GENES RESPOND IN A DOSE-DEPENDENT MANNER TO NUTRITIONAL STRESS IN THE FRESHWATER ZOOPLANKTER, *DAPHNIA PULEX*

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ABSTRACT

Genes respond in a dose dependent manner to nutritional stress in the freshwater zooplankter, *Daphnia pulex*

Anna Patrycja Dlugosz

We studied the physiological and molecular responses of lab-grown *Daphnia pulex* to shifts in dietary (carbon, nitrogen, phosphorus, and cyanobacteria) and growth media (calcium) nutrient supply using a set of potential biomarkers via qPCR. Each examined nutrient had a strong effect on *Daphnia* mass-specific growth rate, and we found significant dose-dependent effects of treatment level (medium and low) on the gene expression of selected indicator genes. Furthermore, linear discriminant analysis models using different combinations of treatment levels could separate the animals between nutritional treatments with 86-100% prediction accuracy. This would suggest that *Daphnia* appear able to respond to nutrient limitation by adjusting their growth rate and associated molecular pathways to deal with an insufficient supply of nutrients. While this study provides valuable information regarding *Daphnia's* ability to adjust physiological and molecular processes under controlled laboratory conditions, more validation needs to be conducted before applying these potential biomarkers to wild populations to assess the type and intensity of nutritional stress.

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Chapter One – General Introduction

Food quality can affect organisms and dictate whether they meet their metabolic and growth demands. Nutrient limitation of primary producers, such as algae and cyanobacteria, leads to poor food quality for primary consumers and results in the animal not receiving enough essential nutrients (Sterner et al. 1993; Elser et al. 2000). Nutritional limitation in primary consumers thus results in adverse physiological effects (e.g., growth and reproductive constraints) that ultimately can lead to population declines and affects food web dynamics (Anderson and Hessen, 2005; Bukovinszky et al. 2012). Many freshwater ecosystems across Canada have been experiencing changing concentrations of ecologically relevant nutrients, including phosphorus, nitrogen, and calcium, due to human-drive environmental change (e.g., acidification, agricultural activities, urban development, etc.), which may be altering patterns of nutrient limitation in aquatic food webs (e.g., Watmough and Dillon, 2002; Prater et al. 2016; Azan and Arnott, 2018).

One species of particular concern is the keystone zooplankton species, *Daphnia.* As a critical component of freshwater ecosystems, *Daphnia* plays an essential role in nutrient cycling and food web dynamics (Frost et al. 2005; Wagner et al. 2013). *Daphnia* have been identified as particularly sensitive to low calcium concentrations in water (Cairns and Yan, 2009; Jones et al. 2020) and appear to be frequently limited by the quality and quantity of food in lakes (Prater et al. 2016). Most of what is known about *Daphnia* nutrition comes from highly controlled feeding experiments that use whole-body responses such as growth, reproduction, and survival as an indicator of nutrient limitation (e.g., Becker and Boersma, 2003; Frost et al. 2006). However, these responses are very similar across multiple limiting nutrients, so it is difficult to tell which nutrient is the limiting one, and the results can be confounded by the presence of other limiting nutrients

(Jones et al. 2020). This results in indirect and circumstantial approaches of assessing *Daphnia* nutrition and limits our ability to assess the nutritional state of wild *Daphnia* populations (Jones et al. 2020). There are emerging molecular methods that might be usable determining the nutritional state of *Daphnia* directly (Jones et al. 2020). However, there has been little to no testing done on these approaches to differentiate animals experiencing different forms of nutritional stress.

Quantitative Polymerase Chain Reaction (qPCR) is a powerful molecular tool for accurately detecting specific DNA sequences, enabling precise quantification of gene expression (Dymond, 2013). It could also prove particularly useful in assessing the nutritional state of animal consumers because it allows for direct, quantitative measurement of gene expression related to nutrient metabolism pathways. Additionally, whole transcriptome analysis facilitates the identification of candidate genes responsive to various forms of stress (e.g., Jones et al. 2020). This combination of qPCR and transcriptome analysis opens avenues for understanding how nutritional factors impact gene expression in *Daphnia*, offering insights into their physiological responses to elemental supply.

Transcriptomic studies of *Daphnia* grown under different food qualities have shown that genes associated with nutritional limitation are responsive to the daphnids' diet and nutrient availability (Jones et al. 2020; Jones et al. 2022). Subsequently, assays were developed for genes significantly differentially expressed under nutrient-deficient conditions, providing a targeted approach to monitor nutritional stress in *Daphnia* (Jones et al. 2023). However, extensive validation and thorough testing of candidate qPCR assays is essential for ensuring their specificity and sensitivity before being employed to monitor nutrient stress in wild populations.

This thesis aims to design and optimize a set of potential qPCR primers from an existing indicator gene database, optimize potential reference genes, and test the dose dependence of selected indicator genes. We will conduct controlled laboratory experiments, exposing *Daphnia pulex* to varying concentrations of carbon (C), calcium (Ca), nitrogen (N), phosphorus (P), and cyanobacteria, and validate the performance of newly developed qPCR assays. This approach strengthens the reliability of qPCR data in nutritional stress studies. If efficient, these biomarkers would present a targeted approach to predicting and monitoring nutrient stress in *Daphnia* populations.

Chapter Two – Genes respond in a dose dependent manner to nutritional stress in the freshwater zooplankter, *Daphnia pulex*

Introduction

Human activities, specifically agriculture and industry, have increasingly affected the supply of dissolved nutrients entering aquatic ecosystems (Bashir et al. 2020). Global warming, coupled with increased loading of growth-limiting nutrients, such as nitrogen (N) and phosphorus (P), has also accelerated the frequency and extent of cyanobacterial blooms (Schindler, 2006). These blooms can be acutely toxic to animals (Smith and Schindler, 2009) and are a low-quality food source for consumers (Ger et al. 2016; Schwarzenberger and Fink, 2018). Changes in the availability of nutrients in aquatic ecosystems can also affect lake food webs by altering the type and intensity of nutritional limitation on freshwater producers and their consumers (Sterner and Elser, 2002).

