How do consumers deal with stoichiometric constraints? Lessons from functional genomics using *Daphnia pulex*

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Abstract

Disaccord between the supply and demand of energy (carbon, C) and certain material elements (e.g. phosphorus, P) across trophic levels is common in most ecosystems and impacts the strength of trophic interactions and ecosystem functions such as productivity and nutrient recycling. Yet, we know little about mechanisms operating at the lower levels of biological organization that drive such higher-level ecological processes. Such information should help refine theories integrating biological processes at multiple levels of organization. Understanding the expression and functions of genes that underlie (to a large degree) physiological adjustments made by organisms to stoichiometric imbalances at trophic interfaces is a first step in this enterprise. Here, we investigate adjustments in gene expression to varying supply and demand of phosphorus relative to other dietary components in the keystone limnetic herbivore, *Daphnia pulex*. Daphniids were fed an algal diet of either LoC-HiP (molar C:P/C24 χ=100) or HiC-LoP (molar C:P/C24 χ=900) for 5 days, resulting in significant growth reductions under HiC-LoP conditions. Microarrays measured the transcriptional regulation of 8217 annotated protein-coding genes under contrasting dietary conditions and revealed 1818 differentially expressed (DE) genes; 19% are genes unique to the Daphnia lineage. We mapped DE genes onto a global chart of metabolic pathways to obtain a systems-level perspective on the responses to stoichiometric imbalances. *Daphnia* differentially regulated pathways were involved in sequestering limiting elements, and in dealing with the products of metabolic adjustments that may be triggered by nutrient stress in primary producers. Functional genomics at trophic interfaces illuminate the complexity of processes underlying stoichiometric constraints on energy and nutrient fluxes in ecosystems.

Keywords: dietary imbalance, ecological stoichiometry, microarrays, phosphorus limitation, secondary production, transcriptomics, trophic interactions

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Introduction

Certain chemical elements are critical to organisms for building and maintaining basic biological structures and maintaining core functions. For example, nitrogen is a necessary component for producing nucleic and amino acids, while carbon-rich substrates are used to fuel respiration and phosphorus-rich rRNA is needed for ribosome biogenesis and thus cellular and organismal growth. The supply of these elements to ecosystems varies considerably, and fluctuations in their relative availability have numerous consequences at multiple levels of organization (Sterner & Elser 2002). Primary producers assimilate inorganic nutrients such as N and P, and impacts of differential supply at higher trophic levels are commonly observed by changes in community structure and secondary production. Such responses to variation in the relative supply of key elements drive the operation of ecological stoichiometry, which sets in motion a host of trophic interactions, from the upper to the lower levels of organization in ecosystems.
elements emphasize the fundamental coupling of energy and materials in food webs, with biological demands being relatively narrow in comparison with the wide variation in the chemical content of the abiotic environment. Ecological stoichiometry (Sterner & Elser 2002) is an approach where organisms are considered to be simply the collections of the elements. While such purposeful abstraction provides predictive power at higher levels of organization, we know little about processes at lower levels of organization that underlie such predictions. We therefore attempt to identify genes whose ultimate protein products are involved in key biochemical pathways that may be relevant during stoichiometric constraints.

Genomic response to altered P-supply has been characterized in some model organisms (Chlamydomonas, Grossman 2000; Saccharomyces, Liu & Sturley 2004; Escherichia coli, Baek & Lee 2007; Arabidopsis, Morcuende et al. 2007) that predominantly obtain P via uptake of inorganic forms. These studies report hundreds of genes and pathways that are differentially expressed by these primary producers when P is depleted. A first category of responding genes are those involved in the mobilization, uptake and transport of P – such as acid phosphatases (e.g. Morcuende et al. 2007) and inorganic P-transporters (reviewed in Jeysingh & Weiden 2007) – thereby enhancing P-uptake and recycling when organisms are P-limited. A second group of responding genes is those involved in central metabolism – such as glycolysis and sucrose biosynthesis – which can regulate internal P-balance under limiting conditions (Hammond & White 2008; Nilsson et al. 2010). Undoubtedly, other categories of responding genes can be listed based on their shared molecular functions. Yet with reference to this second group, reaching homeostasis by altering C-metabolism, respiratory and other compensatory metabolic pathways in relation to P-supply have noticeable consequences for a variety of biological processes. The impacts include production of carbohydrates (Plaxton & Carswell 1999), signalling molecules and organic acids such as malate, citrate and several other products and by-products not produced in P-replete primary producers (Plaxton 2004; Tran & Plaxton 2008). These studies indicate that P-availability in the environment modifies the P-content of primary producer tissue and the concentration of products resulting from alternative metabolic pathways (i.e. a metabolite load).

