

**NUTRIENT METABOLISM OF AN AQUATIC INVERTEBRATE AND ITS  
IMPORTANCE TO ECOLOGY**

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfillment of the  
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Trent University

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## Abstract

### *Nutrient metabolism of an aquatic invertebrate and its importance to ecology*

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Aquatic consumers frequently face nutritional limitation, caused in part, by imbalances between the nutrients supplied by primary producers and the metabolic demands of the consumers. These nutritional imbalances alter many ecological processes including consumer life-history traits, population dynamics, and food web properties. Given the important ecological role of organismal nutrition, there is a need to have precise and specific indicators of nutritional stress in animals. Despite this need, current methods used to study nutrition are unable to distinguish between different types of nutritional limitation. Here I studied nutritional metabolism in the freshwater zooplankter, *Daphnia*. A greater understanding of nutritional metabolism would allow for the development of dietary bio-indicators that could improve the study of the nutritional ecology of animal consumers. Specifically, I addressed the question: ***What affects the biochemical composition of a generalist aquatic consumer?*** My overall hypothesis was that the quantity and quality of the diet affects the biochemical composition in a nutrient specific manner. To test this hypothesis, I examined various response variables involved in nutrient metabolism such as alkaline phosphatase activity, whole metabolome, and free amino acid composition. For each response variable, I grew *Daphnia* under various nutritional stressors and determined if responses are nutrient specific or are a general stress response. I found the current method of measuring alkaline phosphatase was not a phosphorus specific indicator, as activity increased in all nutrient stressed treatments. Analyzing the whole metabolome resulted in nutritional stressors being separated in multivariate space, with many identified metabolites being significantly different from nutrient rich *Daphnia*. Upon further examination the daphnids free amino acids profiles are caused by differences between the supply of amino acids from the

algae and the demand within the *Daphnia*. These differences in supply and demand resulted in the ability to classify the nutritional status of *Daphnia* with the use of discriminant analysis, a classification multivariate model. In addition to a deeper understanding and advanced knowledge of the physiological changes caused by nutrient limitation, this research has provided strong evidence for the application of nutritional biomarkers/profiles to identify the nutritional status of *Daphnia*.

**Keywords:** Ecological stoichiometry, Nutritional limitation, Bio-indicator, Nutritional status, Metabolism,

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## List of Abbreviations

A	Alpha
%	Percent
°C	Degrees Celsius
µl	Microliter
<sup>1</sup> H-NMR	Proton Nuclear magnetic resonance
2-D gel	2 dimensional gel electrophoresis
AA	Amino Acid
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APA	Alkaline phosphatase activity
ATP	Adenosine triphosphate
BAC	Exposed to spores treatment
C	Carbon
C:N	Carbon to Nitrogen ratio by mol
C:P	Carbon to Phosphorus ratio by mol
cDNA	Complementary DNA
D <sub>2</sub> O	Deuterium water
DNA	Deoxyribonucleic acid
DSS	2,2-dimethyl-2-silapentane-5-sulfonate sodium salt
ELISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray ionization mass spectrometry
FA	Fatty acids
FAA	Free Amino Acids
Fe	Iron
GC-MS	Gas chromatography mass spectrometry
GDP	Guanosine triphosphate
GF	Good food/nutrient rich controls
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
HQQ	High food quality and quantity
Hr	Hour
Hz	Hertz
kNN	Non parametric nearest neighbour
L	Liter
LC-MS	Liquid chromatography mass spectrometry
LF	Low food quantity
M	Mol per liter
MF	Methylfluorescein
MFP	3- <i>o</i> -methylfluorescein phosphate

Mg <sup>2+</sup>	Magnesium
miR	Micro RNA
ml	Milliliter
mm	Millimeter
MSGR	Mass specific growth rate
MTE	Metabolic Theory of Ecology
N	Nitrogen
N:P	Nitrogen to Phosphorus ratio by mol
Na	High Sodium treatment
Na <sup>+</sup> /K <sup>+</sup> ATPase	Sodium Potassium adenylpyrophosphatase
NaH <sub>2</sub> PO <sub>4</sub> 2H <sub>2</sub> O	Sodium phosphate monobasic dihydride
NaOD	Sodium hydroxide with deuterium
NL	Nitrogen limited
P	Phosphorus
PL	Phosphorus limited
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RPM	Rotations per minute
S	Seconds
<i>S.l.</i>	<i>Synechococcus leopoliensis</i>
<i>S.o.</i>	<i>Scenedesmus obliquus</i>
SPV	Sulfophosphovanillin
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
UV/VIS	Ultra violet visible spectroscopy
v/v	Volume per volume
w/v	Weight per volume
Zn <sup>2+</sup>	Zinc

# CHAPTER 1

## General Introduction

Aquatic consumers frequently face nutritional limitation (Sterner and Elser 2002), caused by differences between the nutrients provided in their food and those needed to support their growth, reproduction, and maintenance. In general, primary producers are very plastic in their nutrient content while animal consumers are relatively more homeostatic (Frost et al. 2005, Hood and Sterner 2014). These differences are the origin of nutritional imbalances between food and consumer, which can lead to physiological changes within animal consumers. For example, nutritional stress in aquatic invertebrates alters respiration rates (McFeeters and Frost 2011), RNA content (Elser et al. 2000), and lipid content (Tessier and Goulden 1982). These physical changes caused by nutrient stress result in slower growth rates, decreased reproduction and altered body elemental composition of a wide diversity of animal taxa (Frost et al. 2005). Besides changing consumer physiology, nutritional limitation alters consumer population dynamics, and a range of ecosystem processes (Sterner and Elser 2002, Raubenheimer et al. 2009, Yamamichi *et al.* 2015). Given its effects on ecological interactions, there is a need to assess the nutritional state of animal consumers that are confronted with nutrient stress both spatially and temporally.