The effects of poor nutrition are well documented on freshwater zooplankton, including the cladoceran, *Daphnia*. When *Daphnia* consume food with high C:N or C:P ratios, they receive excess C (energy) and too little N or P (Sterner et al. 1993; Sterner and Hessen, 1994). To address the insufficient supply of nutrients, *Daphnia* can adjust their rates of ingestion, metabolism, and excretion of multiple elements, which affects their net incorporation of these elements (Frost et al. 2005). Physiological responses of *Daphnia* to changes in food supply and nutrient availability stem from molecular-level responses in the form of gene expression (Wagner et al. 2013; Jones et al. 2020). *Daphnia* exhibit plasticity in gene expression in response to food availability that is regulated through mechanisms including transcriptional regulation, where transcription factors bind to specific DNA sequences to upregulate (activate) pathways that increase the digestion

and absorption of the limiting nutrient(s) and downregulate (repress) pathways that require disproportionate quantities of the nutrient in short supply (Wagner et al. 2013; Orsini et al. 2018). Specific molecular pathways associated with nutrient limitation, and other stressors, can be targeted using qPCR (Jones et al. 2020). For example, Koussoroplis et. al. (2017) used triacylglycerol lipases as biomarkers of dietary stress in *D. pulex,* which were feeding on cyanobacteria; they found that the gene expression of triacylglycerol lipases increased with more cyanobacterial food in their diet. Quantifying the expression of genes associated with nutritional metabolism could serve as a direct source of knowledge of the type and intensity of nutritional stress (Wagner et al. 2013; Jones et al. 2020) and would improve upon past work that used whole-body animal responses (e.g., growth, reproduction, survival) that typically emerge in response to the low supply of essential nutrients. For these responses, it is difficult to diagnose limitation by one nutrient or another, and the results are potentially confounded by the presence of other limiting nutrients (Wagner et al. 2013; Jones et al. 2020). Previous transcriptome studies of *Daphnia* grown on different quality food have shown that different genes associated with nutritional limitation were responsive to the daphnids' dietary intake of food nutrients (e.g., Jones et al. 2020). This previous work showed the potential to further develop and apply qPCR-based indicators of animal nutritional state.

Here, we measured the responses of six genes putatively connected to molecular pathways associated with nutrient limitation in a clone of *D. pulex*. These genes were chosen based on previous results that Jones et. al. (2022) found were significantly differentially expressed under different nutrient-limiting conditions and that indicated that these genes had potential to characterize the nutritional status of the organism. To examine whether these genes are regulated in a dose-dependent manner, we grew *D.*

pulex under five nutritional treatments (C, N, P, Ca, cyanobacterial) under three levels of supply (high, medium, and low). We also identified a set of candidate reference genes and examined their stability across all our different nutrient-limiting conditions.

Methods

Algae and Daphnia culturing

Scenedesmus obliquus (*S. obliquus* strain 10) and *Romeria leopoliensis* (*R. leopoliensis* strain 102) were purchased from the Canadian Physiological Culture Centre and grown in 2-L culture flasks, receiving continuous aeration and high light intensity (>150 μmol s^{-1} m^{-1}) at 20 °C. *Scenedesmus obliquus* (high abundance of high quality, medium and low P, medium and low N, low and medium Ca, and medium and low food abundance) was cultured in COMBO growth media (Kilham et al. 1998), and *Romeria leopoliensis* (cyanobacterial food) in BG-11 growth media (Rippka et al. 1979). Once algal cells reached their carrying capacity (~1 week of growth), to achieve desired algal nutritional gradients, cultures were diluted daily with differentially enriched P and N-media for ≥ 1 week before harvesting following the dilution rates listed in Table 1 (adapted from Jones et al. 2022).

Daphnia pulex (clone # TCO SOM 1.1) was cultured in P-free COMBO growth media (Kilham et al. 1998) at ~20 °C and fed high-quality *S. obliquus* as needed. Experimental animals were prepared by collecting < 24 hr old *D. pulex* neonates from the 3rd clutch of brood mothers, assigning animals to jars, and feeding following the methods described in Jones et. al. (2020).

Preparation of nutritional treatments

To examine if the gene expression responses of *Daphnia* to nutrient limitation are dose-dependent, we created four diet-based treatments (P, N, food abundance, and cyanobacteria) and one growth media treatment (Ca), totaling five experiments with each consisting of three levels of supply: high, medium, and low. To prepare the Ca treatment, the original COMBO growth media (Kilham et al. 1998) was adjusted for the amount of $CaCl_2 \bullet 2H_20$ from 10 mg/L (high nutrient) to 3 mg/L (medium) and 1.5 mg/L (low). Harvested algae cells (high abundance of high quality, medium and low P, medium and low N, and cyanobacteria) were centrifuged and resuspended in Ca-, P-, and N-free COMBO (Kilham et al. 1998). Subsamples of algal food suspensions were pipetted onto pre-weighed GF/C glass fiber filters (n=3), dried for ≥ 2 hrs at 60°C, and post-weighed to calculate algal dry mass. We saved the post-weighed filters and used an elemental analyzer (Vario EL III, Elementar Inc., Mt Laurel, New Jersey) to verify and determine C and N content. Phosphorus content was estimated using potassium persulfate digestion followed by ascorbic acid-molybdate blue colorimetry (APHA, 1992). During the experiment, algal dry weight was used to calculate feeding amounts, assuming 50% of dry weight is C, to produce 1 mg C/L for the medium food abundance treatment, 0.125 mg C/L for the low food abundance treatment, and 3 mg C/L for the remaining treatments. In all experiments, *Daphnia* were fed a 100% green algae diet, except for the cyanobacterial experiment, where we combined 40% cyanobacteria and 60% green algae for the medium diet and 80% cyanobacteria and 20% green algae for the low nutrient treatment. Food mixtures with varying P and N ratios were calculated using algal dry weight and elemental content data to create C:P 400 (medium P), C:P 800 (low P), C:P

100 (remaining treatments), C:N 16 (medium N), C:N 24 (low N), and C:N 8 (remaining treatments) diets.

Experimental setup

For each experiment (C, N, P, Ca, cyanobacteria), we used 30 replicate jars per treatment (high, medium, and low concentrations) filled with 400ml of COMBO growth media (Kilham et al. 1998). Neonates (<24 hrs old) were collected from the third or fourth clutch of brood mothers, triple rinsed with Ca-, P-, and N-free COMBO (Kilham et al. 1998), and ten *Daphnia* were randomly placed into each jar, with each representing an experimental unit. Additionally, three groups of 20 neonates were saved for each experiment, placed in pre-weighed aluminum cones, dried for ≥ 2 hrs at 60°C, and post-weighed to determine initial *D. pulex* mass. Experiments ran for six days, and *Daphnia* were fed on days 0 and 3. Feeding amounts were doubled for all treatments on day 3 to ensure neonates were receiving enough food to sustain growth (Jones et al. 2020). On day 6 of the experiments, five jars per treatment combination, each containing ten *Daphnia*, were weighed following the above procedure and used to calculate mass-specific growth rate (MSGR). Ten animals from the remaining jars (n=25 jars per treatment) were pipetted into 1.5 ml RNase DNase-free microcentrifuge tubes, and excess COMBO was removed. After adding 100 µl of RNAlater, samples were immediately stored at -80°C until RNA extraction. A summary of the experimental setup (elemental ratios, feeding amounts, etc.) is provided in Table 2.

