher at NBOS-03 and those mayflies had the higher nutrient content.

The decrease in C:P ratio of development class V (Fig. 2.1a and b) at NBOS-05 when compared to NBOS-03 may be the result of the majority of individuals being female, and contributing a disproportional amount of P to the sample. The latter is supported by Markow et al. (1999, 2001) findings on the high P content of female *Drosophila* spp. Consideration of life history strategies and adult feeding status needs to be included in future studies of ontogenetic changes of elemental composition. Villar-Argaiz & Sterner (2002) demonstrated in a freshwater copepod that P deficiency in late stages of development prevented larval *Diaptomus clavipes* from developing into adults, yet younger stages grew just as well as individuals fed a P replete diet. Thus timing of increased P acquisition is probably important. Even in insects that have feeding adults, the quality of food used by adults needs to be investigated in light of larval food quality. Many holometabolous insects have larvae and adults that do not use the same food resources and each stage may face differing elemental imbalances. Future work is needed to determine the variability of C:N:P ratios within *Caenis* spp. development classes and between sexes to better understand the degree of plasticity in body chemistry among development classes and sexes.

Laboratory growth experiment

A high potential for rapid growth provides many potential advantages to species because growth and development rates can affect many life history traits (e.g. age and size at first reproduction) and ecological features (e.g. predation risk) (Elser et al. 2006). Our findings suggest that increased P content of food increases growth rates of smaller development classes of *Caenis* mayfly nymphs (Fig. 2.2). The GRH suggests that growth of organisms with higher potential for rapid growth will be more P limited. Our data supports this hypothesis within the context of the life history of a single taxon. As nymphs mature, growth rates and associated P requirements decrease resulting in less influence of food P content on somatic growth rate. Stream insects typically exhibit higher mortality during early stages of development (Benke & Huryn 2007). While many factors such as density affect survivorship, it is plausible that increased growth rates during early stages of development have the potential to decrease mortality and increase overall production of populations when P is not limited.

Our data showed a significant interaction between P content of food and development class on growth rates of *Caenis* spp. This interaction appears to be driven by P content of food having an effect on growth rates in development class I but not development class II thru IV (Fig. 2.2, Table 2.4). However, in light of our field results it is plausible that later development classes shift P resources from somatic growth to reproductive development. Nymphs within higher P content treatments could have potentially begun developing reproductive structures earlier than nymphs in lower treatments resulting in diminishing growth rates based on HCW measurements earlier than in lower treatments. Thus linear dimensions may not increase even though biomass is accumulated via reproductive development. This supports the hypothesis that not only growth but development may be P limited. Increased development rates have the potential to limit fecundity as it is usually correlated with organism size.

Although P limitation of growth has previously been demonstrated for other mayflies including *Caenis* spp. (Frost & Elser 2002) we provide the first observation of ontogenetic shifts in P limitation of growth related to size and development class within the life cycle of a benthic consumer. Our results agree with studies on terrestrial insects done by Elser et al. (2006) that showed similar shifts in P limitation across the short life cycles of several species of *Drosophila*. However, our experimental design was limited by higher mortality rates than expected in development class IV and the short time period of the experiment. We could not determine whether these shifts were related to size specific growth potential, shifts in P allocation from somatic growth to reproductive development or a combination of the two. Nonetheless, our study demonstrates that P requirements for growth and development can vary across a species life cycle and that P availability has the potential to limit many life history factors within aquatic benthic consumers. Future stoichiometric studies of benthic consumers should consider ontogenetic shifts in P limitation of growth, the mechanisms that control these shifts and the cumulative consequences that varying P limitation across life cycles may have on populations of aquatic organisms.

Acknowledgements

We thank David Lang and Charles Stanley for help collecting water samples from the two field sites. Hui Huang and Thad Scott helped analyze nutrient samples. Steve Dworkin graciously allowed us to use his CN autoanalyzer. The quality and clarity of the manuscript were improved by three anonymous reviewers. JAB and JMT were supported by grants from the U. S. EPA (CP-966137-01) and the Texas Commission on Environmental Quality (TCEQ) (subcontract #470122) to RSK, respectively. Although this research was funded in part by these agencies, it does not necessarily reflect the views of these agencies and no official endorsement should be inferred.

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Table 2.1. Nutrient concentrations for the North Bosque River sample locations corresponding to the field collection of *Caenis* nymphs.

	Site	
Nutrient (μg/L)	NBOS-03	NBOS-05
TP	104	46.1
PO_4-P	10.9	9.3
TN	2058	610
NO_2-N+NO_3-N	2.1	6.6
NH_3-N	50.2	71.3

Table 2.2. Elemental content of periphyton and *Caenis* spp. nymphs across developmental classes at each stream location.

Periphyton or Developmental Class	NBOS-03				NBOS-05			
	n[1]	%C	%N	%P	*n*	%C	%N	%P
Periphyton	---	10.7	1.12	0.116	---	9.70	0.96	0.085
II	504	48.2	11.7	0.93	609	47.0	11.7	0.71
III	231	48.9	12.0	0.81	352	47.2	10.4	0.59
IV	40	51.4	12.0	0.83	40	50.7	10.8	0.74
V	18	53.1	11.5	0.89	9	48.1	10.6	1.17

[1] number of individual nymphs analyzed in each composite sample

Table 2.3. Elemental composition and molar ratios for the control and three P-enriched food treatments used in the growth experiments.

Treatment	%C	%N	%P	C:N	C:P	N:P
+0 (Control)	26.4	2.09	0.07	14.7	960	65
+30μg/L	25.6	2.02	0.26	14.8	254	17
+90μg/L	25.5	2.09	0.70	14.3	95	6.6
+270μg/L	25.2	2.06	1.04	14.3	62	4.4

Table 2.4. One-way ANOVA and Tukey's Studentized Range Test results for the effects of food P content on ranked growth rates for each development class of *Caenis* spp.

Development class	df	*F*	*p*
I	3, 22	8.36	0.0007
II	3, 24	0.74	0.5411
III	3, 21	1.12	0.3650
IV	3, 15	0.18	0.9062

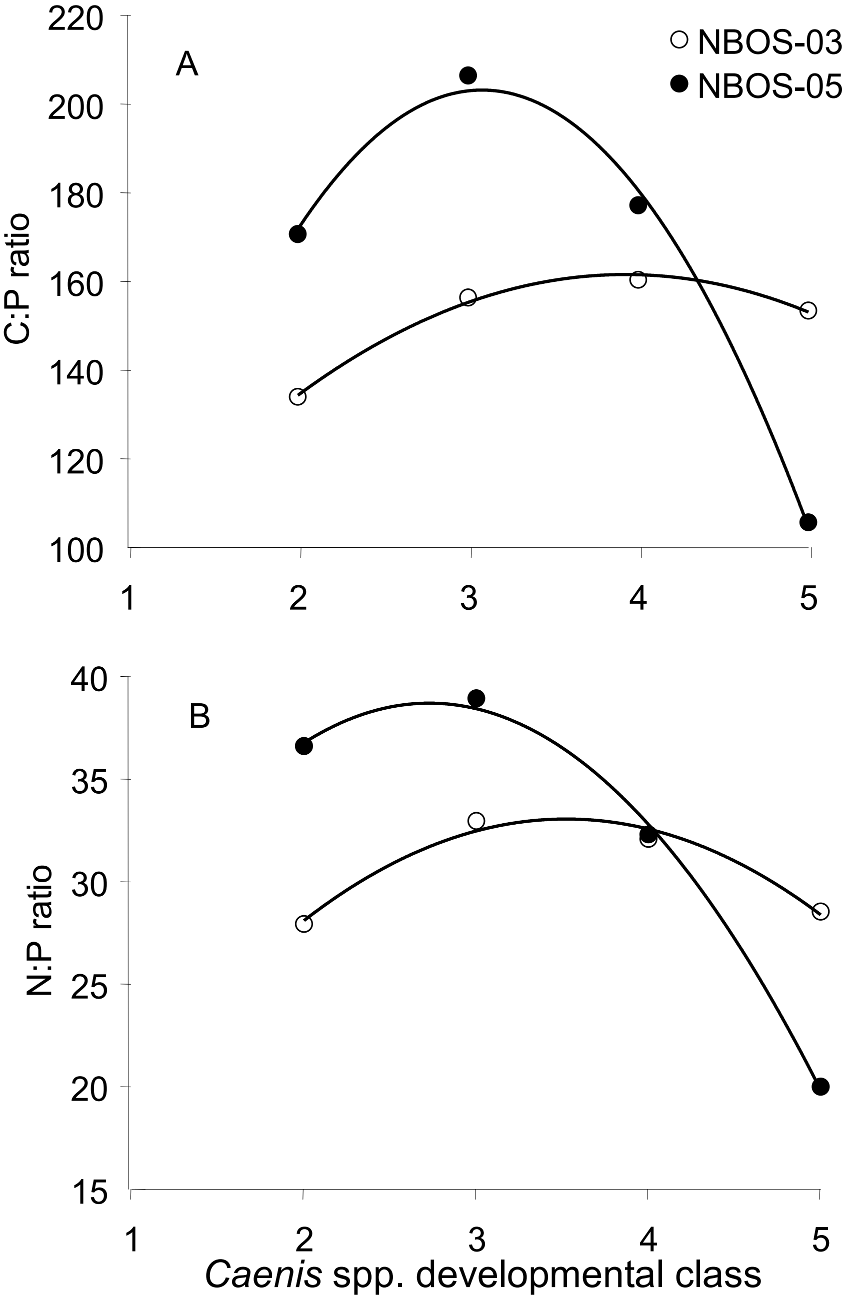

Figure 2.1. Regressions of molar C:P (A), and N:P (B) ratios across *Caenis* spp development classes at NBOS-03 and NBOS-05 collection sites.

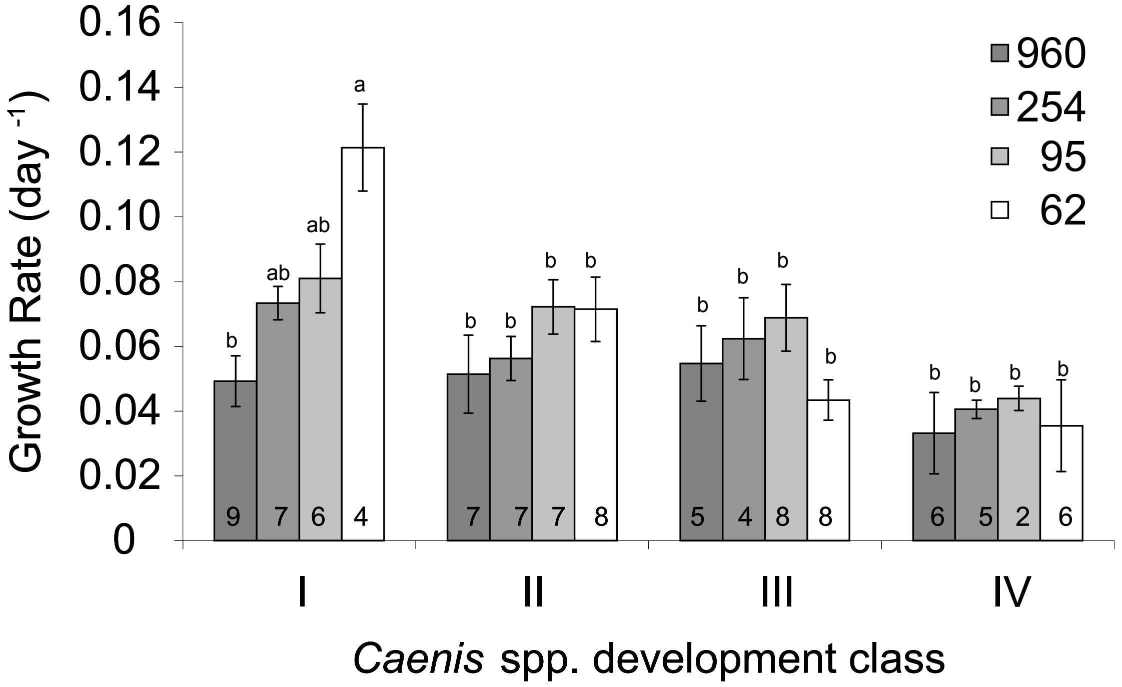

Figure 2.2. (A) Mean ± 1 SE growth rates of individually reared *Caenis* spp nymphs in four different development classes across four different food quality treatments. All food treatments are presented as molar C:P ratios. Sample sizes are indicated within bars. Lower case letters above bars represent significant differences between rank sums of food quality treatments within each development class determined by a Tukey's Stundentized Range Test.