For decades, nutritional ecology focused on single currencies such as energy (carbon) and its trophic transfer through ecosystems (e.g., energetics models; Lindeman 1942). Occurring concurrently to these energetics studies, A.C. Redfield was measuring the relative proportions of macronutrients in marine phytoplankton as a means to

determine how biological processes affect ocean chemistry. He determined marine phytoplankton communities, on average, contained the same ratio of carbon (C), nitrogen (N), and phosphorus (P; i.e., 106:16:1; Redfield 1958). Although the elemental composition of marine seston was assumed to always conform to the Redfield ratio, the nutrient requirements for various species of phytoplankton especially in freshwater were found to vary widely (Kilham 1971). These variations in nutrient requirements lead to Tilman's mechanistic resource competition theory, which states the dominating taxa will have lower requirements for the limiting nutrient and out compete algal species with higher nutrient demands (Tilman 1982). Both Redfield and Tilman advanced nutritional ecology as they used a multi-element perspective. Building on these theories of energetics, nutrient requirements, and competition, a formal framework of ecological stoichiometry was proposed as a means to understand coupled abundant elemental cycles (C:N:P; Reiners 1986). Based on the law of mass conservation, ecological stoichiometry now provides explanations of everything from molecules to ecosystems (Sterner and Elser 2002). Given the scope of this ambitious theory, there is clearly a need to carefully assess its central assumptions and to have empirical tools to test its central theoretical tenets.

A key concept that requires careful examination is whether animal consumers become limited by nutrients other than energy. The nutrient status of consumers is typically assessed using three approaches: 1) simple comparison of the abundant nutrient content of the food and consumer, 2) comparison of elemental threshold ratios to food nutrient content, and 3) measurement of consumer growth in small bioassays. However, each method has limitations. For example, one approach is to determine the nutritional

imbalance between sampled phytoplankton food sources and zooplankton bodies or metabolic requirements (Frost et al. 2006). While informative, such comparisons have difficulty accounting for physiological adjustments (e.g., altered assimilation efficiencies, Frost et al. 2006) or behavioral changes (e.g., food selectivity, Frost & Elser 2002) potentially used by zooplankton to adjust to poor food conditions. Consequently, such comparisons may lead to incorrect conclusions about which nutritional element is limiting animal consumers. A commonly used, alternative approach involves growing zooplankton in whole lake water containing natural food sources and assessing their growth and/or reproduction in bottles (Elser et al. 2001). However, this method can be problematic due to the use of laboratory animals and the artificial environment created by small containers and short incubation periods. Both approaches also face the challenge of co-varying nutritional components. A change in the nutrient content (e.g., phosphorus) of algal food sources is sometimes accompanied by changes in other essential nutritional components (e.g., sterols; Boersma 2000). This covariation creates uncertainty about which food component is the primary limiting nutrient (Boersma 2000) and clouds easy interpretation of experimental and field results. These difficulties are potentially avoided by the development and use of more direct approaches that focus directly on the consumer of interest through the measurement of nutrient-responsive biochemical responses. Hence, there is a need to develop better techniques to assess consumer nutrition that can more precisely identify limiting nutrient or co-limiting nutrients.

One complementary method to assess consumer nutrition is through the use of biochemical and molecular approaches. These approaches have been used to assess the metabolic and physiological effects of nutrient limitation in plants (Hirai et al. 2004),

yeast (Boer et al. 2003, Boer et al. 2010), and bacteria (Hua et al 2004). Such response variables would include aspects of the transcriptome, translational regulators, proteome, metabolome, and lipidome. Particularly good examples are the changes in the transcriptome (Boer et al. 2003) and metabolome (Boer et al. 2010) of yeast subjected to nutritional stress. Yeast have been found to change their gene expression and metabolite composition in a nutrient specific manner in response to phosphorus (P), nitrogen (N) and glucose limitation. While these adjustments appear to be nutrient specific in microbes and plants, it is not known whether these responses are seen in animal consumers.

### **1.1 Metabolic changes in nutrient limited consumers**

Given that many aspects of metabolism are evolutionarily conserved, I would expect animal consumers to show similar responses to nutritional stress as found in yeast. For example, when animals are limited by the quantity of food I would expect the up-regulation of genes and greater production of the proteins involved in the uptake of glucose, amino acids, and fatty acids (Boer *et al.* 2003). Short-term provision of energy for central metabolism (TCA cycle; Wu *et al.* 2004) would likely result from increased oxidation of fatty acids, gluconeogenesis and protein catabolism (Boer *et al.* 2003, Wu *et al.* 2004). As free sugars and other organic pools are consumed for energy, low food quantity should change the metabolite composition of consumers. For example, AMP:ATP ratios should increase due to energetic constraints on ATP production and an increased rate of AMP production through the reduction of adenosine diphosphate (Hardie 2003). Additionally, concentrations of free amino acids (AA) should increase as proteins are catabolized for energy and protein synthesis is limited by low energy supplies (Boer *et al.* 2010). Animal body (or tissue) content of protein and lipids should thus

decrease reflecting the greater breakdown and reduced synthesis rates of these biochemical pools. One might also expect lower RNA:DNA ratios in C-limited animals due to reduced requirements for ribosomal RNA created by the energy-induced constraints on protein synthesis (Wagner *et al.* 1998, Boer *et al.* 2010).

The metabolic responses as described for low food conditions would be different in consumers that are nitrogen (N) stressed. At the genomic level in N-stress consumers, the expression of genes responsible for the production of AA transporters should increase (Boer *et al.* 2003, Hua *et al.* 2004), while genes involved in the uptake of glucose or fatty acids should decrease. There should be increased gene expression of autophagy pathways that are used to recycle unneeded proteins (Boer *et al.* 2003). Genes underlying lipid and carbohydrate storage should also be expressed more as the N-limited consumer stores, excess organic C (Hua *et al.* 2004). Gene expression of and subsequently enzyme activity in metabolic pathways that form ammonium (e.g., cleaving amide bonds) or glutamate from glutathione (Boer *et al.* 2003) should also increase to acquire more free amino acids. The metabolite signature of N-limited consumers should be characterized by lower levels of free amino acids, especially ones containing multiple N atoms (i.e., arginine, histidine, and lysine; Boer *et al.* 2010). Another metabolite intermediate involved in central metabolism,  $\alpha$ -ketoglutarate, should increase as this intermediate can mediate a transamination reaction that produces glutamate (Hua *et al.* 2004, Boer *et al.* 2010). One might also expect greater nucleotide catabolism, as occurs in ammonium-limited bacteria (Hua *et al.* 2004), to decrease protein synthesis and to increase N supply to other key metabolic pathways. The biomolecular content of N-limited consumers would likely be

further characterized by increased lipid content, reduced protein content, and a slightly lower RNA:DNA ratio due to N constraints on ribosomal and protein production.