Daphnia mass-specific growth rate and statistical analysis

For each experiment (C, N, P, Ca, cyanobacteria), we saved animals from nutritional treatments (high nutrient, medium, and low nutrient) and calculated MSGR using the following equation:

$$MSGR = \frac{In(B2) - In(B1)}{time}$$

where B1 stands for the average initial neonate mass, B2 is the average final mass of *Daphnia*, and time is the number of days of growth (i.e., six). To determine if *Daphnia* were growth limited, the effects of each nutritional diet on MSGR were calculated using a two-way analysis of variance (ANOVA) using R (R Core Team, 2019) and the car package (Fox et al. 2013). We ran multiple comparisons using the multcomp package (Hothorn et al. 2008), by comparing the 95% family-wise confidence intervals of the differences between each combination of nutrient levels.

RNA isolation and cDNA synthesis

Following the manufacturer's instructions, RNA from *D. pulex* was extracted for each treatment combination using a PureLink™ RNA mini kit. Before extraction, we removed the RNAlater® from the 1.5ml RNase DNase-free microcentrifuge tubes containing ten *Daphnia*. We included a trizol-chloroform separation phase to maximize yields of genomic material from the chitinous exoskeleton (Athanasio et al. 2016) and an on-column DNase treatment before washing and elution. RNA concentration and purity was measured using a NanoDrop 8000 spectrophotometer. We synthesized cDNA for samples with an absorbance ratio of 260 nm (A260) to 280 nm (A280) of 1.8-2.2 and an A260/A230 ratio > 1.8, which indicated nucleic acid purity. We converted the isolated

RNA to cDNA using the Applied Biosystems High-Capacity RNA-to-cDNA™ kit using 2µg RNA in a 20µL reverse transcription reaction.

Primer design, validation, selection of reference genes, and qPCR

We selected potential nutrient-specific genes for primer design from a previously performed comprehensive transcriptome analysis of *D. pulex* in response to nutritional stress via Illumina sequencing (Jones et al. 2022). We selected five potential indicator genes per nutrient (P, C, N, Ca, cyanobacteria) and used the WFleabase (Colbourne et al. 2005) database to assign predicted functional annotations (Table 4). We obtained gene sequences from the JGI genome portal (Grigoriev et al. 2012). To avoid gDNA amplification, we removed the introns from the input sequences and designed exon junction spanning primers using the Primer3plus (Untergasser et al. 2007) online primer design tool with the following settings differing from the default parameters: amplicon size of 50-250 bp, the maximum length of polynucleotide repeats 3, maximum self-complimentary 2. Using the IDT Oligo Analyzer 3.1 (Integrated DNA Technologies, Inc.), we checked for self-dimers, heterodimers, and secondary structures and ran the primers on BLAST (Altschul et al. 1990) against the genome of *D. pulex* and *D. pulicaria* to check for mispriming (specificity).

Candidate reference genes were identified through literature exploring the genomic responses of *Daphnia* to various stress (Table 5). We used the provided primer sequences to validate the set of reference genes to accurately quantify *D. pulex* gene expression levels using the same protocol. Following this screening, we selected optimal reference genes (Table 6), and further examined the stability of their expression across all treatment combinations through qPCR.

Using our high-nutrient samples, we ran a temperature gradient on each primer pair. We verified the product via 3% gel electrophoresis (1 X TBE buffer and running at 70 V for 1.5 hrs) to obtain optimum annealing temperature and check for appropriate binding, amplification, and product size. We further assessed the specificity of the primers via qPCR by running standard curves (serial dilution of 1:2 and 1:10) and a melting curve analysis. We calculated the standard curve correlation coefficient (R^2), slope, amplification efficiency (E), and limit of quantification (LOQ) (Bustin et al. 2009). Amplification efficiency was calculated from the slope of the standard curve using the following formula:

$$E = 10^{-1/slope}$$

The qPCR amplification product was again assessed via 3% gel electrophoresis to confirm target-specific amplification and find the limit of detection (LOD). Following this initial screening, we selected 1-2 primers per nutritional treatment for the final qPCR assay (Table 3).

The optimized qPCR amplification reactions were performed in 96-well plates using the QuantStudio 3 real-time PCR system with SYBR Green PowerUp™. Reactions were performed in a total volume of 10 µL containing 5 µL of the SYBR Green, 0.9 µL each of forward and reverse primers (10 µM), 1.6 µL ddH_2O, and 2.5 µL of cDNA (10 ng/µL), using five biological replicates, two technical replicates, and a template (positive) and a no-template (negative) control. The qPCR cycling conditions were as follows: 95ºC for 30s, (95ºC for 5s; annealing temperature for 30s) x40, followed by a melting curve analysis and held at 4ºC ∞. Refer to Table 3 for the annealing temperature of primers.

Quantification of gene expression and statistical analysis of qPCR data

To accurately quantify relative gene expression, a single, most stably expressed reference gene was used as a normalizer to compensate for any differences in the amount of starting material. The average of the untreated (high nutrient) samples was used as the control, and gene expression of the target gene in all other samples (medium and low treatments) is expressed as an increase or decrease relative to the control. Since the amplification efficiencies of the reference and target genes were not within 5% of each other, the Pfaffl method was used to calculate relative gene expression (Pfaffl, 2001). To calculate relative gene expression between the test and control samples, normalized to a reference gene, the following formula was used:

$$Ratio = \frac{\left(E_{target}\right)^{\Delta Ct,\ \text{target (average Ct control} - \text{Ct test)}}}{\left(E_{reference}\right)^{\Delta Ct,\ \text{reference (average Ct control} - \text{Ct test)}}}$$

where E_{target} and $E_{reference}$ are the amplification efficiencies of the target and reference genes, respectively, and ΔCt is calculated using the average cycle threshold (Ct) values of the control samples and the Ct value of test sample in the target and reference genes. The $\log_2$ transformed gene expression ratio was used for statistical analysis.

The pheatmap package (Kolde, 2019) was used to create a heatmap of $\log_2$ fold-change to visualize the nutritional phenotypes. To test the capacity of the 7 indicator genes to discriminate between nutritional history, we fit three separate linear discriminant analysis (LDA) models with different combinations of treatment levels. Using the class predictions, we obtained overall model accuracy and created variable loadings plots using ggplot2 (Wickham, 2016). To determine if genes respond in a dose-dependent manner to the level of nutritional stress (medium and low), we completed a two-way ANOVA using

the car package (Fox et al. 2013). Lastly, we completed multiple comparisons on genes with significant interactive effects (nutrient x level). We held the family-wise error rate at 5% when computing confidence intervals of the differences between medium and low nutrient levels for each gene. Due to an unbalanced design, the carbon and cyanobacterial treatments were excluded from analysis. All analyses and plots were completed in R version 4.2.3.

Results

Mass-specific growth rate

Dietary P, N, C, cyanobacteria, and media Ca had a strong effect on *Daphnia* MSGR (Figure 1). *Daphnia* in the high nutrient treatment showed the highest growth rate, and growth rates consistently decreased with more severe nutrient limitation with the lowest rates observed in the low nutrient treatments. Low food supply (carbon) had a strong effect on growth, as did the cyanobacterial diet and low N food. Low Ca concentrations had the smallest effects on MSGR (Figure 1).