CHAPTER THREE

Sex and size matter: Ontogenetic patterns of nutrient content of aquatic insects

This chapter published as:
Back, J. A. and R. S. King. 2013. Sex and size matter: Ontogenetic patterns of nutrient content of aquatic insects. Freshwater Science. 32(3):837-848.

Abstract. C, N, and P content were measured across the ontogeny of lotic aquatic insects representing a diversity of life-history characteristics. The relationship between individual mass and nutrient content was used to show ontogenetic patterns of nutrient content by species. Species analyzed for C and N content exhibited a quasihomeostatic pattern across ontogeny. Percent C and %N varied among taxa irrespective of ontogeny, with %C ranging from 47.4 to 56.2% and %N ranging from 9.6 to 11.6%. P content also varied by species but declined nonlinearly across ontogeny and was best represented by a power function. Percent P varied from >7% in 1st-instar *Tabanus* larvae to only 0.34% in adult male *Ambrysus circumcinctus*. Females had more P per unit mass than males in 6 of the 10 species that could be sexed. In the leptophlebiid mayflies, %P increased in mature female nymphs relative to the penultimate developmental class, whereas %P content of males continued to decline to eclosion. Maximum terminal mass by species was the main factor driving the magnitude of change in %P through their ontogeny. Small-bodied, rapidly growing species exhibited the sharpest decline in P content. Nonhomeostatic patterns in %P across ontogeny and between sexes has important implications for population- and community-level dynamics and ecosystem processes. First, small-bodied, high-%P taxa have faster growth rates than larger individuals, which supports one of the predictions of the growth-rate hypothesis (GRH). Second, elemental imbalance between consumers and their food changes across ontogeny, and therefore, nutrient recycling rate by a species changes with population age structure. Last, community structure may reflect nutrient availability in food such that enriched environments are more likely to be dominated by taxa with high growth rates and thus relatively high P demand.

Key words: ecological stoichiometry, elemental composition, ontogeny, homeostasis, phosphorus, nutrient patterns, aquatic insects.

Ecological stoichiometry is the study of constraints and consequences of elemental imbalances between consumers and their food (Sterner and Elser 2002). Nutrient imbalances arise because the bodies of herbivores and detritivores are typically more enriched in N and P than the plants and detritus they eat (Sterner and Hessen 1994, Fagan et al. 2002, Cross et al. 2003). The C content of plants and detritus usually is similar except that aquatic plants and algae have lower C content than detritus (Kahlert 1998) and terrestrial plants (Sterner and Elser 2002, Shurin et al. 2006). In contrast, predators are more similar in elemental content to their prey (Sterner and Elser 2002). Patterns of CNP content of consumers and their food provides a basis for understanding the effects of elemental imbalances on growth rates of consumers (Elser et al. 2003), nutrient recycling (Vanni et al. 2002, Evans-White and Lamberti 2006, Rothlisberger et al. 2008), population dynamics (Andersen et al. 2004, Moe et al. 2005), foodweb organization, (Vrede et al. 2004), and community structure and function (Elser et al. 2000, Cross et al. 2005).

The CNP content of lotic macroinvertebrates and their food resources has recently received attention (Cross et al. 2003, Bowman et al. 2005, Evans-White et al. 2005, Liess and Hillebrand 2005, Back et al. 2008, Small and Pringle 2010). Organisms accomplish homeostasis by modifying the quality or quantity of organic matter ingested to maintain adequate supply relative to demand of essential elements required for metabolism, growth, and reproduction (Sterner and Elser 2002). Investigators have implicitly assumed that aquatic macroinvertebrates maintain an approximately constant, or homeostatic, elemental composition across life stages or sizes within species. However, the limited number of studies on CNP content of aquatic and terrestrial invertebrates spanning different developmental stages has revealed nonhomeostatic patterns across the ontogeny of species (zooplankton: Andersen and Hessen 1991, Hessen and Lynch 1991, Main et al. 1997, Villar-Argaiz et al. 2002; *Drosophila*: Elser et al. 2006; mayflies:

Frost and Elser 2002, Back et al. 2008, Veldboom and Haro 2011). Specifically for insects, P content of 5 species of *Drosophila* larvae decreased with increasing larval development, and each species had differing P content (Elser et al. 2006). P content decreased across the early developmental stages of *Ephemerella* sp. mayflies (Frost and Elser 2002). P content decreased in early developmental stages of *Caenis* sp. and increased in later developmental stages, whereas C and N content were nearly constant across ontogeny (Back et al. 2008). P content also increased in late-stage larvae, pupae, and adults of the caddisfly *Brachycentris occidentalis* (Veldboom and Haro 2011). However, these investigators compared, at most, the elemental content of several size classes and did not examine individuals across the continuum of ontogeny.

Food elemental content and species development are intimately linked by changing elemental needs across ontogeny. Because elemental content may change across the ontogeny of a species, the severity of elemental imbalance also may change (increase or decrease) assuming constant C, N, and P content of food. This linkage may affect survivorship of specific life-history stages as demonstrated for the copepod *Diaptomus clavipes* (Villar-Argaiz and Sterner 2002).

Understanding how P content varies across ontogeny is of particular importance because P deficiency limits growth of invertebrates (Urabe et al. 1997, Sterner and Elser 2002). Furthermore, growth rates are positively correlated with body %P (Elser et al. 1996, 2003, Frost and Elser 2002, Weider et al. 2005) but negatively correlated with adult invertebrate body size (Woods et al. 2004). Phylogeny also may constrain P (Woods et al. 2004) and N (Fagan et al. 2002) content of invertebrates, independent of ontogeny.

Life-history traits, such as reproductive strategy, probably have a strong bearing on P content during ontogeny. P content of invertebrates that produce a single (usually large) batch of eggs (semelparous taxa) may increase as larvae mature. Percent P of somatic tissue is less than that of gametes (especially eggs) in the few taxa examined (Andersen and Hessen 1991, Markow et al. 1999, 2001, but see Færøvig and Hessen 2003). P content of iteroparous taxa (reproduce multiple times, fewer eggs) probably declines as larvae grow because adults can feed and supply nutrients necessary for maintenance and reproduction. Differences in patterns of P content between iteroparous and semelparous taxa may be because semelparous insect taxa do not feed as adults. Therefore, the burden of P (and C, N, and other elements) acquisition rests entirely on larval feeding. Collectively, these previous studies suggest that sex, reproductive strategy, adult feeding status, trophic level, life-cycle completion time, and metamorphosis type may interact to produce varying patterns in P content across invertebrate ontogeny.

The objectives of our study were to: 1) describe the pattern of %CNP content of aquatic macroinvertebrates across their ontogeny, from egg or 1st instar to mature larva or adult, 2) assess whether nutrient content differs between sexes, and 3) identify consistent patterns (if any exist) in nutrient content related to life-history traits.

Methods

Study area

All insects were collected from 3 to 24 June 2009 from Cowhouse Creek in Coryell County, Texas, USA (lat 31.286122°N, long 97.883994°W). Cowhouse Creek is a tributary of the Brazos River in the Cross Timbers Level III Ecoregion (Griffith et al. 2004). Land cover in the 1180-km^2 catchment consists of shrubland (43%), grassland (34%), and forest (19%) (King et al. 2009). Stream habitats sampled were riffles, runs, and their margins. Riffles and runs consisted of large areas of shallow gravel and cobble substrates overlying limestone bedrock. Insects were collected using D-nets, kick screens, and by hand-picking insects from rocks.

Corydalus cornutus eggs were collected from ash tree (*Fraxinus*) leaves overhanging the creek and *Tabanus* sp. eggs were collected from exposed rocks in riffles. The goal was to collect the entire size range of numerically dominant species. Insects were transported to the laboratory and sorted live under a stereomicroscope.

Chemical analyses

Intact whole insects were dried in Al weighing pans at 50°C for ≥48 h and then stored in a desiccator. The bottoms of the weighing pans were covered with paper towel to: 1) prevent insects from sticking to the pan while drying, and 2) provide evidence of fluid leakage from an insect's body that would lead to its exclusion from chemical analysis. Before chemical analyses, insects were redried for ≥24 h at 50°C. Individual insects were weighed on a microbalance (Mettler Toledo XP-26; Mettler-Toledo AG, Greifensee, Switzerland) to the nearest µg. No method was available to measure simultaneously the C, N, and P content of a single individual. Therefore, C and N content were measured on one set of individuals and P content was measured on a different set of individuals for each species. Enough material was available to measure C, N, and P content on only 8 taxa, whereas P content alone was measured on 10 additional taxa (18 total).

C and N content were measured simultaneously with a Thermo-Finnegan Flash 1200 elemental analyzer (ThermoQuest, Milan, Italy). Individual insects of each taxon were analyzed when possible. In a few cases, small individuals were combined to achieve the minimum 200 µg of dry mass needed for %C and N analysis. Whole insects were placed in Sn capsules, gently crushed with a metal spatula, and sealed in the capsule. Standards of L-cystine (30% C and 11.67% N) and an internal standard of *Anax junius* dragonfly nymphs (49.9% C and 10.8% N) were run with samples for quality assurance/quality control (QA/QC). Mean (SD) % recovery of C and N from L-cystine standards (*n* = 26) were 101.5% (1.30) for C and 95.2% (0.63) for N and from *A. junius* standards (*n* = 22) were 101.1% (1.93) for C and 98.5% (0.45) for N.

For P measurement, individual insects were weighed as above, placed in a 22-mL glass scintillation vials, pulverized with a metal spatula, capped with a lid containing a Teflon septum, and chemically digested in an autoclave for 1 h at 120°C by the method of Færøvig and Hessen (2003). The minimum mass required for P measurement was 10 µg (assuming 1% P content). For sample masses ≤2000 µg, 15 mL deionized (DI) water and 1.8 mL of digestion solution was used. For every 2000-µg increment, an additional 1.8 mL of digestion solution was used in place of 1.8 mL of DI water, up to a 22,000 µg maximum mass digested (19.8 mL digestion solution, no DI water). Individuals that weighed >22,000 µg were broken into subunits, digested in multiple vials, and composited after digestion. All samples with masses >2000 µg were diluted back to the ratio of 15 mL DI:1.8 mL digestion solution with DI water. P content was estimated via colorimetry by the ascorbic acid–molybdate method on a Lachat 8500 flow-injection autoanalyzer with an ASX-520 autosampler (Hach Co., Loveland, CO). Tissue standards of tomato leaf (SRM 1573a, 0.216% P), and bovine liver (SRM 1577c, 1.175% P) and dissolved inorganic standards were run for QA/QC. To ensure no bias among the 11 runs, a wide range of masses for each taxon was analyzed in ≥2 separate runs. The mean (SD, *n*) % recovery for P was 103.9% (14.3, 45) for tomato leaf, 92.7% (6.1, 26) for bovine liver, and 103.6 (6.5, 176) for inorganic P standards.

Data analyses

Patterns of C, N, and P content.—Percent C, N, and P were plotted as functions of dry mass for each taxon separately. C, N, and P curves were evaluated as linear, exponential, logarithmic, and power functions. The goodness of fit of each curve type was assessed using the r^2 value. Linear regressions were tested for slopes equal to 0 in R (version 2.13.1; R Core Development Team, Vienna, Austria) using the CAR library (Fox and Weisberg 2011). A slope equal to 0 is evidence of elemental homeostasis across ontogeny. Mass and % element data were log(x)-transformed prior to analysis to meet assumptions of linear models.

Sexual differences in nutrient content.—Mayfly nymphs were grouped into 5 development classes (DC) based on wing-pad development (Taylor and Kennedy 2006) and sexed based on eye development. Other immature insect taxa and earlier DC mayflies could not be sexed. DCs 3, 4, and 5 were defined by wing pads that reached abdominal segment 1, 2, and 3+, respectively (Fig. 1A–C). Females have simple eyes and males have turbinate eyes (Fig. 1B, C). Adult Coleoptera (*Stenelmis*) and Hemiptera (*Ambrysus*, *Rheumatobates*, *and Rhagovelia*) also were sexed and %P was compared between sexes.