Phosphorus (P) limitation would produce another unique metabolic signature distinct from both low food and N-limited conditions. Inadequate dietary P content should be accompanied by large changes in the acquisition, incorporation, and release of P. As a result, gene expression of P uptake and recycling systems should increase. For example, genes responsible for high affinity sodium phosphate transporters should show greater expression to increase P acquisition from the digestive tract (Sugiura *et al.* 2003). Other genes involved in phosphate uptake, utilization (e.g., PHO pathway) and recycling (e.g., adenosine kinase) should be up regulated to increase the potential for acquiring and retaining P (Wu *et al.* 2004). The metabolic signature of P-limited consumers would be characterized by lower amounts of ATP, phosphorylated sugars, and energy-rich molecules such as nicotinamide adenine dinucleotide (Boer *et al.* 2010). Guanosine, adenosine, and cytosine would all be expected to increase in P-limited consumers resulting from the release of P from the cleavage of RNA. At a biomolecular level, P-deficient consumers should have lower protein content and decreased RNA:DNA ratios caused by reductions in protein synthesis and the reduced production of P-rich rRNA. An increase in lipid content should also be present in P-limited consumer reflecting the storage of excess carbon (Sternner *et al.* 1992).

## **1.2 Objectives**

For my second dissertation chapter, I reviewed the current metabolism literature to explore how similar approaches to study nutrition are being applied to bacteria, yeast and plants. I used this literature to produce a synthesis manuscript describing the need to

identify and develop nutrient specific indicators and their potential uses in studying ecological processes. The development of nutritional indicators would provide advantages over current techniques mainly by being able to identify the limiting nutrient with increased precision and accuracy.

Following the nutritional indicator approach that I advocate in my synthesis chapter, I experimentally explored the applicability of this method to a model organism, *Daphnia*, as *Daphnia* is a keystone species assisting with both top down and bottom up processing of nutrients. Specifically, I examined the biochemical changes caused by C, N and P-limitation in the zooplankton, *Daphnia*. C, N, and P limitation were chosen as types of nutrient limitation because these elements conserved and abundant between abiotic and biotic environments (Sterner and Elser 2002). While *Daphnia* can experience many types of nutritional stress, P-stress has been studied extensively in zooplankton (Sterner & Schulz 1998, DeMott et al. 1998, McCarthy et al. 2010), and, with predicted increases in N deposition, P appears to be an increasingly limiting nutrient (Elser et al. 2010). *Daphnia* is a good animal candidate to experimentally test this approach as its genome has been sequenced (*D. pulex*; Colburne et al. 2011), is likely to experience nutritional deficiencies in nature (Sterner & Schulz 1998) due to high P requirements for growth (Elser et al. 2000), and is a keystone species in many lake ecosystems (Urabe et al. 1995, Elser et al. 2001).

For my third chapter of my dissertation, I examined the alkaline phosphatase (AP) activity in whole body homogenates of *Daphnia*. AP indiscriminately cleaves phosphate from organic phosphates, allowing for the incorporation of P into cells through sodium phosphate channels. Besides AP making P bioavailable, AP activity (APA) increases in

phytoplankton and bacteria specifically in P-limited conditions (North et al. 2007, Sala et al. 2001), while remaining low in other nutritionally stressed conditions. The whole body homogenate of P-limited daphnids also increases their APA in a dose dependent manner, under both field and lab conditions (Elser et al. 2010, McCarthy et al. 2010, Wojewodziec et al. 2010) This led me to the question: ***What affects the specificity of APA in daphnid whole body homogenates?*** I hypothesized the nutrient status of daphnids would influence the APA in whole body homogenates. I predicted increased activity would only be seen when *Daphnia* consume P-limited food. Alternatively, if other elements affect APA in daphnids, then APA will be increased in other nutritional limitations of *Daphnia*, indicating that APA is not a nutrient specific response to P-limitation.

My fourth chapter considered another area of metabolism that may respond to nutritional limitation including the whole metabolome. Metabolomics studies the free metabolites within cells that are not modified by further processing, which provides a more direct approach to determining the nutritional status of the cell compared to gene expression and enzyme activity which may be further modified by post transcriptional/translational regulation. These free metabolites include sugars, intermediates of metabolism (glycolysis, tricarboxylic acid cycle), free amino acids, and nucleotides. Studying the changes in metabolites offers several advantages over current methods to assess daphnid nutrition including the vast number of metabolites that can be quantified and their large dynamic range under different environmental conditions (Boer et al. 2010). Metabolomics has been extensively studied in nutrient limited plants (Huang et al. 2008) and yeast (Boer et al. 2010). This led me to the question: ***What metabolite changes occur in D. magna in response to nutrient limitation?*** I hypothesized the

metabolomic signature would be unique in differentially nourished animals. For example, I predicted decreases in glucose with low food quantity diets as glucose is a major C source for glycolysis. Whereas, I predicted when daphnids are grown under N-limitation, their free amino acids would decrease caused by demand for protein synthesis.

My final chapter examined free amino acid metabolism under various nutrient stressors. Building on the metabolomics results I asked the final question: *What affects the free amino acid composition in Daphnia?* I hypothesized that differential nutrient limitation affects the amino acid profiles. For example, under P-limitation I expected the free amino acids to increase (Boer et al. 2010) as growth would be limited by ribosomal RNA production (Elser et al. 2000). Whereas under C and N limitation, I predicted free amino acids to decrease as both elemental C and N are in free amino acids. Further, changes in amino acid profiles could be used to determine the nutritional status of field caught consumers through a discriminant analysis.

The significance of this research is that it will provide a better understanding of how metabolism is reorganized when consumers are nutritional stressed. Understanding the metabolic changes occurring in nutritionally-stressed consumers provides the initial steps in the development of the nutritional phenotype. I show how this phenotype can be used as an indicator profile by providing evidence that a particular form or intensity of dietary stress can produce metabolic changes in *Daphnia*. While future research will be required to fully develop a nutritional profiling approach, I have provided the necessary framework to develop this important tool in future research.