By contrast to primary producers, heterotrophs obtain their essential elements from uptake or ingestion of complex food resources. While there is little knowledge on the transcriptomic response of heterotrophic consumers to P-availability in the environment, we posit that their transcriptomes should reflect pathways that are directly involved in P-sequestration and scavenging (like those reported above), yet also reflect responses to biochemical changes in primary producers invoked by P-availability. To our knowledge, no transcriptome-level studies have yet examined the consequences of ingesting low P foods by primary consumers. Linking elemental supply to global gene expression between trophic levels promises a high-resolution understanding of the biological basis to adjustments of the flow of energy and materials through food webs. Here, we report results of our microarray investigations into the model herbivore species Daphnia pulex consuming P-replete or P-limited Scenedesmus obliquus, a green alga.

Daphnia are P-rich crustacean herbivores with a somatic C:P ratio around 100 (Elser & Urabe 1999). They are ecologically dominant grazers in lakes and ponds (Lampert 2006) and play a major role in whole-lake energy and phosphorus budgets (Sterner et al. 1992). Seston is suspended particulate matter, such as bacterial and algal cells, that is food for the generalist consumer Daphnia. Freshwater seston C:P varies widely between ~100 to 1000 (Sterner et al. 2008). As a consequence, daphniids frequently ingest diets that contain relatively more C atoms than the amount required for basic life processes (e.g. growth) compared to the available P atoms. These animals are considered to be P-limited. Furthermore, field data indicate that the amount of C available to Daphnia is often more than the amount of calories required to fuel maintenance metabolism (see Sterner & Schulz 1998). Daphnia solve dietary imbalances (most commonly when algal C:P > 268; Frost et al. 2006) by increasing phosphorus-use efficiency and/or by increasing C disposal (e.g. DeMott et al. 1998). Daphniids appear to use both pre- and post-simulation mechanisms to deal with dietary imbalances, particularly in dealing with excess C (reviewed in Hessen & Anderson 2008), with major impacts at higher levels of organization.

The precise and predominant mechanisms that daphniids use to deal with such challenges are poorly understood. Understanding changes in gene expression that at least in part drive such responses of Daphnia will enable integration of subcellular processes to processes at higher levels of organization. Our integrative study employs long oligonucleotide microarrays that are designed from a set of 10 000 uniquely assembled cDNA sequences mapped to 8217 gene models (Colbourne et al. 2005; produced as part of the D. pulex genome sequencing project). We discover differentially expressed (DE) genes by hybridizing labelled cDNA from biological replicates of a single D. pulex clone exposed to elementally balanced and imbalanced diets. By virtue of the quality assembly and annotation of the draft D. pulex genome sequence (Colbourne et al. 2011), we map with confidence the differentially regulated
genes onto the global chart of metabolic pathways (KEGG, Kanehisa et al. 2004) using the iPath interactive tool (Letunic et al. 2008). Based on the current genome annotation, the D. pulex metabolic pathway consists of 1908 genes representing 563 unique enzymes. Our exploration of Daphnia gene expression data begins to identify pathways of relevance under stoichiometric imbalances between resource and consumer, thereby contributes fresh insights to the detailed understanding of the mechanisms underlying the transfer of energy and materials in ecosystems.

Methods

Generation of algae with contrasting C:P

Colonies of the green alga Scenedesmus acutus were grown in continuous flow chemostats at room temperature (~20 °C) in a 18:6 light–dark cycle under low- (5.94 µM) and high-phosphorus (59.37 µM) supply (Kilham et al. 1998). Algae thus grown were harvested and its carbon content estimated spectrophotometrically using a regression equation generated using absorbency and C-content data (estimated using a CHN analyzer, triplicate for estimation of algal C- and P-content. Algal LoC-HiP algae was filtered onto glass fibre filters in triplicate for estimation of algal C- and P-content. Algal C was estimated using an automated CHN analyzer, and %P was estimated using persulfate digestion and ascorbic acid method (APHA 1992).