Analysis of covariance (ANCOVA) was used to assess whether change in %P with mass differed between males and females. Mass was the covariate and sex was the categorical factor in each ANCOVA model. We followed the analysis framework outlined by Engqvist (2005). If the interaction term was significant, then the rate of change of %P differed between males and females (i.e., slopes not equal). The data were analyzed again without the interaction term and the main effects, sex and mass, were examined. The 2 models (significant interaction vs nonsignificant interaction) were then compared with analysis of variance (ANOVA), and a nonsignificant result indicated the simplest model was most appropriate (Engqvist 2005). If the main effects and interaction (sex, mass) were nonsignificant, we concluded that the sexes had the same %P and the regression line had a slope of 0, and thus, %P did not vary with mass. If only mass was significant, the slope was not equal to 0, and %P varied with mass but the sexes had the same %P. If only sex was significant, the sexes had the same slope but one sex had higher %P than the other and the slope of both regression lines was 0. If both sex and mass were significant, then %P changed with mass and the sexes differ in %P, but the slopes of males and females were equal.

Nutrient content and life-history traits.—To test the hypothesis that smaller taxa had sharper declines in %P with increasing size, we plotted the maximum size of each taxon against the P decay rate to assess whether life-history traits influenced patterns in %P. Maximum size was a surrogate for life-cycle completion time. Based on the generation-time law (Bonner 1965, Peters 1983), we predicted smaller maximum-sized taxa would have a faster life-cycle completion time than larger maximum-sized organisms. Reproductive strategy, metamorphosis type, functional feeding group (FFG), and taxonomy were coded into the plot to detect whether patterns emerged based on these life-history traits.

Results

Patterns of %CNP content

Linear regression analysis revealed both %C and N were invariant (i.e., slopes = 0) across the ontogeny of 6 of the 8 taxa examined. *Ambrysus circumcinctus* had slopes < 0 for %C and N, whereas *Baetodes inermis* had slopes < 0 for %C and *Stenelmis* sp. had slopes < 0 for %N. Mean %C ranged from 47.4 to 56.2%, and no trends relative to phylogeny or FFG were evident (Table 1). Mean %N ranged from 9.6 to 11.6%. Trends within FFG showed qualitatively that predators had the highest %N content, followed by filter feeders, collector-gathers, and grazers with the lowest %N content (Table 1). The C:N ratio ranged from 4.9 to 6.4 and predators had the lowest C:N ratios (Table 1). *Tabanus* sp. eggs were 10.9% N and 50.8% C.

The relationship between %P and mass was best represented by a power function across all 18 taxa (Fig. 2A–R). Without exception, small individuals had higher %P than larger individuals within species. Variability in larval %P declined as mass increased. Eggs (not shown in Fig. 2) were available for analysis for 2 species. *Corydalus cornutus* eggs had a mean %P of 1.84% ($n = 21$, range 0.82–3.99%), which was near the largest observed larval %P value (1.86%) for that species. *Tabanus* sp. eggs had a mean %P of 4.67% ($n = 13$, range 1.90–8.19%). First-instar *Tabanus* sp. larvae reared from eggs had a mean %P of 6.11% ($n = 4$, range 4.67–7.27%).

Sexual differences in nutrient content

Among the mayfly species, %P of all leptophlebiids (*Neochoroterpes nanita*, *Thraulodes gonzalesi*, and *Traverella presidiana*) differed significantly between the sexes (Table 2). However, *T. gonzalesi* and *T. presidiana* differences were mass dependent. Females were more P-enriched, and %P was higher in mature DC 5 nymphs than in DC 4 nymphs (Table 3). In contrast, %P of all swimmer mayfly taxa (*Baetis* sp., *B. inermis*, and *Isonychia sicca*) did not differ between the sexes and %P declined as mass increased for all these taxa (Table 3). The slope of %P differed between males and females the hemipterans *Rheumatobates hungerfordi* and *Rhagovelia choreutes*, with %P in females declining less per unit mass than males. Percent P of adult *A. circumcinctus* differed between sexes, and females were P-enriched compared to males. The rate of decline in %P with DC was similar for males and females. Adult *Stenelmis* sp. sexes had the same slope and did not differ in %P content (Table 3).

Nutrient content and life-history traits

No clear patterns in %P were revealed based on life history traits (Fig. 3A–C). However, a strong statistically significant linear relationship ($r^2 = 0.60$, $p < 0.001$) between taxon maximum size and P decay rate (i.e., the slope of the curve for each taxon in Fig. 2A–R) was evident. Species maximum size was the main factor driving the magnitude of changes in %P across ontogeny, with small bodied, rapidly growing species exhibiting the sharpest decline in P content across ontogeny.

Discussion

Patterns of C, N, and P content

The degree to which aquatic macroinvertebrate species change their elemental content across their ontogeny is largely unknown. Our findings show that %C and N are homeostatic across the ontogeny of 6 of the 8 aquatic insect species examined, and in those with significant variation, the changes were small relative to the changes in %P across the ontogeny of all taxa investigated. Both %C and %N were nonhomeostatic in *A. circumcinctus*, whereas only %C was nonhomeostatic in *B. inermis* and %N in *Stenelmis* sp. (Table 1). Even though the slopes were negative, it is unclear to us if they are biologically significant. Overall, the consistency of the %C and N pattern suggests that these elements are quasihomeostatic across the ontogeny of aquatic insects in general. In *Caenis* sp. mayflies, %C increased slightly across size classes, and %N decreased slightly across size classes at 2 sites, results suggesting that %C and %N were more-or-less homeostatic (Back et al. 2008). Percent C increased greatly across the ontogeny of a caddisfly, whereas %N declined gradually (Veldboom and Haro 2011). The increase in %C in the study by Veldboom and Haro (2011) is probably related to the fact that *B. occidentalis* is univoltine, overwinters as mature larvae, and pupates in early spring. Therefore, fat reserves are needed to fuel metamorphosis during pupation and respiration of the adult in the pupal chamber.

Percent C, %N, and C:N changed relatively little across the ontogeny of species in our study. Thus, our results were comparable to values from studies in which only terminal or homogenized life stages were examined (Frost et al. 2003, Evans-White et al. 2005, Leiss and Hillebrand 2005, Lauridsen et al. 2012). In our study, the only pattern in %C was that taxa with sclerotized or armored integuments had greater mean %C than taxa with membranous integuments (Table 1). The slightly higher %N and low C:N of predators relative to other FFGs in our study also agrees with results of other studies (Fagan et al. 2002, Evans-White et al. 2005, Hambäck et al. 2009). The %C and %N of *Tabanus* sp. eggs were similar to %C and %N of larvae, further suggesting ontogenetic homeostasis with respect to C:N.

In contrast, P clearly was not homeostatic across ontogeny. Thus, comparison of our %P data with literature values is difficult. However, %P of late-DC individuals in our study was similar to values reported for mature individuals in other studies (e.g., Evans-White et al. 2005, Hambäck et al. 2009). The pattern of %P was nonlinear (power function) and declined with increasing size for all 18 taxa examined (Fig. 2A–R). Mean %P of eggs was equal to (*C. cornutus*) or less than (*Tabanus* sp.) that of 1st-instar larvae but the range of P content in eggs was large (see Results). The highest egg %P exceeded the %P of the smallest larvae for both species. The large variation in egg %P suggests that not all eggs are created equal. Part of this variation could be because some eggs analyzed were not fertilized. Nutrients derived from males through mating represent a prezygotic investment in reproduction (Zeh and Smith 1985, Boggs 1990) and can influence the number and quality of eggs produced. Markow et al. (2001)

demonstrated that males do contribute to the P content of *Drosophila* eggs during mating. Male contributions to the nutrient content of eggs also have been shown in butterflies (Boggs and Gilbert 1979, Boggs 1990) and beetles (Rooney and Lewis 1999). The fate of low-%P eggs is not known, but perhaps they fail to develop, or they hatch and larvae soon die. The fate of high-%P eggs also is not known, but larvae that hatch probably have an increased somatic growth rate and possibly have higher survivorship resulting from faster growth (Arendt 1997).

We surmise that aquatic insects provision eggs with large amounts of P to promote rapid growth of early instars. However, this strategy is not shared by all insects. The fertilized eggs of the mammalian blood-feeding hemipteran *Rhodnius prolixus* (Reduviidae) contained only 0.61% P (calculated from table 1 in Ramos et al. 2011). Mammalian blood has a high P content (20–85 mg/L P; Rapoport and Guest 1941). After hatching, all sizes of *R. prolixus* feed on mammalian blood, thus a high-P diet may preclude the need for high-P eggs in blood-feeding insects.

The pattern of declining %P across ontogeny has been shown for *Ephemerella* sp. (Frost and Elser 2002) and *Caenis* sp. (Back et al. 2008) mayflies, brachycentrid caddisflies (Veldboom and Haro 2011), and zooplankton (Main et al. 1997). However Back et al. (2008) found greater %P in the largest *Caenis* sp. size class relative to intermediate developmental classes, as did Veldboom and Haro (2011) in *Brachycentris* pupae relative to mature larvae. Back et al. (2008) speculated that the trend in *Caenis* sp. could have been a consequence of a large proportion of females carrying contained high-%P eggs in the sample (but they did not sex individuals in the sample). In our study, all 3 leptophlebiid mayflies had increasing %P in the largest female individuals, but males did not. Declining %P with increasing size fits the predictions of the GRH (Elser et al. 1996, 2006, Main et al. 1997, Sterner and Elser 2002). Small individuals grow faster than large ones of the same species. Thus, growth slows as organisms age (Peters 1983). P is necessary to fuel rapid growth because of the high P content of ribosomes, messenger ribosomal ribonucleic acid (mRNA), and especially ribosomal RNA (rRNA). Unlike deoxyribonucleic acid (DNA), quantities of ribosomes, mRNA, and rRNA are not fixed in cells, and changes in these constituents can alter the amount of cellular P (Sterner and Elser 2002). Transcription rates and protein synthesis are positively correlated with P supply (Acharya et al. 2004, Vrede et al. 2004, Weider et al. 2005).

Nutrient ratios also are important because growth requires different relative amounts of C, N and P, and other elements (Elser et al. 1996, Sterner and Elser 2002). Furthermore, somatic growth may have different elemental requirements than gamete production, especially with reference to P (Vrede et al. 1999, Færøvig and Hessen 2003). Because %C and %N did not vary markedly across ontogeny, the C:N ratio also did not vary much for the 8 taxa examined in our study. However the C:P and N:P ratio obviously increases with declining %P as organism size increases. Because the N:P ratio increases across ontogeny and growth rate slows with increasing size, N must not be limiting for growth when organism P is in high supply (i.e., when organisms are small). Thus, an optimal N:P (or C:P) may not exist for organisms undergoing rapid somatic tissue growth because only %P changes across ontogeny in a significant way.

Sexual differences in nutrient content

In all cases where a difference was detected, females insects were more enriched in P than males. Reproductive strategy does not seem to influence sexual patterns in %P content. Among semelparous mayflies, some taxa have females that are enriched in P relative to males and others have females with the same %P as males. Iteroparous (Hemiptera and Coleoptera) taxa also show both patterns (Table 2). Veldboom and Haro (2011) found 2 populations of

Brachycentris in which male pupae were more enriched in P than female pupae, and 2 populations where the %P was equal between pupae of both sexes. Morehouse et al. (2010) suggested that sexual differences in nutrient content should be expected based on difference in biochemical demand and composition of gametes and other sexually specific structures.

Nutrient content and life-history traits

Phylogeny may an important secondary determinant of %P content in mayfly nymphs across their ontogeny. Mayflies showed 2 distinct patterns in %P that apparently were constrained by phylogeny. Percent P of *Baetis* sp., *B. inermis*, and *I. sicca* did not differ between males and females. However, females of all 3 leptophlebiid taxa (*N. nanita*, *T. gonzalesi*, and *T. presidiana*) were more enriched in P than males. This pattern is consistent with the pattern of increased P in late-instar *Caenis* nymphs documented by Back et al. (2008). Phylogenetic analysis indicates that *Caenis* (Caenidae) is more closely related to Leptophlebiidae (suborder Furcatergalia) and Baetidae is more closely related to Isonychidae (suborder Pisciforma) than Caenidae and Baetidae are related to each other (Ogden et al. 2009).

