THE ECOLOGY AND EVOLUTION OF PHOSPHORUS USE IN *DAPHNIA*

By

RYAN E SHERMAN

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Western New England University
Springfield, Massachusetts
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Dissertation Approved:

Dr. Punidan Jeyasingh

Dissertation Adviser

Dr. Daniel Moen

Dr. Polly Campbell

Dr. Gerald Schoenknecht

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Abstract: Understanding how populations respond to environmental change is an important challenge in contemporary biology. Ecological stoichiometry uses elemental composition of species to make predictions about success in defined conditions of elemental supply. Understanding the success of genotypes within a species as a function of their elemental composition is a first step in understanding the mechanisms generating and maintaining such variation in elemental content. I tested the extent to which P content, a pivotal element in biology, predicted growth and competition in several *Daphnia* genotypes. Further, I measured the use of ^{33}P to understand the extent to which such parameters improved predictions. Genotypes showed significant variation in P content, ^{33}P use, and growth rate. P content alone was a poor predictor of growth and competition. These results suggest that decomposing P content of an individual into physiological components of P use will improve stoichiometric models predicting growth. Further, I examined the link between phosphoglucose isomerase (*PGI*) and P-use efficiency (PUE). *PGI* genotype was shown to underlie differences in PUE in an F2 recombinant *D. pulicaria* population. Additionally, to better understand how multiple elements that comprise biomass covary with PUE, I quantified several elements that comprise biological tissues (i.e. ionomes) within the F2 population. Substantial variation in the ionomes of these genotypes were found, with several correlations among element concentrations. Finally, we know little about how the content and demand for these elements change across ontogeny. I tested how *Daphnia* ionomes vary across species and ontogeny. I found significant effects of age, species, and ontogeny on the ionome content of three species of *Daphnia*. Understanding the genetic basis of these patterns and the ecological implications should contribute to the integration of elements and traits, and the extent to which shifts in elemental contents impact eco-evolutionary dynamics. Ionomic datasets should be a useful diagnostic tool to decipher multi-element constraints on biomass production.

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SYNTHESIS

Background

Understanding how populations respond to environmental change has been a central theme in ecological physiology (Prosser and Bishop 1950, Garland and Carter 1994). The importance of such work has steadily magnified as global temperature and other environmental variables change at a rapid pace (Kareiva et al. 1993), triggering rapid evolution in key traits that can feedback to impact eco-evolutionary dynamics (Schoener 2011). Understanding population-level responses is of particular importance with regards to current global changes such as eutrophication driven by alteration of a single element (e.g. phosphorus; P), as current P application is a doubling of the natural global rate of P inputs for terrestrial ecosystems (Carpenter and Caraco 1998). In fact, P is widely considered to be the primary limiting nutrient in lakes (Schindler 1977, Hecky and Kilham 1988, Tilman and Lehman 2001).

Ecological stoichiometry (ES) is a framework used to understand ecological interactions by using elemental composition of species to make predictions about the growth and fitness of a given species in defined environmental supply conditions (Sterner and Elser 2002). Species differ in body stoichiometry considerably, both among taxa and across trophic levels (Sterner and Hessen 1994, Frost et al. 2006, Persson et al. 2010). Such differences in elemental demand alter relative growth rates of species under constant conditions of supply stoichiometry (Sterner 1993, Andersen et al. 2004, Moe et al. 2005). Further, variation in key life history traits underlies such differences in demand (Elser et al. 1996).

ES has been used effectively to make sense of ecological patterns using interspecific variation in growth, body stoichiometry, nutrient excretion and recycling. However, to make evolutionary links, we need the genetic scope of element-related traits within a population. While there are considerable interspecific differences in elemental content, there is growing evidence that intraspecific (i.e. within population) variation can be considerable as well (DeMott et al. 2004, Bertram et al. 2008, Jeyasingh et al. 2009, El-Sabaawi et al. 2012, Goos et al. 2014, Tobler et al. 2015, Prater et al. 2017, Sherman et al. 2017). Our understanding of the processes generating and maintaining such variation, and associated ecological consequences, is sparse (Jeyasingh et al. 2014). Testing the extent to which stoichiometric models built to predict the success of species perform when extended to genotypes within a species should illuminate important processes that maintain substantial intraspecific variation in stoichiometry.

Study system

To examine the impact P supply has on potential micro-evolutionary shifts in populations, I studied the freshwater zooplankton *Daphnia* (Crustacea: Cladocera). *Daphnia* reproduce parthenogenetically, having clonal reproduction for most of the year and seasonal sexual reproduction resulting in resting eggs that can be preserved in lake sediments for years. Hatching these eggs allows us to compare the genetics and physiology of ancestral genotypes with those of extant descendants. Numerous lab studies have shown relationships between zooplankton growth, specifically *Daphnia*, and P-content in algae (Sterner 1993, Urabe et al. 1997, Sterner and Schulz 1998, Elser et al. 2001, Jeyasingh and Weider 2005, Frisch et al. 2014). Intraspecific variation in growth and stoichiometry in *Daphnia* appears to be correlated with ribosomal (r)DNA structure (Weider et al. 2005). The link between rDNA, P, and growth is formalized in the growth rate hypothesis (GRH; Elser et al. 1996, 2003). Briefly, the GRH posits that rapid growth is P intensive because of increased demand for P-rich ribosomal RNA required for ribosome biogenesis, and thus protein assembly. Therefore, the

GRH predicts a positive relationship between P and RNA quota, RNA quota and growth rate, and thus P quota and growth rate.

Population genetic structure may be affected by such environmental alterations and changes in structure can occur rapidly, affecting the evolutionary trajectory of populations, as gathered from genetic analyses of egg banks (Weider et al. 1997, Brede et al. 2009, Orsini et al. 2013, Frisch et al. 2014, 2017). Specifically, strong shifts in neutral genetic markers (i.e. several microsatellite loci) in South Center Lake (Chisago County, MN, USA) in the *Daphnia pulicaria* population, correlated with anthropogenic environmental change in the region, P enrichment in particular (Frisch et al. 2014). A comparison of the SC dataset to Hill Lake (Aitkin County, MN, USA), where P enrichment has been less prominent, revealed no such population genetic structure shift (Frisch et al. 2017). In SC lake *D. pulicaria* resurrected from resting eggs (i.e. ephippia) separated by thousands of generations exhibited striking differences in P use (i.e. uptake and retention, quantified using 33P assays). These differences in P use were correlated to the amount of P in the lake sediments (Frisch et al. 2014). Further, transcriptomic analyses have illuminated the complexity associated with physiological adjustments made by *Daphnia* to differing P-supply (Jeyasingh et al. 2011, Roy Chowdhury et al. 2014). While *Daphnia* generally retain more assimilated P under P-limiting conditions compared to P-replete conditions (DeMott et al. 1998, He and Wang 2007), such responses have been revealed to be genotype-specific (i.e. exhibit genotype-by-environment interactions Weider et al. 2005, Jeyasingh and Weider 2005, Jeyasingh et al. 2009).

Such microevolutionary dynamics could have important ecological implications since *Daphnia* are P-rich consumers that exhibit rapid growth, and thus play a pivotal role in whole lake P-cycling (Lehman 1980). Moreover, their abundance can control phytoplankton growth, not only via direct grazing, but also indirectly via consumer-driven recycling of P (Sterner 1986). Spatiotemporal P availability (natural or anthropogenic) is variable (Smil 2000) and is fundamental to biology (Westheimer 1987), primary reasons to study biomass production from a P-first perspective, as is

often done in ecosystem-level studies. However, recent work highlights the ecological importance of several other elements represented in biology (reviewed in Jeyasingh et al. 2014, Kaspari and Powers 2016, Penuelas et al. 2019). The ionome is defined as the mineral nutrient and trace element composition of an organism (Salt et al. 2008), which underlies its morphological and physiological state. Additionally, altering supply of a single element to primary producers results in widespread shifts in consumer physiology as *Daphnia* differentially regulate genes involved in the processing of major ions and trace elements under contrasting P supply treatments (Jeyasingh et al. 2011, Roy Chowdhury et al. 2014). With multi-element analyses revealing more about growth and biomass production, especially on the intraspecific level, it is becoming more important to understand the general patterns in ionomic variation within a population.

Overview of Dissertation

The framework ecological stoichiometry (ES) uses elemental composition of species to make predictions about species' success in defined conditions of elemental supply (Sterner and Elser 2002). Although substantial intraspecific differences in stoichiometry have been frequently observed, we are yet to understand the mechanisms generating and maintaining such variation. Extending predictions of stoichiometric models to understand the success of genotypes within a species is a first step in this enterprise. To this end, I measured phosphorus content (%P) within a *Daphnia* species to test the extent of variation in content, and tested the extent to which %P predicted growth rate and competitive ability under contrasting P supply conditions. I also quantified the kinetics of ^{33}P (i.e. acquisition, assimilation, incorporation, retention) under high and low P supply in the algal diet to understand the extent to which such parameters improved predictions of growth and competitive ability. Genotypes showed significant variation in P content, ^{33}P kinetics, and growth rate ($p<0.001$). However, P content alone was a poor predictor of growth and competitive ability. P acquisition, P assimilation, and P retention were the best predictors of growth. Additionally, not all genotypes exhibited the typical growth penalty under P limitation, as would be predicted by the GRH. These

observations indicate that some genotypes are able to maintain growth under P-limited conditions by altering P-use physiology.

To better understand how populations respond to these alterations in P supply, I turned to links previously identified in the field of ecological physiology. Previous work has found strong links between polymorphism at the central metabolic gene locus, phosphoglucose isomerase (*PGI*; EC 5.3.1.9) and environmental variables in arthropods (reviewed in Wheat 2010). The *PGI* locus appears to underlie population genetic responses to various ecological challenges, including responses by daphniids to alterations in P supply, as *PGI* homozygotes have been demonstrated to exhibit higher phosphorus (P) use efficiency (PUE) compared to heterozygotes under P-limiting conditions. However, these studies were confounded by unknown genetic background and evolutionary history (Jeyasingh et al. 2009). Methods in quantitative genetics enable us to partition phenotypic variation in PUE into genetic and environmental components to test the effects of *PGI* genotype on P-use efficiency of *Daphnia* with high genotypic power, while controlling for effects of evolutionary history. To this end, we constructed an F2 recombinant *D. pulicaria* population and found substantial variation in %P and PUE. Homozygotes exhibited higher PUE compared to heterozygotes. These observations indicate important links between the influence of energy and P metabolism on the efficiency of biomass production.

While intraspecific variation in the contents and uptake and retention rates of bulk elements is substantial (e.g., DeMott et al. 2004, Bertram et al. 2008) recent work highlights the ecological importance of several other elements represented in biology (Goos et al. 2017, Jeyasingh et al. 2017, Lind and Jeyasingh 2017, Prater et al. 2018, Rudman et al. 2019). Additionally, correlated changes in multiple elements due to selection for efficient use of one element is center to the field of ionomics (Salt et al. 2008). I quantified the ionomes within the recombinant F2 *D. pulicaria* population. Substantial genotypic variation in the ionomes of these genotypes was found, with several correlations among pairwise element concentrations. Understanding the genetic basis of these patterns

and the ecological implications should contribute to the integration of elements and traits, and the extent to which shifts in elemental contents impact eco-evolutionary dynamics.

While genotypes within the *D. pulicaria* population may differ significantly in their ionomes, we know little about how the content and demand for these elements change across ontogeny. Ontogenetic changes in morphology, behavior, and physiology often rival interspecific differences as organisms require various nutrients to produce new biomass through somatic growth or reproduction. I tested how *Daphnia* ionomes vary across species, age, and ontogenetic stage, as well as how individual elements change with juvenile growth rate. I found significant effects of age, species, and ontogenetic stage on the ionome content of three species of *Daphnia*. For several elements quantified, differences across ontogeny rivaled differences seen between species. These results suggest that variable nutritional demand across ontogeny could play an important role in population dynamics, particularly because of the large role *Daphnia* play in recycling nutrients back to primary producers.

Conclusions and scope

At the intraspecific level, where variation in P content is relatively more constrained, it is evident that growth may be sustained by other mechanisms that do not necessarily result in differences in P content. Differences in gene expression and P use physiology (Misson et al. 2005, Moseley et al. 2006, Dyhrman et al. 2012) should underlie such variation, and represent an important area of further research. For this reason, quantifying elemental use (i.e., acquisition, assimilation, net incorporation, retention), particularly when significant variation is present among genotypes, may be more applicable for examining intraspecific variation, and potentially also variation at broader taxonomic levels.

While such ecologically-important quantitative traits like P-use are under polygenic control, the significant differences among genotypes demonstrates that *PGI* plays a role. Understanding spatiotemporal dynamics of *PGI* polymorphisms in ecologically important taxa like *Daphnia*, may

inform theory predicting shifts in key ecological functions, particularly in the context of anthropogenic environmental changes such as eutrophication. Understanding such responses is particularly important with regards to current global changes such as eutrophication driven by alteration of a single element (e.g. P).

Additionally, differences among species or ontogenetic stages within a species in the contents of multiple elements (i.e. ionome) are indicative of physiological differences among species and adjustments that take place across ontogeny. Changes in an organism's functioning such as allocation changes at maturity, ontogenetic diet shift, or variation in the availability of such elements, may affect the nature of nutrient limitation, having cascading effects on individual life history, biomass production, and nutrient recycling, with potentially important ecological implications.

While a P-first perspective has certainly informed key parameters in ecology such as growth and biomass production, it is clear that ionomes are an important frontier in the field of ecological stoichiometry. Exploring the general covariance patterns among multiple elements that comprise an organism, and links to functional traits such as growth, should aid in furthering our understanding of evolutionary responses to environmental change and, more generally, understanding of the fundamental factors underlying growth and productivity. This elemental view of phenotypic variation simplifies the complexity of understanding the ecological importance of variation in and evolution of traits.

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CHAPTER I

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GENOTYPE-SPECIFIC RELATIONSHIPS AMONG PHOSPHORUS USE, GROWTH, AND ABUNDANCE IN *DAPHNIA PULICARIA*

Ryan E. Sherman, Priyanka Roy Chowdhury, Kristina D. Baker, Lawrence J. Weider, and Punidan D. Jeyasingh

Abstract

The framework ecological stoichiometry uses elemental composition of species to make predictions about growth and competitive ability in defined elemental supply conditions. Although intraspecific differences in stoichiometry have been observed, we have yet to understand the mechanisms generating and maintaining such variation. We utilized variation in phosphorus (P) content within a *Daphnia* species to test the extent to which %P can explain variation in growth and competition. Further, we measured ^{33}P kinetics (acquisition, assimilation, incorporation, and retention) to understand the extent to which such variables improved predictions. Genotypes showed significant variation in P content, ^{33}P kinetics, and growth rate. P content alone was a poor predictor of growth rate and competitive ability. While most genotypes exhibited the typical growth penalty under P limitation, a few varied little in growth between P diets. These observations indicate that some genotypes can maintain growth under P limited conditions by altering P-use, suggesting that decomposing P content of an individual into physiological components of P kinetics will improve stoichiometric models. More generally, attention to the interplay between nutrient content and nutrient-use is required to make inferences regarding the success of genotypes in defined conditions of nutrient supply.

Introduction

The framework of ecological stoichiometry (ES) [1] uses the elemental composition of species to make predictions about the growth and competitive ability of a given species in defined environmental supply conditions. Species differ in stoichiometry considerably [2]. Such differences in elemental demand alter relative growth rates of species under constant conditions of supply stoichiometry (e.g., [3–5]). Further, variation in key life history traits underlies such differences in demand [6]. For example, Iwabuchi & Urabe [7] conducted microcosm experiments on three *Daphnia* species and found that those with low phosphorus (P)-demand species of the freshwater cladoceran genus *Daphnia*, outcompeted others with a high P-demand, under P-limiting conditions. Their results indicated direct effects of supply stoichiometry in determining the growth of populations varying in elemental demand. Within plants, Yu et al. [8] analyzed long-term data in several vascular plants and found that the degree of stoichiometric homeostasis in the face of variable supply stoichiometry (as opposed to elemental content in a single supply environment), best predicts the abundance of a plant species.

While there are considerable interspecific differences in elemental content, there is growing evidence that intraspecific (i.e. within population) variation can be considerable as well (e.g., [9–14]). Our understanding of the processes generating and maintaining such variation, and associated ecological consequences is sparse. Testing the extent to which stoichiometric models built to predict the success of species perform when extended to genotypes within a species should illuminate important processes that maintain substantial intraspecific variation in stoichiometry.

Intraspecific variation in stoichiometry has to be driven by some combination of differential acquisition, assimilation, net incorporation, or retention of elements [15]. Functional genomic analyses of *Daphnia* genotypes within a species reveal substantial differences in gene

expression that could underlie variation in stoichiometry [16]. Multiple genes have been identified to be involved in P homeostasis and that variation of P supply affects the expression of genes involved in P handling (e.g., phosphatases, P transporters; reviewed in Jeyasingh & Weider [17]). Note that substantial genetic variation exists in the expression of such genes in plants (e.g., tomato; [18]; maize; [19]; bean; [20] although little is known in animals. P supply has been shown to significantly alter the expression of ~20% of the genes in the *Daphnia* genome (e.g., [21]). Genotypic comparisons of transcriptomes revealed that genotypes differed in the expression of as much as 30% of the genes in common gardens [22,23]. Differential expression of genes and pathways could alter P allocation to biochemicals (e.g., phospholipids vs sulfolipids; [24–26], tissues (e.g., soft tissue vs carapace; [27], or life-history traits (e.g., growth vs reproduction; [28]) which are known to differ in P demand. While substantial genetic variation in life-history traits is well known [29,30], it is likely that similarly strong genetic variation exists for P allocation to biochemicals and tissues (e.g., bean; [31]; wheat; [32]; rice; [33]; fish [34]. As such, two genotypes can have identical P content, and yet differ substantially in P allocation at the biochemical or tissue levels. Such differences could be manifested in the rates at which P is acquired, assimilated, and excreted. Thus, it is possible that fitness-relevant classical traits (e.g. intrinsic rate of natural increase) can be expressed and maintained in a genotype by altering elemental kinetics without any change in individual body stoichiometry, and yet this results in distinctive competitive or fitness outcomes under the same supply stoichiometry.

We addressed these issues in the freshwater crustacean *Daphnia pulicaria* by testing the extent to which %P predicted growth rate and competitive ability (outcome of intraspecific competition), as well as measured the ^{33}P kinetics (i.e. acquisition, assimilation, incorporation, retention) in high and low P supply. There is substantial intraspecific variation in P-content (e.g., [9,11]). and physiological kinetics of P in *Daphnia* (e.g., [31]). This variation can interact with the P supply environment and affect the relative performance of genotypes [35–37]. The

existence of intraspecific variation in P kinetics is perhaps not surprising, given that P supply alters the expression of about a third of the genes in the *Daphnia* genome [21]. For example, although genotypes did not differ in somatic P-content, Frisch et al. [36] found significant differences in P use of *Daphnia* genotypes that had been hatched from resting eggs, separated temporally by centuries. Specifically, these "resurrected" genotypes differed by as much as 200% in P-retention efficiencies (RetE), and by as much as 300% in the amount of biomass produced per mg P in the body under P-limiting conditions (phosphorus use efficiency; PUE). Such differences in P use, without any change in P content, had predictable impacts on the quantity and quality of algae via recycling by these daphniid grazers [39].

Based on stoichiometric models built to describe performance of species differing in somatic stoichiometry [1], we predicted that genotypes with lower P content will exhibit slower growth rate and be competitively superior in conditions of lower P supply. Moreover, based on the growth rate hypothesis [6], we predicted that P content should be correlated with growth, which is strongly related to competitive ability in *Daphnia* [40]. Further, using physiological models [41], we predicted that all genotypes will increase rates of P acquisition, assimilation, and retention under lower P supply conditions. In addition, we predicted that genotypes with lower P content will acquire, assimilate, and retain P at a slower rate compared to genotypes with higher P content. Finally, we tested the impact of using multi-proxy measures of P kinetics in forecasting genotypic success (growth and competition) using multivariate approaches coupled with model reduction criterion.

Materials and methods

Experimental organisms

The green alga, *Scenedesmus obliquus*, served as the food resource in our experiment. It was grown in continuous-flow chemostats in COMBO media containing concentrations of either

high phosphorus (HP 50 µmol L^{-1}) or low phosphorus (LP 5 µmol L^{-1}) [42] at 20ºC and constant light (~120 µmol m^{-2} s^{-1}). These P supply conditions produced algae with a C: P ratio of ~150 (HP) and ~750 (LP), respectively. *Daphnia pulicaria* resting eggs were isolated from the 20-24 cm sediment layer (sediment dated between ~1967-1977 AD) of South Center Lake, Chisago County, Minnesota (for collection details see Frisch et al. [36]) and propagated parthenogenetically in the laboratory. *D. pulicaria* clonal lineages were stored in a temperature-controlled growth chamber at 20ºC with an 18:6 hr light:dark cycle in COMBO media containing no N or P [42]. Ten genetically distinct genotypes from this resurrected population were used in this experiment, each possessing a unique electromorph marker at the glucose-6-phosphate isomerase (GPI; Enzyme Commission (EC) 5.3.1.9) locus and/or phosphoglucomutase (PGM; EC 5.4.2.2) locus.

Elemental analysis

To determine algal stoichiometry, *Scenedesmus* was filtered from experimental chemostats onto pre-combusted (550ºC for 2hr) and pre-weighed GF/C filters (Whatman, Maidstone, U.K.), and dried at 60 ºC for 72 hr. Carbon (C) content was determined using an automated CHNOS analyzer (VarioMICRO analyzer, Elementar Americas, NJ, U.S.A.). Total phosphorus (P) content was quantified by a modified sulfuric acid digestion method (APHA 1992) and verified using a spinach standard (NIST 1570a). We then converted C and P content by mass into molar C:P for analyses.

To determine body phosphorus content in *D. pulicaria* raised in contrasting P environments, we raised neonates (<24hrs old) individually in jars in 100mL of COMBO media containing no N or P [42]. Individuals were fed 1 mg C L^{-1} per day of either HP or LP *Scenedesmus* algae for a period of 5 days. The culture media was replaced each day. P content of 10 different genotypes were measured following a five-day period under either HP or LP

conditions, with 3 replicates per treatment. To determine *D. pulicaria* P content, pre-weighed samples were combusted (550ºC for 2hr) and dried at 60 ºC for 72 hr. Total phosphorus (P) content was quantified by a modified sulfuric acid digestion method (APHA 1992) and verified using a spinach standard (NIST 1570a). Each sample was a pooled sample consisting of ~0.05mg. P content was calculated as percent of *Daphnia* dry mass.

Radiotracer assays

Radiolabeling algae

Radioisotope assays using ^{33}P allowed us to examine elemental use on a per-atom basis. After ingestion by an organism, we were able to measure the quantity of radioisotopes acquired by the organism. Inorganic radiotracers are primarily introduced to consumers via ingestion of autotrophic periphyton [43]. Therefore, our radiotracer assays provided a robust test of ingestive and post-ingestive P processing. To introduce the radioisotopes into the *Scenedesmus* algae, fresh algae, both HP and LP, were centrifuged at 3600 rpm for 30 min. The supernatant was discarded and the precipitate was resuspended in 100 mL of no N and P COMBO medium [42]. We then added 5.55 Mbq of ^{33}P (as orthophosphate) to each 100mL algal sample. The solutions were kept on a shaker at 20°C, 16:8 light: dark cycle and incubated for 72 hrs to allow the algae to fully incorporate the radioisotope. *Scenedesmus* algae was assumed to be uniformly radiolabeled. Following 72 hrs, the algal solution was centrifuged at 3600 rpm for 30 minutes, after which the supernatant was discarded and the precipitate was resuspended in 100mL of no N and P COMBO medium [42]. Two mL of the radiolabeled algae was then filtered through a GF/F filter (Whatman International Ltd, Maidstone, England). Following an addition of scintillation cocktail (2 mL; Ultima Gold, Perkin Elmer Inc., MA), specific activity was counted in a liquid scintillation counter (Beckman Coulter LS 6500).

^{33}P kinetics

We performed ^{33}P radiotracer assays, following DeMott, Gulati & Siewertsen (1998) and Roy Chowdhury *et al.* (2014). Similarly-sized, ~5 day old *D. pulicaria* were isolated in no N and P COMBO and acclimated to experimental conditions by feeding on 1 mg C L^{-1} of either HP or LP *Scenedesmus* algae daily for 72 hrs. On the day of the experiment, *Daphnia* were pooled in jars of 5, with a minimum of 3, maximum of 5 replicates, and starved for 2 hrs. Following starvation, the *Daphnia* were fed 1 mg C L^{-1} radiolabeled algae, either HP or LP. It is important to note that ^{33}P is a small portion of the total P in the algae, however, it captures general patterns in P processing. Additionally, not all the same animals could not be used to evaluate P kinetics. Four different rates were calculated: acquisition (feeding *Daphnia* radiolabeled algae for 10 min), assimilation (40 min of feeding and 6 hrs of gut clearing), net incorporation (4 hrs of feeding), and retention (10 min of feeding and 12 hrs of gut clearing). When measuring retention, we changed the media every 1, 2, 4, and 8 hrs in order to prevent recycling. We defined acquisition as the intake of P in a given period (10 min), before having been absorbed through the gut wall. Assimilation was defined as absorption of those elements through the gut wall into body tissue, net incorporation as the amount of P allocated following assimilation, and retention as the amount of P retained after 12 hrs of not feeding. *Daphnia* thus collected were placed in scintillation vials for radioactive counting (Beckman Coulter LS 6500). The amount of radioactive ^{33}P was calculated in each organism, correcting for mass in micrograms per microgram dry weight from an average of 5 separate individuals raised in identical conditions. All samples for each genotype were taken and analyzed on the same day of the experiment.

Growth rate

To determine juvenile growth rate under contrasting P supply conditions, *D. pulicaria* individuals were fed 3 mg C L^{-1} per day (to elucidate greater differences in growth) of either HP

or LP *Scenedesmus* algae, with 4 replicates per treatment. The culture media was replaced daily. Growth rates of 10 different genotypes were measured over a five-day period under either HP or LP conditions. Four neonates of each genotype (<24 hrs old)were used for each P-supply treatment, separated individually in 100mL of COMBO media containing no N or P [42]. The length of each neonate in mm was measured from the top of the head to the base of the tail-spine on the first and fifth (final) day of the experiment using a microscope ocular micrometer at 4x magnification (Leica S8APO, Leica Microsystems, IL, USA). Growth rate was determined as the change in length per day from birth to day five. Note that growth rate was also estimated as change in length per day per initial length, to scale for variation in the starting size of individuals. Both approaches showed no differences in any statistical analyses, therefore, all analyses were performed with growth rate defined as change in length per day from birth to day five.