P-starvation experiment

Fifty same-brood sisters were used to generate the experimental Daphnia pulex. Third-brood neonates were collected, pooled and randomly assigned to treatments. Thirty ~48-h-old (~8 h) neonates were kept in glass jars containing 300 mL of COMBO medium (Kilham et al. 1998) without N or P. The jars were placed in a growth chamber at 20 °C, with a 18:6 h light–dark cycle. This experiment was replicated four times totalling eight jars (four HiC-LoP, four LoC-HiP). Animals in each jar were fed with 1 mg C/L of HiC-LoP or LoC-HiP algae, which is well above the incipient limiting concentration of C for Daphnia (Lampert 1987). Animals were carefully transferred into a different set of jars containing the same amount of media and algae every day for the following 5 days. After 5 days, 20 animals from each jar were transferred into separate RNAase-free 1.5-mL microcentrifuge tubes and then flash-frozen in liquid nitrogen after removing excess water. Five hundred (500) µL of Trizol reagent (Invitrogen Life Sciences) was added to each tube, and the tissues were homogenized using a pestle for 5 min, followed by the addition of 500 µL more Trizol reagent before storing at ~80 °C.

Analysis of microarray data

After fabrication of microarrays, RNA purification and amplification, competitive hybridization, imaging and data extraction (see Appendix S1, Supporting information), raw data from Geneexpix were imported into R and analysed using Linear Models for Microarray Data (LIMMA) (Gentleman et al. 2004; R-Project 2009). The data discussed in this publication have been deposited in the NCBI Gene Expression Ominibus and are accessible through GEO accession number GSE27959. Unflagged spots, without background correction, were used for the analysis. We used print-tip loess normalization for within-array and scale normalization across arrays (experimental replicates) for normalization of spot intensities (Smyth & Speed 2003). The identification of statistically significant changes in spot fluorescence (signal) between the experimental conditions was based on the moderated t-statistic using an Empirical Bayes approach, which results in stable inference especially when the number of arrays is small (Smyth 2004). The final list of DE genes was obtained by adjusting for multiple testing by calculating the false discovery rate (FDR) (Benjamini & Hochberg 1995) for each gene using the qvalue package in R (Storey et al. 2004) and applying a 5% FDR criterion.

The genes represented on the array were described during the community-wide manual annotation of the draft D. pulex genome sequence. Following the second revision of the predicted and validated gene set, 8217 probes could be assigned to Dappu version 1.1 gene models (Appendix S2, Supporting information). The predicted protein-coding genes were functionally annotated by homology to annotated genes from the NCBI nonredundant set and classified according to eukaryotic orthologous groups (Koonin et al. 2004), KEGG metabolic pathways (Kanehisa et al. 2004) and phylogenomic gene clustering (Dehal & Boore 2006). Exact binomial tests were conducted between the observed number of DE genes assigned to each KOG and the expected number of DE genes within these functional groups, assuming a random sampling of up-regulated, down-regulated or altogether differentially regulated genes. These same tests were conducted using genes (enzymes) assigned to metabolic pathways, to discover relevant metabolic shifts under stoichiometric imbalances between resource and the Daphnia’s dietary requirements. Enzymes showing transcriptional changes at 5% FDR were mapped onto the overview metabolic
network for D. pulex (Letunic et al. 2008). KEGG networks that contained a greater than expected number of DE genes (P-value <0.05) were finally highlighted to observe functional relationships.

Somatic C:P
Sixty 48-h-old neonates were dried (60 °C) in batches of 15 overnight for estimation of C and P. In addition to the four replicates for isolation of RNA mentioned above, we included two replicate jars containing 30 individuals per treatment for documenting the C and P-content of Daphnia feeding on HiC-LoP and LoC-HiP algae at the end of the experiment. Animals were weighed using a microbalance; their C-content estimated using an automated CHN analyzer. Per cent phosphorus of animals was estimated using persulfate digestion and ascorbic acid method (APHA 1992) after ashing at 550 °C for 2 h.