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## CHAPTER 2

### 2.1 PREFACE

Title: Nutritional indicators and their uses in ecology

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## Chapter 2

### Nutritional indicators and their use in ecology

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#### 2.2 Abstract

The nutrition of animal consumers is an important regulator of ecological processes due to its effects on their physiology, life-history and behavior. Understanding the ecological effects of poor nutrition depends upon correctly diagnosing the nature and strength of nutritional limitation. Despite the need to assess nutritional limitation, current approaches to delineating nutritional constraints can be non-specific and imprecise. Here we consider the need and potential to develop new complementary approaches to the study of nutritional constraints on animal consumers by studying and using a suite of established and emerging biochemical and molecular responses. These nutritional indicators include gene expression, transcript regulators, protein profiling and activity, and gross biochemical and elemental composition. The potential applications of nutritional indicators to ecological studies are highlighted to demonstrate the value that this approach would have to future studies in community and ecosystem ecology.

### 2.3 Introduction

The nutrition of animal consumers varies widely within and among ecosystems. Poor nutrition alters many aspects of consumer performance (e.g., growth), behavior (e.g., foraging rate and strategy), and their ecological interactions within food webs (Sterner and Elser 2002, Frost *et al.* 2005, Raubenheimer *et al.* 2009). For example, poor food quality can slow the growth of aquatic consumers, delays their reproduction and reduces their reproductive rates, alters the quality of their offspring, and increases their mortality rates (Frost *et al.* 2005). Within and among ecosystems, nutrition interacts with the traits and the diversity of organisms in ecosystems to determine the rates and ratios nutrient cycling (Elser and Urabe 1999, Hooper *et al.* 2005). As this important role of nutrition in ecology is relevant across levels from organisms to ecosystems (Dodson 1998), determining the causes and consequences of poor animal nutrition on food webs and ecosystems should be of high priority (Frost *et al.* 2002, Raubenheimer *et al.* 2009).

The evaluation of the nutritional status of consumers has generally relied on imprecise and non-specific response variables. One indication of whether food is providing adequate nutrition is found by comparing the nutrient content of food to a consumer's body content (Sterner and Schulz 1998, Hillebrand *et al.* 2009a). A similar but more refined approach compares food nutrient content to consumer threshold elemental ratios calculated using assimilation efficiencies, metabolic losses, and body content of multiple elements (Urabe *et al.* 1995, Sterner and Elser 2002, Frost *et al.* 2006). While both of these approaches assume consumer growth is constrained by relatively low nutrient content in food, neither provides direct evidence of whether the nutrient of interest is actually limiting consumer performance. In addition, these

approaches rely on parameter estimates (e.g., body nutrient content and assimilation efficiencies) that are also sensitive to low food nutrient content (Frost *et al.* 2006). Comparisons of food nutrient content and consumer nutrient requirements are further open to the criticism that they can neither definitively identify nor characterize diet quality of consumers due to spatial variability in food sources, variable food quality through time, flexible nutrient assimilation efficiencies, and/or selective feeding by consumers on nutrient rich food sources (Frost and Elser 2002).

More direct assessments of consumer nutritional state involve growth bioassays that measure growth and/or reproduction of animals provided with foods that vary in nutrient content (e.g., Elser *et al.* 2001, Lukas *et al.* 2011). These assessments usually provide food carefully formulated under laboratory conditions (e.g., Sterner *et al.* 1993, Von Elert 2002) or collected from different ecosystems (e.g., Tessier and Woodruff 2002), which are suspected to vary in some nutritional component. The identification of nutritional constraints using dietary supplementation approaches have also been widely employed and are relatively straightforward in identifying limiting dietary compounds (e.g., Sperfeld *et al.* 2012). While these approaches are valuable, they usually rely on a response variable (i.e., growth) that can respond to a low supply of *any* nutrient, require laboratory culturing of food and/or animals, and are potentially confounded by changes to food or environment that occur during laboratory or field manipulations. Given these criticisms of both food-animal comparisons and growth bioassays, there is an apparent need for complementary indicators, which can provide an independent and *in situ* characterization of consumer nutritional state. The goal of complementary indicators would be to quantitatively assess the nutritional status of a consumer and thereby

eliminate uncertainty created by the imprecise and non-specific methods described above. Here we present the case that the molecular and biochemical responses of consumers to poor food quality can provide the next generation of nutritional indicators and will play an important role in the further development of nutritional ecology.

*The ideal nutritional indicator.* The development of a reliable and precise nutritional indicator could start by considering what organismal characteristics would avoid or limit the previously described criticisms. At a minimum, an indicator should show a strong and rapid monotonic (increasing or decreasing) response to a single type of nutrition limitation experienced in a consumer. Nutrient-specificity is desired as it would permit individual and interactive roles of multiple nutritional resources to be identified and separated. Indicators that are specific to animal taxa or particular consumers would be useful when separating responses of animals from ingested food or other non-target taxa. Finally, nutritional indicators would ideally be measured directly on the consumer of interest, be relatively non-invasive or non-lethal for larger organisms, require minimal quantities of animal tissues or body mass, and be of low cost and methodological simplicity. One can imagine a suite of such complementary indicators for each limiting nutrient, more or less fitting some or all of these criteria, which could provide a thorough characterization of the type(s) and severity of nutritional limitation being experienced by an animal.

*Identification and development of nutritional indicators.* A potential source of nutritional indicators that possess many of these “optimal” characteristics is based on the physiological responses (i.e., changes in metabolic pathways) of animal consumers to nutrient limitation (DeMott *et al.* 1998, Frost *et al.* 2005, Mutch *et al.* 2005). Specifically

the molecular and biochemical pathways involved in nutrient uptake, incorporation and mobilization would represent a potential source of novel indicators (van Ommen and Stierum 2002). Similar to nutrigenomics approaches used in systems biology (e.g., Ruffel *et al.* 2010), nutritional indicators would include responses and regulators of gene expression, quantity and types of proteins and their activity, and biomolecular and/or elemental content of specific tissues or the whole organism (Table 2.1).