Competitive ability

Six neonates (<24 hrs old) from each genotype were placed in competition with six neonates of a reference genotype. Three reference genotypes were chosen from the 4-8 cm sediment layer (sediment dated between ~2002-2008 AD) from SC Lake, and were used to compare competitive abilities of the experimental genotypes from the 20-24 cm layer of SC. Each reference genotype was paired with each experimental genotyped for a total of 30 trials per P treatment. Neonates were placed in 500 mL flow-through polyvinyl chloride microcosms containing no N, no P COMBO medium [42]. The flow-through chamber microcosms were modeled after Heugens et al. [43] and allowed for a controlled supply of food and nutrients, while eliminating the loss of algae from the water column by sedimentation. *Scenedesmus obliquus* algae were pumped into microcosms one hour before adding neonates, so as to have food available upon immediate transfer. Each 500mL microcosm vessel received 1 mg C L^{-1} per day of either HP or LP algae continuously for a duration of 21 days at a rate of ~1.725 L/day. There was no early mortality within the first 5 days. Following the 21 days, microcosm chambers were

homogenized and 30% (150 mL) of the media was sampled. Twelve *Daphnia* were chosen at random from a 50mL subset of the 150mL sample to test for unique genotype-specific (allozyme) markers. Allozyme electrophoresis followed the protocols of Hebert & Beaton [44], and allowed us to estimate the frequency of each experimental *Daphnia* genotype in competition. Each genotype possessed a unique allozyme (i.e., electromorph) marker at the glucose-6-phosphate isomerase (GPI; Enzyme Commission (EC) 5.3.1.9) locus and/or phosphoglucomutase (PGM; EC 5.4.2.2) locus. Two experimental genotypes (from 20-24 cm sediment layer) share the same GPI electromorph profiles with two of the reference genotypes (from the 4-8 cm sediment layer). Distinguishing these three experimental genotypes from the reference genotypes under competition proved equivocal using the Phosphoglucomutase (PGM; EC 5.4.2.2) locus. Therefore, these two genotypes were excluded from analysis involving competitive ability. By identifying the frequency of each experimental genotype relative to the reference genotypes, we compared the competitive abilities of the experimental genotypes under varying P-supply treatments. Experimental genotype frequencies were calculated by dividing the number of individuals of the target genotype divided by the total number of individuals (experimental and reference) at the conclusion of the experiment. Estimated density values were calculated from the fraction of individuals of the target genotype (out of the 12 sampled) multiplied by the total number of individuals in each microcosm following the 21-day period. Estimated density values are represented in Fig. 1 and Table 1.

Statistical analysis

To test the interactive effects of *Daphnia* genotype and P supply on P content, P- kinetics (acquisition, assimilation, net incorporation, and retention), growth rate, competitive ability, we used separate general linear mixed models (GLMMs) with *Daphnia* genotype as a random effect. All non-normal data were log-transformed for analyses to fit assumptions for GLMMs. Principal component analysis (PCA) was implemented as a dimension reduction technique to visualize our

multivariate data (i.e., growth rate, P content, P kinetics, competitive ability). The PCA was run on a correlation matrix. Correlation matrix PCAs center and standardize the variables so variables with large values and thus higher chance of variation do not have outsized influences on multivariate patterns. To improve interpretability of the components extracted by the PCA, we applied an orthogonal rotation (varimax) to the components, reporting PC axes with an eigen value greater than one. For all traits measured, we did not have an equal number of replicates; therefore, we excluded missing pairwise values for the multivariate analysis (see Table S1 for more details). PC axes scores were plotted to visualize the general relationships between all traits measured in a two-dimensional space. Linear regressions and analyses of covariance (ANCOVA) were run on P content, growth rate, and competitive ability to test predictions based on ES, i.e. that P content and growth rate should be positively correlated, that growth rate predicts competitive ability, and that P content predicts competitive ability. All tests were performed using SPSS (IBM Statistics Version 22).

A model comparison approach was used to evaluate competing models to explain growth rate and competitive ability in the *Daphnia* genotypes. All model comparison data analyses were conducted using the R statistical package MuMIn (R Core Team 2015, version 3.2.3) to measure the relative performance of the models according to Akaike information criterion corrected for small sample sizes (AICc). *Daphnia* P-use variables were included in the full model as potential predictors, with the model comparison approach used to determine their relative importance to growth and competitive ability. These models were analyzed using model selection based on AIC model selection approach to determine which traits best explained growth rate and competitive ability. Using the ΔAICc scores and AICc weights, the top candidate models were identified by removing models with ΔAICc scores greater than 6. Generalized linear models were used to compare a set number of models to the data, measuring the relative support the data gave to each model.

Results

Sources of intraspecific variation

Reaction norm plots (Fig. 1) of the *D. pulicaria* genotypes showed varied responses to P-supply conditions with a significant genotype x P treatment interaction. There was a significant genotype-by-treatment interaction for *Daphnia* %P ($p=0.008$), P acquisition, retention, and assimilation ($p<0.0001$), and P net-incorporation ($p=0.001$) (Fig. 1; Table 1).

Juvenile growth rate showed a significant genotype by treatment interaction ($p<0.0001$; Fig. 1g; Table 1). There was no genotypic variation in competitive ability, though there was a significant treatment effect ($p=0.018$; Fig. 1f; Table 1). There were no significant genotypic or treatment effects on experimental clone density (Fig. 1h; Table 1). There were also no differences in competitive ability among the reference genotypes.

Relationships among P content, P kinetics, growth, and competitive ability

Stoichiometric models predict that individuals with lower somatic P content will be competitively superior under LP conditions [1]. Competitive ability of genotypes was not correlated with somatic P content ($p=0.267$; $R^2=0.006$; Fig. 2a). ANCOVA results revealed that P content and competitive ability did not co-vary ($p=0.146$). P content and competitive ability were not significantly correlated, when separated by treatment (HP, $p=0.917$; LP, $p=0.072$). The growth rate hypothesis predicts that rapid growth should be correlated with high somatic P content [6]. However, P content was not significantly correlated with growth rate ($p=0.144$; $R^2=0.041$; Fig. 2b). ANCOVA results showed that P content and growth rate co-varied ($p=0.002$). Faster growth rate predicts higher competitive ability due to fitness advantages related to size. Linear regressions revealed that competitive ability was significantly correlated with growth rate ($p<0.0001$; $R^2 = 0.28$; Fig. 2c).

Growth rate and competitive ability were not significantly correlated when separating by treatment (HP, p=0.07; LP, p=0.25). P content was not correlated with P acquisition (p=0.412; R^2=0.013; Fig. 3a) nor with P-net incorporation (p=0.111; R^2=0.048; Fig. 3c) or P retention (p=0.357; R^2=0.016; Fig. 3d). P content was significantly correlated with P assimilation (p=0.021, R^2=0.098, Fig. 3b).

The individual loadings on the first three axes of the principal component analysis (PCA) of each data set, summary of variance explained by the PCA, and eigenvalues for each axis can be found in Table S2. Seven of the 10 genotypes are reported in the PCA; one genotype had incomplete P content data and two remaining genotypes shared the same GPI electromorph profiles with two of the reference genotypes. As previously stated, distinguishing these two experimental genotypes from the reference genotypes proved equivocal using the Phosphoglucomutase (PGM; EC 5.4.2.2) locus and therefore, these two genotypes were excluded from the multivariate analysis. Percentage of the total variation explained by the first three axes of the PCA was ~74%. P-use traits explained the most variation on PC1, explaining ~34% of the total variation; PC2 explaining ~26%, and PC3 explaining ~15% (Fig. 4). Plotting phenotypic trajectories revealed differences in the nature and magnitude of change between genotypes in multivariate space in response to altered P supply conditions. In response to LP conditions, genotypes tended to decrease along PC2, representing a decrease in growth rate, competitive ability, and density (Fig. 4a). Genotypes increased along PC1 to varying degrees in response to LP conditions, thus, representing an increase in P acquisition and assimilation as a response to limited P-supply conditions. Notably, clone 36x was did not differ in growth rate between P supply treatments, as it increased its P acquisition and assimilation under LP (Fig. 4a).

Model comparison

The best supported model for competitive ability included P assimilation and P retention (Table 2). The best supported model for growth rate included P acquisition, P assimilation, and P retention (Table 3). With competitive ability as the dependent variable, P content only appeared in one of the top five models (out of 20 models) and in none of the top 5 models when growth rate was the dependent variable (out of 13 models). P assimilation was in all of the top 5 models for predicting both competitive ability and growth rate. P assimilation has the highest relative importance, while P content has the lowest for both competitive ability and growth rate (Table 4). See model selection tables with competitive ability (Table S3) and growth rate (Table S4) in the supplementary materials for more information.

Discussion

The results from this study detail how P acquisition, assimilation, and excretion (i.e., P kinetics) explain variation in growth rate and competitive ability as compared to P content alone in a population of *Daphnia pulicaria*. Genotypes varied in plastic responses in P content and P kinetics to contrasting P supply conditions, differing in their nature and magnitude of plasticity (Fig. 1a-e). Under P limitation (i.e., LP conditions), P acquisition and assimilation increased, and exhibited more variation among genotypes as compared to HP conditions (Fig. 1b-c). P net incorporation and P retention varied significantly between HP and LP treatments (Fig. 1d-e). Although growth rates and competitive abilities varied among genotypes (Fig. 1g), these differences were not correlated with P content or P kinetics (Fig. 2). P content was significantly correlated with P assimilation, but no relationships were found between P content and P acquisition, net incorporation, or P retention (Fig. 3). Genotypes varied in multivariate space, with most genotypes altering growth between high and low P conditions, while one genotype exhibited no difference in growth that was achieved by altering P-kinetics (Fig. 4). P-kinetic traits explained the most variation on PC1, while competitive ability, growth rate, and density

explained the most variation along PC2, and P content explained the most variation along PC3 (Fig.4b).

While most genotypes grew faster under HP conditions, this did not correspond to higher P content. We predicted that genotypes with lower P content would exhibit slower growth and be successful under LP conditions. Under LP conditions, some genotypes had an increase in P-use traits, likely to compensate for P deficiencies; i.e., demand of P for RNA biogenesis under the limiting conditions. Additionally, it may be possible that some genotypes can create more biomass with less P body content (i.e., differential Phosphorus Use Efficiencies – PUE), e.g., [36]. There was less variation in P acquisition under replete P conditions than under P-limited conditions (Fig. 1b). This suggests that under stressful, LP conditions, trade-offs between traits that require a sufficient amount of P are manifested. Large interspecific differences in P content is perhaps important in generating the robust growth-phosphorus couplings [47]. It is worth noting that even studies on species from the same genus do not show support for these predictions [48]. At the intraspecific level, where variation in P content is relatively more constrained, it is evident that growth may be sustained by other mechanisms that do not necessarily result in differences in P content. Differences in gene expression and P use physiology [24–27] should underlie such variation, and represent an important area of further research.

In addition to variable strategies to achieve similar growth values, genotypes appear to use different strategies to achieve similar competitive outcomes against a set of common reference genotypes. Faster-growing individuals were found at higher frequencies in the competition experiment ($p<0.0001$; $R^2 = 0.27$; Fig. 2c), consistent with previous studies (e.g., [38]). While there were significant differences in response to changes in environmental conditions (variation in plastic response to P supply conditions) for the P-use traits, P body content, and growth rate, these did not translate into variation in competitive ability among the *Daphnia* genotypes. The reference genotypes (from the 4-8 cm sediment layer) always

outcompeted the experimental genotypes (20-24 cm sediment layer) in our study, a result that has been demonstrated previously in *D. pulicaria* genotypes [49]. However, genotypic variation in stoichiometry as a result of environmental supply of essential elements can potentially result in differential success. A few studies have reported striking shifts in the frequency of *Daphnia* genotypes depending on nutrient supply stoichiometry [11,35]. Weider et al. [32] showed that specific *Daphnia pulex* genotypes dominate under certain P supply conditions (C:P ~100) vs. others (C:P ~800), demonstrating the large effect P supply can have on competitive outcomes in *Daphnia* populations. However, such competitive outcomes were likely related to other traits not directly related to P content [11]. It is likely that while P content has been useful in predicting the success of species, generating predictions on the success of genotypes is more complex.

P-use traits (i.e. P acquisition, assimilation, net incorporation, and retention) are better at predicting growth rates and competitive abilities than P content alone (Table 2, 3). It is not surprising that a multivariate approach is a better predictor of genotypic performance, as compared to P content alone (Table 4). Nevertheless, using P content alone to make evolutionary inferences, as can be done at the interspecific level (e.g., [49]), could be misleading at the intraspecific level, and perhaps also over longer evolutionary time-scales (e.g., [50]). Genotypes within a species may have the same P content; however, they can vary in P-use, which could lead to a different result in terms of growth and excretion rates. For example, P allocated to tissues such as the *Daphnia* exoskeleton (i.e., carapace; [27] should have slow turnover compared to high turnover molecules such as phospholipids [52], thus imparting distinctive signatures on P retention and excretion rates. For this reason, quantifying elemental use (i.e., acquisition, assimilation, net incorporation, retention), particularly when significant variation is present among genotypes, may be more applicable for examining intraspecific variation, and potentially also variation at broader taxonomic levels. While ecological stoichiometry has provided new insights into how the balance of elements affects ecological interactions, little is known about

evolutionary dynamics in stoichiometric traits [17]. In addition to answering basic questions about the evolutionary genetics of elemental content [53], addressing elemental kinetics is bound to illuminate the evolutionary sources and mechanisms maintaining substantial intraspecific variation in stoichiometry.

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Tables

Table 1 Univariate responses of 10 *Daphnia pulicaria* genotypes to contrasting P supply conditions. Bold font indicates significant results with $p<0.05$.

	Genotype (G)			Treatment (T)			G x T		
GLMM	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Percent phosphorus body content	0.596	8	0.760	3.542	1	0.097	3.164	8	**0.008**
P acquisition	2.178	9	0.131	3.190	1	0.108	26.951	9	**<0.0001**
P assimilation	2.341	9	0.111	26.944	1	**0.001**	10.203	9	**<0.0001**
P net incorporation	4.348	9	**0.020**	0.590	1	0.462	3.687	9	**0.001**
P retention	2.607	9	0.085	0.147	1	0.711	26.02	9	**<0.0001**
Juvenile growth rate	0.829	9	0.608	21.396	1	**0.001**	5.505	9	**<0.0001**
Competitive ability	0.927	7	0.539	9.314	1	**0.018**	0.247	7	0.545
Density	2.728	7	0.104	1.408	1	0.274	0.985	7	0.460

Table 2 Top 5 models for predicting competitive ability.

Variables contained in model				df	Log Lik	AICc	ΔAIC	weight
	P assim.		P ret.	4	2.592	3.9	0.00	0.231
	P assim.	P net.		4	2.064	5.0	1.06	0.136
	P assim.	P net.	P ret.	5	3.262	5.1	1.24	0.124
	P assim.		P ret.	P content	5	2.696	6.3	2.38	0.070
P acq.	P assim.		P ret.		5	2.618	6.4	2.53	0.065

Table 3 Top 5 models for predicting growth rate.

Variables contained in model				df	Log lik	AICc	ΔAIC	weight
P acq.	P assim.		P ret.	5	84.076	-156.5	0.00	0.230
	P assim.		P ret.	4	82.483	-155.9	0.06	0.170
	P assim.	P net.		4	81.988	-154.9	1.59	0.104
	P assim.	P net.	P ret.	5	83.272	-154.9	1.61	0.103
P acq.	P assim.	P net.		5	82.987	-154.3	2.18	0.077

Table 4 Relative importance of the explanatory variables (P-use and P-content) for predicting growth rate and competitive ability. Number of models in which each variable was represented, for the top models with ΔAIC≤6.

Growth rate as dependent variable					
	P acq.	P assim.	P net.	P ret.	P content
Relative Importance (ΔAIC≤6)	0.51	1.00	0.46	0.74	0.21
Contained in top 13 models	7	13	8	8	6
Competitive ability as dependent variable					
	P acq.	P assim.	P net.	P ret.	P content
Relative Importance (ΔAIC≤6)	0.23	0.79	0.53	0.67	0.20
Contained in top 20 models	8	10	12	12	7

Figures

Figure 1 Univariate responses to P-supply conditions in 10 genotypes of *Daphnia pulicaria*. Phosphorus treatment is along the x-axis and phenotypic trait measured along the y-axis (P content (a), P kinetics (b-e), and fitness-relevant traits (f-h)). HP – high phosphorus; LP – low phosphorus. The symbol * indicates a significant genotype-by-treatment interaction with $p<0.01$, ** indicates $p<0.001$, and *** $p<0.0001$.

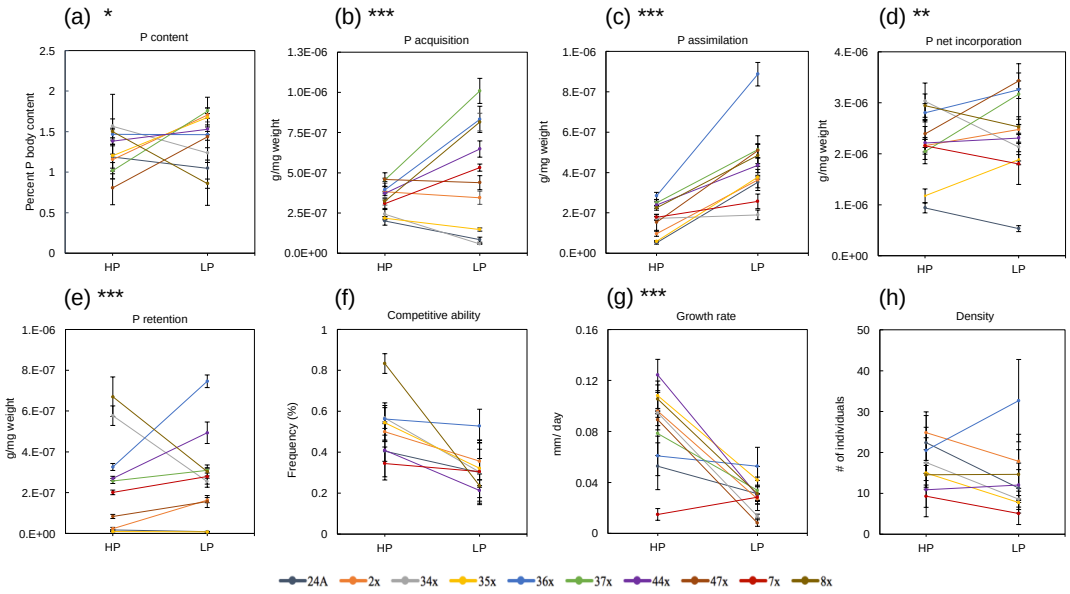


Figure 2 Linear regressions testing predictions based on ecological stoichiometry. Linear relationship between (a) somatic P content and competitive ability; (b) somatic P content and growth rate; and (c) growth rate and competitive ability. Open circles represent Low Phosphorus (LP) treatments; solid circles represent High Phosphorus (HP) treatments. Competitive ability of genotypes was not correlated with somatic P content ($p=0.267$; $R^2=0.01$). P content was not significantly correlated with growth rate ($p=0.144$; $R^2=0.06$). Competitive ability was significantly correlated with growth rate ($p<0.0001$; $R^2 = 0.28$).

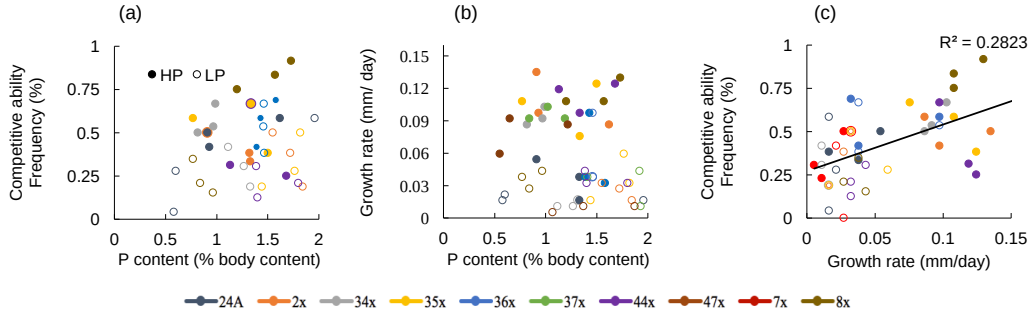


Figure 3 P content and P-use linear relationships, (a) P acquisition, (b) P assimilation, (c) P net incorporation, (d) P retention. Open circles represent Low Phosphorus (LP) treatment; solid circles represent High Phosphorus (HP) treatment.

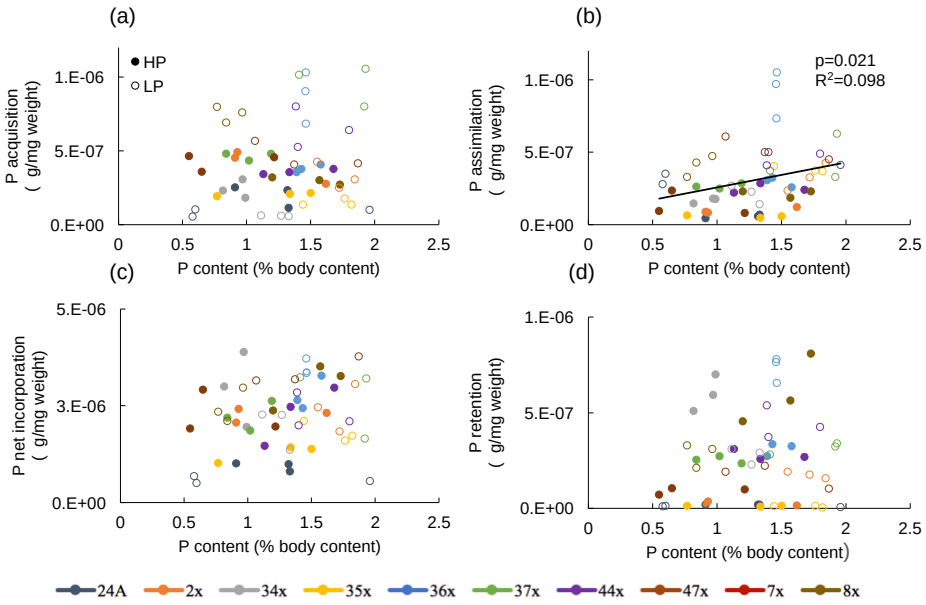
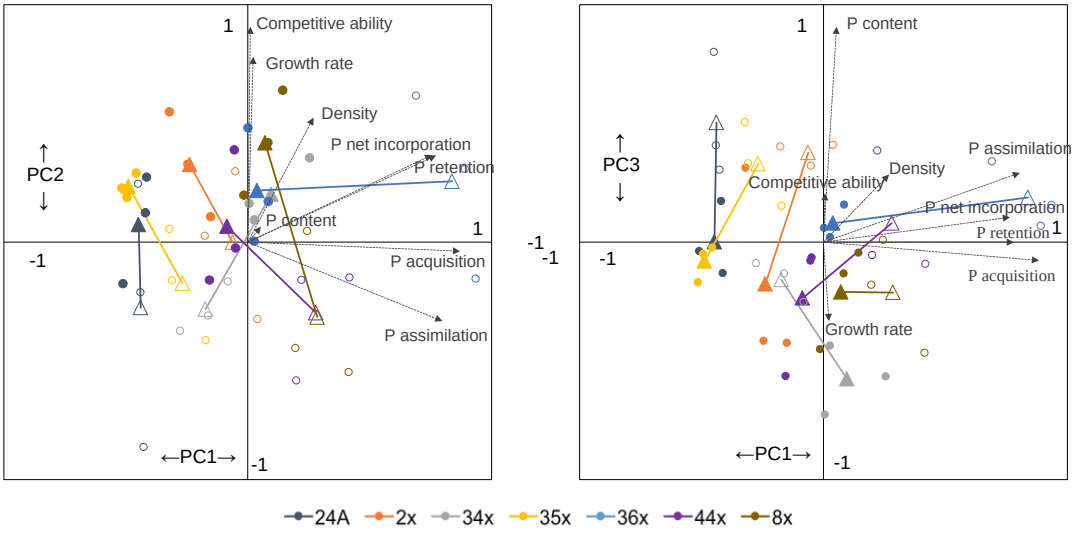


Figure 4 Principal component analysis plots showing as solid lines, the phenotypic trajectories of *Daphnia pulicaria* genotypes (multivariate response to P supply conditions for P content, P kinetics, growth rate, and competitive ability traits). PC1 plotted on the x-axis against (a) PC2 and (b) PC3 on the y-axis. Open shapes represent Low Phosphorus (LP) treatment and solid shapes represent High Phosphorus (HP) treatment. Circles are the raw data values, while triangles represent centroids. HP and LP centroids are connected by a line to show the directionality between HP and LP conditions. Percentage of the total variation explained by the first three axes of the PCA was ~74%. P-use traits explained the most variation on PC1, explaining ~34% of the total variation, with PC2 explaining ~26% of the variation and PC3 explaining ~15%.



Supplementary information

Figure S1 P-use linear relationships. Open circles represent Low Phosphorus (LP treatment; solid circles represent High Phosphorus (HP) treatment. All are significantly correlated ($p<0.0001$). Solid trend lines show significant relationships in HP, and dotted trend lines show significant relationships in LP. All units are represented in micrograms per milligram dry weight.

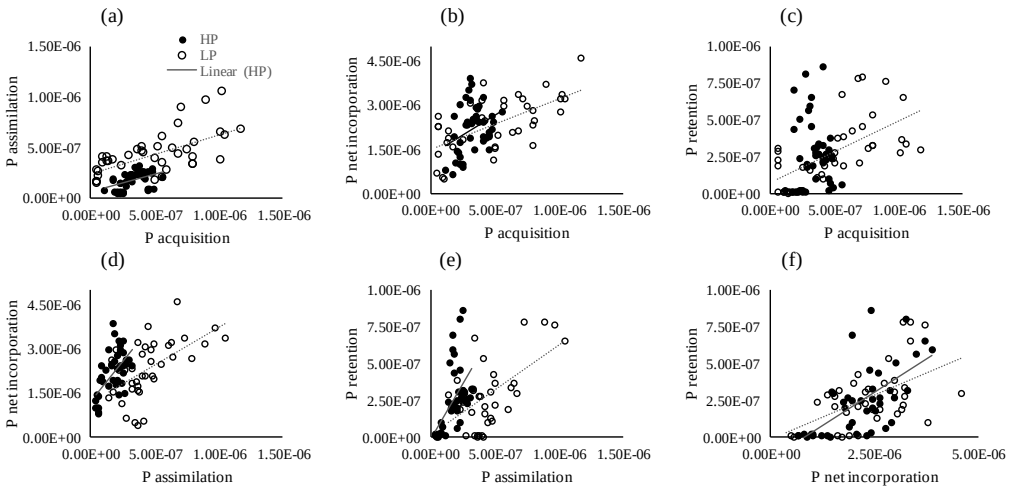


Table S1. Descriptive statistics of all the traits measured, including mean, standard deviation, sample size, and number of samples excluded (pairwise), from the multivariate analyses (PCA).