Growth experiment
Concurrently, we conducted a controlled growth experiment on individuals from the same cohort used in the generation of RNA described above. To track the growth trajectories of individual animals under HiC-LoP and LoC-HiP diet, single 2-day-old (±8 h) individuals were maintained in 100 mL of N- and P-free COMBO medium, and fed 1 mg C/L of HiC-LoP or LoC-HiP algae. This experiment was replicated ten times. Each day, each animal was transferred onto a slide and measured to the nearest 0.01 mm using an ocular micrometer fitted to a microscope. Length of animals was measured from the top of the helmet to the base of the tail spine. After measurement, animals were carefully transferred into another jar containing similar amounts of medium and algae. This procedure was repeated for 5 days to estimate growth performance under HiC-LoP and LoC-HiP diet.

Results and discussion

C:P of algae and Daphnia growth
Scenedesmus obliquus cells grown under low P-supply had a much higher C:P ratio than cells grown under high P-supply (HiC-LoP = 864.66 ± 48.13; LoC-HiP = 118.2 ± 20.49; t = −51.62; d.f. = 14; P < 0.0001). Total C-content of both algae introduced into experimental jars was standardized spectrophotometrically to 1 mg C/mL prior to feeding (see Methods). Chemical analyses revealed that 2-day-old individuals (beginning of the P-starvation experiment) had a somatic C:P of 93 ± 8.2 (mean ± 1SD). After 5 days, the somatic C:P ratio of animals feeding on HiC-LoP algae was 124 ± 12.7, while that of animals feeding on LoC-HiP algae was 114 ± 7 (t = −1.88; d.f. = 2; P = 0.068). Dietary C:P had a major effect on somatic growth rate. Animals raised on LoC-HiP diet grew significantly faster than counterparts reared on HiC-LoP diet (LoC-HiP = 0.22 ± 0.01 mm/5 day; HiC-LoP = 0.08 ± 0.01 mm/5 day; t = −7.88; d.f. = 8; P < 0.0001).

Global patterns of differential gene expression
Eighteen per cent of the surveyed genes on the microarray (1818; see Appendix S3, Supporting information) showed a significant change in gene expression (FDR = 5%) when daphniids were fed algae that differed in the ratio of carbon to phosphorus (Fig. 1). The most current annotation of the Daphnia pulex draft genome assembly v.1.1 suggests that 19% (342) of these 1818 genes have no homology to proteomes of other sequenced organisms. Based on the total average frequency of the number of genes without homology on the microarray (3%), Daphnia-lineage-specific genes are overrepresented in the present set of DE genes (binomial z-ratio = 39.45, P < 0.000001).

Fig. 1 Gene expression changes in Daphnia pulex responding to dietary imbalances of carbon and phosphorus (measured as C:P ratio), based on microarray experiments using four biological replicates. Positive fold change values on a log scale represent 886 transcriptionally up-regulated genes expressed when C:P = 900 (HiC-LoP) compared to C:P = 100 (LoC-HiP). Negative fold change values represent 932 up-regulated genes expressed when C:P = 100 compared to C:P = 900. One thousand eight hundred and eighteen (1818) genes show significant fold change in gene expression (false discover rate = 5%) and are marked red and green. Genes showing no change in gene expression are marked blue.

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For our functional genomics comparisons, we present our experimental results in the context of HiC-LoP. For example, up-regulation of a given gene or pathway under LoC-HiP diet is described here as down-regulation under HiC-LoP. Of the 1818 DE genes, 886 genes were up-regulated and 932 genes were down-regulated under HiC-LoP, suggesting that down-regulation was a slightly more common response to HiC-LoP (binomial $z$-ratio = -1.06, $P = 0.010405$).