Stability analysis of reference genes

Raw Ct values were used to determine gene expression levels of candidate reference genes. To identify the most stable reference gene between treatments, we analyzed the expression stability using five algorithms: RefFinder (Xie et al. 2023), comparative ΔCt method (Silver et al. 2006), geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004). Each stability algorithm ranked the 5 candidate reference genes from the most stable to the least stable gene, with some differences observed (Table 7). With only one exception, Syntaxin-16

ranked as the most stable gene and was selected as the reference gene for the final qPCR assay.

Nutritional phenotypes and predictive capacity of selected variables

Gene expression of the selected indicator genes in the medium and low levels of nutrient supply either showed high within-treatment variability relative to the high nutrient treatment or had poorly differentiated multiple treatments (Figure 2). Differential gene expression was more evident for the gene DAPPUDRAFT_228840 in the P and N limited treatments, where expression was more up-regulated when exposed to the strongest limitation (low supply) compared to weaker limitation (medium) (Figure 2). The opposite effect was seen for DAPPUDRAFT_304085 in the P treatment, which showed greater up-regulation of the gene in response to medium versus low P supply in food. However, due to the high variability among the high nutrient samples, this gene did not clearly differentiate *Daphnia pulex* experiencing different forms of nutritional stress (Figure 2).

We created three LDA models with different combinations of treatment levels to examine whether the gene expression profiles of the selected indicator genes discriminated between high-nutrient and nutritionally stressed *Daphnia* (Figure 3). In model 1, we combined medium and low samples into one treatment (i.e., 'low calcium' and 'medium calcium' are denoted as 'calcium'). In model 2, we examined only medium samples, and in model 3, we used the lowest treatment available (i.e., low where possible, medium where there were no low observations). Based on class predictions, we found that model 1 (merged medium and low observations) differentiated nutritionally stressed *Daphnia* with 86.11% prediction accuracy. However, the variable loadings plot

did not show clear discrimination between treatments except for cyanobacteria (Figure 3A). Similarly, model 2 (medium observations only) had an 88% prediction accuracy, showing clear separation of the cyanobacterial treatment (Figure 3B). Model 3 (the lowest treatment available) had 100% prediction accuracy, with the most apparent level of separation between all treatments but calcium, which overlaps with the high nutrient treatment (Figure 3C).

Dose-dependency of indicator genes

We found significant dose-dependent effects of treatment level (medium and low) on the gene expression of four out of six selected indicator genes. All four genes (DAPPUDRAFT_307316, DAPPUDRAFT_323929, DAPPUDRAFT_299677, and DAPPUDRAFT_228840) had significant differential expression between medium and low supply levels of nitrogen (Figure 4). Additionally, DAPPUDRAFT_299677 responded to phosphorus and DAPPUDRAFT_228840 to calcium levels of supply (Figure 4).

Discussion

Organisms with reduced concentrations of essential nutrients in their diet experience nutrient limitation, which results in adverse physiological effects. This is especially true for filter feeders such as *Daphnia*, who cannot select high-quality food particles and frequently encounter algal food sources that vary in nutritional quality. Here, we studied how *Daphnia pulex* responded to different dietary concentrations of five ecologically relevant nutrients (P, Ca, N, C, and cyanobacteria) on a molecular scale and examined whether selected genes have the potential to discriminate between the

nutritional status of the organism. Our results indicated that *Daphnia pulex* adjusted their metabolic pathways in response to nutrient stress, and the selected indicator genes, when used together, can discriminate between the nutritional history of the organism with a high level of accuracy (Figure 3C).

We used MSGR as a physiological confirmation that *Daphnia* in each treatment responded to varying types and gradients of nutrient limitation and that they were indeed nutritionally limited. As expected, for each nutrient, we found significant reductions in MSGR with a decrease in nutrient availability (Figure 1). These findings on *Daphnia's* growth rate under the examined dietary (C, P, N, and cyanobacteria) and media (Ca) treatments are consistent with other literature (e.g., Sterner et al. 1993; Prater et al. 2016; Jones at al. 2022) and confirms that elemental availability of the algal food source of consumers results in a physiological response.

Dose-dependency and functional annotations of indicator genes

Physiological responses to nutritional stress are controlled by metabolic processes and transcriptome adjustments that can be targeted using qPCR. Ideally, molecular responses would be unique, showing nutrient-specific patterns to each set of treatments that can be distinguished from one another (Wagner et al. 2013). Such patterns were not obvious in the differential expression of individual genes (Figure 2). However, linear discriminant analysis that used all gene expression data was able to separate *Daphnia* exposed to the different nutritional treatments with 100% accuracy (Figure 3C) with similar patterns seen by Jones et al. (2022). This indicates that *Daphnia* responded to changes in nutrient availability by adjusting molecular activity in the form

of gene expression in the bodily tissues, and when considered together, these changes are distinguishable from one another (nutrient-specific).

We found that four of the six potential indicator genes significantly responded to diet or growth media shifts in elemental availability in a dose-dependent manner (Figure 4; Supplementary material Appendix 1, Table S1). Predicted functional annotations were assigned to each gene using the wFleaBase database (Colbourne et al. 2005) to provide insight into the ecology of these species in wild populations. DAPPUDRAFT_307316 showed a significant dose-dependent response to medium and low gradients of nitrogen (Figure 4). The associated protein for this gene is collagen triple helix repeat, associated with phosphate transport and extracellular structure (Colbourne et al. 2005). Collagen supports exoskeleton formation and the maintenance of body shape in *Daphnia* (Huang et al., 2023). While the collagen triple helix gene was downregulated under P and N limitation, there was an upregulation when exposed to Ca (Figure 2), which suggests a decreased demand of P and N required for growth and an increased demand for Ca to aid in exoskeleton formation and hardening of the carapace.

Both DAPPUDRAFT_323929 and DAPPUDRAFT_299677 had a significant dose-dependent response to nitrogen, and DAPPUDRAFT_299677 was also significantly affected by phosphorus gradients (Figure 4), exhibiting a down-regulation of expression patterns (Figure 2). Both genes are associated with the protein vitellogenin fused with superoxide dismutase (VT-SOD), which is involved in trace mineral and lipid transport (Schwerin et al. 2009; Klumpen et al. 2021; Becker et al. 2018). Vitellogenin is synthesized and processed into vitellin, which is part of a significant group of egg yolk proteins involved in embryonic development (Kato et al. 2004), that provide developing embryos with proteins, lipids, carbohydrates, and other resources (Schwerin et al. 2009).

Additionally, in response to environmental stress, *Daphnia* produce ephippia (resting eggs), and VTG-SOD can help with formation of resting eggs that are an important stress response in these animals (Becker et al. 2018). While ephippia were not observed in the study animals, both genes encoding for VT-SOD were up-regulated in response to calcium limitation (Figure 2). *Daphnia* require more calcium than any other zooplankton, allocating up to 90% to their carapace and 26% to physiological processes to support growth and egg production (Porcella et al. 1969). Therefore, the upregulation of these genes with decreasing concentrations of calcium suggests that *Daphnia* are under stress and modifying their growth process by decreasing the use of pathways that require N and P. These findings are similar to those of Becker et al. (2018), where VT-SOD was upregulated under starvation.