Descriptive Statistics				
Trait	Mean	Std. Deviation	Analysis N	Missing N
Growth rate	0.056	0.040	80	16
Competitive ability	0.062	0.018	48	48
Density	15.312	10.373	48	48
P content	0.123	0.030	53	43
P acquisition	<0.001	<0.001	91	5
P retention	<0.001	<0.001	85	11
P net incorporation	0.002	<0.001	86	10
P assimilation	<0.001	<0.001	87	9

Table S2. Principal component analysis of *Daphnia pulicaria* responses to altered P supply conditions.

Rotated Component Matrix			
Trait	Component		
	1	2	3
P acquisition	0.883	-0.039	-0.074
P assimilation	0.806	-0.331	0.294
P net incorporation	0.766	0.367	0.107
P retention	0.781	0.366	0.007
Competitive ability	0.006	0.905	0.209
Growth rate	0.019	0.775	-0.332
Density	0.271	0.522	0.287
P content	0.053	0.066	0.916

Total Variance Explained			
Component	Eigenvalue	% of Variance	Cumulative %
1	3.002	33.781	33.781
2	1.867	25.958	59.739
3	1.090	14.745	74.485

Table S3 Model selection table with competitive ability as dependent variable.

P acq.	P assim.	P net.	P ret.	P content	df	Log Lik	AICc	ΔAIC	weight
	477500		-511000		4	2.592	3.9	0.00	0.231
	412100	-128700			4	2.064	5.0	1.06	0.136
	498200	-67710	-341200		5	3.262	5.1	1.24	0.124
	515300		-538100	-0.22720	5	2.696	6.3	2.38	0.070
-46000	506000		-500100		5	2.618	6.4	2.53	0.065
		-86010			3	-0.412	7.5	3.56	0.039
35110	391400	-132100			5	2.077	7.5	3.61	0.038
	415400	-129200		-0.02435	5	2.065	7.5	3.64	0.037
			-302500		3	-0.494	7.6	3.72	0.036
	536700	-67870	-368300	-0.23060	6	3.373	7.7	3.76	0.035
33520	478400	-70940	-341000		6	3.275	7.8	3.95	0.032
256400		-126200			4	0.554	8.0	4.08	0.030
208700			-408500		4	0.194	8.7	4.80	0.021
-92570	587000		-526300	-0.31220	6	2.788	8.8	4.93	0.020
					2	-2.352	9.0	5.11	0.018
		-52360	-164100		4	-0.147	9.4	5.48	0.015
		-83430		0.34460	4	-0.159	9.4	5.50	0.015
287600		-85320	-223200		5	1.055	9.6	5.66	0.014
			-289000	0.29110	4	-0.317	9.7	5.82	0.013
269700		-125200		0.40450	5	0.918	9.8	5.93	0.012

Table S4 Model selection table with growth rate as the dependent variable.

P acq.	P assim.	P net.	P ret.	P content	df	Log lik	AICc	ΔAIC	weight
52550	-128900		67660		5	84.076	-156.5	0.00	0.230
	-96270		80090		4	82.483	-155.9	0.06	0.170
	-86340	20360			4	81.988	-154.9	1.59	0.104
	-99630	10950	52630		5	83.272	-154.9	1.61	0.103
44910	-112900	16070			5	82.987	-154.3	2.18	0.077
45150	-126400	6599	52860		6	84.349	-154.3	2.19	0.077
57420	-137400		70390	0.03262	6	84.166	-153.9	2.55	0.064
	-92920		77700	-0.02008	5	82.519	-153.4	3.11	0.048
	-79800	19320		-0.04772	5	82.204	-152.7	3.74	0.035
	-96370	10940	50340	-0.01953	6	83.308	-152.2	4.27	0.027
74760	-108000				4	80.579	-152.1	4.41	0.025
43060	-110300	16010		-0.01111	6	82.997	-151.6	4.89	0.020
49410	-133100	6206	55880	0.02558	7	84.403	-151.5	4.97	0.019

CHAPTER II

PHOSPHORUS USE EFFICIENCY OF THE KEYSTONE AQUATIC HERBIVORE *DAPHNIA PULICARIA* IS ASSOCIATED WITH THE PHOSPHOGLUCOSE ISOMERASE LOCUS

Ryan E. Sherman, Rachel Hartnett, Emily L. Kiehnau, Lawrence J. Weider, and Punidan D. Jeyasingh

Abstract

The phosphoglucose isomerase (*PGI*) locus underlies population genetic responses to various ecological challenges. Previous work has shown *PGI* homozygotes of the freshwater grazer, *Daphnia,* exhibit higher phosphorus (P) use efficiency (PUE) and were competitively superior compared to heterozygotes under P-limiting conditions, however, these studies were confounded by unknown genetic background and evolutionary history. We constructed an F2 recombinant *D. pulicaria* population and found a strong effect of *PGI* genotype on an important life-history trait, hatching time of resting/diapausing eggs. Further, F2 recombinants exhibited substantial variation in %P and PUE. Homozygotes exhibited higher PUE compared to heterozygotes. Lower glycolytic efficiency in homozygotes is posited to be advantageous under low P-supply conditions that generate an algal diet with high carbon: P ratios, where disposal of excess carbon is at a premium. These observations indicate important links between energy and P metabolism in influencing the efficiency of biomass production. While such ecologically-important quantitative traits are undoubtedly under polygenic control, *PGI* appears to play a role (i.e., likely as a neutral/quasi-neutral marker). Understanding spatiotemporal dynamics of *PGI* polymorphisms in ecologically important taxa like *Daphnia*, may inform theory predicting shifts in key ecological functions, particularly in the context of anthropogenic environmental changes such as eutrophication.

Introduction

Understanding how populations respond to ecological challenges has been a central theme throughout the history of ecological physiology [1,2]. Seminal work has identified the interactions between polymorphism at the central metabolic gene locus, phosphoglucose isomerase (*PGI*; EC 5.3.1.9), a vital enzyme catalyzing the second step of glycolysis when glucose 6-phosphate is converted into fructose 6-phosphate, and environmental variables in arthropods (reviewed in [3]). For example, allelic variation at the *PGI* locus alters the specific activity and thermal stability of the enzyme, impacting the distribution and thermal ecology of *PGI* variants (e.g., [4–10]). Given the critical role of *PGI* in central metabolism, it is not surprising that polymorphisms at this locus have been found to be associated with a variety of environmental parameters that impact organismal energy budgets (reviewed in [11]).

The importance of such work has steadily magnified as global temperature and other environmental variables change at a rapid pace [12], triggering rapid evolution in key traits that can feedback to impact eco-evolutionary dynamics [13]. As such, integrating lower order processes to higher order ecology is a necessity [14–16]. The framework of ecological stoichiometry (ES; [17]) uses information on energy and key elements (i.e. nitrogen - N and phosphorus - P which are required for protein synthesis and ribosome biogenesis,) to make predictions about rates of biomass production, and excretion of N and P, using the principle of mass balance.

Briefly, because many organisms have species-specific growth rates, they also differ in the concentration of N and P in their bodies (i.e., a given organism's elemental stoichiometry), which impacts the rates at which these elements are excreted, and thus can alter nutrient availability for primary producers (e.g., algae - reviewed in [18]). For example, Elser et al. (1988) [19] found that fast-growing keystone aquatic herbivores like the cladoceran, *Daphnia*, contain

more P compared to slow-growing copepods, and thus recycle P at slower rates. Importantly, a shift in the zooplankton community from copepods to *Daphnia* altered limitation of primary production from N- to P-limitation, thereby linking species-specific physiology to ecosystem processes.

There is growing evidence that intraspecific (i.e., within population) variation in growth and stoichiometry can be considerable in a variety of taxa, often rivalling interspecific differences (e.g. [20–27]), and can alter nutrient supply to algae [28]. It is likely that such variation interacts with environmental changes to impact the fitness of genotypes, although there is still much to learn about the genetic and physiological basis of these interactions [29,30]. Paleogenetic studies have found correlations between the frequency of *PGI* genotypes and the amount of P in a lake [31]. Laboratory experiments with *Daphnia* have revealed that allozymic variants at the *PGI* locus [32] interact with environmental P supply to impact growth and fitness [22,33,34]. Specifically, heterozygotes at *PGI* exhibited faster growth rates (and outcompeted homozygotes) under high P-supply conditions, while homozygotes grew faster and outcompeted heterozygotes under low P-conditions. The authors argued that the mechanism underlying such an interaction is because of lower glycolytic efficiency in *PGI* homozygotes [35], which may be advantageous in low P conditions to dispose of excess dietary carbon associated with P-limited algae [36,37]. In other words, the efficiency with which assimilated P is converted to biomass differs among *PGI* variants [22].

However, these results should be interpreted with caution because of low genotypic replication, and unknown evolutionary histories of the genotypes sampled. Indeed, a paleogenetic study of a *Daphnia* population inhabiting a lake that experienced striking changes in P-loading due to anthropogenic impacts did not show shifts in *PGI*, although strong population genetic structuring (based on microsatellites) was found [38]. Further, transcriptomic analyses have illuminated the complexity associated with physiological adjustments made by *Daphnia* to

differing P-supply [39,40]. Clearly, in the context of the post-genomic era, aiming to identify all the nucleotides that underlie ecologically-relevant variation [41], much remains to be understood about how variation in P-supply shapes the evolution of P-use physiology in metazoan populations living in their natural habitat. Nevertheless, harnessing prior observations about key polymorphisms such as *PGI*, and robustly deciphering their impact on ecologically-relevant traits should continue to inform us [42].

Methods in quantitative genetics enables us to partition phenotypic variation of fitness-related traits in populations (e.g., nutrient-use efficiency) into genetic and environmental components, and track their evolutionary trajectories [43]. Such a design enables us to test the effects of *PGI* genotype on P-use efficiency of *Daphnia* with high genotypic power, while controlling for effects of evolutionary history. To this end, we developed a first-generation F2 mapping panel with ~230 *D. pulicaria* genotypes by crossing a F0 *PGI* heterozygote and a F0 *PGI* homozygote. The resulting heterozygous F1 *PGI* genotype was selfed to produce the F2 lineages. Given the important role of *PGI* in central metabolism, and differences in the enzyme kinetics and energetics of heterozygotes and homozygotes, we expected significant effects of *PGI* genotype on key traits, including: (a) resting-egg hatching phenology, (b) growth rate, (c) phosphorus content, and (d) phosphorous-use efficiency (PUE). We predicted that *PGI* heterozygotes, with higher glycolytic efficiency, would hatch faster than either homozygote. Further, higher glycolytic efficiency of heterozygotes should enable them to grow faster under high phosphorus conditions, and consequently maintain higher phosphorus levels in their bodies, when compared to homozygotes. Finally, we predicted that lower glycolytic efficiency of *PGI* homozygotes would enable them to use phosphorus more efficiently in low phosphorus conditions compared to heterozygotes.

Methods

Constructing the *Daphnia pulicaria* F2 recombinant population

The typical *Daphnia* breeding system is termed cyclical parthenogenesis. It is characterized by an asexual phase of reproduction during the growing season in which females produce clutches of (apomictic) parthenogenetic direct-developing diploid eggs. A switch to sexual reproduction, which includes the production of males (via environmental sex determination) and sexually-receptive females, occurs when environmental conditions deteriorate (e.g., shortened photoperiod signaling on-set of winter). The diploid males produce haploid sperm which fertilize the haploid eggs of the females. Successful matings result in the production of diploid resting eggs, which are encapsulated in a durable sclerotized structure termed an ephippium. For most daphniid species, ephippia are produced sexually with females hatching from resting eggs. Once these hatchlings mature, they start the asexual cycle again.

The F0 lineages used to construct the recombinant *Daphnia pulicaria* population consisted of a female (dam) clone hatched from a ~5-10 year-old resting egg and a male-producing (sire) clone hatched from ~40-50 year-old resting egg isolated from a cyclically parthenogenetic population of *Daphnia pulicaria* inhabiting South Center Lake, Minnesota [38]. These F0 lineages were sexually crossed to produce the F1, which was subsequently propagated parthenogenetically in the laboratory. The F1 clone was induced to produce males under "stressful" conditions (i.e., raised under high population densities in "crowding" water – L.J. Weider, unpublished data). F1 males were crossed with F1 females to produce potential F2 resting eggs (ephippia). F2 ephippial eggs were carefully removed from their casings and eggs (i.e., usually paired) from each ephippium were placed in 24-well cell culture plates containing 1-2 mL of sterile COMBO medium per well [44]. The eggs were then subjected to hatching cues (i.e., incubation for two weeks in cold/dark conditions at 4^{o}C followed by warm/light conditions

at 20ºC) to establish the F2 clone bank. Approximately 7000 F2 eggs were submitted to this hatching protocol. Eggs were isolated and hatched from December 2014 until July 2016. (Note: we recorded the time in days that it took each F2 egg/clone to hatch, after being exposed to the warm/light conditions). The current F2 mapping population consists of ~230 recombinant clonal lines; these clones were maintained in a temperature-controlled growth chamber at 20ºC with an 18:6 hr light:dark cycle in COMBO containing no N or P, and fed the green algae, *Scenedesmus obliquus,* cultured in continuous flow chemostats at stable state.

PGI genotyping

Cellulose-acetate allozyme electrophoresis [32] was used to characterize *PGI* genotypes. Genotyping was based on "fast-slow" electromorphs (e.g., fast-fast, slow-slow are homozygotes; fast-slow are heterozygotes). Electromorphs were scored based on migration distance on the cellulose acetate plates (on a scale of 1-4; with 4 being fastest). The ~5-10 year-old F0 dam clone is a *PGI* heterozygote ("1,4" genotype), while the ~40-50 year-old F0 sire clone is a *PGI* homozygote ("1,1" genotype). These F0 lineages were crossed to produce the F1 clone that was a heterozygote ("1,4"). We genotyped 228 F2 clones (selected randomly) at the *PGI* locus. Two clones of a sister taxon, *D. pulex*, with known *PGI* genotypes [33] were run on each cellulose acetate plate to serve as standards.

Generation of high and low phosphorus algae

Scenedesmus obliquus algae was grown in continuous flow chemostats in COMBO media containing concentrations of either high phosphorus (HP; 50 µmol L^{-1}) or low phosphorus (LP; 5 µmol L^{-1}) [44] at 20ºC and constant light (~120 µmol m^{-2} s^{-1}). These P-supply conditions produced algae with a C: P ratio of ~100 (HP) and ~800 (LP), respectively. To determine algal carbon (C) content, *Scenedesmus* algae was filtered from experimental chemostats onto pre-combusted (550ºC for 2 hr) and pre-weighed GF/C filters (Whatman, Maidstone, U.K.), and dried

at 60 ºC for 72 hr. C content was determined using an automated CHNOS analyzer (Elementar VarioMICRO analyzer, NJ, U.S.A.). To determine algal P content, pre-weighed Whatman cellulose acetate membrane filters (0.45µm; GE Healthcare Life Sciences, Pittsburgh, PA, USA) containing algal samples were digested with a 2:1 *v/v* solution of trace metal grade nitric acid and hydrogen peroxide for at least 24 hr in 15 mL polypropylene tubes, and then diluted to a volume of 10 mL with ultrapure (Type 1) water. Digested samples were then analyzed through Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; Thermo Scientific iCAP 7400, Waltham, MA). Validation and calibration of the ICP-OES was achieved by using multi-element external reference standards (CCV Standard 1, CPI International, Santa Rosa, CA, USA). Additionally, an in-line Yttrium internal standard (Peak Performance Inorganic Y Standard, CPI International, Santa Rosa, CA, USA) was used to correct for any instrument drift or matrix effects. We then converted mass specific carbon and phosphorus content into molar values for analyses.

Measurement of *Daphnia* growth rate

To determine juvenile growth rate (JGR), *D. pulicaria* were raised individually in 100 mL of COMBO media containing no N or P, and fed 1 mg C L^{-1} per day of either HP or LP *Scenedesmus* algae. Growth rates of genotypes were measured over a three-day period under either HP or LP conditions. The length (mm) of each neonate was measured from the top of the head to the base of the tail-spine on day zero and day three of the experiment using a microscope at 4x magnification (Leica S8APO, Leica Microsystems, IL, USA). Growth rate was calculated by the difference in length from day zero to day three, divided by the number of days and corrected for initial (day zero) length. This protocol was replicated six times for each genotype for each P-supply treatment. We calculated the difference in growth rate between P-supply treatments by subtracting the growth rate of *Daphnia* under LP supply conditions from the growth rate under HP conditions.

Measurement of *Daphnia* phosphorus use efficiency (PUE)

To determine %P and PUE, *Daphnia* were raised in the same environment as outlined above for JGR, however only under the LP algal supply treatment. Three-day-old daphniids were dried in a drying oven at 60°C for at least 72 hr and weighed using a microbalance (Mettler Toledo XP2U). Total phosphorus content and PUE of the *Daphnia* were quantified by a modified sulfuric acid digestion method (APHA 1992) and verified using a spinach standard (NIST 1570a). PUE is defined as the amount of biomass per unit of somatic P concentration under P-limiting conditions where PUE = M/P_C, where, M = dry mass (mg), P_C = P concentration of dry biomass (mg). P-traits were quantified in triplicate per clone.

Statistical analysis

A Kolmogorov-Smirnov test was used prior to analysis to test response variables for normality; all variables, with the exception of difference in growth rate between P-supply treatments, did not meet the assumptions of linear models and were log-transformed prior to analysis of either general linear models (GLMs) or general linear mixed models (GLMMs) to meet normality assumptions. To test the effects of *PGI* genotype on hatching phenology and JGR, GLMs were used followed by a Tukey's post-hoc test of differences between *PGI* genotype. A GLMM with *Daphnia* clone as a random effect and P-supply treatment as a fixed effect, was used to test the interactive effect of *Daphnia* clone and P-supply treatment on JGR, as well as effect of *Daphnia* clone on difference in growth rate between P treatments. Following log transformation, %P and PUE data still did not fit the assumption of normality. Therefore, Kruskal-Wallis tests were performed to test the effect of *PGI* genotype on *Daphnia* %P and PUE. A Kruskal-Wallis test, the non-parametric equivalent to a one-way analysis of variance (ANOVA), uses rank-transformed data prior to analysis. A Dunn-Bonferroni post-hoc analysis was performed on this rank-transformed data to determine significant differences between *PGI* genotypes for %P and

PUE. Binomial logistic regression analysis was used to test the effect of *PGI* on the difference in JGR between HP and LP algal treatments, categorized binomially as a positive or negative growth response. A linear regression model was used to compare the correlation between %P and growth rate among all F2 clones and by *PGI* genotype. All tests were performed using SPSS (IBM Statistics v. 22).

Results

Approximately 7000 F2 eggs were produced, out of which ~1700 were hatched, and 280 hatchlings were successfully established as clonal lines. We genotyped 228 of these 280 F2 recombinants for *PGI*. There was a highly significant difference in resting egg hatching phenology among the three *PGI* genotypes (Fig. 1). *PGI* "1,1" clones tended to hatch most rapidly, while *PGI* "4,4" clones took the longest to hatch. The heterozygous "1,4" clones were intermediate in hatching phenology, as predicted by an additive model of gene action. These results are contrary to our original prediction of *PGI* heterozygotes hatching faster due to higher glycolytic efficiency. Post-hoc analysis (Tukey) revealed *PGI* heterozygotes were significantly different from each of the homozygotes and likewise, the two homozygous genotypes differed significantly from each other in hatching time ($F_{2, 225} = 11.001$; $p < 0.001$).

We were unable to measure all three traits (i.e. JGR, %P, PUE) on all F2 recombinant clones genotyped for *PGI*. Moreover, there were a few F2 clones where we had trait measurements, but were unable to genotype them due to loss of clonal lines. We quantified JGR of 121 clones of which we knew the *PGI* genotype for 109. We quantified %P and PUE for 120 clones of which 116 were genotyped for *PGI*.

Cellulose acetate allozyme electrophoresis revealed 131 "1,4" heterozygotes at *PGI* and 97 homozygotes (38 "1,1" + 59 "4,4") of the 228 F2 clones screened. Frequencies of these genotypes were as follows: "1,1" =0.167, "1,4" =0.575, and "4,4" =0.259. These frequencies

deviated from Hardy-Weinberg expectations of a 1:2:1 ratio with heterozygote excess (df =1; critical chi-square value = 5.755>5.025; p<0.025). There was a significant effect of treatment on JGR in the 109 clones assayed (15 "1,1" + 30 "4,4" + 64 "1,4"; $F_{1,1312}$ =28.688; p<0.001), although JGR was not significantly different among *PGI* genotypes ($F_{2,1312}$ =0.869; p=0.153; Fig 2b). Average difference in growth rate between the P treatments showed a significant overall clone effect ($F_{120,1354}$ =1.606; p<0.001; Fig 2a). Logistic regression analysis revealed no effect of *PGI* genotype on growth rate response (positive or negative difference in growth rate between HP and LP) under P treatments (Table S1).

%P among the 116 clones (16 "1,1" + 29 "4,4" + 71 "1,4") ranged from 0.49% to 1.97%, with a mean of 1.17% (Fig 3a), while PUE ranged from 51 to 208 mg biomass/mg P with a mean of 103 (Fig 4a). We found a significant effect of *PGI* genotype on %P and PUE (Figs. 3b & 4b). %P and PUE were significantly lower in "1,1" homozygotes compared to heterozygotes. There was no significant difference in %P between "4,4" and "1,4" homozygotes nor between "1,1" and "4,4" (Fig. 3b). There was no significant correlation between growth rate and %P for the entire dataset (R^2 = 0.0166, p=0.171; Fig. S1), or for each *PGI* genotype (Fig. S2, test statistics reported in Table S2).

Discussion

Measurement of ecologically-relevant traits in this *Daphnia pulicaria* F2 mapping population revealed the power of recombination in generating substantial phenotypic diversity. Allelic variation at *PGI* impacting efficiency of central (energetic) metabolism, was correlated with such phenotypic diversity.

Variation at *PGI* impacts traits such as parthenogenetic egg production, with important population genetic consequences [45]. Specifically, deviations from Hardy-Weinberg Equilibrium (HWE) at the *PGI* locus in *Daphnia* have been shown to be common, appearing most often as

heterozygote excesses [46], as was the case for our study. Additionally, we found fewer "1,1" homozygotes in the F2 population than would be expected by HWE, suggesting fitness differences. Such lower frequency of the "1,1" homozygotes is surprising since they hatched earlier than the "4,4" homozygote and the heterozygote (Fig 1). As such, earlier hatching time does not appear to influence frequency of *PGI* genotypes, at least as measured in our laboratory setting.

PGI variants are maintained in populations because of differential fitness depending upon the environment, notably by life-history trade-offs in response to food stress involving resource allocation [47–49]. The interactions between food stress and genotype indicate that trade-offs occur across *PGI* genotypes and performance, which may help to explain the high variation found at this locus, along with rapid genetic changes [46,50,51]. *Daphnia* studies have suggested that ecological generalists and seasonal specialists may coexist within a single *Daphnia* population [50]. For example, previous work on *Daphnia* found *PGI* homozygotes produced a more thermally-stable form of the enzyme compared with heterozygotes [35], and were distributed predictably as a function of temperature in a lake [52]. In addition to temperature, changes in key ecosystem parameters such as phosphorus supply have been found to shift *PGI* genotype frequencies. Specifically, Mort and Jacobs (1981) [53] found that P enrichment of a lake increased the frequency of *PGI* heterozygotes from 80% up to almost 98% in the span of only a few weeks. Similarly, Weider et al. (1997) [31] used a paleogenetic approach and found significant correlations among total P in Lake Constance (Germany) and the frequency of *PGI* alleles represented in the resting egg bank of *Daphnia galeata* over a 30-year period. Because P-supply to lakes impacts C:P content of the algal diet of *Daphnia* [18], differences in glycolytic efficiency among electromorphs may interact with P-supply to impact fitness (i.e., presence of genotype-by-environment interaction; [22,33]). *PGI* homozygotes have lower specific activity, thus some energy is lost as heat [35], and should be advantageous in low P-supply conditions that

generate algae with high C:P diet, where there is a premium to dispose of excess C [36,37,54].

Such microevolutionary dynamics could have important ecological implications because *Daphnia* are P-rich consumers that exhibit rapid growth, and thus, play a pivotal role in whole lake P-cycling [55]. Moreover, their abundance can control phytoplankton growth, not only via direct grazing, but also indirectly via consumer-driven recycling of P [56]. Previous work has documented interspecific differences in *Daphnia* body %P ranging from ~0.8-1.9 %P [20,57]. We found that %P ranged from ~0.5-2.0% among the recombinants with *PGI* genotype underlying such differences (Fig. 3). Such substantial variation in P content among *Daphnia* genotypes of the same species, raised in a common garden, to our knowledge has not been previously reported. In addition to differences in P content, we found substantial variation in the efficiency at which assimilated P is converted into biomass (i.e. PUE), and significant effects of *PGI* genotype in explaining such variation. Simulation models of individual daphniids with increased PUE has shown to improve the *Daphnia*'s ability to survive and grow, whereas the assumption of constant nutrient ratios was unable to replicate experimental observations of *Daphnia* growth under nutrient-limited conditions [58]. Our observations are consistent with previous work reporting substantial variation in PUE among *Daphnia* genotypes [59], and lower PUE for *PGI* heterozygotes [22]. These results indicate that the quantity and quality of *Daphnia* biomass produced can be impacted by genetics, and feedback to impact P-supply to algae in microcosms [28], although we know nothing about such effects under natural conditions. Attention to *PGI* may be informative, especially for work that explores eco-evolutionary dynamics using the framework of ecological stoichiometry [30].

Intraspecific variation in growth and stoichiometry has been observed before in several taxa, including *Daphnia*, and appears to be correlated with rDNA structure [60]. The link between rDNA, P, and growth is formalized in the growth rate hypothesis (GRH; [61,62]). Briefly, the GRH posits that rapid growth is P intensive because of increased demand for P-rich

ribosomal RNA required for ribosome biogenesis, and thus protein assembly. Therefore, the GRH predicts a positive relationship between P and RNA quota, RNA quota and growth rate, and thus P quota and growth rate. Of the tripartite links of the GRH, the link between %P and growth rate was not evident in our study. It is important to note that this does not disprove the GRH, which proposes links at the biochemical level that manifest as patterns in organismal stoichiometry and growth, particularly at broad taxonomic scales [62]. As such, data on RNA quotas are required to directly test the GRH. It is possible our method to measure growth (using repeated length measurements on the same individual) was not sensitive enough to detect differences in growth compared to dry mass estimates on (clonal) sisters over time. Nevertheless, length-mass regressions on *D. pulicaria* isolated from several Minnesota lakes did not vary among genotypes and between HP and LP treatments, although significant size variation among clutch mates was observed (Jeyasingh *unpublished data*). Regardless, it should be noted that the GRH predicts associations among P, RNA, and growth (which is used as a surrogate for protein synthesis). As such, protein data may be needed to pick up finer scale differences in growth among genotypes [63].