Mature *Baetis* and *Caenis* nymphs are both small and can complete their life cycles and reproduce in ≤2 wk (Edmunds et al. 1976, Brittain 1982, Taylor and Kennedy 2006), yet they differ in ontogenetic patterns in %P. The %P of the largest *Caenis* sp. size classes increased in a manner similar to its slower growing larger relatives, the leptophlebiids, whereas %P of *Baetis* sp. did not increase with size. In contrast, *B. inermis* and its close relative *Baetis* sp. share the same ontogenetic %P pattern despite *B. inermis* differing in morphology and behavior. *B. inermis* has spines and tubercles on its legs and abdomen and does not swim actively (Edmunds et al. 1976).

The causal factors responsible for the difference in %P between the suborders are not known, but warrant further study.

Reproductive strategy, type of metamorphosis, and FFG did not appear to be associated with the pattern of decreasing %P with increasing maximum size (Fig. 3A–C). Patterns did not differ between semelparous and iteroparous taxa, between pauro- or holometablous taxa, or among FFGs. Moreover, the expected pattern of that %P of FFGs would reflect the quality of food sources (i.e., increasing %P in collector-gatherers, filterers, scrapers, and predators) was not observed. Thus, the maximum size of a taxon drove the rate at which %P declined (Fig. 3A–C). The smallest taxa had the greatest decline in %P and the fastest life-cycle-completion times. The only exception was *Stenelmis* sp., which can take from 6 mo to 2 y to complete its life cycle (White 1978, Brown 1987).

Nonhomeostatic patterns in %P across ontogeny have important implications for population- and community-level processes and will affect predictions about consumer-driven nutrient recycling. The difference between the nutrient content of food and the nutrient demand of a species determines recycling rates (Elser and Urabe 1999, Evans-White and Lamberti 2006, Rothlisberger et al. 2008). For a given species, small individuals may be a sink and hoard nutrients (especially P) whereas larger individuals become a source so that organisms advance along a sink–source continuum as they grow. The high %P of small individuals may profoundly influence their perceived quality to predators. Small individuals may not contain as much P (by mass) as larger individuals, but shorter prey-handling times may benefit predators that target small prey. Thus predatory taxa may preferentially target small taxa and decrease P excretion rates to conserve P for egg production. Nonpredatory taxa may hoard P across ontogeny to help meet P demands for reproduction. Although the actually mechanisms employed by organisms to meet P demands for growth and reproduction are not clear, some combination of altered P excretion rates, P uptake, and switching to higher quality food or increased food ingestion rates are likely involved.

The effect of nutrient subsidies on patterns of ontogenetic nutrient content should be investigated. Nutrient subsidies may increase insect nutrient content across ontogeny or might result in larger individual size, egg size, or fecundity without altering %P. Subsidies can be passed up the food chain, thereby influencing community structure and function. Furthermore, high %P content of small taxa means high P demand, a possible mechanism for the proliferation of small, rapidly growing taxa in nutrient-enriched ecosystems (Miltner and Rankin 1998, King and Richardson 2007, Wagenhoff et al. 2011).

Acknowledgements

We thank the Center for Reservoir and Aquatic System Research at Baylor University for use of the microbalance and the Lachat QC 8500 FIA instrument used for P analyses and S. Dworkin for use of the elemental analyzer for C and N analyses. Funding was provided by the Center for Reservoir and Aquatic Systems Research and the Department of Biology at Baylor University. We thank Theodore Angradi, Pamela Silver, and two anonymous reviewers for helpful comments that greatly improved the manuscript.

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Table 3.1. Results of analysis of covariance of slopes of regressions for C and N content of aquatic insects across their ontogeny. [1] = predator, [2] = filter feeder, [3] = collector-gather, [4] = grazer, SE = standard error, CI = confidence interval.

Taxon	*n*	Mass range (µg)	Nutrient	Mean % (SE)	Slope	r^2	*p*	Slope CI (5%, 95%)	C:N
Ambrysus circumcinctus[1]	24	50–15,110	C	50.0 (0.50)	–0.0003	0.3305	0.004	–0.0004, –0.0001	5.0
			N	11.6 (0.12)	–0.00003	0.0935	0.05	$-0.000089, -6 \times 10^{-8}$	
Rhagovelia choreutes[1]	14	200–1680	C	51.1 (0.60)	0.0009	0.0369	0.5108	–0.002, 0.004	5.2
			N	11.5 (0.15)	0.00003	0.0008	0.9255	–0.0007, 0.0008	
Tabanus sp.[1]	21	740–21,750	C	47.9 (0.50)	–0.0001	0.1744	0.0596	–0.0003, 0.000006	4.9
			N	11.3 (0.10)	–0.00001	0.0225	0.5164	–0.00004, 0.00002	
Chimarra sp.[2]	31	170–3710	C	50.0 (0.66)	–0.0007	0.0188	0.4625	–0.002, 0.001	5.3
			N	11.0 (0.17)	–0.0003	0.0609	0.1809	–0.0008, 0.0001	
Stenelmis sp.[3]	17	31–750	C	54.0 (1.06)	–0.0022	0.0236	0.5552	–0.01, 0.005	5.7
			N	11.0 (0.29)	–0.002	0.2534	0.0393	–0.004, –0.0001	
Neochoroterpes nanita[3]	35	53–3390	C	47.4 (0.55)	0.0004	0.0126	0.2724	–0.0008, 0.002	5.3
			N	10.5 (0.13)	–0.0002	0.0364	0.5209	–0.0004, 0.00013	
Baetoides inermis[3]	31	140–1560	C	56.2 (0.61)	–0.0057	0.3328	0.0005	–0.0075, –0.0023	6.4
			N	10.2 (0.13)	–0.0001	0.005	0.99	–0.0006, 0.0006	
Psephenus texanus[4]	30	370–5070	C	52.8 (0.63)	0.0005	0.026	0.1838	–0.0006, 0.0006	6.4
			N	9.6 (0.13)	–0.0001	0.0648	0.1748	–0.0003, 0.00007	

Table 3.2. Results of analysis of covariance comparing the relationship between body mass and %P of individuals with respect to sex. ns = not significant, $^{\#}$ = 0.1 > $p \geq 0.5$, * = 0.5 > $p \geq 0.01$, ** = 0.01 > $p \geq 0.001$, *** $p < 0.001$.

Taxon	df	Mass	Sex	Mass × sex
Neochoroterpes nanita	$F_{1,55}$	2.38 ns	4.859*	5.191*
Thraulodes gonzalesi	$F_{1,51}$	125.136***	1.859 ns	28.908***
Traverella presidiana	$F_{1,53}$	138.922***	1.110 ns	7.276**
Baetis sp.	$F_{1,53}$	74.082***	3.475 ns	0.854 ns
Baetodes inermis	$F_{1,53}$	12.928***	2.516 ns	0.059 ns
Isonychia sicca	$F_{1,38}$	59.326***	2.931 ns	1.468 ns
Rheumatobates hungerfordi	$F_{1,12}$	11.285**	8.155*	$3.491^{\#}$
Rhagovelia choreutes	$F_{1,4}$	54.281**	52.584**	13.963*
Ambrysus circumcinctus	$F_{1,6}$	6.162*	30.565***	0.861 ns
Stenelmis sp.	$F_{1,6}$	0.399 ns	0.383 ns	1.075 ns

Table 3.3. Mean mass (n) and %P (SE) of male and female mayfly nymphs and adult Hemiptera and Coleoptera.

Taxon	Development class	Mass (µg)		%P	
		Males	Females	Males	Females
Neochoroterpes nanita	3	235 (13)	308 (13)	0.96 (0.02)	0.96 (0.02)
	4	412 (4)	469 (8)	0.87 (0.02)	0.91 (0.03)
	5	669 (6)	900 (15)	0.87 (0.03)	0.97 (0.02)
Thraulodes gonzalesi	3	294 (10)	374 (8)	1.40 (0.06)	1.19 (0.04)
	4	635 (5)	713 (8)	1.08 (0.05)	1.05 (0.03)
	5	831 (12)	1009 (12)	0.99 (0.03)	1.10 (0.04)
Traverella presidiana	3	310 (17)	402 (10)	1.32 (0.05)	1.18 (0.04)
	4	560 (5)	948 (8)	1.14 (0.05)	0.97 (0.03)
	5	1492 (10)	2019 (7)	0.96 (0.02)	0.99 (0.02)
Baetis sp.	3	81 (4)	76 (11)	1.92 (0.20)	1.93 (0.09)
	4	108 (4)	101 (12)	1.16 (0.02)	1.29 (0.06)
	5	153 (9)	135 (7)	1.10 (0.03)	1.15 (0.02)
Baetodes inermis	3	456 (10)	504 (10)	0.78 (0.03)	0.83 (0.02)
	4	700 (6)	783 (10)	0.81 (0.04)	0.81 (0.02)
	5	1175 (10)	1244 (10)	0.74 (0.02)	0.75 (0.02)
Isonychia sicca	3	2175 (12)	2509 (8)	1.15 (0.02)	1.15 (0.02)
	4	5500 (5)	7668 (5)	1.03 (0.05)	1.00 (0.05)
	5	6802 (5)	13,768 (6)	0.99 (0.07)	0.98 (0.04)
Rhagovelia choreutes	Adult	346 (4)	571 (4)	0.62 (0.17)	0.84 (0.14)
Rheumatobates hungerfordi	Adult	309 (5)	381 (11)	1.15 (0.03)	1.18 (0.10)
Ambrysus circumcinctus	Adult	10747 (4)	12,674 (5)	0.40 (0.05)	0.60 (0.02)
Stenelmis sp.	Adult	660 (4)	847 (5)	0.57 (0.07)	0.59 (0.09)

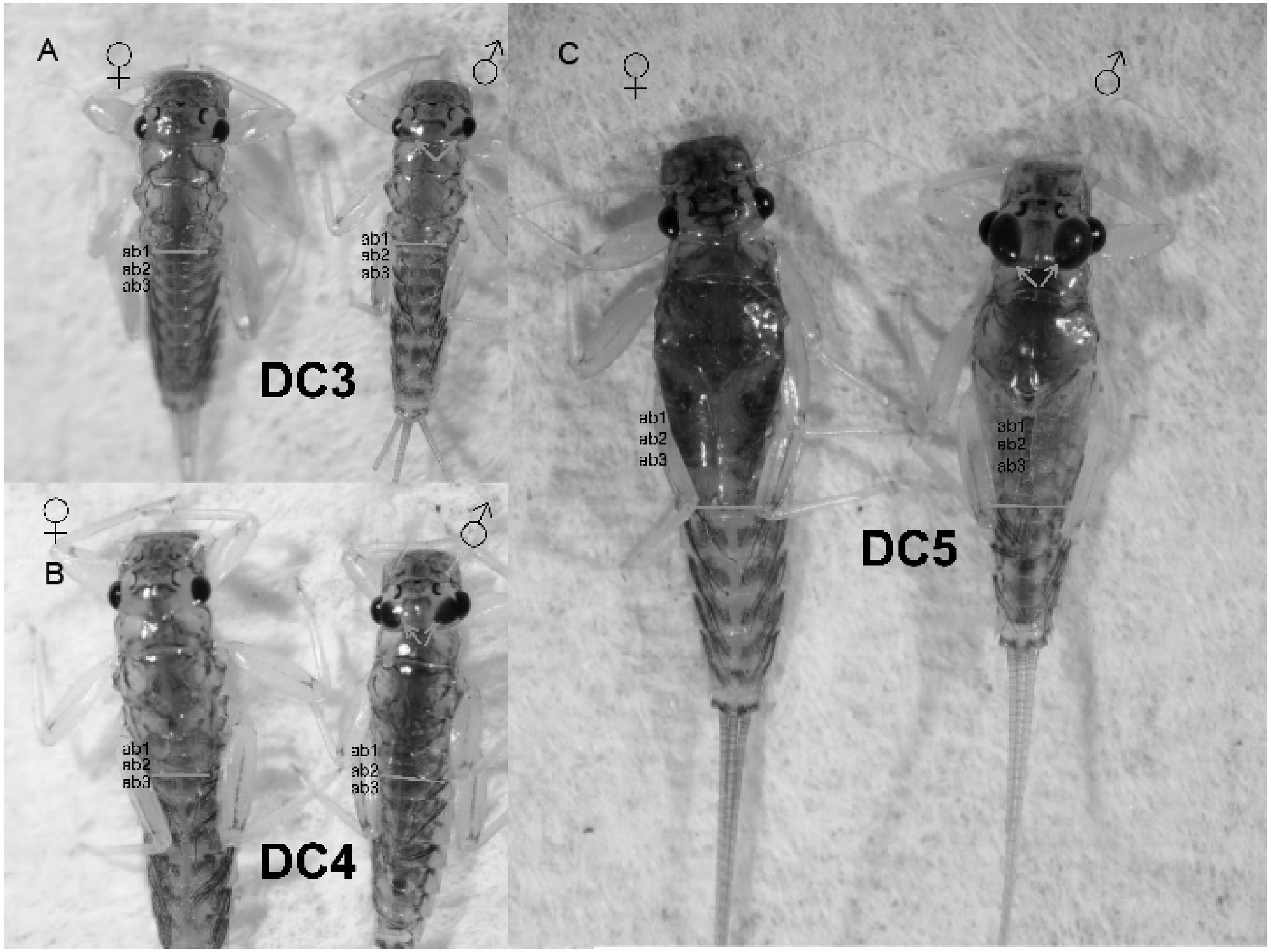

Fig. 3.1. Early (A), intermediate (B), and late (C) development classes (DC) for the mayfly *Neochoroterpes nanita*. Turbinate eyes of males are indicated by arrows. Horizontal line is the furthest extent of the wing pads. The first 3 abdominal segments are denoted as ab1, ab2, and ab3.