The development and use of nutritional indicators could follow a common approach: 1) profiling of system responses to nutritional stress, 2) identification and isolation combinations of responses variables unique to each nutrient, and 3) careful validation of nutritional profiles in animals. During the initial phase, the identification of nutritional indicators would clearly benefit from emerging information rich fields (e.g., genomics, transcriptomics, proteomics) due to their ability to profile systematic changes in consumer metabolism in response to nutrient deprivation (van Ommen and Stierum 2002). Profiling or descriptive shotgun studies of consumer nutrition stress can capture whole-scale responses of organisms to different nutritional stresses (Tanzer *et al.* 2003). Characterization of such system-wide responses has permitted the isolation and use of specific indicators that are intimately connected to different nutrients of interest in studies of plants and microbes (Table 2.1).

While large quantities of data are produced by ‘-omic’ approaches, data reduction techniques could be employed to identify unique responses to each nutritional stressor. Data reduction techniques, including multivariate statistics (principle components analysis) or non-linear black box modeling techniques (artificial neural network), are typically employed to identify unique stress indicators (Lancashire *et al.* 2009). For

example, identification of nutrient-specific changes from system wide responses could follow the approach described by Boer *et al.* (2003, 2010), who used cluster analysis to identify unique gene expression responses to specific forms of nutrient limitation. The precise growth limiting metabolites can also be identified when they exhibit positive relationships with the external supply of the nutrient in question (Boer *et al.* 2010). These techniques could be used separately or together to identify nutrient specific ('key') indicators that could then be used to diagnose the strength and nature of consumer nutrient limitation.

Following their identification, potential nutrient specific indicators would require further validation by examining their responses to different degrees of nutrient-stress and to limitation by other nutrients. This validation process would also need to verify the nature and strength of responses in different animal genotypes because genetic heterogeneity among individuals or populations could produce different responses to the same type or degree of limitation. In addition, some indicators may vary among ontogenetic stages as nutrient demands change with life-history stage or due to changing metabolic demands for nutrients. The nature of this ontogenetic plasticity would need to be examined and accounted for prior to wide scale or routine use of this approach. Some nutritional indicators have already seen wide use within and among animal taxa, which indicates that these potential complications may not pose irremediable obstacles to the development and application nutritional profiling. For example, the blood content of hemoglobin and ferritin are widely used as indicators of iron status in humans and in other mammalian taxa (National Research Council, 1979, Denic and Agarwal 2007) despite considerable potential for ontogenic, population, and species level variability in

these indicators. In addition, nutritional indicators would need to be examined for their interactions with confounding environmental variables. Such environmental sensitivity would require special consideration given the wide physico-chemical conditions animals experience within and among the diverse ecosystems of Earth. After validation, biochemically based indicators could be added routinely to studies of the nutritional ecology of animal consumers (Figure 2.1).

Once developed and validated, nutritional profiles could be used to carefully track the form and intensity of nutritional limitation in animal consumers. A nutritional profile would necessarily contain multiple indicators to aid in the differentiation of various potential forms of nutrient limitation (i.e., food quantity, N, P, or fatty acid/sterol limitation). Ideally, these sets of indicators would show high nutrient specificity of their responses, be relatively invariant to variability caused by ontogeny or genetics, and exhibit minimal or predictable changes to changes in the ambient environment. Provided this, profiles derived from animals consuming unknown diets could be quantitatively compared to the data matrix produced by known types of nutritional limitation. This type of analysis would assess the similarity between the nutritional profile of animals consuming known diets and that of the unknown consumer of interest. This approach would generate likelihood statements about which element is limiting the animal under study and thereby provide a quantitative assessment of an animal's nutritional state.

#### **2.4 Nutritional indicators**

The development and application of biochemically based nutritional indicators will involve the measurement of organismal responses (e.g., metabolomics) that may not be commonly used or familiar to many ecologists. Here we present brief descriptions of

molecular and biochemical responses that could provide important information regarding the nutritional state of animal consumers. For each, we review the nature of these response variables of interest and explain how it connects nutrition and metabolism. We further present examples of how these biochemical responses have been used in previous studies that examined links between nutrient supply and the ecology of different species. While this work has largely been completed on non-animal taxa (i.e., microbes and plants), it nonetheless illustrates the potential usefulness and value of this approach for those studying animal consumers. We also briefly highlight the benefits of and potential constraints to the application of each class of indicator to ecological studies of nutrition.

*Gene expression.* Patterns of gene expression could be a rich source of information on the nutritional state of animal consumers. Previous work with model organisms, including *Escherichia coli* (Hua *et al.* 2004), *Saccharomyces cerevisiae* (Boer *et al.* 2003, Wu *et al.* 2004), *Arabidopsis thaliana* (Morcuende *et al.* 2007), and *Daphnia pulex* (Jeyasingh *et al.* 2011), has demonstrated strong links between nutrition and gene expression. These studies have all identified groups of genes that are highly induced or suppressed by a single type of nutrient-limitation. For example, there were 484 nutrient-specific gene expression responses in yeast (*S. cerevisiae*) subjected to low supplies of glucose, nitrogen (N), phosphorus (P), and sulfur (Boer *et al.* 2003). Many of these gene-based indicators of nutrition involve nutrient transport, regulation of nutrient fluxes and pools (e.g., increases in amino acid transporters), and mobilization of stored nutrients mainly through increased catabolism (Boer *et al.* 2003).

Given the considerable number of genes that appear linked to nutrient stress, it seems likely that at least a small number of individual genes could be identified and used

to diagnose the nature and strength of animal nutrient limitation (Table 2.1). This approach of tracking the expression of a single or few genes is currently being used to provide evidence of nutritional limitation in fish and has been advocated for its potential uses to aquaculture (Panserat and Kaushik 2010). Specifically, greater relative expression of sodium phosphate co-transporter genes has been linked to P-limitation in rainbow trout and provides strong evidence of this form of nutrient limitation in fish growing in aquaculture (Sugiura *et al.* 2003). Beyond this, patterns of gene expression are being used more widely to study the role of nutrients in ecology. For example, aquatic consumers have recently been found to increase the production of digestive proteases in response to cyanobacterial food sources (Schwarzenberger *et al.* 2010) and to increase the expression of genes underlying the eicosanoid synthesis pathway in response to higher fatty acid content of their diet (Schlotz *et al.* 2012). As the tools to measure these response variables become more common, nutrient-specific indicators based on gene expression will be of considerable utility to ecologists.