Our observations on the effects of *PGI* genotype on %P and PUE indicate the importance of considering the energetic costs of protein synthesis in the context of the GRH. Peptide assembly is energetically expensive (i.e., four ATP molecules per bond are required) and dominates energetic budgets of fast-growing cells [64]. Thus, differences among *PGI* genotypes in ATP generation may impact the efficiency with which assimilated P is converted into proteins, and this may alter one or more relationships posited by the GRH. Indeed, several central metabolic enzymes, including *PGI*, have been identified as robust quantitative trait loci, often with strong pleiotropic effects, for carbon, nitrogen, and phosphorus metabolism in crops (e.g., [65–67]). We have yet to decipher the precise role of *PGI* on P-use efficiency and growth of ecologically-important taxa like *Daphnia*. Results reported herein, along with prior observations,

rationalize such work. Identifying the genetic basis of key parameters in stoichiometric models necessitates attention to energetics, together informing us about the evolutionary responses of biota to global environmental changes and potential feedbacks to ecology.

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Figures

Figure 1 Mean (± 1 S.E.) number of days prior to hatching of F2 *Daphnia* resting eggs. Letters above the bars represent significant ($p<0.05$) Tukey's post-hoc differences between *PGI* genotype as the result of a GLM ($F_{2, 225} = 11.001$; $p < 0.001$).

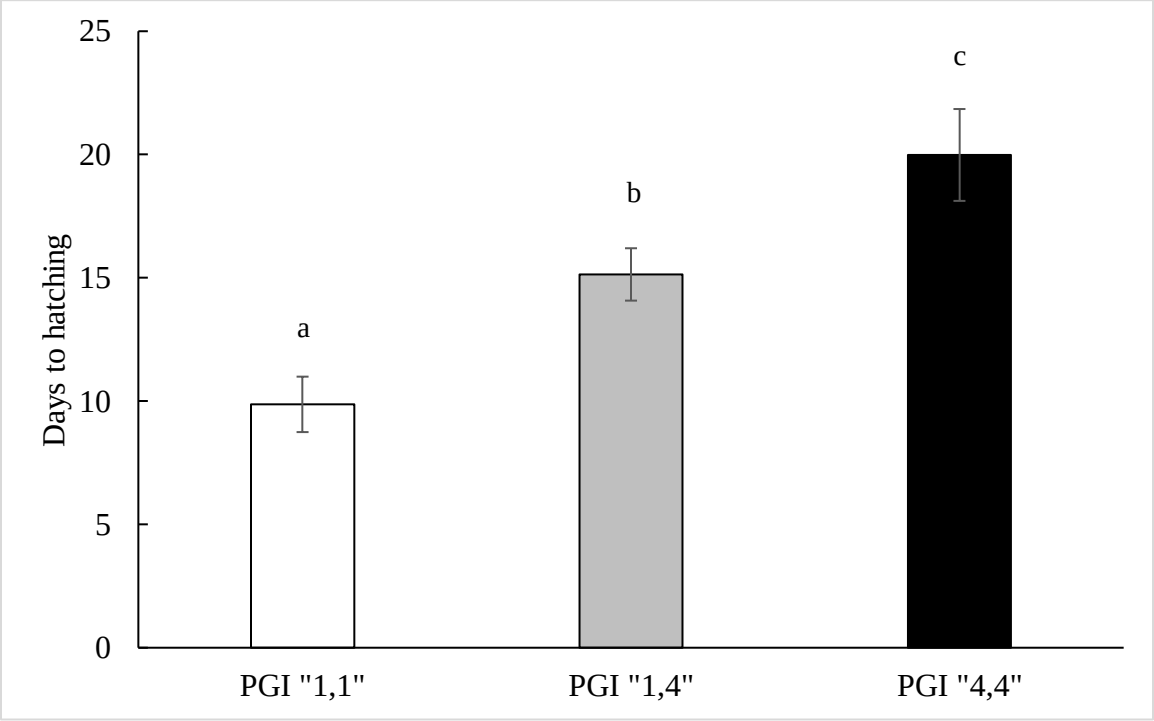
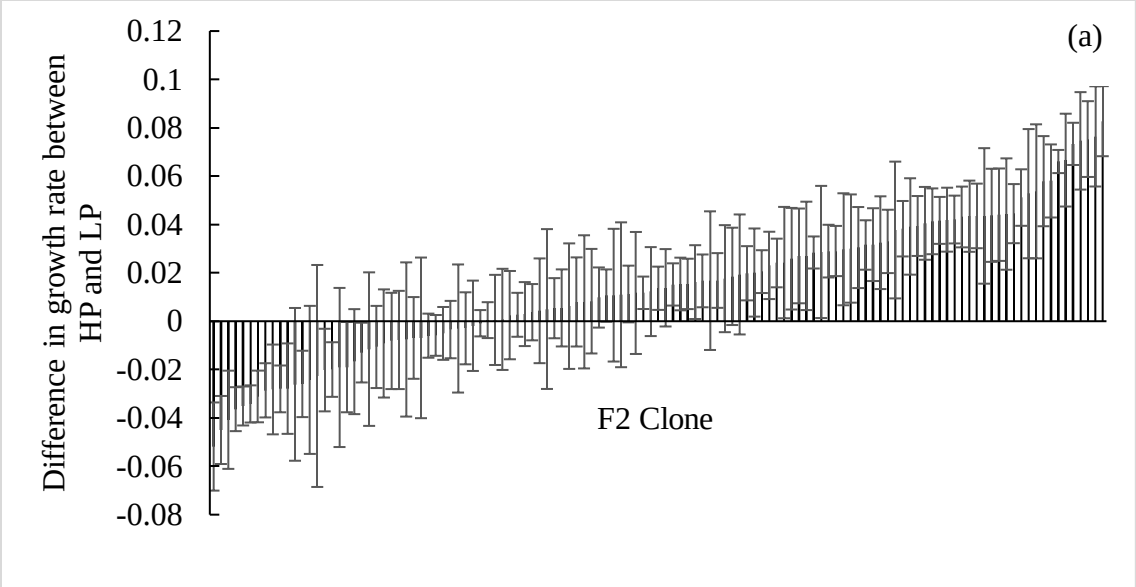


Figure 2 (a) Average difference in growth rate between high phosphorus (HP) and low phosphorus (LP) treatments for the F2 clones has a significant F2 clone effect as indicated by the GLMM ($F_{120,1354}$ =1.606; p<0.001). Difference in growth rate between P supply treatments were ordered from smallest to largest; negative values represent clones that had higher average growth rate under LP conditions compared to HP conditions. Errors bars represent 1 S.E. **(b)** Effect of P supply treatment and *PGI genotype* on mean (± 1 S.E.) growth rate. GLMs were used to determine P supply treatment effect ($F_{1,1312}$ =28.688; p<0.001), *PGI* genotype effect ($F_{2,1312}$ =0.869; p=0.153), and P treatment**PGI* effect ($F_{2,1312}$ =0.153; p=0.858).


(a)
Difference in growth rate between HP and LP
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
F2 Clone

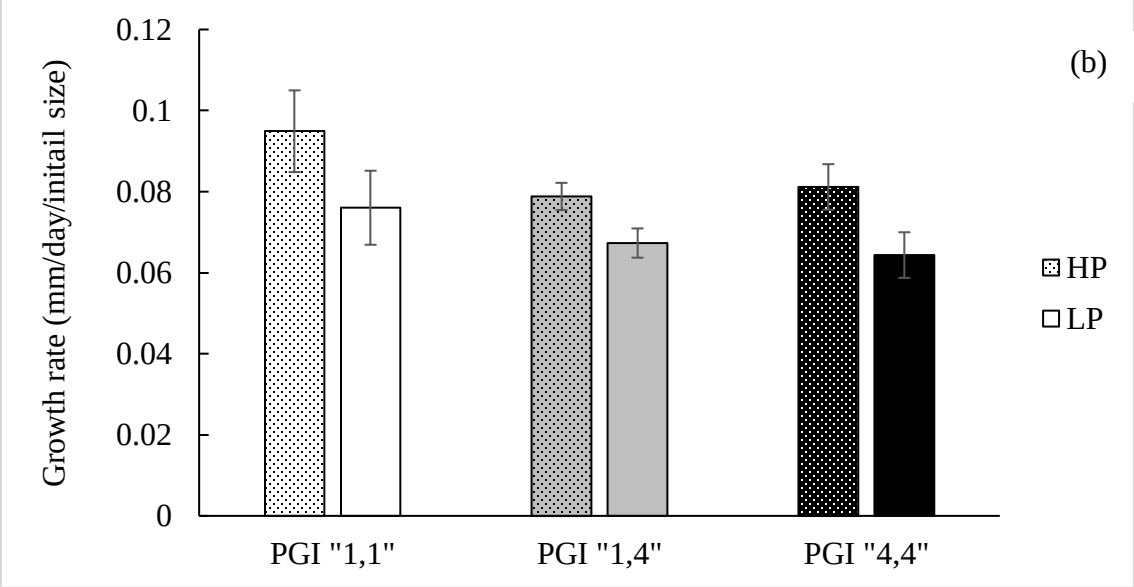
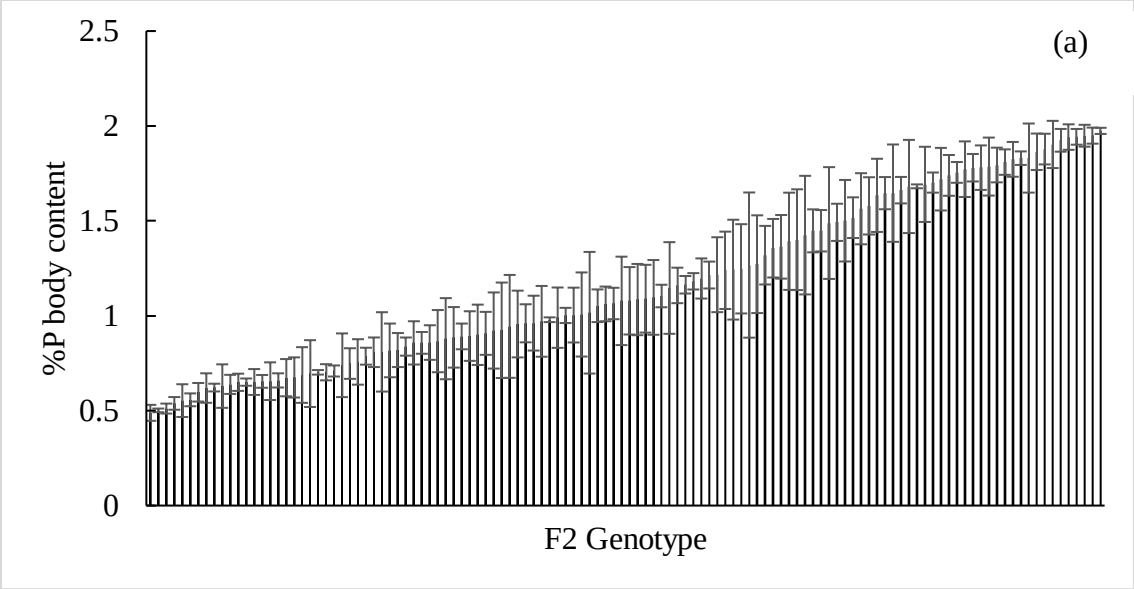

(b)
Growth rate (mm/day/initail size)
0.12
0.1
0.08
0.06
0.04
0.02
0
HP
LP
PGI "1,1"
PGI "1,4"
PGI "4,4"

Figure 3 (a) Mean percent phosphorus body content of F2 clones show a significant genotype effect as a result of a Kruskal-Wallis test ($p=0.009$). Errors bars represent 1 S.E. **(b)** Differences in mean (± 1 S.E.) %P between *PGI* genotypes. Letters above the bars represent significant ($p<0.05$) Dunn-Bonferroni post-hoc differences between *PGI* genotype as the result of a Kruskal-Wallis test ($p =0.009$) on rank-transformed data ("1,1" rank mean= 103.59, "1,4" rank mean= 141.57, "4,4" rank mean= 116.45).


(a)
2.5
2
1.5
1
0.5
0
%P body content
F2 Genotype

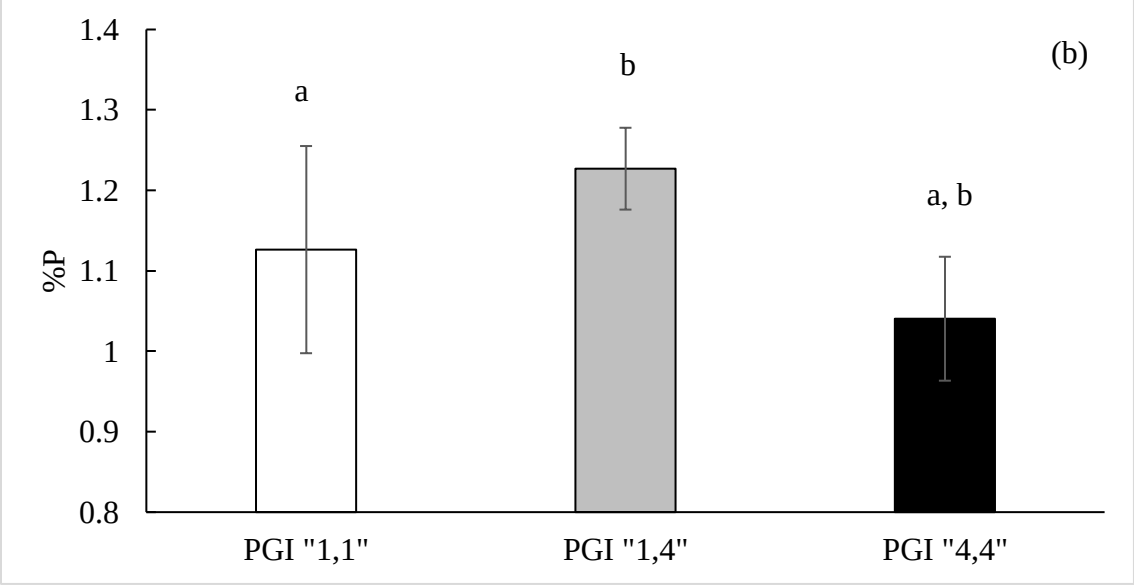
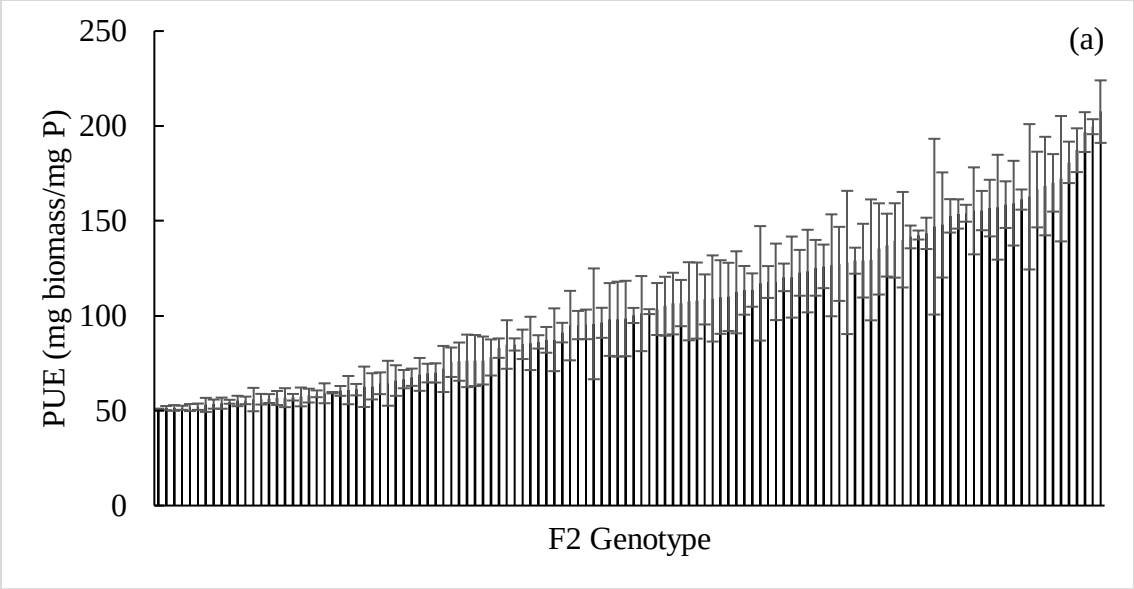
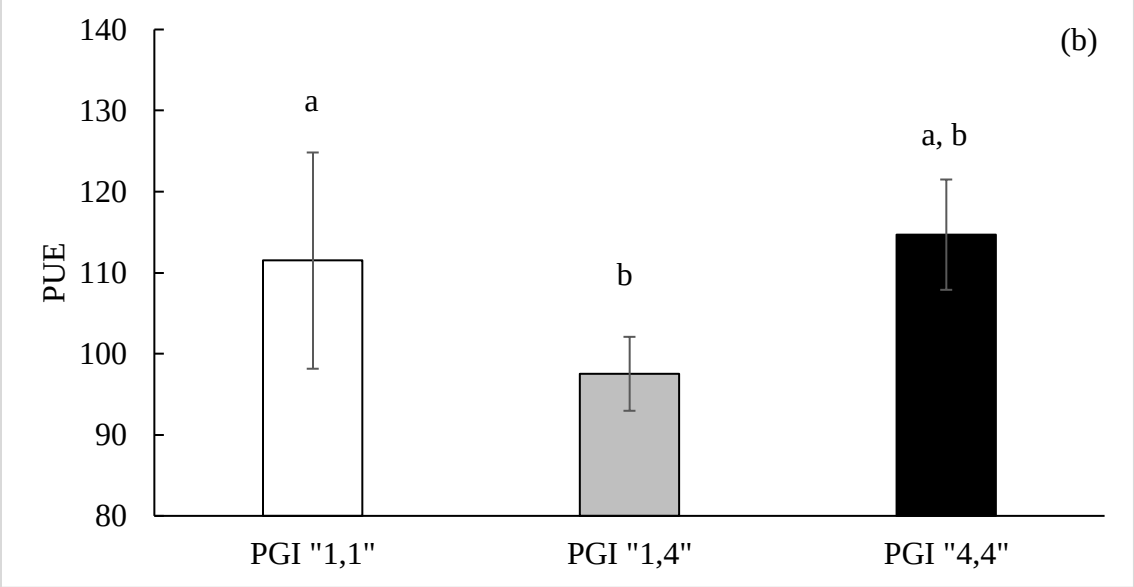

(b)
1.4
1.3
1.2
1.1
1
0.9
0.8
%P
a
b
a, b
PGI "1,1"
PGI "1,4"
PGI "4,4"

Figure 4 (a) Mean phosphorus-use efficiency (PUE) for F2 clones show a significant genotype effect as a result of a Kruskal-Wallis test ($p=0.009$). Errors bars represent 1 S.E. **(b)** Mean (± 1 S.E.) differences in PUE between *PGI* genotypes. Letters above the bars represent significant ($p<0.05$) Dunn-Bonferroni post-hoc differences between *PGI* genotype as the result of a GLM ($p=0.009$) on rank-transformed data ("1,1" rank mean= 158.41, "1,4" rank mean= 120.43, "4,4" rank mean= 145.55).


(a)
250
200
150
100
50
0
PUE (mg biomass/mg P)
F2 Genotype


(b)
140
130
120
110
100
90
80
PUE
a
b
a, b
PGI "1,1"
PGI "1,4"
PGI "4,4"

Supplemental information

Figure S1 Linear regression for JGR and %P for F2 recombinant *Daphnia* population, regardless of *PGI* genotype. There is no significant correlation between growth rate and %P (p=0.171)

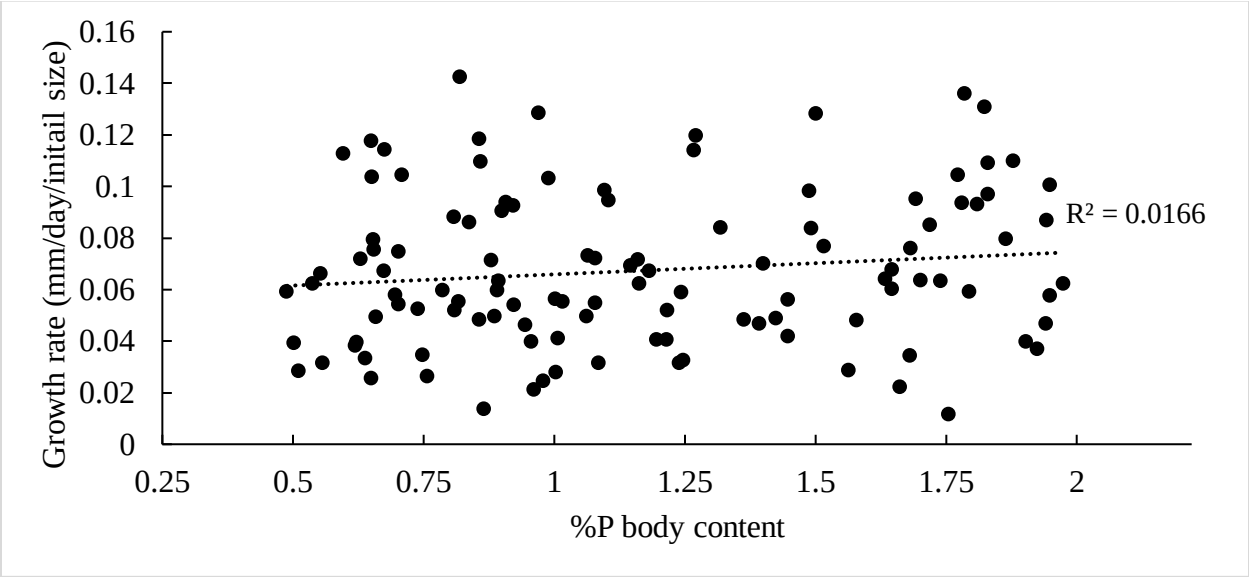


Figure S2 Linear regressions for %P and GR for each *PGI* genotype.

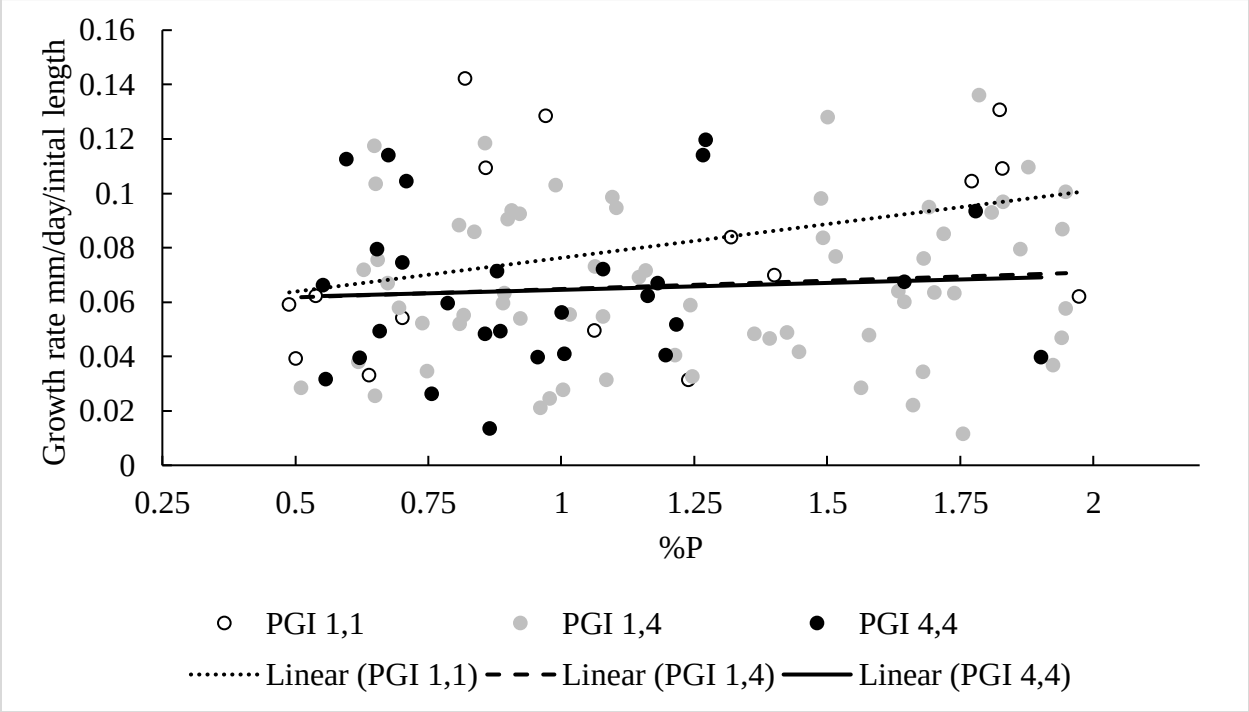


Table S1 Binomial logistic regression. There is no effect of *PGI* genotype on the growth rate response under P treatments (positive/negative difference in growth rate between HP and LP).

Predictor	B	SE	Wald's Chi squared	df	p	Odds ratio
PGI			0.203	2	0.903	
PGI (1)	0.164	0.707	0.054	1	0.816	1.179
PGI (2)	-0.110	0.475	0.053	1	0.817	0.896
Constant	0.847	0.398	4.523	1	0.033	2.333

Table S2 Linear regressions for %P and JGR

PGI genotype	F	df	p
1,1	2.570	1	0.133
1,4	0.417	1	0.521
4,4	0.118	1	0.734

Table S3 *PGI* genotype effect on days to hatching

GLM	F	df	p
PGI genotype	11.001	2	**<0.001**
Error		225	

Post-hoc (Tukey's)	*PGI* genotype		p
	1,1	1,4	**0.012**
	1,1	4,4	**<0.001**
	1,4	4,4	**0.016**

Table S4 Effect of P Treatment and *PGI* genotype on growth rate

GLM	F	df	p
Treatment	28.688	1	**<0.001**
PGI genotype	0.869	2	0.420
Treatment * *PGI* genotype	0.153	2	0.858
Error		1312	

Table S5 *PGI* genotype effect on %P and PUE

	%P		PUE	
GLM	df	p	df	p
PGI genotype	2	**0.009**	2	**0.009**

Post-hoc (Dunn-Bonferroni)	%P		PUE	
	PGI genotype	p	*PGI* genotype	p
	1,1 1,4	**0.046**	1,1 1,4	**0.046**
	1,1 4,4	1.000	1,1 4,4	1.000
	1,4 4,4	0.061	1,4 4,4	0.061

CHAPTER III

QUANTITATIVE GENETICS OF *DAPHNIA PULICARIA* ELEMENTAL CONTENTS

Ryan E. Sherman, Punidan D. Jeyasingh

Abstract

Ecological stoichiometry integrates traits of abundant taxa and the concentration of elements that encompass the bulk of biomass to make predictions about higher-order processes. Although there is substantial intraspecific variation in the contents of some elements, we know little about the nature of such variation. Similar work in autotrophs revealed that loci explaining the most variation in a given element can be co-localized for other elements, indicative of the fundamental interconnectedness among elements encompassing an individual (i.e. its ionome). We report data on variation in the ionomes of a recombinant F2 population of the dominant freshwater grazer, *Daphnia pulicaria*. We found substantial genotypic variation in ionomes. Correlations among pairwise element concentrations revealed several significant positive relationships, although the strength of correlations differed substantially. Genotypes significantly differed in population density, which was negatively related to the concentrations of most elements. These observations demonstrate the power of recombination in generating ionomic diversity, and the importance of considering elemental correlations while making functional inferences from elemental data.