Lastly, DAPPUDRAFT_228840 was significantly upregulated in response to nitrogen and downregulated in response to calcium gradients (Figure 4). The magnitude of response matched the proportion of nutrient limitation in their diet (i.e., lower N, higher upregulation; Figure 2). Additionally, while the effect was not significant between the medium and low concentrations, the same pattern was seen for the phosphorus treatment (Figure 2). This is expected as DAPPUDRAFT_228840 corresponds to glycoside hydrolase and is involved in carbohydrate metabolism (Colbourne et al. 2005). Carbohydrate reserves maintain adequate glucose levels in the hemolymph and provide cells with materials to produce ATP (Klumpen et al. 2021). In response to a lack of food, glycogen stores are first degraded to supply glucose (Klumpen et al. 2021). Results from this study support previous findings on P limitation in *Daphnia*, which results in increased phosphorus uptake and use efficiency, accompanied by an upregulation of

associated metabolic pathways (Jeyasingh et al. 2011; Jones et al. 2020), and this can also be inferred regarding *Daphnia* exposed to nitrogen limitation.

Our study indicates that *Daphnia pulex* shows that the physiological response of reduced growth, resulting from insufficient nutrients from their diet and surrounding environment are accompanied by changes in gene expression. While we found that *Daphnia* alters specific pathways in response to nutrient limitation, these responses did not appear to be nutrient-specific and were muted by high variability among control animals. Consequently, these findings show the limited potential to identify single biomarkers that determine the type and intensity of nutrient limitation in *D. pulex*. It is clear that more development of this approach is needed before biomarkers can be used to study nutritional limitation in lake zooplankton populations.

Tables and Figures

Table 1: Daily dilution rates and nutrient composition of algae growth media.

Treatment	**Daily dilution rate (ml)**	**N concentration (µM as $NaNO_3$)**	**P concentration (as µM $NaHPO_4$)**
High Nutrient	800	1000	50
Cyanobacterial food	800	1000	50
Calcium	800	1000	50
Carbon	800	1000	50
Medium N	500	275	50
Low N	200	485	50
Medium P	600	1000	17.75
Low P	300	1000	7

Table 2: Algae species, elemental ratios, feeding amounts and culture media for each treatment.

Treatment	**Gradient**	**Food level (mg C/L)**		**Food C:P ratio**	**Food C:N ratio**	**Algae species**	**Calcium concentration of COMBO* (mg/L)**
		Day 0	**Day 3**				
Control	High nutrient	3	6	~100	~8	*Scenedesmus obliquus*	10
Calcium	Medium	3	6	~100	~8	*Scenedesmus*	**3**
	Low	3	6	~100	~8	*obliquus*	**1.5**
Nitrogen	Medium	3	6	~100	**~16**	*Scenedesmus*	10
	Low	3	6	~100	**~24**	*obliquus*	10
Phosphorus	Medium	3	6	**~400**	~8	*Scenedesmus*	10
	Low	3	6	**~800**	~8	*obliquus*	10
Carbon	Medium	**1**	**2**	~100	~8	*Scenedesmus*	10
	Low	**0.12**	**0.25**	~100	~8	*obliquus*	10
Cyanobacterial	Medium	3	6	~100	~8	***Scenedesmus***	10
food	Low	3	6	~100	~8	***obliquus* & *Romeria leopoliensis***	10

Table 3: Details of qPCR primers used in assay including melting temperature (T_m), limit of quantification (LOQ) and limit of detection (LOD).

Gene ID	Primer sequence (5′ → 3′)	Nutrient	T_m (ºC)	Annealing temp (ºC)	Product size (bp)	R^2	Slope	Efficiency (%)	LOQ (ng/uL)	LOD (ng/uL)
DAPPUDRAFT_302545	F: GCTCTCCCAACTTGGATGCC R: TGGGAGGCAACAGGTCGAAT	Carbon	61.3	50.0	159	0.99	-3.01	115	0.50	0.12
DAPPUDRAFT_302545	F: GCTCTCCCAACTTGGATGCC R: TTGGGAGGCAACAGGTCGAAT	Carbon	61.5	63.8	160	0.99	-3.61	89	0.25	0.01
DAPPUDRAFT_299677	F: CTACATCGGCAACATCAAGA R: ACTTCAGTGGTAGTAAGCAGG	Nitrogen	56.3	61.0	213	0.99	-3.13	108	0.01	0.01
DAPPUDRAFT_323929	F: GGTCCATCGTCGTCAAGCTG R: GGTGACGAATGACGAAGGGT	Nitrogen	60.6	50.4	153	0.99	-2.94	118	0.01	0.01
DAPPUDRAFT_228840	F: TCGATGGCGGTTGCTATGTG R: TACAATGTCGATCTCGCCCG	Phosphorus	60.4	58.9	215	0.99	-3.07	111	0.1	0.01
DAPPUDRAFT_307316	F: TCAATCGACAGTGCGAAGAT R: TGGAATACCTGGCTCTCCTG	Calcium	57.9	61.0	242	0.99	-2.64	138	0.25	0.06
DAPPUDRAFT_304085	F: TGGGTACGGTCGTCTTCAGG R: CATCAGAGCAGCCCACATGA	Cyanobacteria	60.7	50.4	215	0.98	-3.80	83	0.25	0.03
Dappu-194044	F: CACATTGGTCGTCCTTAGTCTTG R: TGCTATACGTTACGCTTGTCCTTAC	Reference Stx16	-	58.9	148	0.99	-2.89	122	0.01	0.01

Table 4: Details of predicted gene function and potential links to biological processes for the 6 indicator genes.

Gene name	Protein family	Predicted gene function	Potential links to biological processes	Source
DAPPUDRAFT_302545	Sulfotransferase	Sulfotransferase activity	Sulfation is involved in cellular processes such as cell communication, growth, development, and defence mechanisms. Food deprivation leads to a downregulation of digestive enzymes. Involved in defense mechanisms when exposed to toxins (e.g., upregulated when exposed to plastics).	Trotter et al. (2021)
DAPPUDRAFT_299677	Vitellogenin (VTG) fused with superoxide dismutase (SOD)	Lipid transport	Serves as a trace mineral transport protein. Vitellogenin is processed and synthesized into vitellin forming a significant group of egg yolk proteins involved in embryonic development that provide the developing embryos with proteins, lipids, carbohydrates, and other resources and is a sign of oocyte maturation. During oxidative stress, SOD converts reactive oxygen species into less harmful molecules which can be degraded. *Daphnia* produce ephippia in response to environmental stress and VTG-SOD can help with formation by increasing stress tolerance.	Becker et al. (2018) Kato et al. (2004)
DAPPUDRAFT_323929	Vitellogenin (VTG) fused with superoxide dismutase (SOD)	Lipid transport	The amount of vitellogenin proteins is directly associated with number of offspring. Involved in lipid transport and upregulated under starvation.	Chatterjee et al. (2019) Schwerin et al. (2009)
DAPPUDRAFT_228840	Glycoside hydrolase	Carbohydrate metabolism	Downregulated under stress. Carbohydrate reserves are used to maintain adequate glucose levels in the hemolymph and provides cells materials to produce ATP used for energy. In response to a lack of food, glycogen stores are first degraded to supply glucose.	Klumpen et al. (2021)
DAPPUDRAFT_307316	Collagen triple helix repeat	Phosphate transport and extracellular structure	Collagen supports exoskeleton formation and the maintenance of body shape.	Huang et al. (2023)
DAPPUDRAFT_304085	Membrane protein	Signal transduction mechanisms	No relevant research in *Daphnia spp.*	N/A

Table 5: Candidate reference genes used for stability analysis, including predicted functions. Annealing temperature is between 60-61ºC.