*Translational regulators.* Similar to gene expression, translational regulators are a potential source of nutritional indicators. Translational regulators can modify the amount of protein produced per gene transcript and can be quite responsive to organismal nutrition. In particular, several microRNAs (miR) have been found to be robust and nutrient-specific indicators of nutritional stress in plants (reviewed in Scheible *et al.* 2011). The best described example is miR 399, which has only been found in strongly P-limited plants and shows no cross reactivity with other nutritional stressors (Scheible *et al.* 2011). While miRs have also been used to study stress responses in animals, whether they respond to nutrient stress in consumer taxa has yet to be determined. Nutrient

specific miR responses in consumers, if found, would be extremely valuable to ecologists because miR are highly conserved within and among taxa (Sempere *et al.* 2006, Wheeler *et al.* 2009). The conserved nature of miRs would allow for the examination of nutrient stress in different animal taxa with little to no susceptibility to contamination by plant food material (Pasquinelli *et al.* 2000, Sempere *et al.* 2006).

*Protein composition and activity.* Proteins represent another potentially rich source of indicators of nutritional stress in animal consumers. Within consumers, poor diet is known to change proteins involved with nutrient uptake, incorporation and storage, and degradation and recycling of cellular structures (Kolkman *et al.* 2006). These changes are well-documented in many organisms experiencing nutrient stress and have been used to infer their nutritional state under natural conditions. For example in *Pseudomonas putida*, unique protein composition was found in cells grown under C-stress compared to those grown under N or P-stress (Givskov *et al.* 1994). Following the identification of such nutrient-stress proteins, nutritional indicator profiles could be developed for routine use in ecological studies. This approach has been particularly useful in the study of iron-limitation of phytoplankton. For example, under Fe-limitation, diatoms replace the Fe-containing electron transport protein, ferredoxin, with a flavin containing protein, flavodoxin (LaRoche *et al.* 1996). In Lake Superior, greater flavodoxin levels are present in offshore areas, which indicate more intense Fe-limitation of diatoms in these areas (McKay *et al.* 2004). While protein-based indicators require some methodological development, this approach could ultimately be extremely cost effective and involve minimal complexity, which would increase its usefulness for studies of nutritional studies of animal consumers and their ecological interactions.

The activities of enzymes are another potential indicator of consumer nutritional status. Enzyme activity has been routinely used to detect nutrient-stress in phytoplankton (e.g., North *et al.* 2007) and bacteria (e.g., Sala *et al.* 2001), and, more recently, has been used to study aquatic consumers (McCarthy *et al.* 2010, Elser *et al.* 2010, Wagner and Frost 2012). Elser *et al.* (2010) used this approach to study the intensity of P-limitation in zooplankton communities grown under different levels of N-loading. They found an increased alkaline phosphatase activity in *Daphnia* associated with higher N:P ratios in their food. Enzymatic indicators benefit from the existence of many well-developed enzyme assays that could be readily adopted for *in situ* studies. On the other hand, this approach may be confounded by enzyme cross-reactivity to other substrates and suffer from a lack of nutrient-specificity. For example, Wagner and Frost (2012) found apparent increases in alkaline phosphatase activity in P-, N-, and food-limited *Daphnia*, which plausibly resulted from the cleavage of fluorometrically-labeled substrates by other non-P sensitive enzymes. Nevertheless, enzyme activities could be an important complementary component of animal nutritional profiling and its use in ecology.

*Metabolite analysis.* The composition of metabolites could provide valuable information of the type and intensity of nutrient stress in animal consumers. Metabolite responses to nutrient stress are well-described in plants (Bölling & Fiehn 2005), bacteria (Yuan *et al.* 2009), and yeast (Boer *et al.* 2010). For example, a few key metabolites respond strongly to C (glucose)-, N-, and P-limitation in yeast with different metabolite responses observed among the different types of nutrient limitation (Boer *et al.* 2010). Specifically pyruvate, glutamine, and adenosine triphosphate were found to be nutrient-specific growth limiting metabolites in glucose-, N-, and P-limited yeast respectively

(Boer *et al.* 2010), which means they each decreased substantially in cells experiencing each respective type of nutrient limitation. One major advantage of using metabolite composition is that it can be applied to any consumer with little method development (e.g., no requirement to design consumer-specific primers or produce protein-specific antibodies), which increases its immediate applicability to different animal taxa and nutritional stressors. However, the distinct disadvantage of this method is that without consumer gut clearing or the analysis of specific tissues, food material can contribute metabolites to the sample of interest and could confound easy interpretation of resulting data.

*Lipid analysis.* Lipids are a well-known dietary requirement of animals for growth and development. For example, some animal taxa have a restricted ability to synthesize sterols or  $\omega$ 3 and  $\omega$ 6 polyunsaturated fatty acids (PUFA) *de novo* (e.g. Stanley-Samuelson *et al.* 1988). The quantity and relative composition of these structurally and functionally diverse lipids in consumers could provide information on the nature and intensity of nutritional limitation. Although our knowledge of the underlying molecular mechanisms is rather poor, the responses of polar and non-polar lipids, fatty acids (FA) and sterols to different types of nutritional stress in algae have been systematically examined. Under both nutrient-limiting conditions and at high light intensities, non-polar lipids and saturated FA accumulate in algal cells (Guschina & Harwood 2009). In contrast, sterols respond to light-nutrient interactions in algae such that they increase with light intensity under low nutrient supply but decrease with increasing light intensity under high nutrient supply (Piepho *et al.* 2010). In animal consumers, the ratio of non-polar storage to polar structural lipids in whole bodies or isolated tissues would likely closely

match food quantity (Elendt 1989). More specifically, the composition and relative quantities of FA and sterols in animal consumers may illuminate the type and strength of biochemical limitation (i.e., either direct FA or sterol limitation) or mineral limitation.