Introduction

Trait-based approaches in community ecology focus on functional traits (e.g., morphology, physiology, life history) of species in order to understand the mechanisms by which species interact with each other and the abiotic environment (e.g., Litchman and Klausmeier 2008). Since all traits are constructed using energy and materials, ecological stoichiometry (ES; Sterner and Elser 2002), which aims to explain the distribution and abundance of taxa based on fundamental traits such as metabolic demands for energy and materials, is a particularly relevant framework (e.g., Meunier et al. 2017).

The foundations of ES can be ascribed to interspecific differences in elemental demands for growth that impact community structure and nutrient supply (e.g., Elser et al. 1988, Sterner et al. 1992). These observations catalyzed questions about evolutionary processes that underlie diversity in elemental contents and demand (e.g., Kay et al. 2005, Morehouse et al. 2010, Snell-Rood et al. 2015). Such work, focusing on key elements that are abundant in biomass (i.e. bulk elements such as carbon, nitrogen, phosphorus), has discovered important patterns.

Autotrophs at the base of food webs are more flexible in their carbon: nitrogen: phosphorus (C:N:P) stoichiometry, reflecting environmental supply of mineral elements, arguably due to storage mechanisms (e.g., vacuoles, polyphosphates) and the fact that C fixation continues during mineral limitation, compared to heterotrophic metazoans occupying higher trophic levels (Sterner and Elser 2002). Nevertheless, there is variation in the relative contents of C, N, and P among heterotrophic taxa that lack storage mechanisms for minerals (e.g., González et al. 2018), and this is thought to be related to the expression of key traits such as growth rate (Elser et al. 2003). Such discoveries have not only contributed to trait-based approaches in ecology, but have also illuminated the ecological relevance of evolutionary change (e.g., Jeyasingh et al. 2014).

Evolutionary change in any trait is made possible by genetic variation in a population. The underlying genetic variation in quantitative traits can have large impacts on the rates at which such traits

can evolve (Lynch and Walsh 1998). Intraspecific variation in the contents of bulk elements is substantial (e.g., DeMott et al. 2004, Bertram et al. 2008), although the design of previous studies precludes the isolation of genetic and environmental contributions to such variation (e.g., Sherman et al. 2017, in review). Similar work on model autotrophs such as *Arabidopsis* (Buescher et al. 2010) and crop plants (Zhang et al. 2014) resulted in rather surprising discoveries. Particularly, loci explaining the most variation in a given element is co-localized for other elements, which is indicative of the fundamental interconnectedness among elements (Baxter 2015).

Non-specificity and/or multi-element transporters are thought to underlie such correlated changes in multiple elements due to selection for efficient use of one element and is central to the field of ionomics (Salt et al. 2008). While understanding the mechanisms underlying such correlations is beyond the realm of ecology, information on the general tendencies of covariance of elements is clearly important because it impacts the biochemistry of biomass production at lower trophic levels and the stoichiometry of diet for higher trophic levels. We know little about the ecological importance of elements beyond those that are thought to commonly limit ecosystem productivity (Kaspari and Powers 2016, Jeyasingh et al. 2017, Penuelas et al. 2019). Furthermore, understanding the ecological consequences of evolutionary change in the content of an element often requires information on multiple elements (Jeyasingh et al. 2014, Leal et al. 2017).

To our knowledge, rigorous quantitative-genetic studies documenting the impacts of genetic recombination on the entire set of elements comprising an organism (i.e. its ionome; Salt et al. 2008) have not been done in ecologically important taxa. Here, we report data on ionomic variation in a recombinant F2 population of the dominant freshwater grazer, *Daphnia pulicaria.* This breeding design isolates the effect of genetic recombination, while controlling for effects of evolutionary history and, to some extent, the environment, on the contents of multiple elements among genotypes. We specifically aimed to find: (i) the extent of variation in elemental contents among recombinants, and (ii) identify correlations among elements. We expected significant genetic variation in the contents of some elements, although we

predicted that the magnitude of variation would be element-specific. Furthermore, we expected that some of this variation in elemental content and correlations among them would be explained by growth.

Methods

Constructing the Daphnia pulicaria F2 recombinant population

The F0 lineages used to construct the recombinant *Daphnia pulicaria* population consisted of a female (dam) clone hatched from a ~5-10 year-old resting egg and a male-producing (sire) clone hatched from a ~40-50 year-old resting egg isolated from a cyclically parthenogenetic population of *Daphnia pulicaria* inhabiting South Center Lake, Minnesota (Frisch et al. 2014). The generation of the F2 recombinant population is detailed in Sherman et al. (in review). Briefly, F0 lineages were used to produce the F1, which was subsequently crossed to produce the F2 population. These clonal F2 lineages were maintained in a temperature-controlled growth chamber at 20ºC with an 18:6 hr light:dark cycle in COMBO containing no N or P (Kilham et al. 1998), and fed the green algae, *Scenedesmus obliquus.*

Estimating genotype-specific population growth rate and carrying capacity

The following procedures were conducted on individual F0, F1 and F2 genotypes. Ten *D. pulicaria* neonates (<24-hours-old) were collected, and placed in a 100 mL jar of COMBO media and fed 1 mg carbon (C) L^{-1} day^{-1} of high phosphorus (C:P~150) *Scenedesmus obliquus* algae grown in continuous flow chemostats at 20ºC and constant light (~120 µmol m^{-2} s^{-1}). Neonates were grown for 5 days prior to density counts to limit the effect of early mortality on population growth rate. After 5 days, the neonates were transferred to 500 mL of COMBO and fed 1 mg C L^{-1} day^{-1}. These *D. pulicaria* cultures were maintained at ~20 ºC, and 16:8 light: dark cycle. Density counts were conducted three days a week and media changes conducted one day a week. These counts were conducted by filtering entire jars (using filter mesh) and the total number of *Daphnia* counted within the 500 mL jar. Following the 25 days in the 500 mL jar, final counts were conducted and *Daphnia* were sorted by size class (>710 µm;

425 µm to 710 µm). Filtered, size separated *Daphnia* samples were then dried in a drying oven at 60ºC for at least 72 hrs.

Daphnia ionome quantification

Ionomes were quantified from the dry biomass collected at the end of the experiment. Using an automated CHN analyzer (varioMicro Cube; Elementar Americas, Mt. Laurel, NJ, USA), we determined C and N concentrations for all *Daphnia* samples, while the concentrations of other elements was quantified using an automated ICP-OES analyzer (iCAP 7400; Thermo Scientific, Waltham, MA, USA) following previously published methods (Goos et al. 2017, Jeyasingh et al. 2017, Prater et al. 2018, Rudman et al. 2019) adapted for *Daphnia*. Briefly, *Daphnia* samples were digested in 15 ml trace metal-free polypropylene conical centrifuge tubes (VWR International, Radnor, PA, USA), then diluted to a final volume of 5 mL with ultrapure (Type 1) water. Validation and calibration of the ICP-OES was achieved by using multi-element external reference standards (CCV Standard 1, CPI International, Santa Rosa, CA, USA). Additionally, an in-line Yttrium internal standard (Peak Performance Inorganic Y Standard, CPI International, Santa Rosa, CA, USA) was used to correct for any instrument drift or matrix effects. Digestion blanks were also run to correct for background concentrations. Thirteen elements (B, Ca, Cu, Fe, K, Li, Mg, Mn, Na, P, S, Sr, and Zn) were above detection limits, and the concentrations of these elements along with C and N (in $\mu g\ g^{-1}$) were $\log_{10}$-transformed before statistical analyses.

Statistical analysis

Analysis of variance (ANOVA) was performed to determine the effect of F2 genotype and size class on the biomass (total dry mg *Daphnia*) at the end of the experiment. To account for multiple comparisons of the ionomes of *Daphnia* (i.e. 15 ANOVAs on individual element contents) we considered the result of these ANOVAs to be significant with a $p<0.001$. This significance level is below the threshold that would be set by Bonferroni correction (i.e. alpha level of 0.05/15 = 0.003). Size classes ($F_1=38.189$, $p<0.001$) and genotype ($F_{61}=2.633$, $p<0.001$) both differed in dry biomass. However, there

was no interactive effect of size class x genotype (F_{57}=0.535, p=0.997). Therefore, size class was excluded as a factor from future analyses, as the proportion of the size classes did not differ between genotypes.

We ran two principal component analyses (PCA) on all $\log_{10}$-transformed element concentrations for F0s & F1 and F2 genotypes to determine how genotypes differ in multidimensional space. Centroids (multivariate centers of distribution) for each genotype were plotted post hoc (i.e. as inactive, supplemental variables in the analysis) to visualize these relationships among genotypes. We then retained the first two principal component (PC) scores from the F2 PCA for further analysis to test for the occurrence of multivariate separation due to genotype by running a MANOVA and separate ANOVAs on the first two PCs with genotype as a factor. Additionally, univariate analyses (separate ANOVAs) with genotype as a random factor were performed to determine individual elements that differed in the F2 genotypes. Element-by-element pairwise correlations were performed to determine relationships among elemental concentrations.

Logistic growth models were fit to the *Daphnia* density counts over time, for each replicate jar within each genotype. Growth rate (*r*) and carrying capacity (*K*) were calculated based on these logistic growth curves. However, not every experimental culture fit the logistic growth curve; therefore, a binary logistic regression was performed to test if genotype was a significant predictor of whether or not the *Daphnia* density counts over time fit a logistic growth model (i.e. *r* and *K* coded as either 0, if no significant parameter could be fit, and 1 if a significant parameter did fit). For the data for which significant *r* and *K* values could be estimated ($p<0.05$), ANOVA was performed to test the effect of genotype on *r* and *K*. ANOVA was also performed to determine any genotype effect on final *Daphnia* density count.

To determine the relationship between element content and *r*, *K*, and density, linear regressions were performed with *r*, *K*, and density as the independent variable, and element concentration as the

dependent variable. ANOVAs, MANOVA, and binary logistic regression were performed using SPSS v.22. Logistic growth modeling was performed in RStudio (R package *growthcurver*). Pairwise correlations, linear regressions, and PCA were performed using JMP Pro 14.

Results

The PCA of elemental composition of the F0s and F1 revealed that the first two principal components (PCs) explain ~63% of the total variation (Table S1, Fig S1), and individual element ANOVAs show B, Fe, and S to differ among the F0s and F1 genotypes (Table S2). The PCA of elemental composition of the 58 F2 recombinants revealed that the first two PCs explain ~54% of the total *Daphnia* elemental variation (Table S3; Fig. S2). MANOVA results on the factor scores of these two PCs and individual ANOVAs on each PC reveal a significant difference among genotypes in their ionomes (Table S4). Univariate analysis on the F2 ionomes revealed that the contents of Cu, Fe, K, Li, Mg, Na, P, S, Sr, and Zn were significantly affected by genotype (Table 1). Coefficients of variation (standard deviation/mean) differed among elements, ranging from ~0.06 for carbon to ~0.6 for strontium (Figure 1).

We found several significant relationships among elements, the vast majority of which were positive (Table 2; Figure S3). Strength of correlations also varied, with strong correlations ($R > 0.68$) observed between Mg-Ca, Mn-Cu, and P-Mg.

To understand the extent to which ionomic variation is related to growth parameters, we calculated values for *r* and *K* by fitting data from each jar to a logistic growth model. Of the 244 total jars, we were able to reliably estimate *K* for 120 jars, and *r* for 84 jars. The remaining jars either did not fit a logistic growth model, or had non-significant estimates of *r* or *K* ($p<0.05$). A logistic binary regression revealed that genotype was a poor predictor of model fit. For the *Daphnia* cultures that we could estimate *r* and *K* (i.e. $p<0.05$), ANOVA revealed no significant genotype effect on either *r* ($F_{39}=1.146$, $p=0.329$) or *K* ($F_{47}=1.391$, $p=0.102$). However, genotype did have a significant effect on density ($F_{61}=4.842$, $p=<0.001$) in the *Daphnia* culture at the end of the experiment.

Several significant negative correlations between element contents and carrying capacity (*K*) and density were observed (Table S5, Fig. 2, Fig. S4). Additionally, both K and Zn were positively correlated with and density (Table S5, Fig. 2, Fig. S4). Population growth rate *(r*) had a no significant relationship ($p<0.001$) with any element concentration.

Discussion

We found substantial genotypic variation in the ionomes of recombinant F2 *Daphnia pulicaria* (Figure 1). Correlations among pairwise element concentrations revealed several significant positive relationships, although the strength of correlations differed substantially (Table 2). Genotype had a significant effect on density at the end of the experiment when animals were sacrificed for ionomic analyses, and density was negatively related to the concentrations of most elements (Fig. 2). Given the breeding design employed, such diversity is most likely a function of sexual recombination.

The F1 was located between the two F0s in multidimensional space (Fig. S1), although the genotypes did not differ significantly in the contents of individual elements. In contrast, F2 recombinants differed significantly in 10 of the 15 elements measured (Table 1). These results indicate that the contents of some elements (i.e. B, Ca, Mn, C, N) may be more robust to genetic disturbances compared to others. At least two such elements (C, N) are also known to exhibit strong physiological homeostasis in *Daphnia* (Andersen and Hessen 1991, Sterner and Hessen 1994), while Ca concentration can vary depending on environmental supply (Tan and Wang 2009). As such, it is likely that while some elements exhibit different degrees of genetic and physiological homeostasis, potentially with important ecological and evolutionary implications.

Coefficients of variation in each element among F2 recombinants indicate that recombination did not affect the contents of all elements equally (Fig. 1). This is perhaps expected given differences in the number of metabolic pathways that utilize different elements (e.g., C is required by all, while trace elements are required by few), potentially impacting the fitness relevance of such recombination. In other

words, deleterious recombination in genomic regions associated with bulk elements could be "rescued" by recombination at several other loci to maintain homeostasis in that element and prevent severe fitness penalties. In comparison, recombination at fewer loci involved in trace element processing may have lower probability of rescue by other loci with severe fitness penalties (i.e. such recombinants do not develop or reproduce, and thus are not part of the F2 population). Regressing concentration of an element in *Daphnia* and its coefficient of variation on a log-log scale revealed a positive relationship (R^2= 0.6; Fig. S5), potentially indicative of such mechanisms, although further study is required test whether this hypothesized mechanism that may shape ionomes.

While such evolutionary genetic mechanisms that shape ionomes remain to be discovered, it is clear that recombination generates rich ionomic diversity potentially due to correlated shifts in multiple elements. Of 59 (out of a possible 105) relationships among elements in the pairwise correlation matrix, strong correlations (R > 0.68) were only found between 3 pairwise comparisons: Mg & Ca, Mn & Cu, and P & Mg, all being positive relationships. Of these three strong correlations, two may be explained by ion transport, as many Mg transporters do not discriminate well against Ca (Maguire 2006) and the ionic radii of Mn2+ and Cu2+ are not that different (Kayestha and Hajela 1995). The Mg and P relationship may be related to electroneutrality, as many negative phosphate ions are electrically compensated by positive Mg2+, this relationship playing a significant role in ion catalysis and substrate specificity as reviewed in (Yang et al. 2006). Moderate correlations (0.36 < R < 0.67) were found between 33 pairwise comparisons (Table 2). Importantly, these correlations were not between bulk elements that encompass the majority of proteins and biomass (i.e. C, N) and other elements. This suggests that either C:N contents may be a poor indicator of protein content in *Daphnia* (Wilder and Jeyasingh 2016), or protein content does not impact quotas of other elements. We are unable to test these alternative because we did not measure protein content or accrual in this population-level study.

Concentration of elements in *Daphnia* that were significantly correlated with estimated *K*, and actual density at the time of sampling for ionomic analyses revealed somewhat similar patterns. All

elements that significantly varied with *K* also varied with density, but not vice versa (Table S4). Of the 15 possible relationships, the concentration of 11 elements decreased with density. This observation indicates that most material resources are partitioned among individuals as density increases, which could be simple exhaustion of available elements. As such, when *Daphnia* density is high, predators of daphniids may intake lower concentrations of several elements per daphniid, with potentially important impacts on their foraging behavior and associated ecological consequences.

In summary, we report the extent to which genetic recombination impacts the ionome of an ecologically important taxon, and the general tendencies in covariance among multiple elements encompassing an organism. Understanding the genetic basis of such patterns and its ecological implications should have much to contribute toward integrating elements and traits, and the extent to which evolutionary shifts in elemental contents of taxa feedback to impact ecology.

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Tables

Table 1 Univariate analysis (ANOVA) of F2 *Daphnia* genotypes log_{10}-transformed elemental concentrations ($\mu g\ g^{-1}$) with genotype as a random factor. F, df, and p-values are reported with significant effect of genotype in bold ($p<0.001$). B and C are the only elements that have no significant differences among the F2 genotypes.

ANOVAs				
Element	Factor	F	df	p
B	Genotype	1.040	57	0.405
	Error		314	
Ca	Genotype	1.389	57	0.043
	Error		314	
Cu	Genotype	2.192	57	**<0.001**
	Error		314	
Fe	Genotype	2.503	57	**<0.001**
	Error		313	
K	Genotype	2.692	57	**<0.001**
	Error		313	
Li	Genotype	1.999	57	**<0.001**
	Error		314	
Mg	Genotype	2.064	57	**<0.001**
	Error		314	
Mn	Genotype	1.541	57	0.012
	Error		314	
Na	Genotype	2.032	57	**<0.001**
	Error		314	

P	Genotype	2.960	57	**<0.001**
	Error		314	
S	Genotype	2.028	57	**<0.001**
	Error		314	
Sr	Genotype	3.150	57	**<0.001**
	Error		314	
Zn	Genotype	6.345	57	**<0.001**
	Error		314	
C	Genotype	1.628	28	0.055
	Error		65	
N	Genotype	2.292	28	0.003
	Error		65	

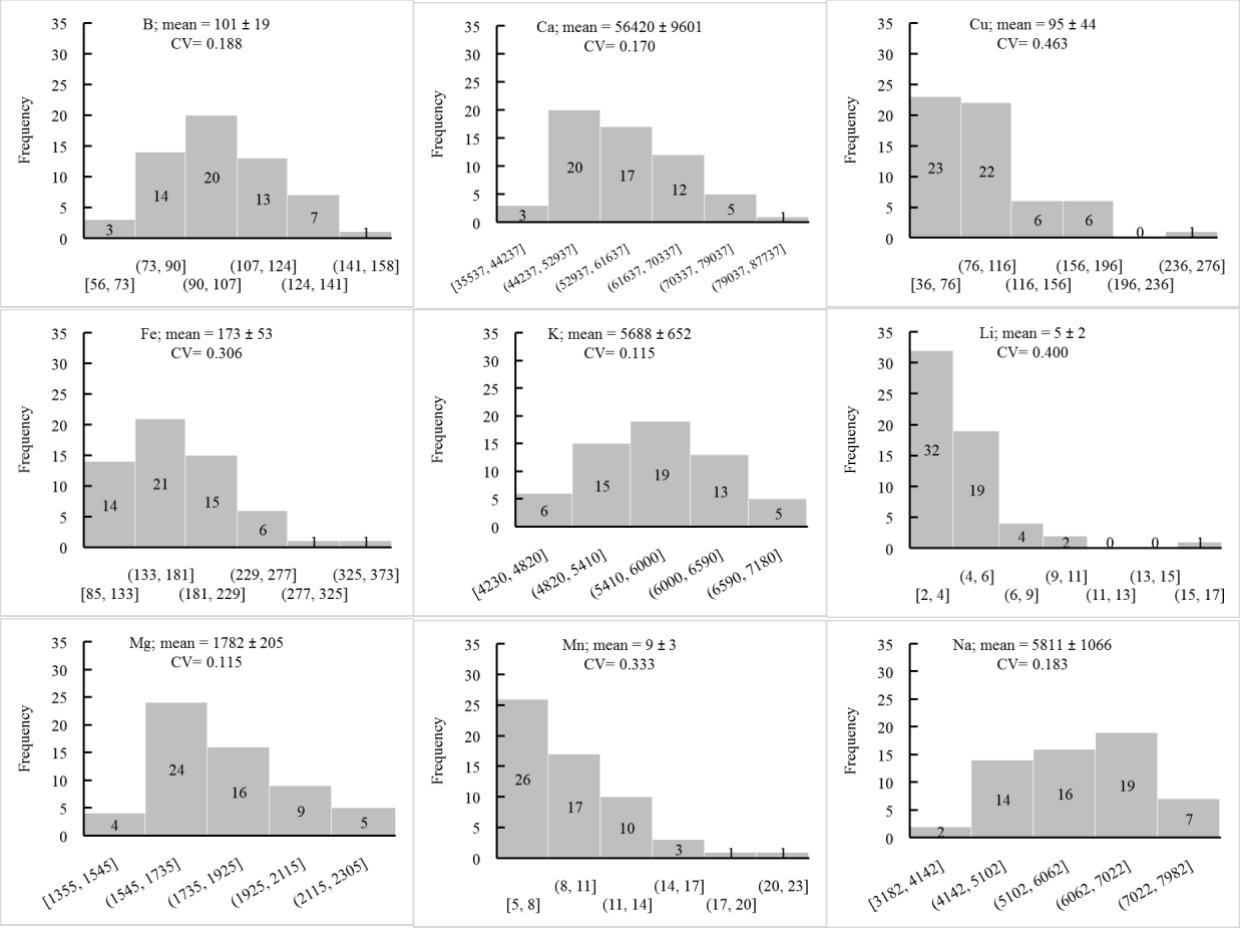
Table 2 Element-by-element pair-wise correlations. Correlation coefficient (R) and p-value reported for each pair-wise comparison. Moderate correlations ($0.36 < R < 0.67$) are denoted with "*", strong correlation ($R > 0.68$) are denoted with "**." Significant p-values are in bold ($p<0.001$). 59 pair-wise correlations were found to be significant out of 105 comparisons; of these 3 had a strong correlation, 33 had a moderate correlation, and 30 had a weak correlation.

Variable	by Variable	Correlation	p-value	Variable	by Variable	Correlation	p-value
log Ca	log B	0.4949*	**<0.0001**	log S	log Na	0.2091	**<0.0001**
log Cu	log B	0.4254*	**<0.0001**	log S	log P	0.5643*	**<0.0001**
log Cu	log Ca	0.5428*	**<0.0001**	log Sr	log B	0.3135	**<0.0001**
log Fe	log B	0.1498	0.0027	log Sr	log Ca	0.3626*	**<0.0001**
log Fe	log Ca	0.2401	**<0.0001**	log Sr	log Cu	0.2872	**<0.0001**
log Fe	log Cu	0.2770	**<0.0001**	log Sr	log Fe	0.0519	0.3020
log K	log B	0.2150	**<0.0001**	log Sr	log K	-0.0481	0.3386
log K	log Ca	0.1422	0.0045	log Sr	log Li	0.3992*	**<0.0001**
log K	log Cu	-0.1258	0.0121	log Sr	log Mg	0.3445	**<0.0001**
log K	log Fe	0.0136	0.7877	log Sr	log Mn	0.2507	**<0.0001**
log Li	log B	0.5370*	**<0.0001**	log Sr	log Na	0.1926	**0.0001**
log Li	log Ca	0.6068*	**<0.0001**	log Sr	log P	0.3917*	**<0.0001**
log Li	log Cu	0.5130*	**<0.0001**	log Sr	log S	0.4398*	**<0.0001**
log Li	log Fe	0.2032	**<0.0001**	log Zn	log B	0.3311	**<0.0001**
log Li	log K	0.0328	0.5150	log Zn	log Ca	0.5186*	**<0.0001**
log Mg	log B	0.5246*	**<0.0001**	log Zn	log Cu	0.1877	**0.0002**
log Mg	log Ca	0.7983**	**<0.0001**	log Zn	log Fe	0.1995	**<0.0001**
log Mg	log Cu	0.4654*	**<0.0001**	log Zn	log K	0.4035*	**<0.0001**
log Mg	log Fe	0.1903	**0.0001**	log Zn	log Li	0.1249	0.0126
log Mg	log K	0.3568	**<0.0001**	log Zn	log Mg	0.3493	**<0.0001**
log Mg	log Li	0.5448*	**<0.0001**	log Zn	log Mn	0.0759	0.1300
log Mn	log B	0.5357*	**<0.0001**	log Zn	log Na	-0.2339	**<0.0001**
log Mn	log Ca	0.4802*	**<0.0001**	log Zn	log P	0.4071*	**<0.0001**
log Mn	log Cu	0.7203**	**<0.0001**	log Zn	log S	0.3774*	**<0.0001**
log Mn	log Fe	0.3107	**<0.0001**	log Zn	log Sr	0.0918	0.0671
log Mn	log K	-0.0316	0.5297	log N	log B	0.0359	0.7328

log Mn	log Li	0.6068*	**<0.0001**	log N	log Ca	0.1022	0.3297
log Mn	log Mg	0.4834*	**<0.0001**	log N	log Cu	0.0729	0.4874
log Na	log B	0.1155	0.0210	log N	log Fe	-0.0019	0.9858
log Na	log Ca	0.0409	0.4149	log N	log K	0.0373	0.7241
log Na	log Cu	-0.0281	0.5761	log N	log Li	0.0623	0.5530
log Na	log Fe	0.0926	0.0648	log N	log Mg	0.1086	0.3001
log Na	log K	0.1728	**0.0005**	log N	log Mn	0.1052	0.3155
log Na	log Li	0.1378	0.0058	log N	log Na	-0.1250	0.2326
log Na	log Mg	0.3505	**<0.0001**	log N	log P	0.2004	0.0541
log Na	log Mn	-0.0168	0.7375	log N	log S	0.1595	0.1268
log P	log B	0.4689*	**<0.0001**	log N	log Sr	0.0258	0.8061
log P	log Ca	0.6226*	**<0.0001**	log N	log Zn	0.0591	0.5738
log P	log Cu	0.4321*	**<0.0001**	log C	log B	0.0780	0.4571
log P	log Fe	0.0347	0.4900	log C	log Ca	-0.1007	0.3367
log P	log K	0.4012*	**<0.0001**	log C	log Cu	0.0640	0.5420
log P	log Li	0.5112*	**<0.0001**	log C	log Fe	0.0170	0.8715
log P	log Mg	0.7245**	**<0.0001**	log C	log K	0.0180	0.8644
log P	log Mn	0.4305*	**<0.0001**	log C	log Li	-0.0545	0.6037
log P	log Na	0.0484	0.3345	log C	log Mg	-0.0330	0.7533
log S	log B	0.4104*	**<0.0001**	log C	log Mn	-0.0682	0.5163
log S	log Ca	0.5143*	**<0.0001**	log C	log Na	0.1128	0.2818
log S	log Cu	0.3346	**<0.0001**	log C	log P	0.0112	0.9155
log S	log Fe	0.3663*	**<0.0001**	log C	log S	-0.0262	0.8028
log S	log K	0.2888	**<0.0001**	log C	log Sr	-0.0788	0.4525
log S	log Li	0.4630*	**<0.0001**	log C	log Zn	-0.0975	0.3523
log S	log Mg	0.5506*	**<0.0001**	log C	log N	0.2318	0.0254
log S	log Mn	0.2432	**<0.0001**				

Figures

Figure 1 Histograms of the 15 elements measured in the 58 F2 *Daphnia* genotypes. Mean concentration (μg g^{-1}) ranges for each genotype are reported on the x axis and frequency on the y axis. Overall mean ± standard deviation and coefficient of variation (CV) is reported above each graph. The number of genotypes within each mean element range is reported within each bar.