Even though this experiment revealed a rather large number of DE genes, the magnitude of the difference of expression seemed modest with relatively few genes being strongly up- or down-regulated in response to LoC-HiP. Indeed, only six genes were up-regulated by more than twofold (Fig. 1, see Appendix S3, Supporting information). The low dynamic range is likely a feature of DNA quantity printed on this microarray platform. Among these genes are three Daphnia-lineage-specific genes of unknown function. Another more than twofold up-regulated gene is a member of a paralogous group of six up-regulated lipoproteins. The final two strongly responding genes area: (i) a homolog to the beta subunit of a Sec61 protein translocation complex and (ii) a homolog to a reticulon gene. Based on their homology to functionally annotated genes in laboratory model species, these genes likely function by trafficking proteins across cell membranes; both are found on the surface of the endoplasmic reticulum (Yang & Strittmatter 2007). A comparatively larger number of genes (28) were down-regulated by more than twofold when animals were challenged with HiC-LoP. Among these strongly down-regulated genes are two Daphnia-lineage-specific genes as well as multiple members of several multilocus gene families (Fig. 1, see Appendix S3, Supporting information). Clearly, when only strongly responding genes are considered, down-regulation of affected genes appears to be the predominant response to HiC-LoP, i.e. 82% of genes responding by more than twofold were down-regulated while 18% were up-regulated. Furthermore, the Daphnia-lineage-specific genes are markedly overrepresented in the group of strongly up-regulated genes (50% vs. 3%, exact binomial test: $P = 0.000493$), while their representation in the group of strongly down-regulated genes does not significantly exceed their average representation on the microarray (7.1% vs. 3%, exact binomial test: $P > 0.10$).

**Differential expression of genes belonging to eukaryotic orthologous groups (KOGs)**

To be able to link differential expression in response to HiC-LoP with molecular function, we focused our attention on a subset of genes that could be assigned KOG categories based on orthology (Dehal & Boore 2006; Colbourne et al. 2011). On our array, 3234 genes were represented by 1055 KOGs with the number of genes per KOG ranging from 1 to 83. We used exact binomial tests to identify multilocus gene families that deviate from the average expected number of DE genes per KOG ranging from 1 to 83. We used exact binomial tests to identify multilocus gene families that deviate from the average expected number of DE (average: 1402/3234 = 0.4335), up-regulated (average: 765/3234 = 0.2365) or down-regulated (average: 637/3234 = 0.1970) genes.

We identified three multilocus gene families that showed an excessive number of DE genes (KOGs 2650, 0628, and 0022, see Appendix S4, Supporting information); in each case, the deviation was because of a larger than expected number of down-regulated genes. The most remarkable example of excessive down-regulation is provided by KOG2650, a multilocus gene family of zinc carboxypeptidases, enzymes that hydrolyse peptide bonds and play key roles in separating individual amino acids from polypeptides, and also in protein maturation. Of the 14 genes that were on the array, 10 were down-regulated under HiC-LoP diet. Our result may provide a direct molecular link to previous observations of significant down-regulation of protein synthesis under P-limitation (see Sterner & Elser 2002 for detailed discussion).

Another multilocus gene family showing a larger than expected number of down-regulated genes is represented by KOG4258, a family of insulin/growth factor receptors (see Appendix S4, Supporting information). Seven of the ten genes on our array were down-regulated in response to HiC-LoP, revealing that algal P-limitation causes down-regulation of growth factors in daphniid consumers and underlining the significant relationship between organismal growth and P-demand (Sterner & Elser 2002).

We observed only one example of a multilocus gene family exhibiting a larger than expected number of up-regulated genes, represented by KOG1971, a gene family of procollagen-lysin 5-dioxygenases that are involved in lysine degradation. All three genes on the array were up-regulated in response to HiC-LoP. However, because of low statistical power, this result should be considered merely suggestive of a possible molecular mechanism.

**Differential expression of metabolic pathways**

We aggregated sets of DE genes into functional categories using the KEGG database (Kanehisa et al. 2004) and the iPath (Letunic et al. 2008) interactive explorer (http://pathways.embl.de/ipath1/data_mapping.html). We recommend that readers upload Appendix S5 (Supporting information) into this interactive explorer to help guide the results and discussion that follow. The KEGG categories are presented in *italics* below, to
help the reader locate the region of the metabolic chart in discussion (both in Fig. 2, as well as in the interactive map recommended above).