*Biomolecular content.* While recent advancements in molecular biology provide an array of novel and extremely promising nutritional indicators, previously developed and currently available biochemical responses should continue to be considered for use in ecological studies. These responses would include the biomolecular composition (e.g., protein, lipids, DNA, and RNA) of tissues and organismal bodies (e.g., Figure 2). While responses of biomolecular pools are unlikely to be entirely nutrient-specific, they could nonetheless assist with the diagnosis of specific types of nutrient limitation. For example, low lipid content, high free amino acid content, and low body %C would together indicate limitation by food quantity. The application of such approaches, in an absolute sense, would likely require species-specific information on maximum growth rate and biochemical composition during non-limiting conditions. For example, relatively fast growing animal taxa may exhibit relatively large changes in RNA:DNA ratios under P-limitation, whereas this ratio may be less sensitive in slower growing taxa (Elser *et al.* 2000). Given differences in clones/species or life history strategies and the potential lack of nutrient-specificity, the usefulness of these biomolecular data alone as indicators of nutrient stress may be limited. However, the ease and low cost of sample analysis nevertheless will continue to make these responses a viable complementary source of nutritional information.

*Physiological processes.* Key physiological traits involved in nutrient metabolism could also be used as complementary indicators of nutrient stress (Figure 2.2). Nutrient-

poor food is well known to alter metabolic rates, digestive and absorptive efficiencies and rates and ratios of nutrient excretion and egestion (Sterner and Elser 2002, Frost *et al.* 2005). For example, DeMott *et al.* (1998) found lower body P content, increased P assimilation, and reduced P excretion in *Daphnia* grown with P-deficient diets. When appropriate, addition of these types of response variables to ecological studies of nutrition would strengthen conclusions regarding the identity and severity of elemental limitation. Physiological processes involved in nutrient metabolism (e.g., assimilation efficiencies or nutrient release rates) would be particularly useful in determining animal nutritional state. The value of these measurements is that they are already quite commonly used, are of low cost, and are relatively easy to interpret. On the other hand, some of these variables (i.e., growth and respiration) are known to respond to all forms of nutritional limitation, show low or no consumer specificity, and are of less value when used alone in the absence of other nutritional indicators.

While much insight into the nature and type of nutrient-specific responses described above is from past work examining bacteria, yeast and plants, this information nonetheless can be used to illustrate how metabolic profiles of animal consumers could be altered by nutritional stresses and to create a detailed nutritional profile (Figure 2.2; supplementary information). Together, systematic compilations of these molecular responses, such as described here, will provide greater and more defensible conclusions regarding the nutritional status of an animal consumer whether collected from nature or used in an experiment.

## **2.5 Application of nutritional indicators to ecological studies.**

There is growing recognition within the field of ecology of the fundamental importance of the relative availability of multiple resources cycling within and among ecosystems (Sterner and Elser 2002). For example, consumer-food mismatches in nutrient content alter individual (e.g., feeding behavior) and population-wide (e.g. grazing rates) processes (Frost *et al.* 2005, Hillebrand *et al.* 2009a). Despite the broad importance of understanding the role of nutrients in ecosystems, methods used to evaluate the degree and type of nutritional limitation experienced by animal consumers remain relatively simple and unrefined. Improved nutritional indicators are thus needed and would add value to many types of ecological studies. Nutritional indicators would be of considerable use in determining how nutrient supply ratios alter diverse and varied ecological dynamics by way of changes in animal physiological condition and performance (Figure 2.1).

*Occurrence of nutritional limitation.* One area that nutritional indicators could immediately be applied is the determination of the type, frequency, and strength of nutritional limitation of animals *in situ*. Previous determinations of nutrient limitation in consumers have relied on indirect inferences based on stoichiometric differences between food and tissue (e.g., Frost and Elser 2002) and/or animal growth responses in bioassays (e.g., Müller-Navarra *et al.* 2000). While these approaches have seen wide use, their ability to differentiate among multiple forms of nutritional limitation has been repeatedly questioned (Hartwich *et al.* 2012). Because animals ingest resource packages (in contrast to plant uptake of single resources), consumers may face frequent co-limitation by multiple nutritional components in their food (Lukas *et al.* 2011, Sperfeld *et al.* 2012),

especially when these nutrients strongly co-vary in food material (Hartwich *et al.* 2012). Nutritional indicators would provide a means to study the relative importance of co-varying nutritional components. For example, divergent responses of consumers to P-, specific types of FA-, sterol-, or amino acid-limitation could provide a defensible and relatively easy means to separate these types of nutritional limitation (Wacker & Marin-Creuzburg 2012). Indicator profiles could thus quickly illuminate which nutritional components, alone or in combination, are limiting consumer performance. Detecting resource-specific limitation signals would also provide a method to determine the general applicability of multiple resource limitation to consumers as in autotrophs (Harpole *et al.* 2011). In addition, this approach could be applied to individual animals, allowing for the assessment of variability in nutritional state within populations. Substantial variability among animals would indicate fine-scale heterogeneity in food sources or foraging success and could be included in ecosystem models that often consider only the average nutritional state of animals within populations. Variability in nutritional profiles associated with genotype or species would be of interest in and of itself as it would provide evidence for the evolution of differential sensitivity to poor food quality. If found, this inter- and intraspecific variability in nutrient stress would be fertile territory for future studies aiming to link local scale selective pressures created by dietary stress to population genetics and the consequences on phenotypic variation among populations. At larger scales, studies could use this approach to better assess the nutritional states of consumers and their populations through time (e.g., within a lake or field across seasons) or across space (e.g., among streams or forest plots across resource gradients).

Descriptive data on the frequency and severity of animal nutritional stress could also be used in studies examining the environmental drivers of animal nutrition. In particular, nutrient supply ratios, plant taxonomic composition, and other important environmental variables (e.g., temperature) all can affect the quantity and quality of food available to consumers, which could be verified using a nutritional profile to assess the nutritional state of consumers. Nutritional indicators could also be used to examine the propagation of nutrient limitation signals across trophic levels. With an experiment on marine pelagic food webs, Malzahn *et al.* (2007) found that predators responded to the nutrient content of the algae consumed by their herbivorous prey even though the body stoichiometry of the herbivores did not change. Nutritional indicators, in this case applied to herbivores and predators, would provide an improved evaluation of proximate nutritional mechanisms that transfer resource limitation up through the food web (Figure 1).