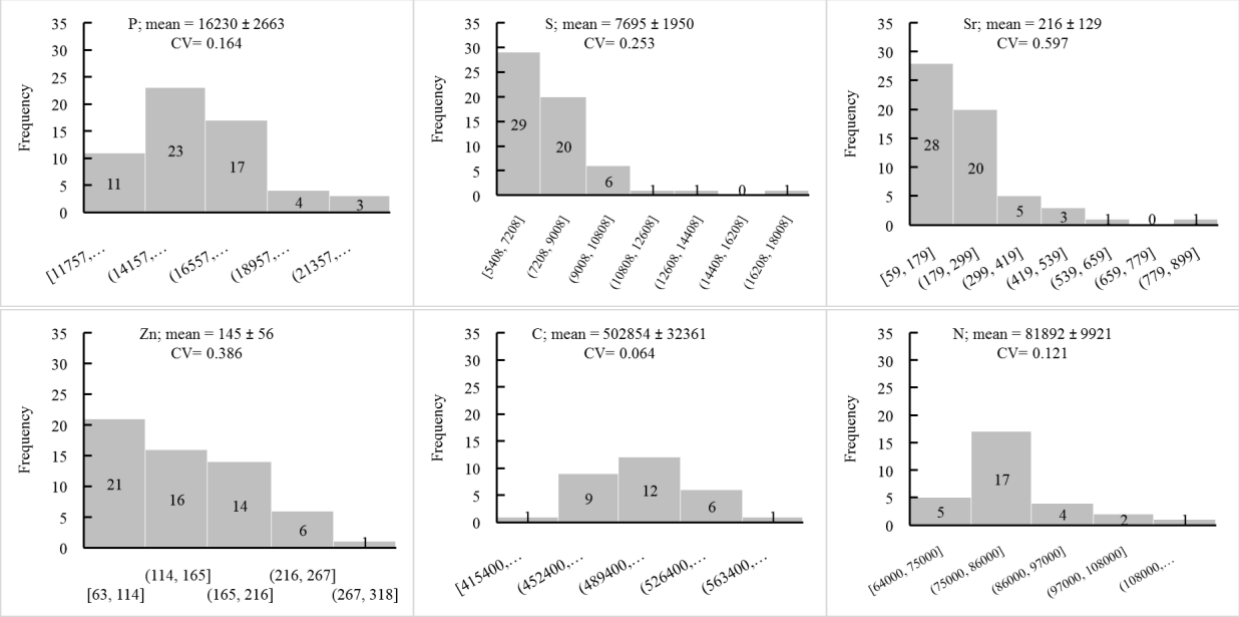
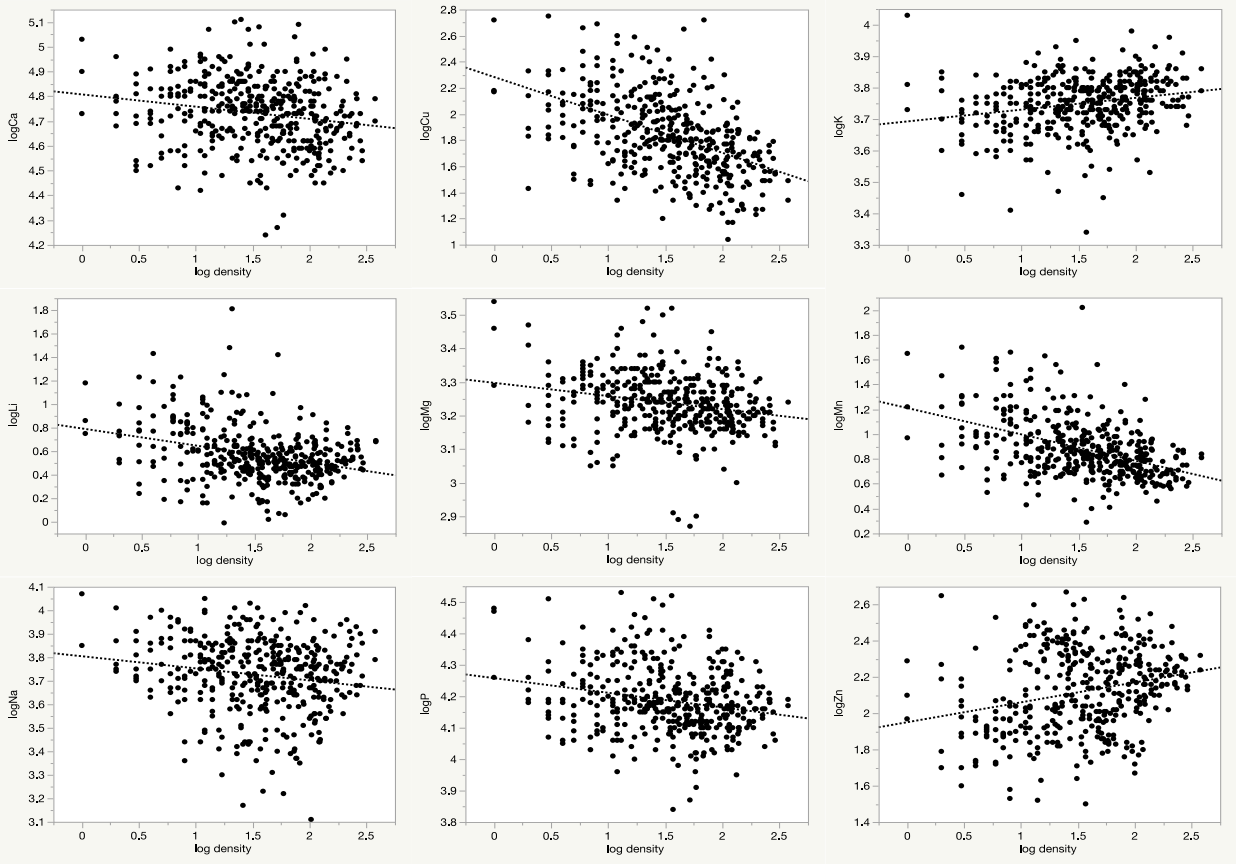

P; mean = 16230 ± 2663
CV= 0.164
Frequency
11
23
17
4
3
[11757,…
(14157,…
(16557,…
(18957,…
(21357,…
S; mean = 7695 ± 1950
CV= 0.253
29
20
6
1
1
0
1
[5408, 7208]
(7208, 9008]
(9008, 10808]
(10808, 12608]
(12608, 14408]
(14408, 16208]
(16208, 18008]
Sr; mean = 216 ± 129
CV= 0.597
28
20
5
3
1
0
1
[59, 179]
(179, 299]
(299, 419]
(419, 539]
(539, 659]
(659, 779]
(779, 899]
Zn; mean = 145 ± 56
CV= 0.386
21
16
14
6
1
[63, 114]
(114, 165]
(165, 216]
(216, 267]
(267, 318]
C; mean = 502854 ± 32361
CV= 0.064
1
9
12
6
1
[415400,…
(452400,…
(489400,…
(526400,…
(563400,…
N; mean = 81892 ± 9921
CV= 0.121
5
17
4
2
1
[64000, 75000]
(75000, 86000]
(86000, 97000]
(97000, 108000]
(108000,…

Figure 2 *Daphnia* density (log_{10}-transformed) at the end of the experiment by elemental concentrations (log_{10}-transformed) for all genotypes. Each point represents a single replicate *Daphnia* culture. All plotted graphs have significant correlation ($p<0.001$).



Supplementary information

Table S1 Principal component analysis of F0s and F1 ionomes. Percent variance explained and eigenvalues are reported for the first two principal components (PC1 & PC2). Loading scores are also reported for each element. The first two PCs explain ~63% of the total variation in the ionomes of these genotypes.

Principal component	PC1	PC2
% variance	38.700	24.149
Eigenvalue	5.0310	3.1394
Loadings		
B	0.59097	0.49321
Ca	0.86293	-0.20051
Cu	0.85813	0.16990
Fe	-0.43528	0.41244
K	-0.42749	0.70637
Li	0.69891	0.31608
Mg	0.33282	0.62700
Mn	0.79184	0.09218
Na	-0.63012	0.63146
P	0.53004	0.44247
S	-0.16146	0.85492
Sr	0.32675	0.54571
Zn	0.88962	-0.18237

Table S2 Univariate analysis (ANOVA) of F0s and F1 *Daphnia* genotypes log_{10}-transformed elemental concentrations ($\mu g\ g^{-1}$) with genotype as a random factor. F-statistic (F), df, and p-values are reported with significant effect of genotype in bold ($p<0.001$).

ANOVAs				
Element	Factor	F	df	p
B	Genotype	3.696	2	0.040
	Error		24	
Ca	Genotype	1.128	2	0.340
	Error		24	
Cu	Genotype	0.894	2	0.422
	Error		24	
Fe	Genotype	4.925	2	0.016
	Error		24	
K	Genotype	1.931	2	0.168
	Error		24	
Li	Genotype	0.409	2	0.669
	Error		24	
Mg	Genotype	0.159	2	0.854
	Error		24	
Mn	Genotype	3.389	2	0.051
	Error		24	
Na	Genotype	1.906	2	0.170
	Error		24	
P	Genotype	1.524	2	0.238
	Error		24	
S	Genotype	4.051	2	0.030
	Error		24	

Sr	Genotype	1.178	2	0.325
	Error		24	
Zn	Genotype	2.612	2	0.094
	Error		24	

Table S3 Principal component analysis of the F2 ionomes. Percent variance explained and eigenvalues are reported for the first two principal components (PC1 & PC2). Loading scores are also reported for each element. The first two PCs explain ~54% of the total variation in the ionomes of these genotypes.

Principal component	PC1	PC2
% variance	41.120	12.749
Eigenvalue	5.3456	1.6574
Loadings		
B	0.71901	-0.03962
Ca	0.85390	0.00297
Cu	0.67314	-0.50485
Fe	0.34779	-0.15453
K	0.30892	0.77684
Li	0.75125	-0.29668
Mg	0.85455	0.18662
Mn	0.68112	-0.51564
Na	0.18377	0.22921
P	0.79064	0.24158
S	0.71388	0.24449
Sr	0.51031	-0.07516
Zn	0.49242	0.45663

Table S4 MANOVA and separate ANOVAs with genotype as a factor on the individual factors scores retained from the first two principal components (PCs) of the PCA on F2 *Daphnia* ionomes.

Wilk's Lambda	F	df	Error	p
Genotype	2.660	114	622	**<0.001**
ANOVA PC1				
	F	df	p	
Genotype	1.742	57	0.002	
Error		312		
ANOVA PC2				
	F	df	p	
Genotype	3.711	57	**<0.001**	
Error		312		

Table S5 Correlations of carrying capacity (*K*), density, growth rate (*r*) and element concentration. All variables were $\log_{10}$-transformed prior to analysis. Slope, intercept, R^2, and p-values are reported, with significant correlations in bold (p<0.001).

	carrying capacity (*K*)				density				growth rate (*r*)			
Element	Slope	Intercept	R^2	p	Slope	Intercept	R^2	p	Slope	Intercept	R^2	p
B	-0.058	2.08	0.022	0.037	-0.053	2.05	0.026	0.001	+0.041	1.98	0.005	0.355
Ca	-0.043	4.81	0.015	0.084	-0.049	4.80	0.034	**<0.001**	+0.025	4.72	0.002	0.560
Cu	-0.323	2.38	0.224	**<0.001**	-0.290	2.28	0.235	**<0.001**	-0.026	1.68	0.001	0.769
Fe	-0.004	2.21	0.000	0.914	-0.045	2.26	0.013	0.021	+0.127	2.28	0.028	0.034
K	+0.046	3.66	0.047	0.002	+0.038	3.69	0.051	**<0.001**	+0.029	3.78	0.007	0.291
Li	-0.063	0.66	0.016	0.074	-0.143	0.79	0.099	**<0.001**	-0.062	0.47	0.005	0.362
Mg	-0.041	3.30	0.035	0.008	-0.039	3.30	0.053	**<0.001**	-0.010	3.21	0.001	0.674
Mn	-0.230	1.28	0.164	**<0.001**	-0.213	1.21	0.207	**<0.001**	+0.052	0.83	0.003	0.456
Na	-0.015	3.75	0.002	0.575	-0.052	3.80	0.028	**<0.001**	-0.026	3.70	0.003	0.584
P	-0.063	4.29	0.048	0.002	-0.047	4.26	0.050	**<0.001**	-0.030	4.13	0.006	0.311
S	-0.027	3.89	0.007	0.247	-0.027	3.89	0.012	0.031	-0.017	3.80	0.003	0.491
Sr	+0.121	1.96	0.014	0.099	-0.019	2.20	0.001	0.580	+0.0002	2.13	0.000	0.999
Zn	+0.131	1.93	0.050	0.002	+0.110	1.95	0.061	**<0.001**	+0.152	2.22	0.030	0.026
C	-0.022	5.74	0.045	0.059	-0.010	5.71	0.020	0.178	-0.019	5.68	0.012	0.348
N	-0.079	5.05	0.162	**<0.001**	-0.044	4.97	0.107	0.001	-0.040	4.85	0.018	0.248

Figure S1 PCA plot of F0s and F1 ionomes with PC1 on the x-axis and PC2 on the y-axis. Centroids (multivariate centers of distribution) for each genotype were plotted post hoc (i.e. as inactive, supplemental variables in the analysis) to visualize these relationships among genotypes. The first two PCs explain ~63% of the total variation in the ionomes of these genotypes.

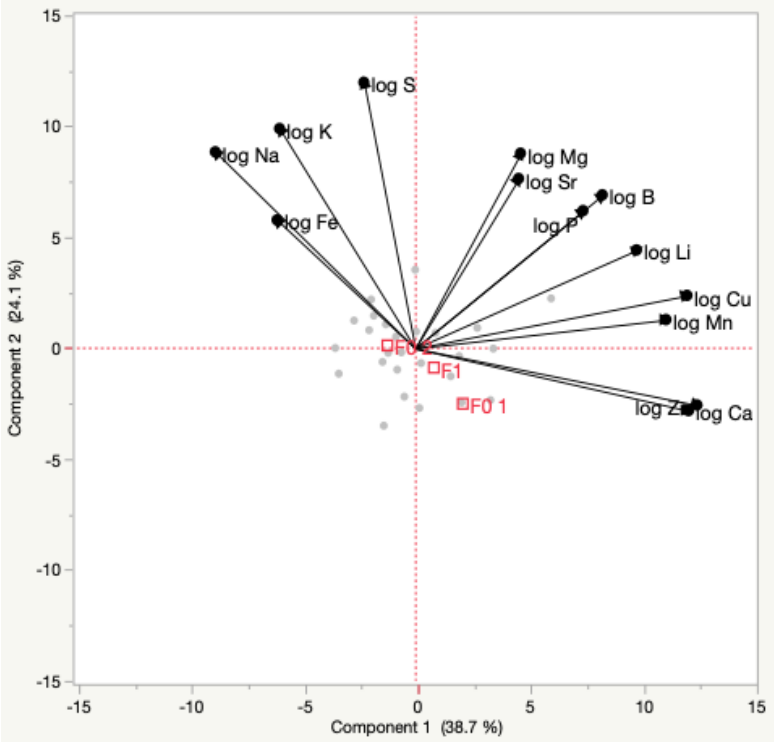


Figure S2 PCA plot of F2 ionome, with PC1 on the x-axis and PC2 on the y-axis. Centroids (multivariate centers of distribution) for each genotype were plotted post hoc (i.e. as inactive, supplemental variables in the analysis) to visualize these relationships among genotypes. The first two PCs explain ~54% of the total variation in the ionomes of these genotypes.

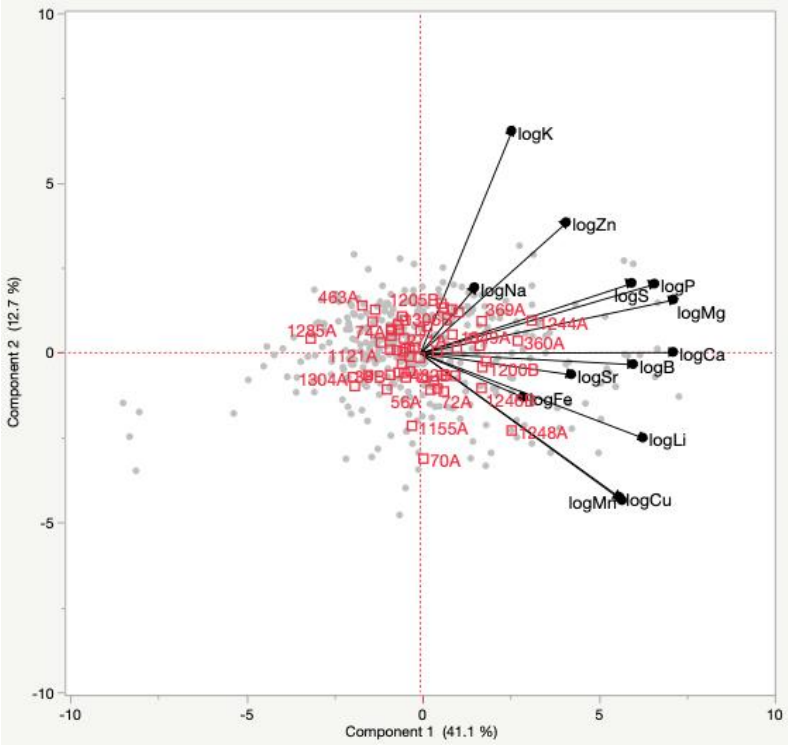


Figure S3 Element-by-element (log_{10}-transformed) pair-wise correlation matrix. Correlation coefficient (R) and trendline plotted for each correlation.

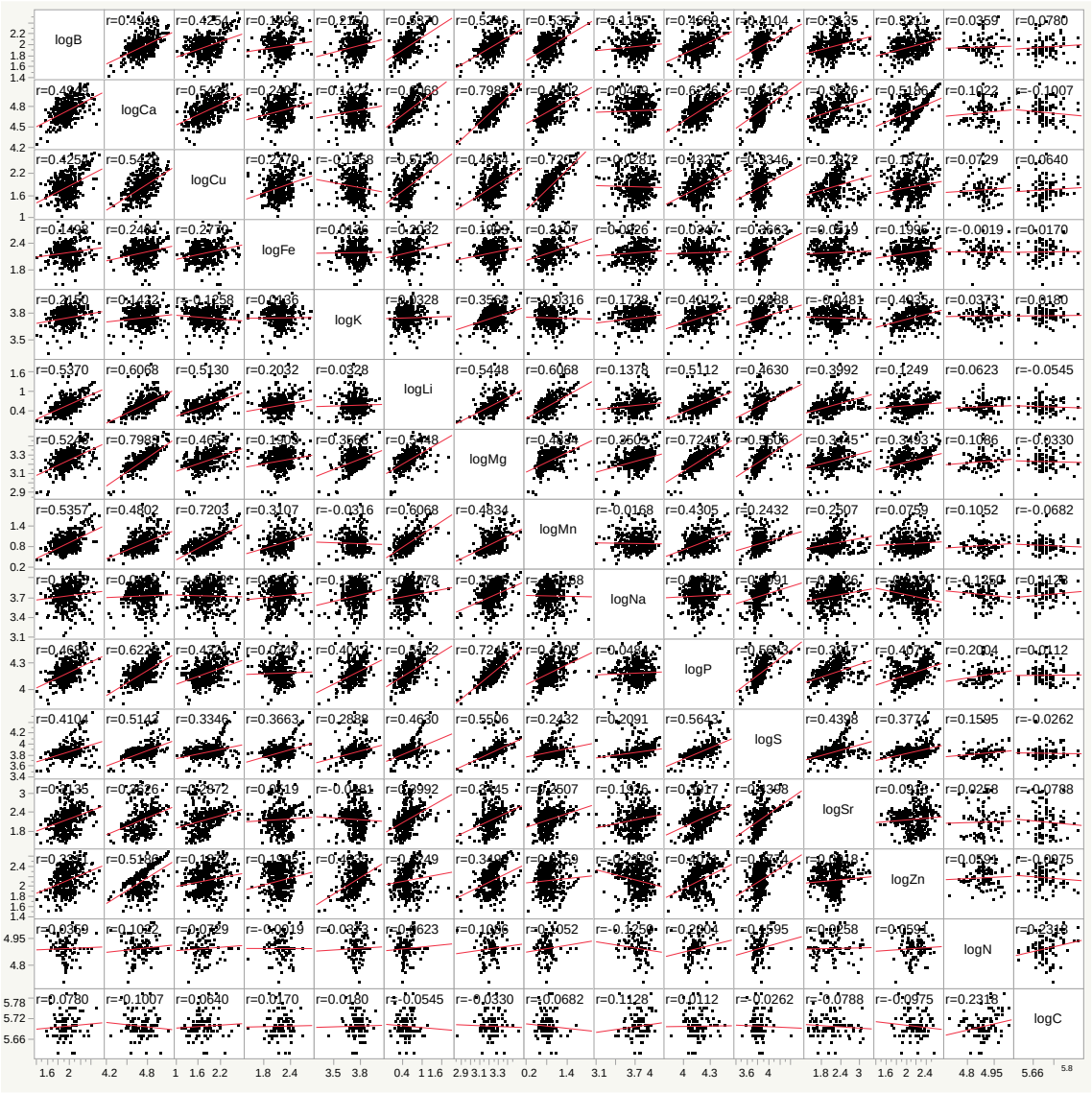


Figure S4 Linear regressions of *Daphnia* carrying capacity (*K*) (log_{10}-transformed) on the x-axis by elemental concentrations (log_{10}-transformed) on the y-axis for all genotypes. Each point represents a single replicate *Daphnia* culture. All plotted graphs have significant correlation ($p<0.001$).

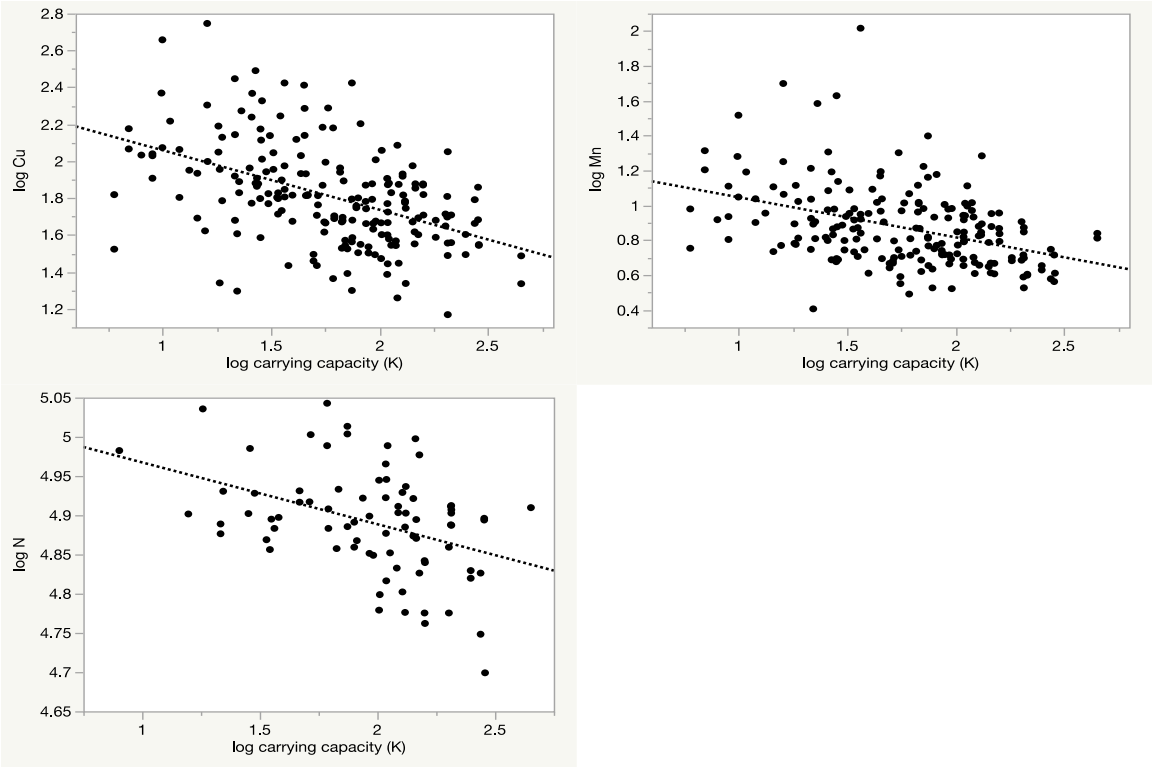
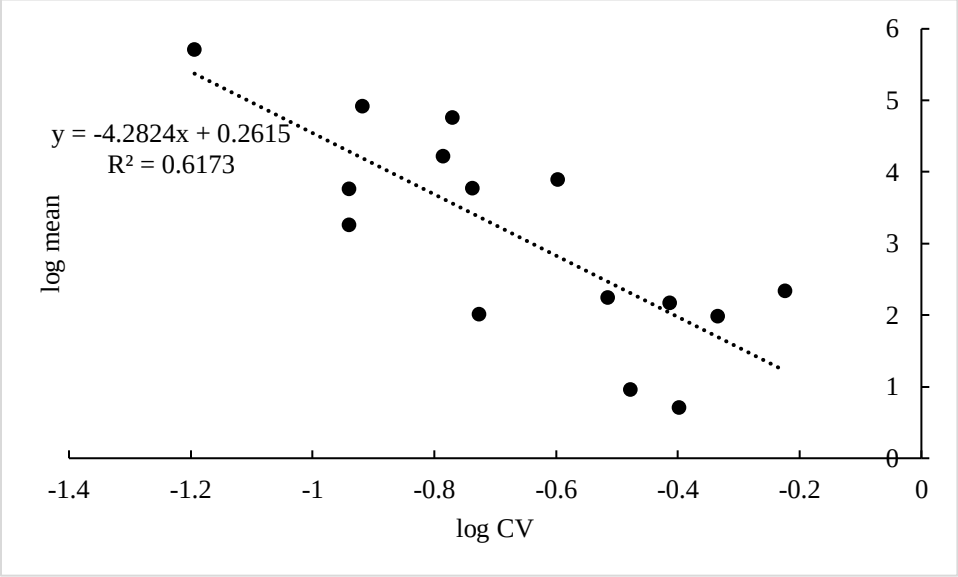


Figure S5 Regression of element concentration in *Daphnia* (log_{10}-transformed) and its coefficient of variation (log_{10}-transformed). The equation for the trendline and R^2 value reported on the figure.