There are a total of 896 probes on the microarray representing 313 different enzymes that can be mapped onto the 12 broadly defined KEGG categories (including the non-DE enzymes). Of the 1818 DE genes, 224 genes (135 different enzymes including duplicated genes) participate in one or more of the 130 KEGG pathways; 111 of these genes were up-regulated under HiC-LoP condi-

![Diagram of metabolic pathways](image)

**Fig. 2** Differentially regulated enzymes represented on the microarray and involved in metabolic pathways in *Daphnia pulex*. When *Daphnia* are fed a HiC-LoP diet, 173 up-regulated genes are indicated by red edges, while 159 down-regulated genes are indicated by green edges. Genes that have paralogs with opposite transcriptional responses to dietary changes are represented by yellow edges. White edges represent enzymes on the microarray that show no transcriptional change. Six highlighted pathways are enriched by differentially expressed genes as a fraction of the unique enzymes in the pathways represented on the microarray: (A) biosynthesis of alkaloids; (B) tryptophan metabolism; (C) androgen and oestrogen metabolism; (D) arachidonic acid metabolism; (E) glycerolipid metabolism; (F) phenylpropanoid biosynthesis. Five enriched pathways are not visible at this resolution. See Table 1 for details.
tions, while the remaining 113 genes were down-regulated (see Appendix S6, Supporting information). For 17 enzymes, one or more duplicates responded to the experimental conditions in opposite polarity (Fig. 2).

We performed exact binomial tests for each pathway to identify those that deviate from the expected number of DE genes based on random assortment among pathways (see Methods). We began by identifying pathways that are enriched or impoverished with responding members, irrespective of gene copy numbers. We found six pathways that are down-regulated and five pathways that are up-regulated under HiC-LoP conditions at a significance level <0.05 (Table 1).

Several genes involved in biosynthesis of alkaloids derived from histidine and purine were up-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone A). Products of such pathways include pilocarpine, which is known to increase secretion of digestive enzymes in vertebrates (Wiedmeier et al. 1987). It is possible that daphniids feeding on HiC-LoP algae utilize pilocarpine to increase digestibility of P-limited algal cells that are known to have thicker cells walls (Tillberg & Rowley 1989). Similarly, pathways resulting in alkaloids derived from terpenes and polyketides were also down-regulated under HiC-LoP conditions. The concentration of several secondary metabolites such as terpenes and polyketides in autotrophs is impacted by the amount of mineral nutrients available (e.g. Ormeno et al. 2008). Thus, differential phosphorus supply to algae may well have altered the amount of such secondary metabolites, eliciting responses in Daphnia.

Phosphorus supply has a large impact on the tryptophan content of autotrophs (e.g. Singh 1981). We observed that many genes involved in tryptophan metabolism were down-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone B). Tryptophan is an essential amino acid for most secondary consumers, and thus, its availability impedes protein synthesis. Whether P-limited Scenedesmus contain lower amounts of tryptophan remains to be seen and could result in further growth penalties under P-limited conditions (i.e. in addition to the growth rate hypothesis; Elser et al. 1996). Interestingly, we found an up-regulation of genes involved in aminocyt-tRNA biosynthesis under HiC-LoP conditions. While overall protein synthesis in a cell decreases with P-availability (Boer et al. 2010), tRNA synthesis has been observed to be uncoupled from rRNA synthesis under limiting conditions (Oliver & McLaughlin 1977), potentially indicating translational control of protein synthesis (Weiss 1973; Lodish 1976).

Pathways involved in androgen and oestrogen metabolism were down-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone C). Daphniids feeding on HiC-LoP algae exhibit delayed sexual maturity (Jeyasingh & Weider 2005). Thus, one would expect down-regulation of hormones involved in growth and maturity (Mitchell 2001) under P-starvation. Moreover, such sex hormones appear to be tightly linked to mobilization of P during reproduction to supplement key reproductive tasks such as synthesis of yolk phosphoproteins in other organisms (Gardner & Pfeiffer 1943).

We found that the majority of the known genes involved in biosynthesis of plant hormones were up-regulated under HiC-LoP conditions (Table 1). Several plant hormones are known to promote P-uptake and translocation (e.g. Machackova et al. 1986), many of which are found in physiologically relevant concentrations in algal as well (Tarakhovskaya et al. 2007). It is possible that P-limited algal cells accumulate higher concentrations of hormones which upon ingestion invokes daphniids to up-regulate metabolic machinery to deal with changes in phythohormones.