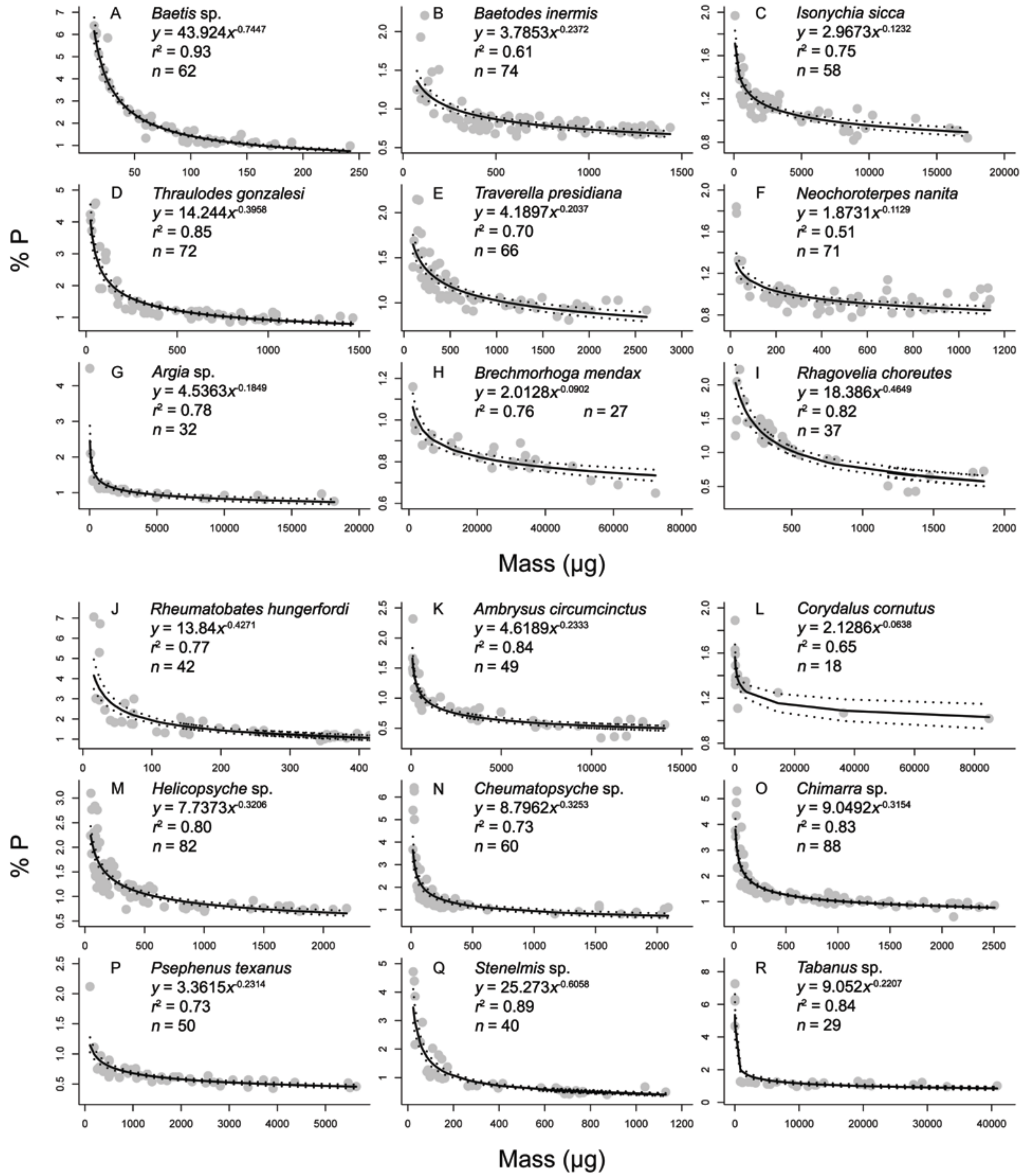

Fig. 3.2. Relationship between individual mass and %P for *Baetis* sp. (A), *Baetodes inermis* (B), *Isonychia sicca* (C), *Thraulodes gonzalesi* (D), *Traverella presidiana* (E), *Neochoroterpes nanita* (F), *Argia* sp. (G), *Brechmorhoga mendax* (H), *Rhagovelia choreutes* (I), *Rheumatobates hungerfordi* (J), *Ambrysus circumcinctus* (K), *Corydalus cornutus* (L), *Helicopsyche* sp. (M), *Cheumatopsyche* sp. (N), *Chimarra* sp. (O), *Psephenus texanus* (P), *Stenelmis* sp. (Q), and *Tabanus* sp. (R). Raw data were plotted to illustrate the nonlinear pattern of data. Solid line is a regression line, and dotted lines are 95% confidence intervals.

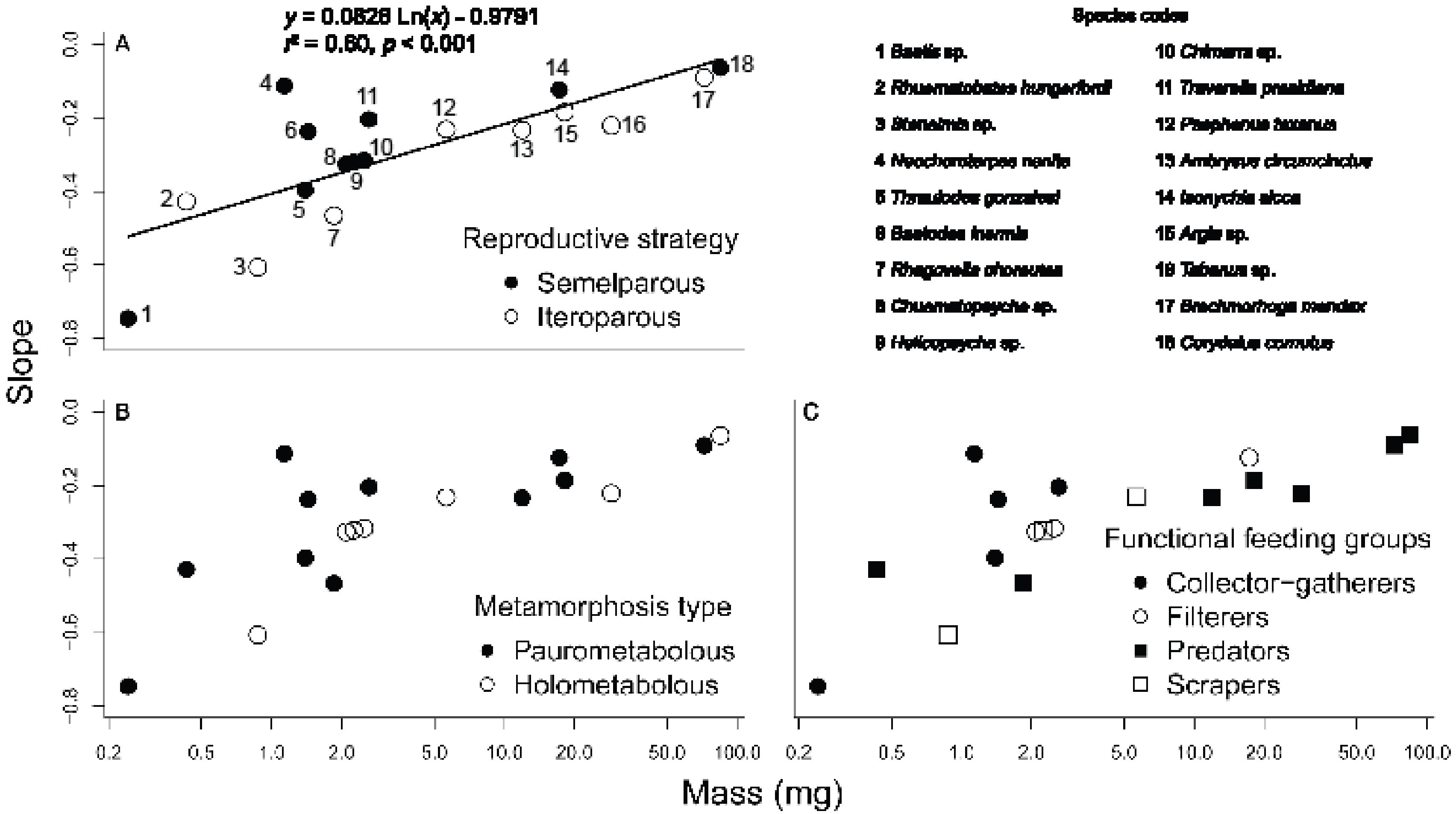

Fig. 3.3. Relationship between the largest mass observed for a taxon and the slope of the power function (from Fig. 3.2) that describes the decrease in %P (slope) across ontogeny by reproductive strategy (A), type of metamorphosis (B), and functional feeding group (C). Lines were fitted by least squares regression.

CHAPTER FOUR

Sex and size - subsidized: phosphorus enrichment dramatically increases body size of two mayfly species, and disproportionately so for females

Abstract Nutrient requirements for growth and reproduction vary widely among and within major groups of animals. Organisms perceive deficiencies or surpluses in food quantity or quality based on the organisms' energetic and nutritional needs, which reflect its physiological state. I examined the effects of stream nutrient enrichment on body size and %P content in 2 mayflies: *Baetis* sp. and *Neochoroterpes nanita*. Mayfly nymphs were collected from 7 sites spanning a steep P gradient in both stream water and periphyton. Effects of the gradient were examined between sexes and within sexes across different development classes (DC). Mean individual mass increased abruptly in both species within DCs with increasing stream nutrient enrichment, with *N. nanita* responding at a lower level of enrichment than *Baetis*. Females of both species exhibited a greater increase in mass in response to enrichment relative to males within the same DC. The %P of male and female *Baetis* did not differ within sites and DCs. However, in *Baetis* sp, %P declined with increasing DCs, and individuals from lower P streams had higher %P relative to individuals from enriched locations in the same DC. In contrast, P content across DCs was different between sexes for *N. nanita.* Only male *N. nanita* had declining %P across all DCs. Females declined in %P between DC 3 and 4, and then increased in %P between DC 4 and 5. These results show that stream nutrient enrichment dramatically increases individual mass for both mayfly species, particularly females. These results further suggest that enrichment is accelerating the growth rate without clearly altering the developmental rate given that mayflies from enriched sites were much larger at the same level of development as nymphs from unenriched sites. These findings may have important implications to the effect of enrichment on population genetics (has enrichment caused directional selection toward larger individuals?) as well as community structure (are taxa that grow larger and/or more quickly in response to enrichment more competitive?).

Key words: nutrient subsidies, nutrient stoichiometry, phosphorus, mayflies, ontogeny, life-history tradeoff

Nutrient requirements for growth and reproduction vary widely among and within major groups of animals (Sterner and Elser 2002, Elser et al. 2006). Organisms perceive deficiencies or surpluses in food quantity or quality based on the organisms' energetic and nutritional needs, which reflect its physiological state. Natural energy and material subsidies are common occurrences in many ecosystems (Polis et al 1997). For example, allochthonous inputs of leaf litter from deciduous trees provide a seasonal pulse of organic material and nutrients that can fuel shredder (Cummins et al. 1989) and detritivore food webs (Hall et al 2000, Cross et al. 2003, Danger et al. 2013). Terrestrial insects from riparian habitats can subsidize stream fishes during periods of low aquatic insect emergence (Wipfli 1997, Nakano et al. 1999, Wurtsbaugh 2007). Further, anadromous fishes can subsidize resident stream fishes and invertebrates via eggs and carcasses (Wipfli et al 2003, Moore et al 2008) and provide dissolved nutrients to streams (Levi et al. 2013). All of these subsidies are of short duration, and thus provide a pulse of increased resources.