Gene name	Gene ID	Function	Primer sequence (5′ → 3′)	Product size (bp)	Efficiency (%)	Source
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Dappu-302823	Enzyme that catalyzes glycolysis which breaks down glucose for energy	F: TGGGATGAGTCACTGGCATAC R: GAAAGGACGACCAACAACAAAC	136	93	Spanier et al. (2010) Altshuler et al. (2015) Tang et al. (2015)
TATA binding protein (TBP)	Dappu-194512	Plays a role in transcription initiation by recruiting transcription factors and RNA polymerase	F: CTACGATGCATTCGATAACATATACC R: AGAACCAGCAATGAGTTAAACAAAG	144	90	Spanier et al. (2010) Coldsnow et al. (2017) Kaupinis et al. (2017)
Syntaxin 16 (Stx16)	Dappu-194044	Protein involved exocytosis	F: CACATTGGTCGTCCTTAGTCTTG R: TGCTATACGTTACGCTTGTCCTTAC	148	93	Spanier et al. (2010) Altshuler et al. (2015) Coldsnow et al. (2017) Kaupinis et al. (2017)
Matrixmetallo-proteinases (MMP)	Dappu-303491	Enzymes involved in the breakdown of extracellular matrix components	F: CGAAACATGGACGCATAACTC R: GTCCCAAAGTGTGACCGAAC	80	92	Spanier et al. (2010)
C-terminal pdz ligand of neuronal nitric oxide synthase (CAPON)	Dappu-100564	Protein that interacts with neuronal nitric oxides synthase and modulates its activity	F: TAACGAGTCGGGAGGAAGTG R: GCTGGACTTGAGCCAGTATCTC	140	94	Spanier et al. (2010)

Table 6: Slope, efficiency and R^2, LOQ, and LOD for optimal candidate reference genes.

Gene	Annealing temp (ºC)	Slope	Efficiency (%)	R^2	LOQ (ng/uL)	LOD (ng/uL)
GAPDH	58.9	-3.60	90	0.99	0.001	0.001
TBP	56.4	-3.06	112	0.99	0.01	0.01
Stx16	58.9	-2.89	121	0.99	0.01	0.01
MMP	61.0	-2.94	118	0.99	0.25	0.01
CAPON	58.9	-2.87	123	0.98	0.125	0.125

Table 7: Stability rankings of candidate reference genes using 5 stability algorithms: BestKeeper, NormFinder, comparative ΔCT method, RefFinder, and geNorm. Genes are ranked from most to least stable.

Rank	geNorm		NormFinder		BestKeeper		RefFinder		ΔCt	
	Gene	geNorm M	Gene	SV	Gene	SV	Gene	SV	Gene	SV
1	Stx16	0.653	Stx16	0.327	CAPON	0.605	Stx16	1.19	Stx16	1.15
2	TBP	0.653	TBP	0.327	Stx16	0.626	TBP	2.00	TBP	1.20
3	CAPON	0.930	CAPON	0.924	MMP	0.689	CAPON	2.28	CAPON	1.37
4	MMP	1.077	MMP	1.407	TBP	0.740	MMP	3.72	MMP	1.62
5	GAPDH	1.487	GAPDH	2.026	GAPDH	1.083	GAPDH	5.00	GAPDH	2.10

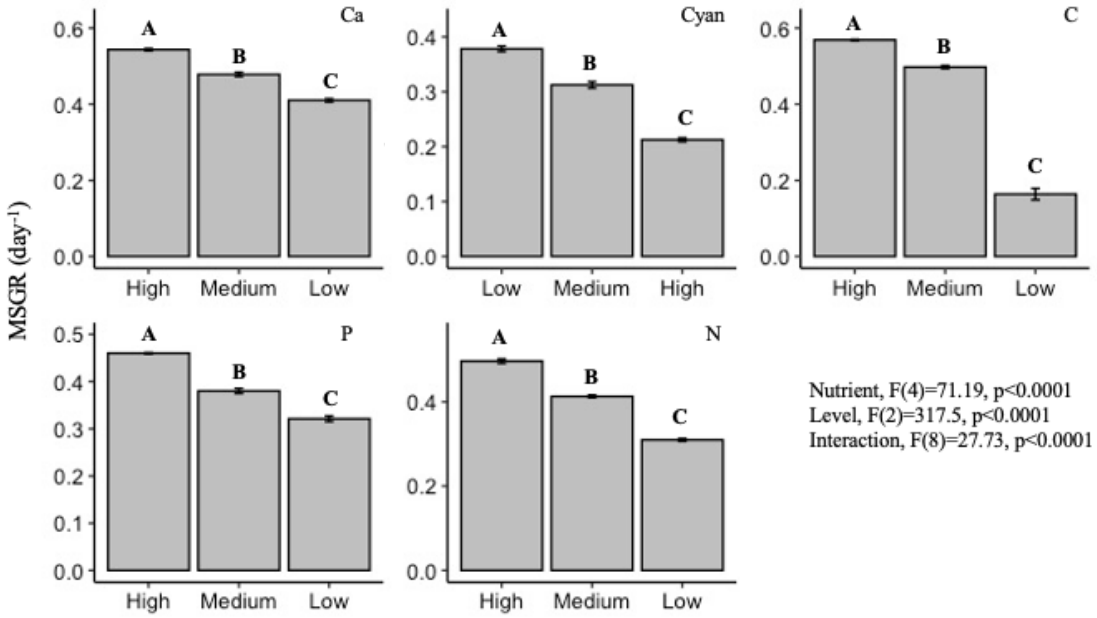

Figure 1. Mean mass-specific growth rate (MSGR) of *Daphnia pulex* grown under high, medium, and low levels of calcium (Ca), carbon (C), cyanobacteria (Cyan), nitrogen (N), and phosphorus (P). Error bars represent ± 1 s.e. Two-way ANOVA results show an interaction between nutrient type and level. The bold letters are results of pair-wise contrasts indicating significant ($P < 0.05$) differences in growth rates between nutrient levels (high vs low, high vs med, med vs low).