A majority of the genes involved in arachidonic acid metabolism were down-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone D). Arachidonic acid is a polyunsaturated fatty acid (PUFA). Several studies have shown that P-availability decreases PUFA content of algal cells (Muller-Navarra 1995). Therefore, the down-regulation of such pathways under P-limited (HiC-LoP) conditions is not surprising. Essential PUFAs play several important physiological roles, including immunity, metabolism and reproduction (Stanley 2006). Increased P-supply enhances algal eicosapentaenoic acid (EPA) content (Muller-Navarra 1995), a potentially limiting biochemical for Daphnia growth (Brett et al. 2009). Thus, down-regulation of arachidonic acid metabolism may be expected because EPA is an intermediary metabolite in this pathway. Closer analyses of the enzymes involved in this pathway revealed that prostaglandin E-synthase (PGE synthase, EC 5.3.99.3) was down-regulated under HiC-LoP conditions. PGE synthase is involved in eicosanoid metabolism, suggesting that lower levels of EPA in P-limited algal cells could underlie this observation. On the other hand, prostaglandin D-synthase (PGD synthase, EC 5.3.99.2) was up-regulated, perhaps indicating more complex changes in eicosanoid metabolism in Daphnia feeding on HiC-LoP algae.

Genes involved in glycerolipid metabolism were largely up-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone E). Glycerolipids in autotrophs are primarily phospholipids (Hammond et al. 2003), the amounts of which have been observed to be reduced by as much as 50% under P-limitation (Chlamydomonas, Moseley & Grossman 2009), possibly by the replacement of phospholipids with galactolipids (Arabidopsis, Li et al. 2006). Consumers feeding on such P-starved plant cells are therefore likely to experience more galactolipid content,
which does not contain P. It could be possible that daphniids are metabolizing glycerolipids either in their food or even those stored in their body to increase P-use efficiency.

Phenylpropanoid biosynthesis was significantly down-regulated under HiC-LoP conditions (Table 1, Fig. 2 – zone F). Phenylpropanoids are a diverse group of molecules derived from the shikimate pathway and perform multiple functions in plants.

Table 1 KEGG pathways on the array that are enriched (responsive) or impoverished (relatively robust) with differentially expressed (DE) genes when daphniids cope with HiC-LoP conditions, irrespective of the response of paralogs.

<table>
<thead>
<tr>
<th>KEGG category</th>
<th>Pathway code</th>
<th>Total unique genes from pathway on array</th>
<th>No. up-regulated genes</th>
<th>No. down-regulated genes</th>
<th>Total no. differentially regulated (DE) genes</th>
<th>Expected Prob. Pathway name</th>
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<td>1</td>
<td>9</td>
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<td>2</td>
<td>4</td>
<td>6</td>
<td>1.39 0.0467 Phenylpropanoid biosynthesis</td>
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<td>2.50 0.0533 Pentose phosphate pathway</td>
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<tr>
<td><strong>Amino acid</strong></td>
<td>ec00250</td>
<td>12</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>3.74 0.0612 Alanine, aspartate and glutamate metabolism</td>
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<td>ec01064</td>
<td>18</td>
<td>4</td>
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<td>5.00 0.0644 Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid</td>
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<td>2.81 0.0835 Drug metabolism – other enzymes</td>
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<td>14</td>
<td>2</td>
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<td>9</td>
<td>4.37 0.0996 Starch and sucrose metabolism</td>
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Expected numbers of DE genes (expected no.) were compared to the observed number of up- or down-regulated genes using a binomial test, and their significance levels (prob.) are presented.
key functions in plants, including signalling of nutrient stress (Vogt 2010). The induction of key phenylpropanoids such as anthocyanins is strongly up-regulated under N limitation in Arabidopsis (Scheible et al. 2004), with P-limitation also playing a key mediating role (Peng et al. 2008). Although we are unaware of studies reporting such responses to mineral limitation in algae, gene expression in Daphnia indicates that the products of such secondary metabolic pathways in algae may differ depending on P-supply. Products of secondary metabolism may be important in determining the quality of an algal cell, because we found another pathway linked to the larger shikimate pathway, tryptophan metabolism, that was also down-regulated. Daphnia contain about 0.02% (of wet mass) tryptophan (Bogut et al. 2010). Down-regulation of tryptophan metabolism in Daphnia feeding on HiC-LoP algae indicates that P-limitation may have altered the quantity or quality of the reactants (e.g. shikimic acid) required for tryptophan synthesis.