Nutrients and organic matter from waste-water treatment plant (WWTP) effluent and agricultural runoff are anthropogenic sources of resource subsidies to streams. These subsidies can be virtually continuous in the case of waste water treatment plants (WWTP) effluent. Thus the background concentrations of dissolved nutrients can become elevated far beyond the natural condition and provide an excess of nutrients for primary producers and heterotrophic bacteria. These inputs can change the quality of resources available to consumers (Bowman et al. 2005, Singer and Battin 2007). Resources with increased nutrient content can cause consumers to increase growth rates (Urabe and Sterner 2001, Frost et al. 2006), reproductive output and egg survivorship, (Urabe and Sterner 2001), and the nutrient content of the consumers themselves (Cross et al. 2003, 2005, Back et al. 2008, Small and Pringle 2010).

A species' resource requirements can change across its ontogeny. An ontogenetic approach (Back et al. 2008, Back and King 2013) to nutrient subsidies can provide age specific responses to food quality. This approach is valuable because organism can encounter resource bottlenecks that are life stage specific, and result in decreased survivorship of life stages, as seen in the copepod *Diaptomus clavipes* (Villar-Argaiz and Sterner 2002). Resource demand and nutrient content of consumers can vary with gender also, as shown for several *Drosophila* sp. by Markow et al. (1999, 2001) and various aquatic insects by Back and King (2013).

Phosphorus (P) is of particular importance in freshwater aquatic systems because it often limits primary production and can limit growth in aquatic invertebrates (Sterner and Elser 2002, Andersen et al. 2007, Davis et al. 2010, Danger et al 2013). In contrast to nitrogen and carbon, which vary little across the ontogeny of aquatic insects, P content and may vary substantially from egg to adult (Frost and Elser 2002, Back et al. 2008, Veldboom and Haro 2011, Back and King 2013). The non-homeostatic pattern of P content coupled with the fact that P can limit growth and reproduction suggest that P subsidies in freshwater ecosystems may effect individual growth, survivorship, and fecundity. Thus an investigation of organisms which reside in streams that span a gradient of resource availability can provide insight into what level of resource availability is recognized as a subsidy by resident consumers.

Here, I studied the effects of a steep gradient of surface water and periphyton P on nymphs of 2 mayfly species (*Baetis* sp. and *Neochoroterpes nanita*) shown previously to differ in the pattern of P content across their ontogeny, particularly between sexes (Back and King 2013). Specifically, I determined 1) if nymphal mayflies of these 2 species respond to increased P content in their food by increasing in size and/or P content, and 2) if patterns in size and P content across the P gradient were the same for males and females.

Methods

Study area

This study was conducted among 7 streams spanning a steep gradient in water column nutrient concentrations and periphyton nutrient content (Table 1). Study streams were located within the Cross Timbers Level III Ecoregion (Griffin et al. 2004) and Brazos River Basin in north central Texas and were similar in size, substrate, light, and other environmental characteristics other than nutrients (King et al. 2009). The primary sources of nutrients to these streams were point source discharges from WWTPs and nonpoint runoff from concentrated animal feeding operations (CAFOs) (Pease et al. 2012).

Mayflies were collected from 8 to 23 September 2008 and 3 to 27 June 2009. Rocks from each sample site were collected from riffles and gently rinsed with stream water to remove sediment and invertebrates. Periphyton was removed by brushing rocks with a toothbrush, then dried 50 °C for at least 48 hours and pulverized using a Mini-Bead beater (Biospec Products, Bartlesville, Oklahoma) and then stored in a desiccator. Nymphs of *Baetis* sp. and *Neochoroterpes nanita* were collected from riffles, runs and their margins using D-nets, kick screens, and hand picking insects from rocks. Mayflies were transported to the lab and sorted to species, sex and development class (DC) live under a stereomicroscope. Mayfly nymphs were grouped into DCs using wing pad development following Taylor and Kennedy (2006) and sexed based on eye development (Back and King 2013). Development classes 3, 4 and 5 were defined as wing pads reaching abdominal segment 1, 2 and 3+ respectively. Females have simple eyes and males have turbinate eyes (see Fig. 1 in Back and King 2013).

Chemical Analyses

Unfiltered water samples were digested with potassium persulfate/boric acid for total nitrogen (TN) and potassium persulfate for total phosphorus (TP). TN was estimated via colorimetry using sulfanilamide via cadmium reduction method and TP was estimated using the ascorbic acid-molybdate method on a Lachat 8500 flow-injection autoanalyzer with an ASX-520 autosampler.

The procedure used to estimate periphyton and insect phosphorus content is detailed in Back and King (2013). Briefly, intact whole mayflies were dried in aluminum weighing pans at 50 °C for at least 48 hours and then stored in a desiccator. Individual insects were weighed on a microbalance (Mettler Toledo XP-26) to the nearest μg, placed in 22 ml glass scintillation vials, pulverized with a metal spatula, capped with a lid with a teflon septa, and chemically digested in an autoclave for 1 h at 120°C using the method described in Færøvig and Hesson (2003).

Percent P was estimated via colorimetry using the ascorbic acid-molybdate method on a Lachat 8500 flow-injection autoanalyzer with an ASX-520 autosampler. Tissue standards of tomato leaf (SRM 1573a, 0.216% P), and bovine liver (SRM 1577c, 1.175% P) as well as dissolved inorganic standards were run for QA/QC purposes. To assure no bias among the runs, a wide range of mass for each taxon was analyzed on at least two separate runs. The mean % recovery of tomato leaf P was 103.9% (SD = 14.3, n =45), bovine liver P, 92.7% (SD = 6.1, n=26), and inorganic P standards was 103.6 (SD = 6.5, n=176).

Data Analyses

Neither *Baetis* sp. nor *N. nanita* were collected from every sample site in both September 2008 and June 2009 (Table 4.2). Because the pattern and magnitude of mass and % phosphorus content between years at a sample site were similar, data were combined for mayflies that occurred in both years at a sample site. The analysis path used is as follows: mass and % phosphorus content were analyzed singly with ANOVA starting with a full factorial model (i.e. 3-way interaction, 3 2-way interactions, and 3 single factors). If the 3-way interaction was not significant, a simpler model (i.e. 3 2-way interactions, and 3 single factors) was analyzed. This stepdown technique was continued until the simplest model with all significant factors remained. No single factor was eliminated if it were part of a significant interaction (Engqvist 2005, Crawley 2007, Tabachnick and Fidell 2007). Mass and % P were log(x)-transformed prior to analysis to meet assumptions of linear models. All analyses were performed with R (version 2.13.1; R Core Development Team, Vienna, Austria) using the CAR library (Fox and Weisberg 2011).

Results

Water samples for total P ranged from 10 – 1804 µg/L and total N ranged from 197 – 6165 µg/L. Periphyton %P ranged from 0.02 – 0.25%. Thus the 7 sample sites represent an enrichment gradient for water column total P and N as well as periphyton P (Table 4.2).

Mayfly Mass –Baetis sp.

Mass of *Baetis* was significantly influenced by 2-way interactions between all 3 main effects (Table 4.3, Fig 2A). Neils Creek, Paluxy River, Cowhouse Creek and Lampasas River are referred to as unenriched sites, and the North Bosque, Leon and Nolan Rivers are considered enriched. First, mean individual mass increased across DCs within sites. This result is expected because DC is a measure of nymph morphological development and by definition the mass of DC5 > DC4 > DC3 for any mayfly species. However, within a DC, the magnitude of differences between sexes increases with increasing periphyton phosphorus enrichment. Within DC 3, masses across sites and within sex showed a slight increase across the enrichment gradient. No consistent pattern between male and female masses was evident, with males being heavier than females at 3 of the 4 unenriched sites, and one of the enriched sites. Males on average were heavier than females for every DC at Neils Creek, the least enriched site. Within DC 4, differences in mass relative to sex are very evident at the enriched sites. Not only are both sexes heavier at the enriched sites, but females are much bigger than males. Females from the unenriched sites weighed on average 1.3 µg more than males, and weighed 45 µg more than males at the enriched sites. In fact, DC 4 males and females from the enriched sites are the mass of DC 5 *Baetis* sp. from the unenriched sites. Female DC 5 weighed on average 11 µg more than males from the unenriched sites, and 107 µg. more at enriched sites.

Mayfly Mass –N. nanita.

Neochoroterpes nanita mass was influenced significantly by all 2-way interactions between DC, sex, and site. For *N. nanita*, Neils Ck, Paluxy R. and Cowhouse Ck. are referred to as unenriched sites, and the Lampasas, North Bosque, Leon and Nolan Rivers are considered enriched. Within DC 3, masses across sites and within sex showed a slight increase across the enrichment gradient (Table 4.3, Fig. 2B). Females were on average heavier than males for all DCs at every site except Neils Ck. Males on average were heavier than females for every DC at Neils Creek, which is the same pattern seen for *Baetis* sp. Within DC 4, differences in mass relative to sex are very evident at the enriched sites. However, in contrast to *Baetis* sp., only females are heavier at the enriched sites. Females from the unenriched sites weighed on average 26 µg more than males, and weighed 185 µg more than males at the enriched sites. DC 4 females from enriched sites are the mass of DC 5 males from unenriched sites. Female DC 5 weighed on average 155 µg more than males from the unenriched sites, and 535 µg. more at enriched sites.

Mayfly % P –Baetis sp.

Baetis sp. P content was influenced significantly by site*DC, and an interaction between these 2 main effects (Table 4.4, Fig. 3A, Appendix A). All sites had a decline in %P content from DC3 to DC5. However, the unenriched sites had higher %P than the enriched sites. No differences in %P content based on sex were detected, and males and females had the same pattern in %P content across DCs.

Mayfly % P–N. nanita

Neochorterpes nanita P content was influenced significantly by site, DC, and an interaction between these 2 main effects (Table 4.4, Fig. 3B, Appendix B). As seen in *Baetis* sp., the unenriched sites had the highest %P content, but the magnitude of the difference was much less than the *Baetis* sp. response, and only seen in DC3 from the Paluxy R. and Cowhouse Ck. Males declined in %P content from DC 3 -5 for every site except Cowhouse Ck., where DC 5 was slightly greater than DC 4 (see circled points in Fig. 3B). However, female DC 5 nymphs were always more P rich than female DC 4 nymphs. Moreover, female DC 4 and 5 nymphs were always more P rich than males DC 5 nymphs.

Discussion

Mayfly Mass

The overall response for both species was an increase in mass with increasing enrichment. The mass increase was greatest with larger DCs, and females showed the largest increase in mass across the enrichment gradient. However, the magnitude and onset of mass increase across DC and sex was species specific (Fig. 2). Nutrient demand for growth, reproduction and metabolism is considered a species specific property (Frost et al. 2006). If mayflies were nutrient limited at unenriched sites, then growth rates would be slower (Frost and Elser 2002, Sterner and Elser 2002, Back et al. 2008), and as a consequence, ultimate size could be smaller compared to those from enriched sites. Although we did not measure growth rates in this study, Fig. 2 clearly shows that P enrichment is positively correlated with larger mayfly size, and females are affected more than males. Singer and Battin (2007) also found increased insect body size at P enriched sites. It should be noted that Small et al. (2011) found chironomids from dosed high P streams were unable to use additional P in their food, evidenced by higher P excretion rates and no change in growth rate relative to chironomids from low P streams. Boersma and Elser (2006) found increases in growth rate with increasing dietary P up to point, and then growth rates declined (a subsidy-stress type response). They believe the decline in growth rates at high P levels is related to energetic costs related to carbon:phosphorus ratios. All declines in growth rate were associated with a minimum food P content of 0.5%, which is twice the level found in this study. The exact mechanism that results in larger mayfly size at enriched sites is unknown. However, resource quantity and quality are known to change life-history traits such as growth rate and organism size at time of reproduction (Arendt 1997, Urabe and Sterner 2001). It is plausible that small increases in dietary P results in faster growth at low levels of enrichment, but at higher levels of enrichment size is maximized at the expense of faster growth. For insects in general (Honěk 1993) and mayflies in particular (Brittian 1982), fecundity scales positively with body size. Because mayflies are semelparous (one reproductive event in their lifetime), the fact that fecundity can be potentially maximized by two different strategies based on food quantity and quality makes biological sense. Iteroparous taxa (reproduce multiple times) have the potential to follow up a low fecundity season with a high fecundity season based on resource quantity and quality changes over time.