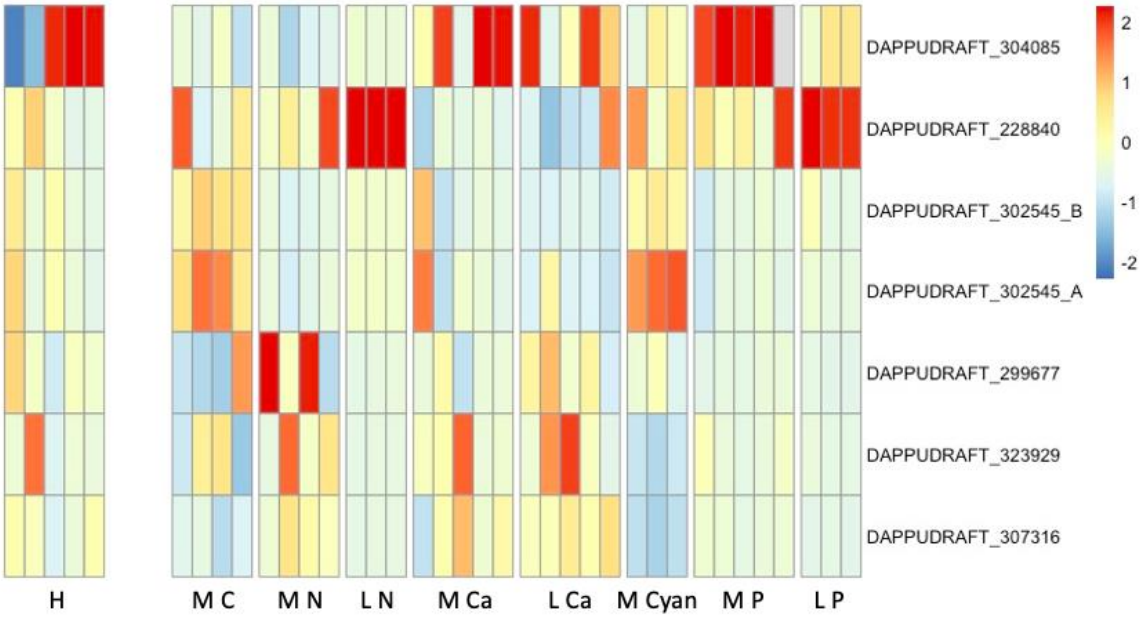

Figure 2. Heatmap of log_2-transformed gene expression ratios for the 7 potential biomarkers for *Daphnia pulex* grown under high (H), medium (M), and low (L) levels of calcium (Ca), carbon (C), cyanobacteria (Cyan), nitrogen (N), and phosphorus (P). Each rectangle represents one biological replicate. Positive values indicate upregulation and negative values indicate downregulation of gene expression.

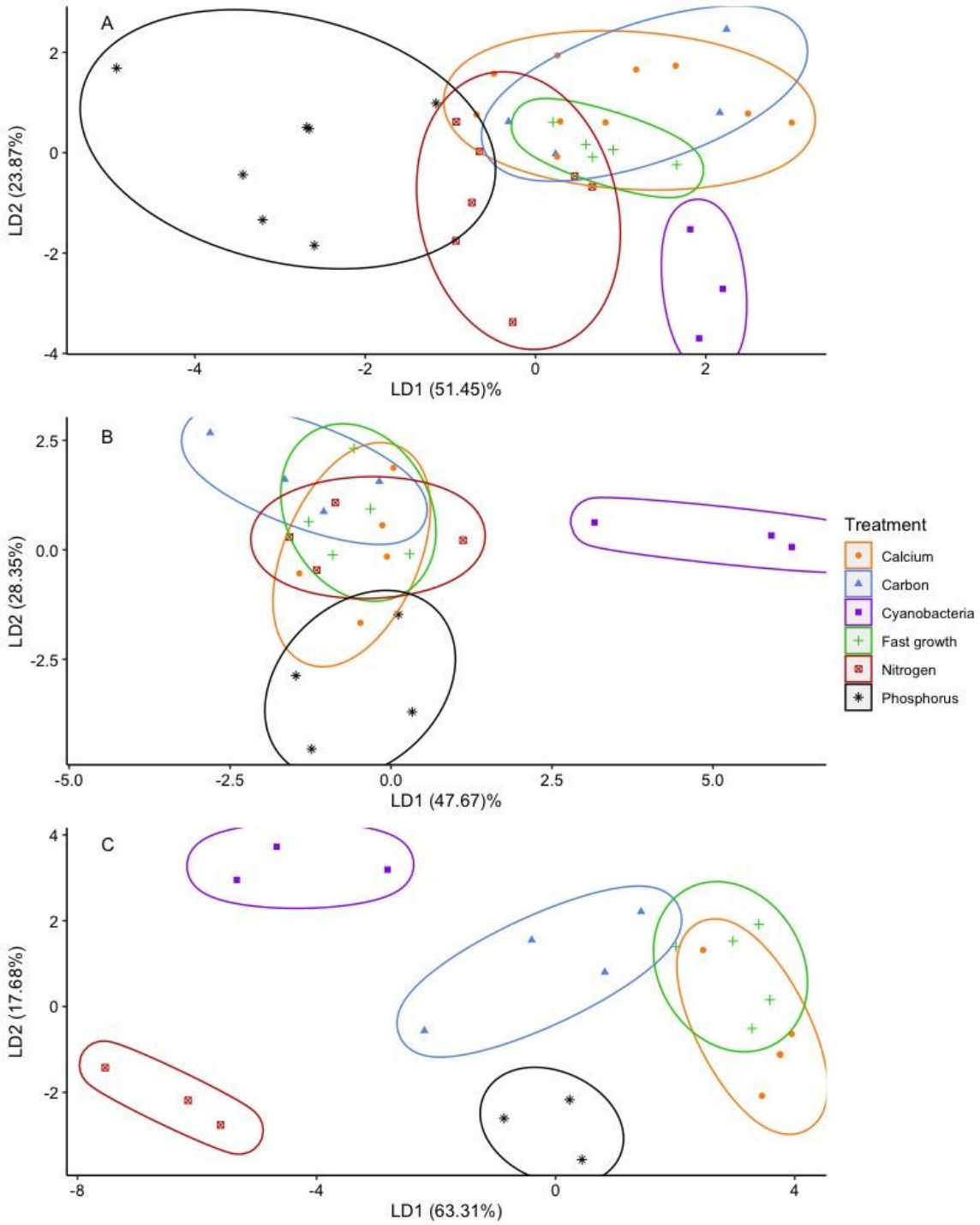

Figure 3. Nutrient specific linear discriminant analysis (LDA) models for the 7 potential biomarkers of high nutrient and nutritionally stressed *Daphnia.* A) Includes all observations however low and medium are combined into a single nutritional treatment (Model prediction accuracy = 86.11%). B) Includes only high and medium observations (Model prediction accuracy = 88%). C) Includes high observations and the lowest treatment available (Model prediction accuracy = 100%).