CHAPTER IV

INTERSPECIFIC AND ONTOGENETIC VARIATION IN THE IONOMES OF *DAPHNIA*

Ryan E. Sherman[1], Punidan D. Jeyasingh[1]

Abstract

Changes across ontogeny often rival interspecific differences in classical traits (e.g., morphological, physiological, behavioral). Such variation is often associated with changes in the contents of chemical elements in an organism. However, beyond carbon, nitrogen, and phosphorus, little is known about how elemental quotas vary across ontogeny. Here, we investigated the extent to which species and ontogeny impact the contents of multiple elements in an individual (i.e. the ionome). We found significant effects of species and ontogeny on the ionomes of three species of the keystone freshwater herbivore, *Daphnia*. Of the 11 elements quantified, three elements (K, Mn, Zn) did not vary with species or ontogeny, while 8 significantly varied among species, ontogeny, or their interaction. For several elements quantified, differences across ontogeny rivaled differences seen among species. Significant differences among species with age in the contents of multiple elements are indicative of physiological differences among species and adjustments that take place across ontogeny. As such, variation in the availability of such elements among lakes could impact *Daphnia* life history and population dynamics, with potentially important ecological implications.

Introduction

Diversity in traits among ontogenetic stages and species should have elemental signatures. Understanding such signatures enables predictions about the biogeochemical roles of organisms, thus linking trait expression and ecological functions (Reiners 1986). The framework of ecological stoichiometry uses information on the organismal content of biologically important elements to make predictions about key ecological processes, including individual growth and ecosystem productivity (Sterner and Elser 2002). With its foundations in ecosystem science, the dynamic interaction between trait expression and biogeochemical cycles has been studied in the context of bulk elements that comprise the majority of biomass (e.g., carbon, nitrogen, phosphorus). One powerful insight of this enterprise is arguably the growth rate hypothesis (GRH; Elser et al. 1996, 2003), which posits that fast-growing species and life stages should have high mass-specific phosphorus (P) contents because rapid growth is P intensive due to increased demand for P-rich rRNA required for ribosome biogenesis, and therefore protein synthesis. Specifically, the GRH predicts a positive relationship between P and RNA quota, RNA quota and growth rate, and thus P quota and growth rate (Elser et al. 1996). Although the GRH was formulated for invertebrate species without large P storage mechanisms (e.g., bone in vertebrates; Elser et al. 1996) that are <1 mg in body mass (Gillooly et al. 2005), studies in a variety of taxa lend support to at least some links (e.g., P content and growth rate) predicted by the GRH (reviewed in Elser et al. 2003; Hessen et al. 2013).

Given large changes in mass-specific growth over ontogeny (Arendt 1997), the GRH should be most evident in stages that prioritize allocation of resources to growth (e.g., juveniles). Studies have found support for some of the relationships (e.g., growth rate and P content) predicted by the GRH across ontogenetic stages (e.g., Main et al. 1997; Elser et al. 2006; Pilati and Vanni 2007; Back and King 2013). Such work revealed substantial ontogenetic variation in

elemental contents, often rivalling interspecific differences, with important implications for ecosystem-level processes (e.g., Tiegs et al. 2016).

The predictions of the GRH should have important implications for the quotas of other elements, particularly trace metals. While C, N, and P are central for protein synthesis and growth, proteins thus synthesized perform a variety of functions, often with the aid of cofactors (e.g., ~10% of most proteomes are Zn-proteins; Sousa et al. 2009). As such, higher rates of protein production should result in higher quotas of these trace metals. Unlike bulk elements, trace elements need not be constantly supplied in diet, as such demands can be met via reallocation within cells and tissues (e.g., Worms et al. 2006). However, an excess of trace metals in cells can cause oxidative stress (Sevcikova et al. 2011). Thus, trace metal content has to be tightly regulated, potentially impacting their quotas (i.e. concentrations in a cell or individual) and the ATP quotient available for protein synthesis and growth.

In addition, it is possible that the GRH, while central to individual growth, may not always be relevant to population growth, particularly in multicellular organisms if gametogenesis places distinct elemental demands. Transcriptomic and proteomic profiles of individuals investing in oogenesis has been shown to be distinct from other stages (Song et al. 2006, Von Wyschetzki et al. 2015). Given such widespread physiological adjustments, it is likely that ontogeny impacts the contents of multiple elements. For example, Fe is known to be important because of its role in oogenesis in mosquitoes (Zhou et al. 2007), and differential supply of Fe disproportionally impacts reproductive traits in zooplankton (Chen et al. 2011), including daphniids (Lind and Jeyasingh 2017). Moreover, Prater et al. (2018), in their study on salamanders, found that adults had higher concentration of some elements (i.e. Ca, P, S, Mg, Zn and Cu) compared to metamorphic juveniles, which had greater amounts of C, Fe and Mn.

While we have yet to understand even the general physiological tendencies underlying such ionome-wide shifts, recent work demonstrates the importance of considering such variation in the entire suite of elements encompassing an individual (i.e. its ionome; Salt et al. 2008) to understand key ecological and evolutionary patterns (e.g., Goos et al. 2017; Jeyasingh et al. 2017; Prater et al. 2018; Rudman et al. 2019). Inferences made using data on one or two elements is often misleading because of strong covariance among elements (Baxter 2015). To date, little is known about interspecific and ontogenetic variation in the ionomes of ecologically important metazoans. Here, we characterized the ionomes of three species of *Daphnia* over key ontogenetic stages. Daphniids vary remarkably in life history because those lives are shaped by size-selective predation (Lynch 1980). *D. magna* is a large-bodied species belonging to the subgenus *Ctenodaphnia* (Colbourne and Hebert 1996), inhabiting ponds where gape-limited predators are more abundant than visual predators such as fish. *Daphnia pulex* and *D. pulicaria* are relatively smaller-bodied sister species in the subgenus *Daphnia* that are diverging based on ecotype. *Daphnia pulex* inhabits fishless ponds, while *D. pulicaria* inhabits lakes with fish, and as such the two species have evolved distinct life histories (Dudycha and Tessier 1999). Generally, under fish predation, daphniids invest earlier in reproduction compared to gape-limited invertebrate predation where the opposite occurs (Wellborn et al. 1996). We expected substantial variation in multiple axes of the ionome, and predicted that species and ontogeny will explain some of that variation.

Methods

Study organisms

Daphniids within each species were single clonal lineages. The *D. pulicaria* clone (SC4) originated from South Center Lake, Chisago County, MN (Frisch et al. 2014). The *D. pulex* clone (LL4-15) originated from a fishless pond (Weider et al. 2004). The *D. magna* clone was

purchased from a commercial vendor (Aquatic Biosystems, Ft. Collins, CO), although there is no information about its origin. All three *Daphnia* species were cultured in COMBO medium (Kilham et al. 1998) without N and P, and maintained in a growth chamber at ~20^0C and a 16:8 light:dark cycle.

Growth experiment

The animals were fed the green alga, *Scenedesmus obliquus,* cultured in continuous flow chemostats in COMBO medium at the rate of ~1mg C L^{-1} day^{-1}. Gravid daphniids were collected from stock jars and grown in 100mL of COMBO. Neonates (<24-hr-old) released from these gravid individuals were placed in 100mL of COMBO without N and P and fed 1mg C L^{-1} day^{-1} of high phosphorus *Scenedesmus obliquus* algae (C:P ~150). To characterize ontogenetic changes in the ionome, daphniid samples from these jars were collected by their age (in days) at either day 0, 3, 5, 7, 9, 11, or 13. Twenty individual neonates were placed in the same 100mL jar for five days. On the 6^{th} day, daphniids were split into 100mL jars in groups of ten. When daphniids reached day 9, they were split into jars with 5 individuals. Media replacement occurred every 3 days. Once daphniids of respective ages were sampled, they were placed in a drying oven at 60°C for at least 72 hrs and then weighed using a microbalance to the nearest 0.1 µg (Metter-Toledo XP2U). The dry mass per individual was calculated by dividing the dry weight of each sample by the number of individuals per sample. Mass-specific growth rate (MSGR) was calculated by subtracting the natural log of the initial individual mass of the previous collection day from the natural log of the average individual mass on each collection day and divided by the days between each collection day (Tessier and Goulden 1987); i.e. day 3 MSGR for an individual = ln(average day 3 mass)-ln(mass of day 0 individual)/3, day 5 MSGR for an individual = ln(average day 5 mass)-ln(mass of day 3 individual)/2.

Ionome quantification

Using a combination of inductively coupled plasma-optical emission spectrometry (ICP-OES) and carbon & nitrogen (CN) analysis, ionomes were quantified from the dry biomass collected at the aforementioned timepoints (daphniid age in days). Carbon and nitrogen contents were quantified using an automated CHN analyzer (varioMicro Cube; Elementar Americas, Mt. Laurel, NJ, USA), and the concentrations of other elements were quantified using a ICP-OES analyzer (iCAP 7400; Thermo Scientific, Waltham, MA, USA). *Daphnia* ICP-OES samples were digested in 15 ml trace-metal-free polypropylene conical centrifuge tubes (VWR International, Radnor, PA, USA) by adding 200 µL of trace-metal-grade 67-70% HNO_3 (BDH Aristar ® Plus, VWR International, Radnor, PA, USA) and 100 µL of trace metal grade 30-32% H_2O_2 (BDH Aristar ® Ultra, VWR International, Radnor, PA, USA). Each sample was then allowed to digest overnight at room temperature (~22°C), until the solution ran clear. All digested *Daphnia* samples were then diluted to a final volume of 5 mL with ultrapure (Type 1) water. Validation and calibration of the ICP-OES was achieved by using multi-element external reference standards (CCV Standard 1, CPI International, Santa Rosa, CA, USA). Additionally, an in-line Yttrium internal standard (Peak Performance Inorganic Y Standard, CPI International, Santa Rosa, CA, USA) was used to correct for any instrument drift or matrix effects. Digestion blanks were also run to correct for background concentrations. Eleven elements (P, Ca, S, Na, K, Mg, Zn, Fe, and Mn) were found above detection limits in all samples. Mass-specific ($\mu g\ g^{-1}$) concentrations of these elements, including C and N were $\log_{10}$-transformed to meet normality assumptions before statistical analyses.

Statistical analyses

ANOVAs were performed with species and age as fixed factors to identify mass-specific ionome-wide differences among species, ontogenetic stage (same-day gravid vs. pre-reproductive individuals), and age groups. ANOVAs were also performed to determine the effect of species and age on individual element content (log-transformed), and differences between pre-

reproductive and gravid individuals. To account for multiple comparisons of the ionomes of *Daphnia* (i.e. 11 ANOVAs on individual element contents) we considered the result of these ANOVAs to be significant with a $p<0.001$. This significance level is below the threshold that would be set by Bonferroni correction (i.e. alpha level of 0.05/11 = 0.0045). Tukey's post hoc analysis was performed for all significant ANOVAs to identify factor effects. All statistics were performed using SPSS v.22.

Results

Interspecific differences in life-history traits

Univariate analysis (ANOVA) showed that <24-hr-old neonate mass differed among species (Table 1), with *D. magna* neonates having more mass than both *D. pulex* and *D. pulicaria* (Fig. 1). The same trend was seen when examining mass at maturity (Table 1, Fig. 1). ANOVA of *Daphnia* mass across age demonstrated significant species and age effects (Table 1) but no interactive effect, as *D. pulex* and *D. pulicaria* did not differ in mass across age as compared to *D. magna*. ANOVA of age of maturity revealed no significant effect of species (Table 1).

Effects of age and ontogenetic stage on ionomes

ANOVAs revealed a significant effect of species on Na and P concentrations in *Daphnia*, while significant age effects were evident for C, Fe, Na, P, and S (Fig. 2, Table 2). There were significant interactive effects on the concentrations of N (Fig. 2, Table 2).

When examining the differences between non-reproductive and gravid *Daphnia*, ANOVAs revealed a significant effect of species on Ca, Mg, Na, and P contents (Fig. 3, Table 3). Significant ontogenetic stage effects (non-reproductive compared to gravid) were revealed on Fe *Daphnia* body concentration (Fig. 3, Table 3). The effects of age and stage on the ionomes of

each species is presented in the supplemental information (Tables S1 & S2) and mean ± 1SE reported in Tables S3 and S4.

Discussion

Our results demonstrate that species, age, and ontogenetic stage can have an effect on the ionomes of *Daphnia*. Both ANOVAs performed to analyze ionomic data revealed that of the 11 elements quantified, three elements (K, Mn, Zn) did not vary with species or age, while 8 significantly varied among species, age, or by a species*age interaction. In both analyses (Tables 2 and 3), species differed in content of Na and P. When comparing non-reproductive and gravid individuals of the same age (ontogenetic stage), species also differed in the content of Ca and Mg, in addition to Na and P. Age alone impacted the contents of C, Fe, Na, P, and S, while ontogenetic stage impacted the content of Fe. N content was significantly affected by species and age.

While several studies have found P content to vary interspecifically, even among closely related taxa (e.g. Andersen and Hessen 1991; Sterner 1995; Seidendorf et al. 2007, 2009), we know little about species variation in Ca, Mg, and Na. Although all of the elements quantified here have never been measured in concert on the same clonal lineages, studies have examined the content of these elements in *Daphnia* independently. While the contents of Na and P differed among species in both ANOVAs, the contents of Ca and Mg only differed among species when comparing non-reproductive and gravid daphniids. Na content differences among species could represent species differences in Na uptake or demand, although little is known among *Daphnia* species.

Within the *Daphnia* genus, Ca content has been shown to vary interspecifically (Tan and Wang 2010) and has been shown to increase with body size of the species (Wærvågen et al. 2002). Within species, juvenile *Daphnia* have higher relative Ca content compared with larger

bodied adults (Hessen and Rukke 2000). We found similar Ca contents in <24h-old neonates of all three species, although *D. pulex* increased while *D. pulicaria* decreased in Ca content around maturity (Fig. 2). These two species are known to differ in age-specific fecundity, with *D. pulex* investing earlier in life compared to *D. pulicaria* (Dudycha 2003). Such differences could explain the divergent pattern in Ca content between these species, although we did not collect age-specific fecundity data in our study.

Unlike Ca, we are unaware of studies on the role of Mg in *Daphnia* nutrition, although studies have documented its importance in mitigating toxicity by other elements (e.g., Ha et al. 2017). On a cellular level, Mg stabilizes enzymes, including those involved in ATP-generating reactions (Aikawa 1981, Jahnen-Dechent and Ketteler 2012), specifically when ATP is synthesized from ADP and inorganic phosphate (Ko et al. 1999, Williams 2000, Gout et al. 2014), and known to limit growth of microbes (e.g., (Lusk et al. 1968). As such, one should expect an increase in Mg quotas as growth rate increases, which may in part explain the species difference observed as these species differ in their MSGR. Understanding the mechanisms underlying this pattern is certainly worthy of further study.

The contents of C, Fe, Na, P, and S were affected by daphniid age as well as an interactive effect of age and species on the content of N (Table 2). The fastest period of growth in *Daphnia* happens between birth and sexual maturity when they are also known to be P rich (e.g., Villar-Argaiz et al. 2002; Andersen and Hessen 1991; DeMott 2003). In other taxa, such as copepods, gradual decreases have been observed in body P as individuals grew and an increase of C and N with size was affected by growth rate (Villar-Argaiz et al. 2000). Hessen (1990) found that juvenile *Daphnia magna* had more body P than did adults, probably due to the high growth rate experienced by small bodied organisms as predicted by the GRH (Elser et al. 2000). Certainly, in our study, juvenile stages had higher MSGR (day 3 and 5) compared to that of older daphniids (Fig. 4). In crustaceans, C allocation usually undergoes a shift when somatic growth

slows at the onset of reproduction (Andersen 1997). As somatic growth ceases, much (~80%) of the allocable carbon is invested in reproduction (Lynch et al. 1986, Andersen 1997). Additionally, the proportion of resources allocated to reproduction varies with body size (Peters 1983), which could underlie the observed differences across age. We know considerably less about how Na and S demand and content change with age in *Daphnia*. Age differences in Na content could in part be explained by whole-body Na^+ uptake differing according to the life stage, with a shift from Na^+/Cl^- exchanger in juveniles to a $Na^+/K^+/2Cl^-$ cotransporter in adults (Bianchini and Wood 2008). The significant effect of age on Fe may be driven by allocation changes at maturity, as will be discussed in the next section.

Individuals may produce tissues of different compositions at different stages of their life. The most prominent example of such a change is that juvenile development implies the production of somatic tissues, whereas adult reproduction may require different nutrients to produce eggs. We observed ontogenetic differences in Fe (Fig. 3, Table 3). Specifically, gravid daphniids had significantly higher Fe compared to the pre-reproductive individuals. Indeed, we know demand for some metals may change ontogenetically, specifically Fe, where dietary Fe is heavily allocated to eggs (Zhou et al. 2007) and has major impacts on fecundity (Chen et al. 2011, Lind and Jeyasingh 2017). Differences in body and egg elemental (C, N, P) composition have been reported for many species (e.g. Sterner and Schulz 1998; Færøvig and Hessen 2003; Ventura and Catalan 2005; Visanuvimol and Bertram 2010) as pronounced changes in nutritional physiology takes place during reproduction to support gametogenesis, although we did not see significant differences in these elements when comparing non-reproductive and gravid daphniids.

While identifying the physiological mechanisms underlying the ionomic patterns observed here are outside the scope of any one study, these patterns do illuminate the elemental consequences of species- and stage-specific physiological differences in *Daphnia*. These data will be useful in guiding more targeted studies on the dynamics of some elements and its implications

for the expression of fitness-relevant traits among species and life stages. Whether such shifts bear any ecological relevance remains to be seen. Significant effects of species and life stage on the contents of 8 of the 11 measured elements, at minimum, indicate that the nutritional value of daphniids for predators should depend on the species and stage of individuals captured, perhaps with important implications for trophic interactions as discovered for relative supplies of bulk elements (e.g., C:P; Elser and Urabe 1999). Furthermore, substantial ionomic variation among taxa (e.g., Jeyasingh et al. 2017, Prater et al. 2018, Penuelas et al. 2019, Rudman et al. 2019) and spatiotemporal variation in the supplies of multiple elements, beyond the commonly studied bulk elements, are becoming apparent, potentially underlying signatures of co-limitation at the ecosystem-level (e.g., Kaspari and Powers 2016, Penuelas et al. 2019). Ionomic datasets should be a useful diagnostic tool to decipher the entire set of elemental constraints on biomass production at multiple levels.

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Acknowledgments

We are grateful to P Campbell, D Moen, and G Schoenknecht for their support and comments.

Tables

Table 1 *Daphnia* life-history variables

GLM				
Neonate mass				
	Factor	F	df	p
	Species	29.062	2	**<0.001**
	Error		17	
Mass at maturity				
	Factor	F	df	p
	Species	6.881	2	0.002
	Error		72	
Mass by age (day)				
	Factor	F	df	p
	Species	97.826	2	**<0.001**
	Age	57.533	6	**<0.001**
	Species*Age	6.945	12	**<0.001**
	Error		297	
Age at maturity				
	Factor	F	df	p
	Species	1.348	2	0.266
	Error		76	

Table 2 Effect of *Daphnia* species and age (in days) on *Daphnia* ionome (ANOVAs on individual elements)

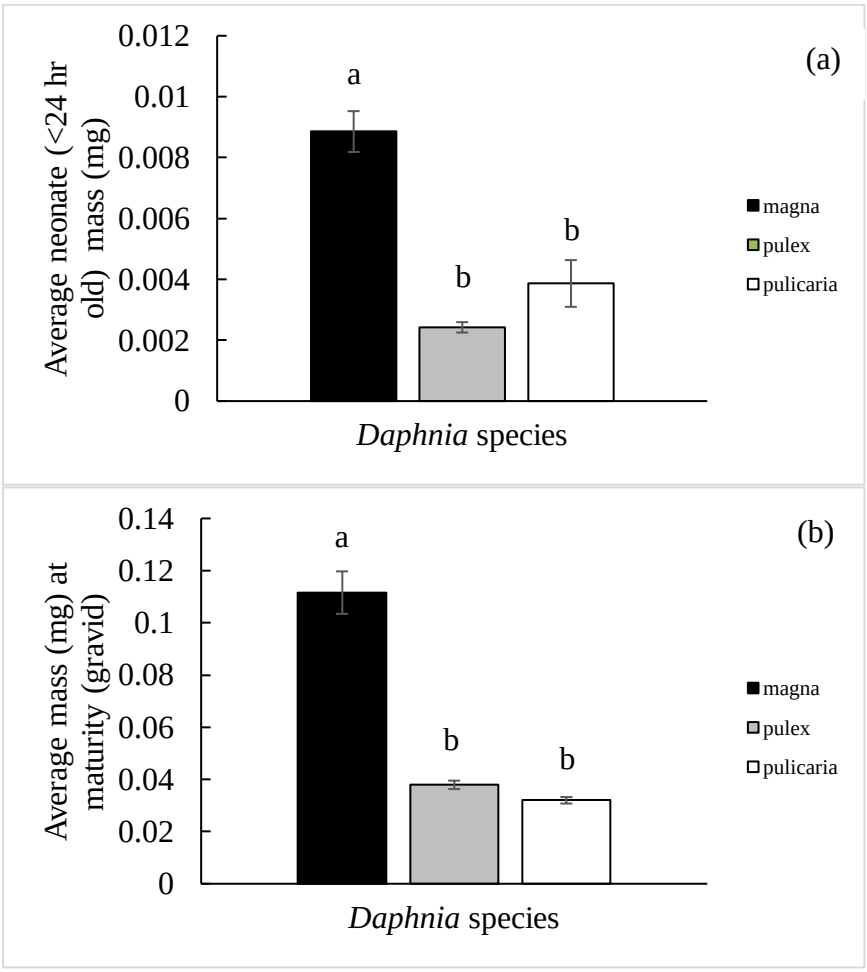
ANOVAs				
Element	Factor	F	df	p
C	Species	2.677	2	0.075
	Age	6.757	6	**<0.001**
	Species*Age	2.638	12	0.005
	Error		75	
N	Species	2.945	2	0.059
	Age	9.753	6	**<0.001**
	Species*Age	5.543	12	**<0.001**
	Error		75	
Ca	Species	4.756	2	0.010
	Age	1.939	6	0.079
	Species*Age	2.158	12	0.017
	Error		131	
Fe	Species	0.855	2	0.428
	Age	5.818	6	**<0.001**
	Species*Age	1.230	12	0.270
	Error		131	
K	Species	3.179	2	0.045
	Age	4.323	6	0.001
	Species*Age	1.450	12	0.151
	Error		131	
Mg	Species	6.626	2	0.002
	Age	1.217	6	0.302
	Species*Age	1.519	12	0.125
	Error		131	
Mn	Species	1.340	2	0.265
	Age	1.829	6	0.098
	Species*Age	0.702	12	0.748
	Error		131	
Na	Species	31.979	2	**<0.001**
	Age	8.852	6	**<0.001**
	Species*Age	1.160	12	0.318
	Error		131	
P	Species	9.564	2	**<0.001**
	Age	5.336	6	**<0.001**
	Species*Age	0.757	12	0.693
	Error		131	
S	Species	0.701	2	0.498
	Age	16.024	6	**<0.001**
	Species*Age	0.744	12	0.706
	Error		131	
Zn	Species	1.357	2	0.261
	Age	1.773	6	0.109
	Species*Age	0.566	12	0.866
	Error		131	

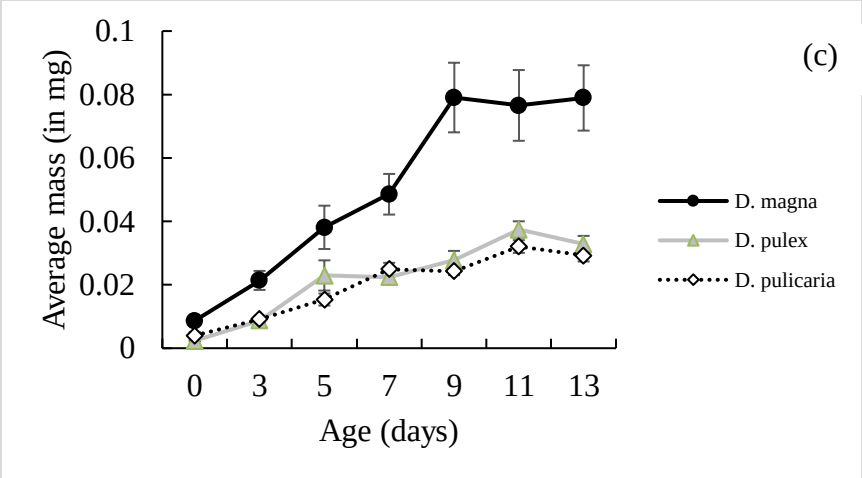
Table 3 Effect of species and ontogenetic stage on *Daphnia* ionome (ANOVAs on individual element concentrations)

ANOVAs				
Element	Factor	F	df	p
C	Species	3.073	2	0.054
	Stage	1.534	1	0.221
	Species*Stage	4.291	1	0.043
	Error		54	
N	Species	3.367	2	0.042
	Stage	4.316	1	0.043
	Species*Stage	1.766	1	0.189
	Error		54	
Ca	Species	18.965	2	**<0.001**
	Stage	6.978	1	0.010
	Species*Stage	1.176	2	0.313
	Error		106	
Fe	Species	2.304	2	0.105
	Stage	14.235	1	**<0.001**
	Species*Stage	1.369	2	0.259
	Error		106	
K	Species	6.079	2	0.003
	Stage	2.063	1	0.154
	Species*Stage	0.364	2	0.696
	Error		106	
Mg	Species	12.291	2	**<0.001**
	Stage	3.590	1	0.061
	Species*Stage	1.130	2	0.327
	Error		106	
Mn	Species	2.227	2	0.113
	Stage	0.367	1	0.546
	Species*Stage	0.363	2	0.696
	Error		106	
Na	Species	55.398	2	**<0.001**
	Stage	8.056	1	0.005
	Species*Stage	1.549	2	0.217
	Error		106	
P	Species	9.228	2	**<0.001**
	Stage	5.575	1	0.020
	Species*Stage	0.612	2	0.544
	Error		106	
S	Species	0.017	2	0.701
	Stage	8.581	1	0.004
	Species*Stage	1.617	2	0.203
	Error		106	
Zn	Species	0.726	2	0.486
	Stage	2.781	1	0.098
	Species*Stage	0.129	2	0.879
	Error		106	

Figures

Figure 1 Life-history (mean ± 1SE) differences among *Daphnia* species. **(a)** average neonate (<24 hr old) mass, **(b)** mass at maturity (gravid), **(c)** average mass by day, **(d)** average age of maturity (gravid). Letters above bars indicate significant differences based on Tukey's post hoc test.