Neither species showed a gradual increase in mass across the periphyton P gradient. The response was a rather abrupt increase in mass when an apparent periphyton P content threshold was reached. *Baetis* sp. was most evident at the three most enriched sites (periphyton %P 0.13%, fig 2A) but for *N. nanita* the effect appeared at a lower level of enrichment (periphyton %P 0.07%, Fig 2B). The largest changes were found in *Baetis* sp., with increases in mass being greater than those seen in *N. nanita* within sex for each DC. Female *Baetis* sp. increased mass more than *Baetis* sp. males. Our data suggest that small taxa have a higher potential for mass increase relative to larger taxa. The obvious benefit to larger size is an increase in fecundity for females (Brittain 1982) and perhaps a competitive advantage for larger males which can increase breeding success (Peckarsky et al. 2002).

Mayfly % Phosphorus

The patterns in %P across ontogeny and between sexes previously described by Back and King (2013) for *Baetis* sp. and *N. nanita* also were observed in this study, but here were shown repeatedly across several streams differing in P enrichment. Males of both species declined in %P across their ontogeny, as did female *Baetis* sp. However female *N. nanita* increased in %P content between DC4 and DC5 at every location, regardless of level of P enrichment (Fig 3 and Table 4.5). The large increase in mass at enriched sites experienced by *Baetis* sp. were accompanied by large percent declines in %P at these enriched sites. Although *N. nanita* also increased in mass at enriched sites, the %P decline in males was much smaller than that of *Baetis* sp. males, and females actually increased in %P content. So an apparent tradeoff exists between mass gains and %P changes across ontogeny and the pattern and magnitude of changes are species specific.

A comparison of fig. 2 and 3 reveals that a tradeoff occurs *within* each species as well. For both species, unenriched sites had higher %P than enriched sites when comparing within a DC. Masses increase at the enriched sites for DC4, and especially DC5, although the effect is much stronger for females (Table 4.5, Appendix A and B). We hypothesize *Baetis* sp. modify their life history by allocating P to faster growth and smaller body size (Fig. 2A) at unenriched sites. This faster growth is accompanied by large declines in body P content across DCs. The decline in body P is much smaller at enriched sites (Fig. 3A). This will result in more generations per year, which will increase annual fecundity. At enriched sites, speed of development is exchanged for an increase in mass, which probably increases individual fecundity, and possibly egg size. It is not known if P allocation to gametes is different, which could have implications for egg hatching and early nymph survivorship and growth.

The life history strategy hypothesized for *N. nanita* is somewhat different. Males behave just like *Baetis* sp. males: they increase in size at enriched sites (Fig 2B), and decline in %P content across their ontogeny (Fig 3B), however the magnitude of both is less than that observed for *Baetis* sp. males. Females increase in mass at enriched sites (Fig 2B), and also increase in %P between DC4 and DC5 (fig. 3B) on average 5% more at enriched sites (Table 4.5). *N. nanita* may also exchange fast growth rates at unenriched sites for large size at enriched sites, but females do not entirely sacrifice larger body size for a decline in body P content.

Both taxa share the same tradeoff when faced with nutrient (P) limitation. Nutrient poor sites have populations that are smaller in size but more enriched in P, especially for small individuals. This most certainly leads to a fast life cycle completion time. When P becomes more plentiful, taxa maximize their size, and hence their fecundity, but probably take longer to mature. Thus to maximize egg production, these species reproduce quickly with short generation times, or increase generation time and maximize fecundity. Both strategies will maximize reproductive effort over time, especially when populations are multivoltine. Our findings further confirm that an ontogenetic approach is a more robust methodology for investigating linkages among food and organism stoichiometry and life history traits.

Acknowledgements

We thank the Center for Reservoir and Aquatic System Research at Baylor University for use of the microbalance and the Lachat QC 8500 FIA instrument used for P analyses. Funding was provided by the Center for Reservoir and Aquatic Systems Research and the Department of Biology at Baylor University.

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Table 4.1. Watershed characteristics for the 7 sample sites (From King et al. 2009). n = number of outfalls, MGD = outfall permitted discharge in Million Gallons per Day.

Sample site	Sample Dates	Coordinates °N/°W	Area (km^2)	Outfalls (n/MGD)	Developed (%)	Forest (%)	Shrub (%)	Grassland (%)	Pasture (%)	Crop (%)
Neils Ck.	28 Sept 2008	31.697610/	357	0/0.00	0.2	35.6	1.4	57.7	0.7	2.2
	14 June 2009	97.531697								
Paluxy R.	15 Sept 2008	32.250462/	933	0/0.00	1.1	32.9	11.0	49.8	3.1	0.8
	8 June 2009	97.845545								
Cowhouse Ck.	18 Sept 2008	31.286122/	1180	1/0.06	0.7	18.8	43.0	33.8	1.6	1.5
	25 June 2009	97.883994								
Lampasas R.	8 Sept 2008	31.117936/	1571	0/0.00	0.7	14.0	53.6	29.2	1.0	1.1
	3 June 2009	98.056610								
N. Bosque R.	13 Sept 2008	31.977147/	925	2/3.50	4.3	13.3	22.6	44.6	7.1	6.0
	5 June 2009	98.040383								
Leon R.	11 Sept 2008	31.424955/	6180	12/6.08	2.7	13.5	30.6	37.6	6.8	7.2
	19 June 2009	97.749983								
Nolan R.	30 Sept 2008	32.251880/	451	5/6.73	12.3	3.2	0.1	65.7	8.8	5.9
	11 June 2009	97.406301								

Table 4.2. Phosphorus content of periphyton, water quality data and presence of mayfly taxa in 2008 and 2009. TP total phosphorus, TN total nitrogen, P present, NP not present.

Site	Year	Periphyton % phosphorus	TP (µg/L)	TN (µg/L)	*Baetis* sp.	*N. nanita*
Neils Creek	2008	0.03	14.6	197	NP	NP
	2009	0.01	10.4	401	P	P
Paluxy River	2008	0.04	11.8	201	NP	NP
	2009	0.01	11.8	212	P	P
Cowhouse Creek	2008	0.05	14.2	307	P	P
	2009	0.04	14.0	239	P	P
Lampasas River	2008	0.07	11.4	245	NP	P
	2009	0.07	43.2	463	P	P
North Bosque River	2008	0.11	36.8	404	P	P
	2009	0.15	208	510	P	P
Leon River	2008	0.22	348	1664	P	P
	2009	0.20	135	879	NP	NP
Nolan River	2008	0.25	1804	6165	P	P
	2009	0.13	631	2748	P	P

Table 4.3. Results of analysis of variance for body mass of *Baetis* sp. and *N. nanita* mayflies. ns = not significant, * = 0.5 > $p \geq 0.01$, ** = 0.01 > $p \geq 0.001$, *** $p < 0.001$.

	Baetis sp.		*N. nanita*	
Factor	df	F	df	F
Site	$F_{6,571}$	116.63***	$F_{6,652}$	32.05***
DC	$F_{2,571}$	800.90***	$F_{2,652}$	1112.60***
Sex	$F_{1,571}$	15.80***	$F_{1,652}$	72.47***
Site*DC	$F_{12,571}$	9.88***	$F_{12,652}$	7.60***
Site*Sex	$F_{6,571}$	4.56***	$F_{6,652}$	5.76***
DC*Sex	$F_{2,571}$	7.63***	$F_{2,652}$	3.18*

Table 4.4. Results of analysis of variance for % phosphorus of *Baetis* sp. and *N. nanita* mayflies. ns = not significant, * = 0.5 > $p \geq 0.01$, ** = 0.01 > $p \geq 0.001$, *** $p < 0.001$.

	Baetis sp.		*N. nanita*	
Factor	df	F	df	F
Site	$F_{6,571}$	55.69***	$F_{6,652}$	7.11***
DC	$F_{2,571}$	163.10***	$F_{2,652}$	99.43***
Sex			$F_{1,652}$	14.88***
Site*DC	$F_{12,571}$	4.26***		
DC*Sex			$F_{2,652}$	26.20***

Table 4.5. Comparison of percent change in mean mass and %P among development classes (DC) between unenriched and enriched sites for male and female *Baetis* sp. and *N. nanita*.

DC	% difference males mass	% difference females mass	% difference males %P	% difference females %P
		Baetis sp.		
3	27	46	-59	-57
4	53	99	-36	-42
5	85	140	-30	-13
		N. nanita		
3	15	27	-10	-8.7
4	30	62	-1.1	0.0
5	26	63	-3.7	5.0

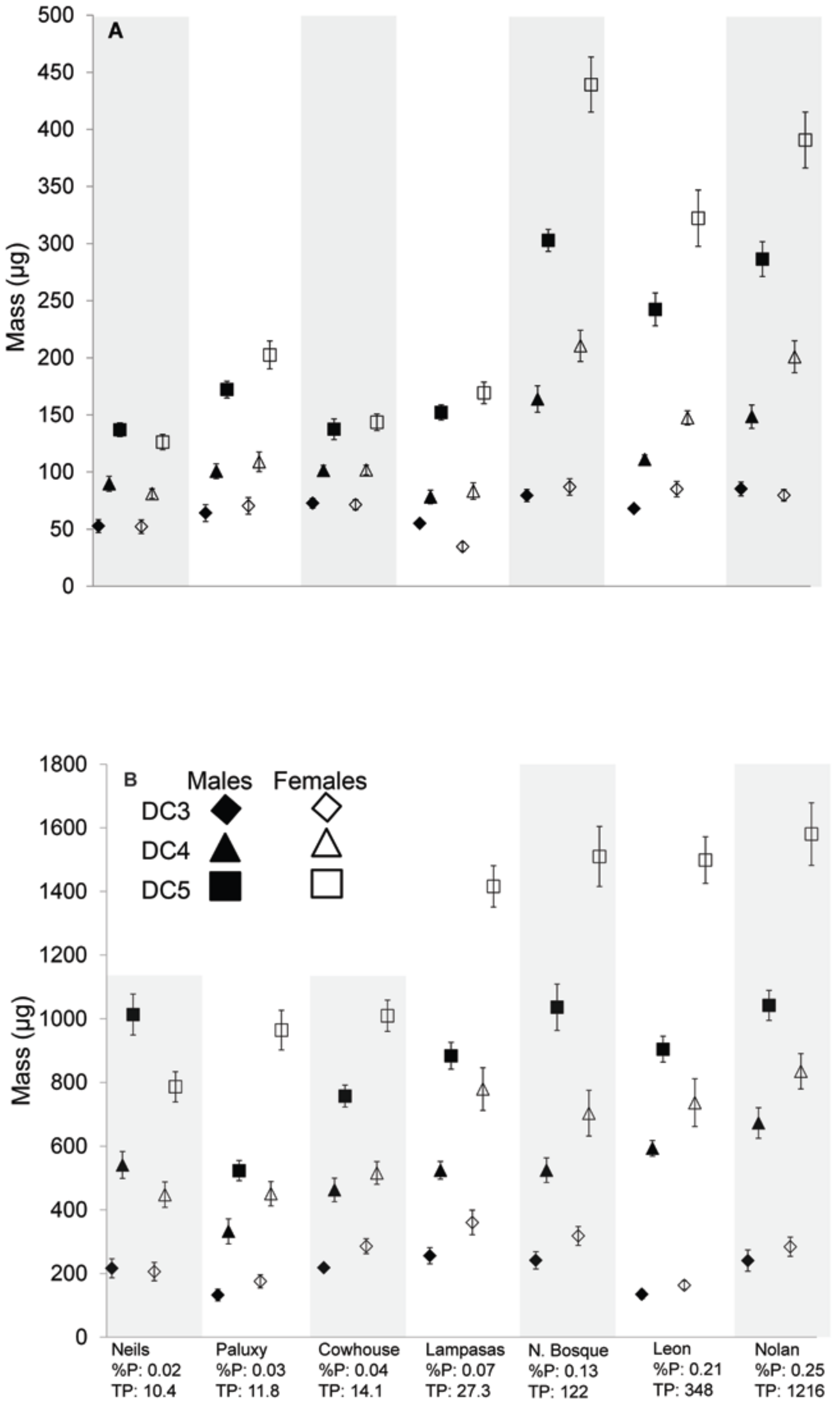

Figure 4.1. Masses for sexes and development classes (DC) for *Baetis* sp. (A) and *N. nanita* (B). Note that x-axis follows the phosphorus enrichment gradient; most unenriched to most enriched , left to right.