Concluding remarks

Research in ecological stoichiometry is pursued by asking whether the complexities of biochemical processes can be subsumed into a simple, mass balance framework (Sterner & Elser 2002). Such models are highly desirable from the standpoint of making predictive statements from as small an information base as possible, for example, predicting which species will be successful in a given environment from information about their elemental contents alone. However, this simplification purposely overlooks the potentially complex biochemical and metabolic adjustments that must be made by organisms in contrasting elemental supply conditions. The contrast in information content between simple mass balance models and systems-level analysis is enormous. Ecological stoichiometry can be credited with simplifying and making tractable a hugely complicated set of processes, but tools such as those utilized here can help begin to reveal the entire scope of biological functionality (from genes to ecosystems) associated with elemental imbalances. It is important to note the potential confounding factors in our experiments. For example, dietary C:P also affects size and growth rate of animals; thus, DE of some genes may be caused by such correlated impacts of P-supply. Nevertheless, daphnids inhabiting ecosystems with differential P-supply are also likely to experience such variation in correlated responses.

Results from our functional genomics study show that P-supply to algae impacts Daphnia in many and complex ways, ultimately resulting in the well-documented effects at higher levels of organization (Sterner & Elser 2002). The transcriptomic responses of organisms such as bacteria, algae and higher plants to P-limitation reveal differential regulation of hundreds of pathways and thousands of genes (e.g. Grossman 2000; Liu & Sturley 2004; Misson et al. 2005; Baek & Lee 2007). Much of the attention is rightly paid to a systems-level understanding of phosphorus use efficiency (i.e. transport, scavenging). From an ecological viewpoint, organisms such as bacteria, algae and plants are fundamentally different in the mode by which they interact with the nutritional environment, compared to metazoan consumers such as Daphnia. But P-limitation is a significant ecological factor for metazoans too. When P is in limited supply in an ecosystem, organisms at the base of the food web respond in diverse ways (e.g. see Jeyasingh & Weider 2007 and references therein). Thus, consumers at higher trophic levels in P-limited ecosystems should be exposed to not only lower supply of inorganic P, but also to the products of biochemical adjustments in producers in response to P-limitation. Our data have illuminated the rich complexity underlying the response of Daphnia to P-sufficient (LoC-HiP) and P-deficient (HiC-LoP) algal cells. Several of the pathways that were differentially expressed between dietary conditions involve the products and by-products of metabolic responses of algae to P-limitation. Further research on the impacts of these pathways on the performance of Daphnia is required to fully understand the impact of stoichiometric constraints imposed by large-scale ecological processes on the fine-scale subcellular processes, and vice versa. This first transcriptomic analysis of consumer response to limited supply of P into the food web indicates that indirect responses, mediated by biochemical alterations at the producer level, may play a significant role in determining the transfer of energy and materials in ecosystems.

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The authors are members of the Daphnia Genomics Consortium. They combine their diverse expertise and interests to develop theory at the interface of ecology and evolution.

**Supporting information**

Additional supporting information may be found in the online version of this article.

**Appendix S1** Description of the Daphnia microarray design, microarray production, target synthesis, hybridization, image acquisition, and data extraction. Microarray platform GPL13280 and data GSE27959 are deposited at National Center for Biotechnology Information Gene Expression Omnibus database.

**Appendix S2** Table with all annotated genes, including the alignment statistics of oligo-probe sequences to Dappu version 1.1 frozen gene models, paralog IDs, KOG, KEGG, address in assembled genome sequence, and including the microarray results for each probe/gene.

**Appendix S3** The 1818 differentially expressed probes on the Daphnia microarray. The probes were selected controlling for the False Discovery Rate (Qvalue) at 5%. Positive fold change implies up-regulation of expression while negative fold change implies down-regulation.
Appendix S4 Summary of the number of microarray probes (genes) that were mapped to EuKaryotic Orthologous Groups (KOG). This table provides information on the number of differentially expressed probes that are up-regulated and down-regulated under HiC-LoP conditions.


Appendix S6 Summary of the number of enzymes, ignoring paralogs, that were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. This table provides information on the number of differentially expressed enzymes that are up-regulated and down-regulated under HiC-LoP conditions.

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