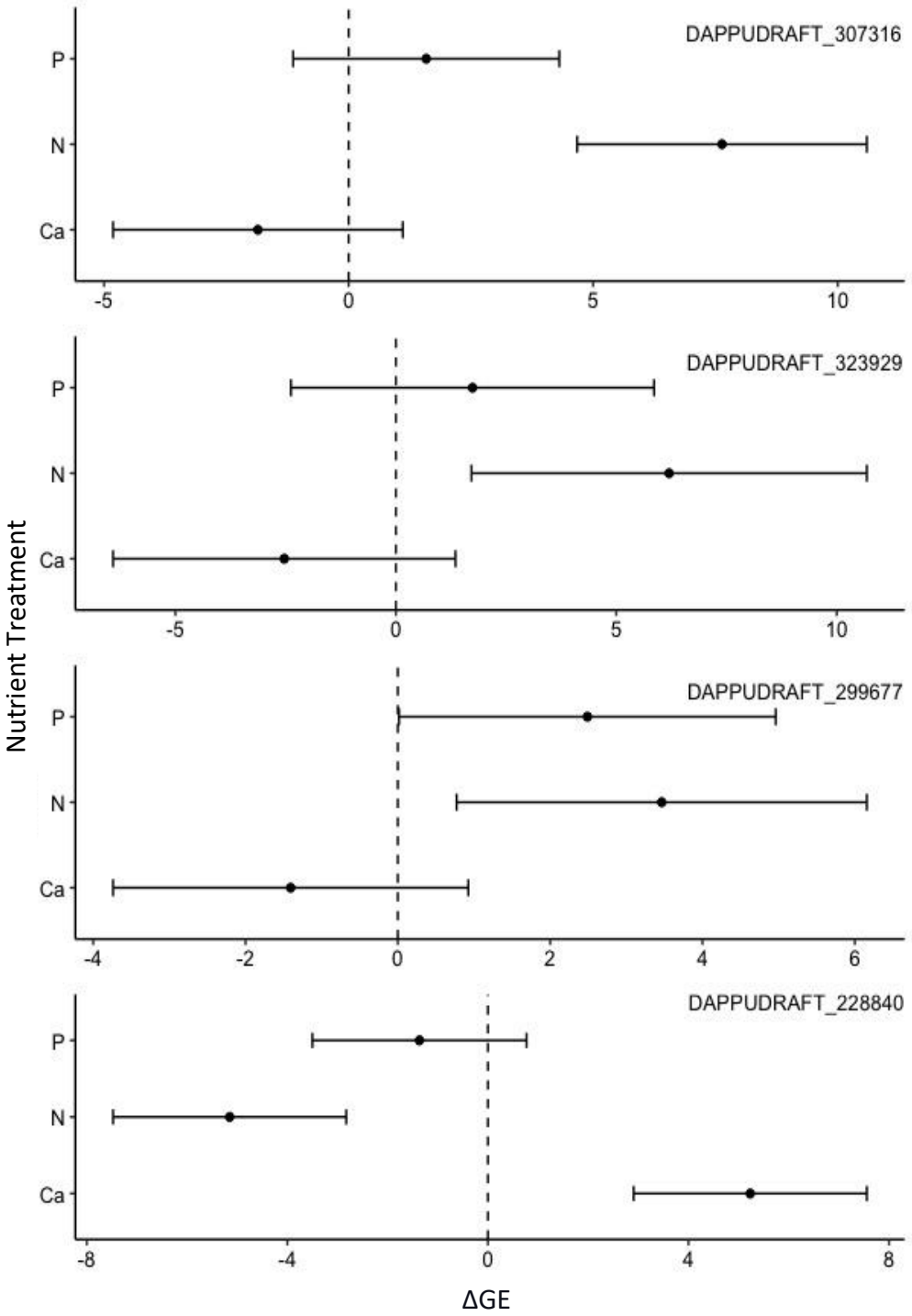

Figure 4. Confidence intervals of the differences in gene expression (ΔGE) between medium and low nutrient levels of phosphorus (P), nitrogen (N), and calcium (Ca). Overlap with zero indicates no differences between medium and low levels of the nutrient in question. Positive values indicate an upregulation of the gene and negative values indicate downregulation. The sum error rate for the family of comparisons (for each gene) is 5%. Gene ID is labelled in the top right corner of each plot.

Chapter Three – General Conclusion

I examined the gene expression responses of six genes involved in nutrient uptake and metabolism in *Daphnia pulex*, with a goal of identifying biomarkers that determine the type and intensity of nutrient limitation. To do so, I first designed qPCR primers for the candidate indicator genes and completed initial testing to ensure their accuracy and reliability for the qPCR assay. I then exposed *Daphnia* to different concentrations of essential nutrients (C, N, P, Ca, and cyanobacteria) in their food and growth media and determined whether gene expression changes in both a dose-dependent and a nutrient-specific manner. While we found that some genes showed a dose-dependent effect, they could not entirely separate differentially nutrient-stressed organisms with a high level of accuracy.

While this study provides valuable information regarding *Daphnia's* ability to adjust physiological and molecular pathways, more vigorous validation of primers remains before we can extend these laboratory findings to natural ecosystems and employ them as indicators of nutritional stress, as gene responses did not appear to be nutrient-specific and were muted by high variability among control animals. The development of qPCR primers for monitoring nutritional stress in *Daphnia* represents a critical advancement in understanding zooplankton's physiological and ecological responses to changing environmental conditions. As future studies continue to refine and expand the database of qPCR primers, integrating molecular techniques into ecological research promises to enhance our ability to monitor and mitigate the effects of nutritional stress on freshwater ecosystems.

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Appendix

Supplementary information

Table S1: Two-way ANOVA results calculated using log_2 transformed gene expression ratios of animals exposed to medium and low levels of calcium, nitrogen, and phosphorus.

Gene ID	Nutrient			Level			Interaction		
	d.f.	**F**	***p***	**d.f.**	**F**	***p***	**d.f.**	**F**	***p***
DAPPUDRAFT_302545_A	2	0.912	0.4186	1	2.974	0.0518	2	3.473	0.0518
DAPPUDRAFT_302545_B	2	0.928	0.4126	1	2.902	0.1048	2	4.340	0.0280
DAPPUDRAFT_299677	2	3.858	0.0374	1	4.103	0.0557	2	5.694	0.0106
DAPPUDRAFT_323929	2	4.195	0.0293	1	5.750	0.0259	2	3.727	0.0412
DAPPUDRAFT_228840	2	33.59	<0.0001	1	7.100	0.0153	2	4.692	0.0221
DAPPUDRAFT_307316	2	11.60	0.0005	1	20.32	0.0002	2	15.09	0.0001
DAPPUDRAFT_304085	2	14.04	0.0002	1	1.932	0.1815	2	1.117	0.3489