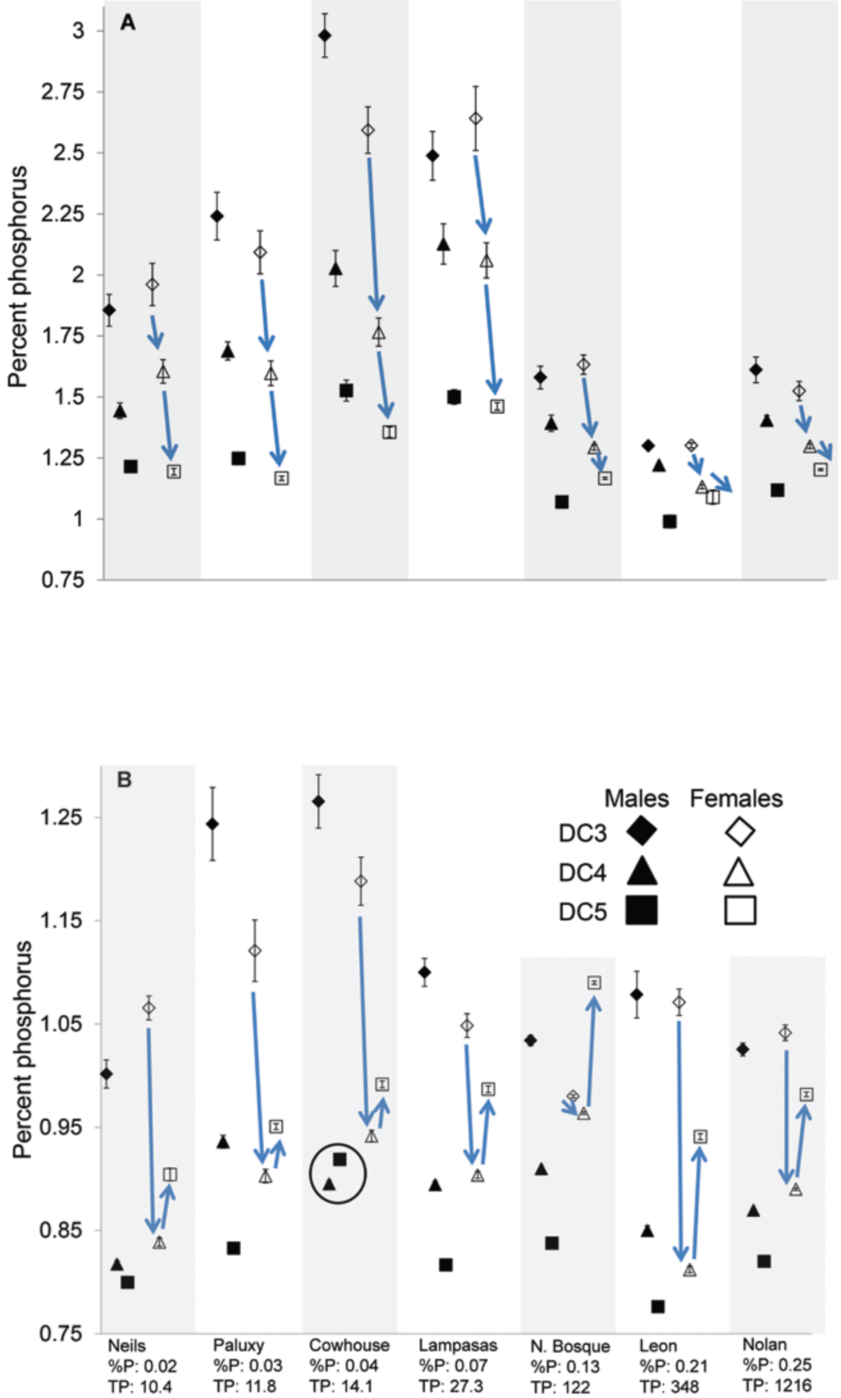

Figure 4.2. Percent body phosphorus for sexes and development classes (DC) for *Baetis* sp. (A) and *N. nanita* (B). Note that x-axis follows the phosphorus enrichment gradient; most unenriched to most enriched , left to right.

CHAPTER FIVE

Conclusions and Synthesis

Ecological stoichiometry has been a hot research topic over the past 10-15 years, with 100's, if not 1000+ peer reviewed paper published. The research presented herein represents one of the very few attempts to take a detailed look at stoichiometry from a fine scale ontogenetic perspective. It is also one of the few studies that have examined stoichiometric differences based on sex. Lastly, this study has had a large taxonomic breadth that examined aquatic insect stoichiometry of 19 aquatic insect species representing 7 orders of aquatic insects.

Results from these studies show that carbon and nitrogen content of aquatic insects varies little across insect ontogeny and is considered quasihomeostatic. Phosphorus content was decidedly non-homeostatic across all 19 insect species examined. Nutrient enrichment affected insect phosphorus content and size. Male and females differed in phosphorus content in 6 of the 10 taxa examined. In each case where males and females differed in phosphorus content, females were more enriched than males. Female mayflies were the same size or larger than males at unenriched sites, but were always larger than males at enriched sites. Patterns in phosphorus content showed a phylogenetic pattern as well; male and female baetid mayflies declined in phosphorus content across their ontogeny. In contrast, male leptophlebiid mayflies declined in phosphorus content, but females increased in phosphorus content across all sample location, but the effect was enhanced at enriched sites. The take home message is this: sex, size, ontogeny and resource quality interact to effect organism stoichiometry.

Failing to take into account the non-homeostatic or sex specific pattern of phosphorus content could change the interpretations of stoichiometric studies. A wide variety of population, community and ecosystem level processes and functions could benefit from an ontogenetic approach to ecological stoichiometry. As demonstrated, organism size is increased across nutrient gradients. Because body size is positively correlated with fecundity, high fecundity is expected at enriched sites due to the increase in female body size. It is unknown is egg size, quality, and survivorship are increased at enriched sites. Size specific foraging may be important in nutrient limited systems; with small sized organisms have a higher phosphorus content. Thus short handling times and high nutrient content may optimize foraging. Energy and material transfer in food webs may be enhanced. The rate of nutrient recycling probably varies across enrichment gradients, but across ontogeny as well.

APPENDICES

Appendix A – Mass (+/- SE) and % phosphorus (+/- SE) for *Baetis* sp.mayfly nymphs. *n* = number of observations, DC = development class.

Site		*Baetis* sp.					
		males			females		
	DC	*n*	mass (μg)	% P	*n*	mass (μg)	% P
Neils Creek	3	10	52.7 (5.7)	1.86 (0.07)	10	52.2 (6.0)	1.96 (0.09)
	4	10	89.7 (6.5)	1.44 (0.03)	10	81.1 (4.2)	1.61 (0.05)
	5	10	137.0 (6.0)	1.22 (0.01)	10	126.2 (6.7)	1.19 (0.01)
Paluxy River	3	10	64.1 (7.4)	2.24 (0.10)	10	70.4 (7.2)	2.09 (0.09)
	4	8	100.6 (6.6)	1.69 (0.04)	8	108.9 (8.6)	1.60 (0.05)
	5	11	172.3 (7.3)	1.25 (0.02)	10	202.5 (12.2)	1.17 (0.01)
Cowhouse Creek	3	18	72.8 (4.3)	2.98 (0.09)	20	71.5 (4.5)	2.59 (0.10)
	4	13	101.1 (4.6)	2.03 (0.07)	23	101.7 (4.2)	1.77 (0.06)
	5	17	137.5 (9.0)	1.53 (0.04)	12	143.6 (7.2)	1.36 (0.02)
Lampasas River	3	6	55.0 (3.4)	2.49 (0.10)	8	34.6 (3.8)	2.64 (0.13)
	4	10	78.2 (6.0)	2.13 (0.08)	9	83.3 (7.2)	2.06 (0.07)
	5	15	152.2 (6.7)	1.50 (0.03)	15	169.3 (9.5)	1.46 (0.02)
North Bosque River	3	19	79.4 (5.3)	1.58 (0.05)	20	86.9 (7.1)	1.63 (0.04)
	4	17	163.9 (11.6)	1.39 (0.03)	18	210.5 (13.6)	1.29 (0.01)
	5	20	302.9 (9.6)	1.07 (0.01)	19	439.3 (24.1)	1.17 (0.01)
Leon River	3	6	68.0 (2.7)	1.30 (0.01)	8	85.0 (6.7)	1.30 (0.01)
	4	10	111.0 (3.9)	1.22 (0.01)	9	147.4 (6.2)	1.13 (0.01)
	5	11	242.4 (14.4)	0.99 (0.03)	8	322.2 (24.6)	1.09 (0.03)
Nolan River	3	20	85.2 (6.0)	1.61 (0.05)	25	79.7 (5.0)	1.52 (0.04)
	4	20	148.5 (10.3)	1.40 (0.02)	19	201.0 (14.0)	1.30 (0.01)
	5	18	286.4 (15.3)	1.12 (0.01)	16	390.7 (24.4)	1.20 (0.00)

Appendix B – Mass (+/- SE) and % phosphorus (+/- SE) for *N. nanita* mayfly nymphs. *n* = number of observations, DC = development class.

Site		*Neochoroterpes nanita*					
		males			females		
	DC	*n*	mass (µg)	% P	*n*	mass (µg)	% P
Neils Creek	3	10	216.4 (30.0)	1.00 (0.01)	10	205.9 (29.0)	1.07 (0.01)
	4	10	540.8 (42.4)	0.82 (0.00)	9	447.4 (39.9)	0.84 (0.00)
	5	10	1013.6 (64.2)	0.80 (0.00)	13	786.5 (47.4)	0.90 (0.01)
Paluxy River	3	19	132.2 (18.4)	1.24 (0.04)	11	175.5 (20.7)	1.12 (0.03)
	4	10	332.3 (39.1)	0.94 (0.01)	14	450.7 (38.3)	0.90 (0.01)
	5	10	523.1 (31.6)	0.83 (0.00)	14	964.5 (62.0)	0.95 (0.00)
Cowhouse Creek	3	25	218.0 (9.0)	1.27 (0.03)	24	285.6 (23.0)	1.19 (0.02)
	4	6	462.5 (37.2)	0.90 (0.00)	13	515.8 (35.8)	0.94 (0.01)
	5	20	757.2 (34.0)	0.92 (0.00)	27	1009.5 (49.0)	0.99 (0.00)
Lampasas River	3	20	255.7 (25.4)	1.10 (0.01)	22	360.0 (38.7)	1.05 (0.01)
	4	19	524.2 (27.7)	0.89 (0.00)	20	779.4 (66.9)	0.90 (0.00)
	5	29	883.7 (42.4)	0.82 (0.00)	40	1415.9 (64.9)	0.99 (0.00)
North Bosque River	3	10	241.3 (27.1)	1.03 (0.00)	6	318.0 (29.4)	0.98 (0.00)
	4	7	524.4 (38.6)	0.91 (0.00)	8	703.4 (71.7)	0.96 (0.00)
	5	9	1036.4 (72.8)	0.84 (0.00)	7	1509.9 (94.5)	1.09 (0.00)
Leon River	3	19	134.6 (14.1)	1.08 (0.02)	18	163.2 (13.2)	1.07 (0.01)
	4	6	593.2 (24.7)	0.85 (0.00)	9	736.6 (74.7)	0.81 (0.00)
	5	11	904.5 (40.8)	0.78 (0.00)	20	1498.7 (73.2)	0.94 (0.00)
Nolan River	3	17	240.4 (33.4)	1.03 (0.01)	17	284.0 (30.0)	1.04 (0.01)
	4	17	673.0 (48.0)	0.87 (0.00)	18	835.0 (55.7)	0.89 (0.00)
	5	26	1042.1 (17.0)	0.82 (0.00)	24	1580.8 (98.2)	0.98 (0.00)

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