(c)
Average mass (in mg)
0.1
0.08
0.06
0.04
0.02
0
0 3 5 7 9 11 13
Age (days)
D. magna
D. pulex
D. pulicaria

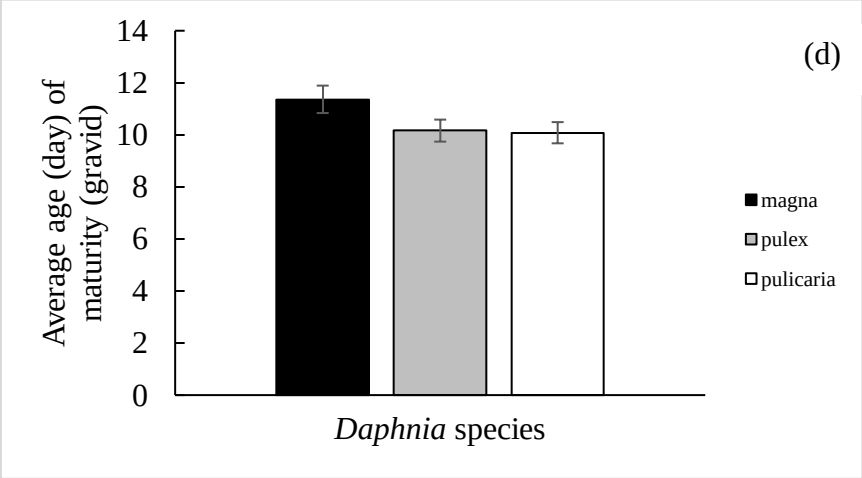
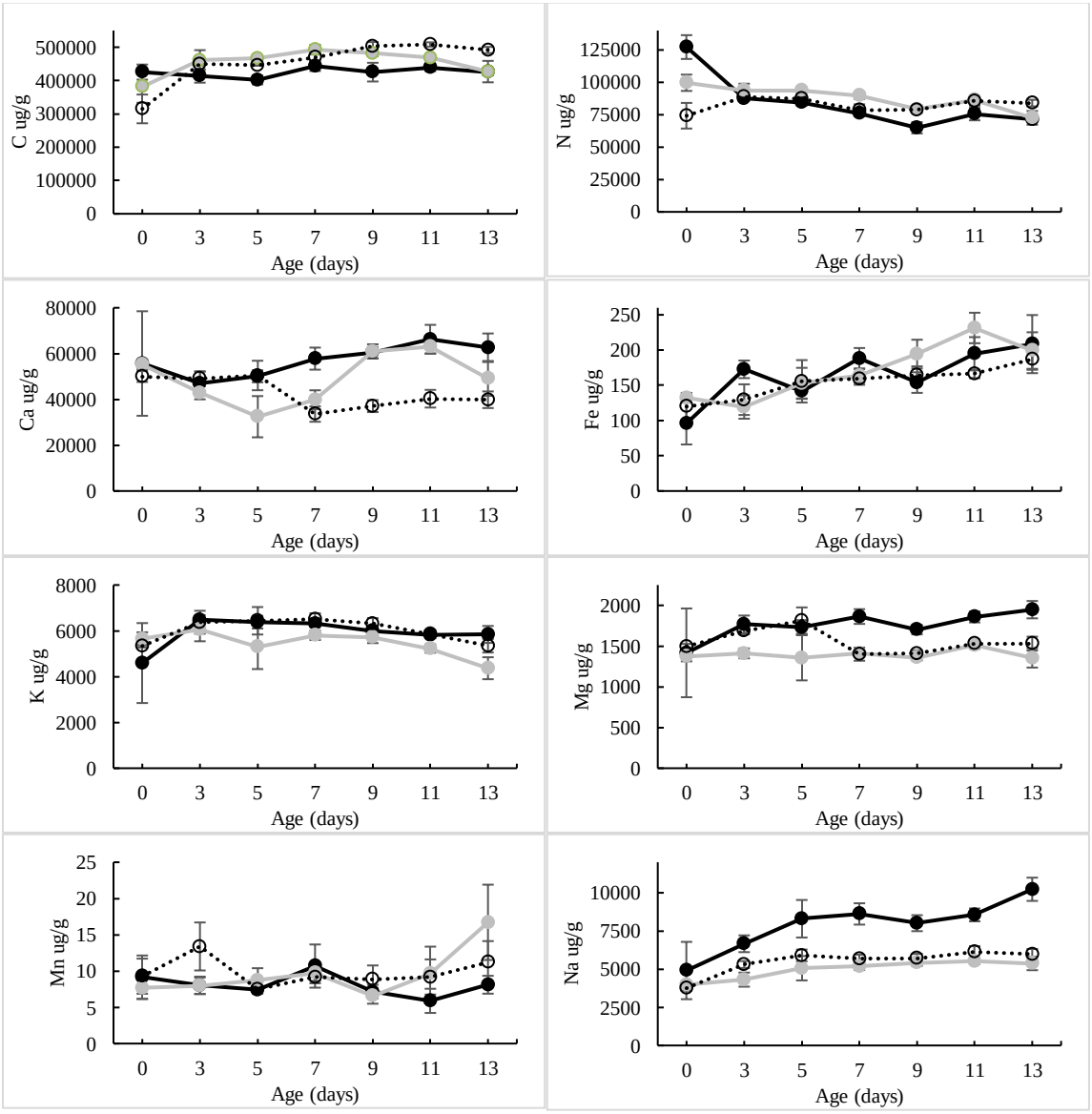

(d)
Average age (day) of maturity (gravid)
14
12
10
8
6
4
2
0
Daphnia species
magna
pulex
pulicaria

Figure 2 Effects of age on the ionome of three *Daphnia* species. Mean of x-y samples ± 1SE are depicted. See Table 2 for ANOVA results.



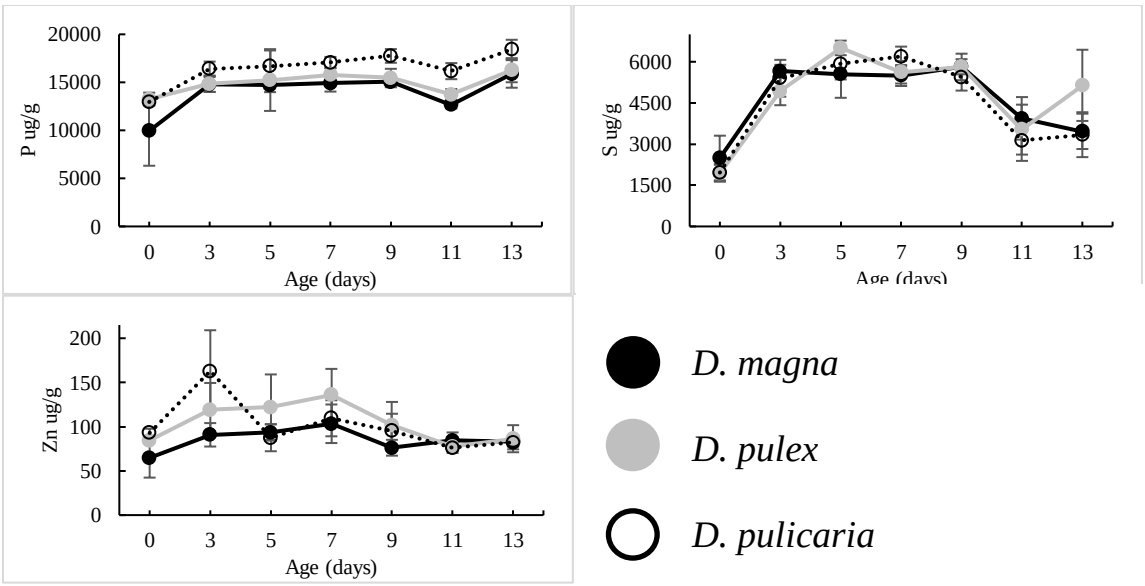

P ug/g
20000
15000
10000
5000
0
0 3 5 7 9 11 13
Age (days)
S ug/g
6000
4500
3000
1500
0
0 3 5 7 9 11 13
Age (days)
Zn ug/g
200
150
100
50
0
0 3 5 7 9 11 13
Age (days)
D. magna
D. pulex
D. pulicaria

Figure 3 Comparison of *Daphnia* body element concentration of pre-reproductive and gravid stages. Note that measurements are averages of aged 7-13 days, with individuals within each age having an equal representation of pre-reproductive and gravid individuals. See Table 3 for GLM results. C and N missing for *D. magna* due to missing data for gravid individuals.

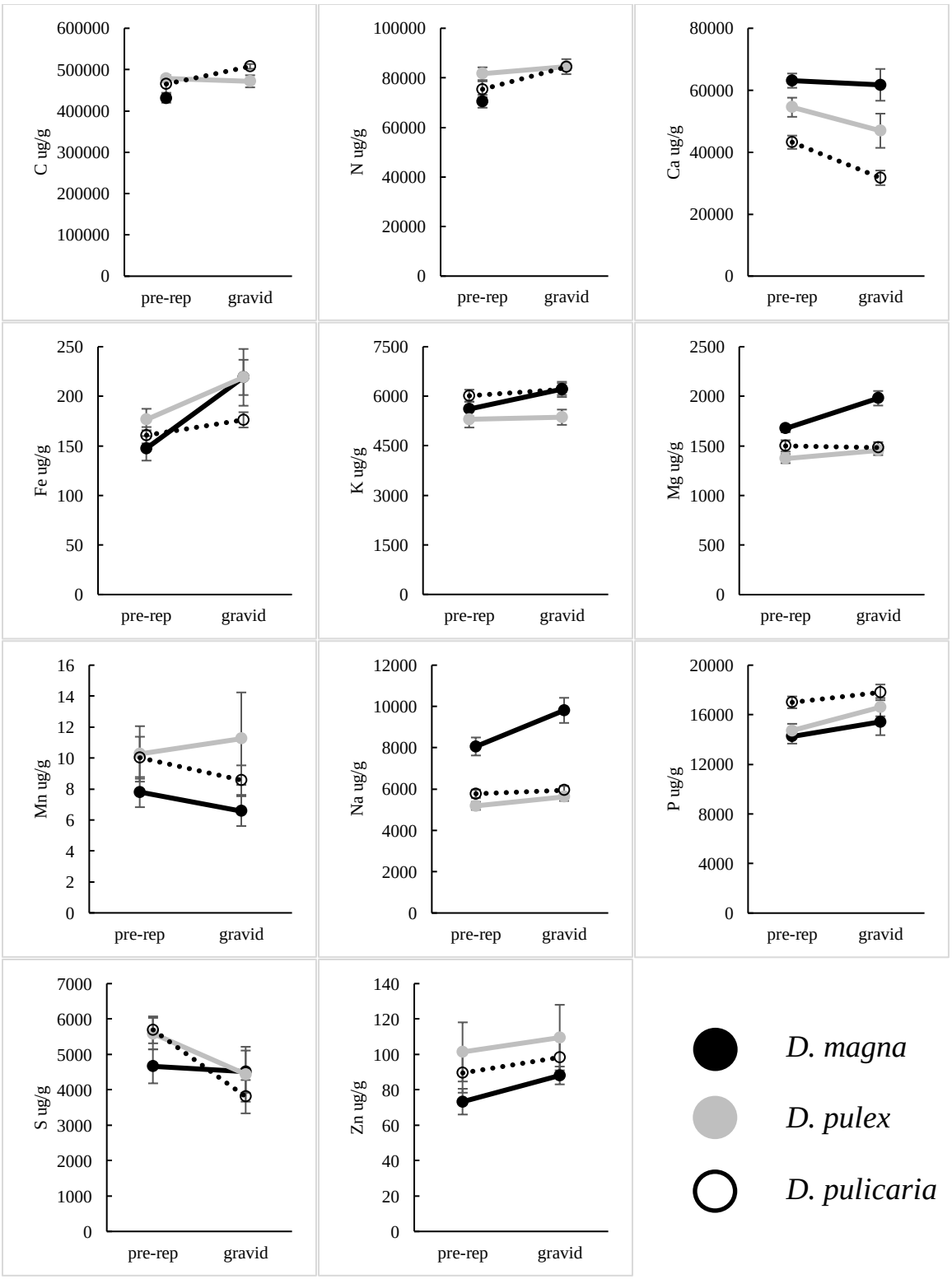
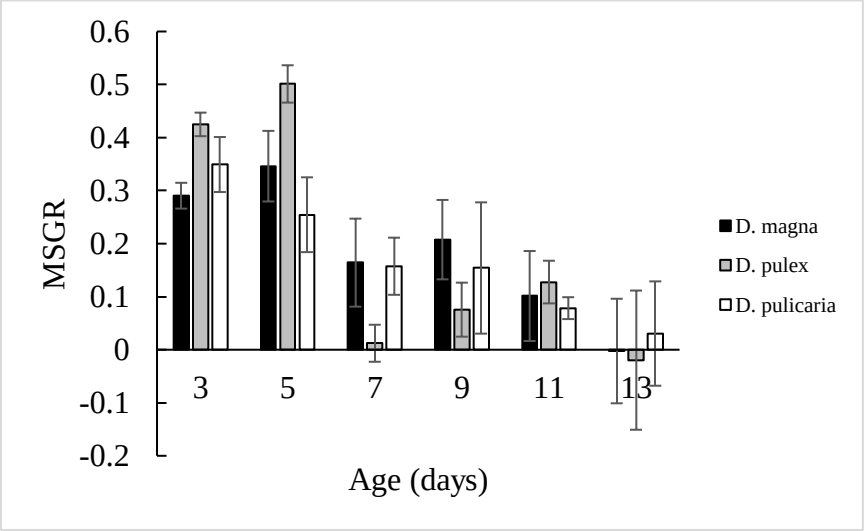

C ug/g
600000
500000
400000
300000
200000
100000
0
pre-rep
gravid
N ug/g
100000
80000
60000
40000
20000
0
pre-rep
gravid
Ca ug/g
80000
60000
40000
20000
0
pre-rep
gravid
Fe ug/g
250
200
150
100
50
0
pre-rep
gravid
K ug/g
7500
6000
4500
3000
1500
0
pre-rep
gravid
Mg ug/g
2500
2000
1500
1000
500
0
pre-rep
gravid
Mn ug/g
16
14
12
10
8
6
4
2
0
pre-rep
gravid
Na ug/g
12000
10000
8000
6000
4000
2000
0
pre-rep
gravid
P ug/g
20000
16000
12000
8000
4000
0
pre-rep
gravid
S ug/g
7000
6000
5000
4000
3000
2000
1000
0
pre-rep
gravid
Zn ug/g
140
120
100
80
60
40
20
0
pre-rep
gravid
D. magna
D. pulex
D. pulicaria

Figure 4 Mass-specific growth rate (MSGR) of *Daphnia* species by age.



Supplemental Information

Table S1 Effect of age on *Daphnia* ionome within species

		GLMs			
Element	Species	Factor	F	df	p
C	*D. magna*	Age	0.486	6	0.811
		Error		20	
	D. pulex	Age	3.256	6	0.015
		Error		28	
	D. pulicaria	Age	6.080	6	**<0.001**
		Error		27	
N	*D. magna*	Age	18.553	6	**<0.001**
		Error		20	
	D. pulex	Age	5.481	6	0.001
		Error		28	
	D. pulicaria	Age	1.248	6	0.314
		Error		27	
Ca	*D. magna*	Age	1.232	6	0.313
		Error		36	
	D. pulex	Age	3.095	6	0.014
		Error		41	
	D. pulicaria	Age	2.085	6	0.070
		Error		54	
Fe	*D. magna*	Age	2.416	6	0.046
		Error		36	
	D. pulex	Age	2.753	6	0.024
		Error		41	
	D. pulicaria	Age	2.425	6	0.038
		Error		54	
K	*D. magna*	Age	2.082	6	0.080
		Error		36	
	D. pulex	Age	2.465	6	0.040
		Error		41	
	D. pulicaria	Age	2.592	6	0.028
		Error		54	
Mg	*D. magna*	Age	1.864	6	0.114
		Error		36	
	D. pulex	Age	0.493	6	0.810
		Error		41	
	D. pulicaria	Age	1.476	6	0.204
		Error		54	
Mn	*D. magna*	Age	0.770	6	0.599
		Error		36	
	D. pulex	Age	1.454	6	0.218
		Error		41	
	D. pulicaria	Age	0.664	6	0.679
		Error		54	
Na	*D. magna*	Age	3.918	6	0.004
		Error		36	
	D. pulex	Age	1.658	6	0.156
		Error		41	
	D. pulicaria	Age	3.886	6	0.003
		Error		54	
P	*D. magna*	Age	2.839	6	0.023

		Error		36	
	D. pulex	Age	0.633	6	0.703
		Error		41	
	D. pulicaria	Age	2.972	6	0.014
		Error		54	
S	*D. magna*	Age	5.617	6	**<0.001**
		Error		36	
	D. pulex	Age	5.166	6	**<0.001**
		Error		41	
	D. pulicaria	Age	8.323	6	**<0.001**
		Error		53	
Zn	*D. magna*	Age	1.030	6	0.422
		Error		36	
	D. pulex	Age	0.710	6	0.643
		Error		41	
	D. pulicaria	Age	1.238	6	0.301
		Error		53	

Table S2 Effect of ontogenetic stage on *Daphnia* ionome within species. C and N missing for *D. magna* due to missing data for gravid individuals.

		GLMs			
Element	Species	Factor	F	df	p
C	*D. pulex*	Stage	0.384	1	0.542
		Error		22	
	D. pulicaria	Stage	4.500	1	0.045
		Error		22	
N	*D. pulex*	Stage	0.262	1	0.614
		Error		22	
	D. pulicaria	Stage	6.602	1	0.017
		Error		22	
Ca	*D. magna*	Stage	0.355	1	0.558
		Error		21	
	D. pulex	Stage	2.524	1	0.121
		Error		35	
	D. pulicaria	Stage	8.494	1	0.005
		Error		50	
Fe	*D. magna*	Stage	5.383	1	0.030
		Error		21	
	D. pulex	Stage	4.099	1	0.051
		Error		35	
	D. pulicaria	Stage	2.485	1	0.121
		Error		50	
K	*D. magna*	Stage	4.098	1	0.056
		Error		21	
	D. pulex	Stage	0.141	1	0.709
		Error		35	
	D. pulicaria	Stage	0.360	1	0.551
		Error		50	
Mg	*D. magna*	Stage	14.628	1	0.001
		Error		21	
	D. pulex	Stage	1.289	1	0.264
		Error		35	
	D. pulicaria	Stage	0.010	1	0.920
		Error		50	
Mn	*D. magna*	Stage	0.743	1	0.398
		Error		21	
	D. pulex	Stage	0.080	1	0.779
		Error		35	
	D. pulicaria	Stage	0.451	1	0.505
		Error		50	
Na	*D. magna*	Stage	5.628	1	0.027
		Error		21	
	D. pulex	Stage	2.304	1	0.138
		Error		35	
	D. pulicaria	Stage	0.375	1	0.543
		Error		50	
P	*D. magna*	Stage	0.938	1	0.344
		Error		21	
	D. pulex	Stage	4.631	1	0.038
		Error		35	
	D. pulicaria	Stage	0.975	1	0.328
		Error		50	

S	*D. magna*	Stage	0.140	1	0.712
		Error		21	
	D. pulex	Stage	3.703	1	0.062
		Error		35	
	D. pulicaria	Stage	11.812	1	0.001
		Error		50	
Zn	*D. magna*	Stage	4.589	1	0.044
		Error		21	
	D. pulex	Stage	0.357	1	0.554
		Error		35	
	D. pulicaria	Stage	0.971	1	0.329
		Error		50	

Table S3 Ionome mean ±1 SE rounded to the nearest whole number for *Daphnia* species at different ages. All element concentrations are represented as µg element per g of dry mass, and the units for age is days since birth.

Species	Age	C	N	Ca	Fe	K	Mg	Mn	Na	P	S	Zn
magna	0	425250 +/- 22668	127250 +/- 9187	55666 +/- 22818	96 +/- 30	4592 +/- 1746	1417 +/- 545	9 +/- 3	4907 +/- 1878	9945 +/- 3626	2494 +/- 813	65 +/- 22
pulex	0	381367 +/- 48202	99667 +/- 6297	55299 +/- 2191	132 +/- 7	5648 +/- 287	1373 +/- 60	8 +/- 2	3977 +/- 224	13255 +/- 674	1939 +/- 307	84 +/- 1
pulicaria	0	314767 +/- 43140	74233 +/- 9897	49990 +/- 2369	120 +/- 19	5338 +/- 66	1495 +/- 32	9 +/- 2	3752 +/- 165	12959 +/- 307	1962 +/- 310	93 +/- 5
magna	3	413900 +/- 20342	87550 +/- 2001	47010 +/- 5151	173 +/- 12	6486 +/- 201	1768 +/- 107	8 +/- 1	6660 +/- 549	14777 +/- 779	5660 +/- 229	91 +/- 13
pulex	3	461325 +/- 29910	93675 +/- 5281	42878 +/- 2847	119 +/- 17	6056 +/- 513	1410 +/- 61	8 +/- 1	4311 +/- 461	14852 +/- 841	4924 +/- 507	119 +/- 30
pulicaria	3	449500 +/- 4325	88750 +/- 741	49101 +/- 3114	130 +/- 22	6375 +/- 500	1686 +/- 41	13 +/- 3	5300 +/- 226	16421 +/- 743	5399 +/- 671	163 +/- 46
magna	5	401867 +/- 13420	84367 +/- 2369	50073 +/- 2612	142 +/- 11	6375 +/- 276	1729 +/- 89	7 +/- 0	8300 +/- 1228	14724 +/- 743	5546 +/- 859	93 +/- 9
pulex	5	466800 +/- 868	93825 +/- 1685	32437 +/- 9000	153 +/- 22	5305 +/- 976	1356 +/- 277	9 +/- 2	5058 +/- 798	15241 +/- 3215	6505 +/- 265	122 +/- 37
pulicaria	5	445700 +/- 1249	87267 +/- 1746	50434 +/- 6438	156 +/- 30	6439 +/- 599	1813 +/- 162	7 +/- 1	5897 +/- 384	16690 +/- 1631	5929 +/- 572	88 +/- 15
magna	7	443580 +/- 15151	76120 +/- 2263	57799 +/- 4818	188 +/- 15	6319 +/- 150	1863 +/- 90	11 +/- 3	8614 +/- 699	14919 +/- 881	5499 +/- 381	103 +/- 22
pulex	7	492622 +/- 14498	89944 +/- 2743	39866 +/- 4108	164 +/- 10	5807 +/- 207	1408 +/- 39	10 +/- 1	5184 +/- 243	15784 +/- 682	5620 +/- 413	136 +/- 30
pulicaria	7	469188 +/-	78463 +/-	33581 +/- 3344	159 +/- 8	6509 +/- 255	1401 +/- 81	9 +/- 1	5685 +/- 261	17074 +/- 573	6193 +/- 359	110 +/- 20

		26636	4552									
magna	9	425175 +/- 28088	64900 +/- 4330	60446 +/- 2417	154 +/- 15	5990 +/- 212	1702 +/- 59	7 +/- 1	8009 +/- 523	15044 +/- 556	5819 +/- 276	76 +/- 9
pulex	9	482000 +/- 8846	79214 +/- 2847	60943 +/- 3121	194 +/- 20	5723 +/- 259	1358 +/- 34	7 +/- 1	5421 +/- 211	15480 +/- 930	5811 +/- 484	102 +/- 26
pulicaria	9	502700 +/- 12784	78775 +/- 1318	37073 +/- 2605	164 +/- 13	6321 +/- 232	1411 +/- 38	9 +/- 2	5710 +/- 291	17751 +/- 715	5443 +/- 494	95 +/- 19
magna	11	438725 +/- 10564	75475 +/- 4782	66259 +/- 6291	195 +/- 24	5820 +/- 135	1858 +/- 67	6 +/- 2	8550 +/- 422	12644 +/- 364	3938 +/- 776	84 +/- 9
pulex	11	468225 +/- 4661	86375 +/- 2685	63013 +/- 2948	231 +/- 22	5225 +/- 182	1512 +/- 46	10 +/- 4	5525 +/- 206	13733 +/- 576	3527 +/- 910	78 +/- 7
pulicaria	11	508233 +/- 6440	85733 +/- 3730	40297 +/- 3856	166 +/- 6	5853 +/- 178	1531 +/- 48	9 +/- 2	6124 +/- 381	16168 +/- 837	3132 +/- 744	76 +/- 4
magna	13	425800 +/- 10054	71433 +/- 1233	62552 +/- 6228	208 +/- 41	5842 +/- 370	1947 +/- 107	8 +/- 1	10229 +/- 762	15876 +/- 1434	3465 +/- 645	83 +/- 6
pulex	13	426775 +/- 32136	72575 +/- 5316	49158 +/- 7611	199 +/- 26	4367 +/- 480	1355 +/- 119	17 +/- 5	5371 +/- 441	16265 +/- 1253	5141 +/- 1297	86 +/- 15
pulicaria	13	491560 +/- 9399	84000 +/- 2563	39844 +/- 3626	187 +/- 15	5347 +/- 295	1531 +/- 85	11 +/- 3	5974 +/- 344	18426 +/- 1009	3344 +/- 818	82 +/- 8

Table S4 Ionome mean ±1 SE rounded to the nearest whole number for gravid and pre-reproductive (pre-rep) *Daphnia* species. All element concentrations are represented as µg element per g of dry mass.

Species	Stage	C	N	Ca	Fe	K	Mg	Mn	Na	P	S	Zn
magna	gravid	n/a	n/a	61734 +/- 5127	219 +/- 29	6215 +/- 178	1980 +/- 73	7 +/- 1	9811 +/- 609	15429 +/- 1075	4513 +/- 595	88 +/- 5
magna	pre-rep	430273 +/- 10385	70527 +/- 2586	63091 +/- 2303	148 +/- 12	5613 +/- 224	1675 +/- 38	8 +/- 1	8062 +/- 436	14246 +/- 574	4663 +/- 480	73 +/- 7
pulex	gravid	471600 +/- 14653	84514 +/- 3035	46918 +/- 5521	219 +/- 18	5366 +/- 231	1454 +/- 47	11 +/- 3	5622 +/- 195	16611 +/- 736	4440 +/- 775	110 +/- 19
pulex	pre-rep	478520 +/- 6941	81660 +/- 2572	54502 +/- 3086	177 +/- 11	5302 +/- 248	1372 +/- 46	10 +/- 2	5187 +/- 193	14725 +/- 543	5588 +/- 443	101 +/- 17
pulicaria	gravid	507700 +/- 5824	84386 +/- 1225	31726 +/- 2362	176 +/- 8	6207 +/- 231	1483 +/- 55	9 +/- 1	5942 +/- 206	17805 +/- 634	3806 +/- 471	98 +/- 11
pulicaria	pre-rep	464980 +/- 21040	75370 +/- 3206	43210 +/- 2141	161 +/- 8	6016 +/- 183	1500 +/- 57	10 +/- 1	5773 +/- 199	16999 +/- 478	5694 +/- 381	90 +/- 11

VITA

Ryan E Sherman

Candidate for the Degree of

Doctor of Philosophy

Dissertation: ECOLOGY AND EVOLUTION OF PHOSPHORUS USE IN *DAPHNIA*

Major Field: Integrative Biology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Integrative Biology at Oklahoma State University, Stillwater, Oklahoma in May, 2019.

Completed the requirements for the Bachelor of Science in Biology at Western New England University, Springfield, Massachusetts in May, 2013.