*Linking nutrition to organism physiology.* The development of nutritional indicators would also feed into better and more complex experimental designs aimed at understanding how nutritional limitation affects consumer physiology, life history, and behavior. For example, the study of acclimation to poor nutrition by animal consumers and its time scales would be improved by nutrient-specific indicators. The measurement of key metabolite fluxes would add great value to time scale experiments and provide a better understanding of how nutrient limitation affects consumers. In addition, nutritional profiling would be useful for studies of consumer feeding rates and food selection, mass conversion, and for determining how fluctuating food quantity and quality affects the growth and reproduction of consumers in natural ecosystems. For example, one could

determine if nutrient stress occurs during short periods of low food quality, even if growth rates are not affected (e.g., Hood and Sterner 2010). This decoupling of nutrient stress and growth rates would imply that there are compensatory physiological processes and changes in mass conversion efficiency. The mechanisms linking food quality and optimal foraging could also be studied using nutritional indicators. For example, one could take simultaneous and repeated measurements of food quality, ingestion rates, assimilation efficiencies, and nutritional profiles. Such data would show whether changes in the downstream nutritional state of the animal precede or simply follow alterations to feeding behavior caused by food of declining quality. The use of nutrient-specific indicators would also aid in studies of consumer habitat selection, differential use of resource patches of varying quantity and quality, and selectivity for certain food items.

Indicators of animal nutritional state could also be used to generate an improved mechanistic understanding of nutrition with the metabolic theory of ecology (MTE; Brown *et al.* 2004). MTE seeks to understand ecological processes through the use of metabolic scaling of individuals and populations primarily as a function of temperature and body size. Plasticity in metabolic scaling appears common (Glazier 2005) and recent work suggests nutritional limitation can change mass-metabolism scaling (see Jeyasingh 2007). Most simply, nutritional indicators could be used to show whether scaling changes systematically with the type and strength of dietary stress in consumers. Alternatively, nutritional profiles of animals *in situ* could be used to adjust the application of metabolic power laws to derive population-level parameters. Integrated nutritional profiling would provide an avenue to determine the cause of variable metabolic scaling either under laboratory (e.g., Jeyasingh 2007) or field (e.g., McFeeters *et al.* 2011) conditions.

*Ecological interactions.* Nutritional indicators could also be used to improve studies of the population dynamics of animals and their interactions in food webs. While changes in food quantity and quality affect key demographic parameters, the translation of these effects into population gains/losses depends upon the interactions with other factors such as mortality from predators and disease (Dodson 1998). Indicators applied to multiple species could help sort out how competitive abilities change with nutritional state and affect interactions among similar species. Specific indicators could thus address the question of whether apparent differences in consumer nutrient requirements actually translate into different competitive abilities and altered fitness at population scales. In this regard, competition experiments among two or more taxa could be improved by better tracking of the nature and severity of nutritional stress in multiple consumer species through time. For example, two common zooplankton species, *Daphnia* and *Bosmina*, appear to have different requisite P requirements and sensitivity to low P food (Schulz and Sterner 1999). In competition experiments, one might expect that the high P requirements of daphnids would produce more frequent and intense periods of P-limitation in these animals under conditions of low external P supply and thereby provide a competitive advantage to relatively P-poor *Bosmina*.

Nutritional indicators could be incorporated into studies that consider how populations or communities respond to variable resource supply ratios. For example, they would be well suited to determine whether coexistence of multiple consumers on single prey items can result from the limitation by separate nutritional components (Loladze *et al.* 2004). In this example, different species would show limitation by different nutrients even though they are consuming the same prey item. Additional ecological questions of

interest that link nutrients and trophic interactions would also be amendable to this approach including: does reduction of consumer density by predation yield decreased limitation by food quantity and greater constraints by poor food quality? Or how does host nutritional quality alter parasite population establishment and growth? For these types of studies, nutritional profiles would provide information about the nature and strength of limitation in changing population and communities as they respond to varying food quantity and quality.

*Ecosystem processes.* Nutritional indicators and their application to consumers *in situ* would provide new information on the controls of ecosystem structure and function. The flux and storage of matter, including the rates and ratios nutrient cycling, appear to strongly depend on the traits and the diversity of organisms in ecosystems (Hooper *et al.* 2005). Nutritional profiling would enable ecologists to more quickly and easily link functional traits affecting consumer nutritional demand and status to their effects on the movement and retention of elements in ecosystems (Figure 1). Recent evidence from autotrophs suggests that: i) resource availability and resource ratios constrain both biodiversity and biomass production and ii) increased producer biodiversity subsequently enhances the retention and transfer efficiency of available resources into new production (Cardinale *et al.* 2009, Hillebrand & Lehmpfuhl 2011). Similar work has not been completed thus far for consumers, which precludes assessing whether nutritional complementarity is intimately tied to consumer biodiversity or determines how nutrients affect emergent ecosystem processes. This particularly includes understanding biodiversity effects on nutrient recycling, which have been predicted (McIntyre *et al.* 2007) and described (Hillebrand *et al.* 2009b), but remain poorly linked mechanistically

to the nutrient status of consumers. One could also determine how the strength and nature of nutritional limitation varies between herbivores and detritivores, who often encounter distinctly different types of food (Olf *et al.* 2009). Such work would help explain variable rates of organic matter processing and storage in ecosystems. Ultimately, nutritional indicators could be used to determine how food quality constraints on individual consumers or their populations affect the movement and storage of multiple elements through the environment.

## **2.6 Conclusions**

The use of nutritional indicators will advance the study of ecology by providing more explicit and greater evidence of a consumer's nutritional state. There are strong and specific effects of poor nutrition on cellular and molecular mechanisms underlying animal metabolism, which will become easier to identify and study using rapidly advancing information-rich fields of study in biochemistry and molecular biology. Nutritional ecology is poised to benefit from greater and more ambitious use of these approaches by integrating and adopting this information to devise novel and precise methods of studying consumer nutritional state. The greater ability to study relationships between food quantity and quality, consumer nutrition and performance, and ecological processes promises to revolutionize the study of ecology and its nutritional bases.

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## 2.9 Tables and Figures

Table 2.1. Potential indicators of animal nutritional state

Type	Example response variable	Analytical methods/tools	Consumer-specific	Nutrient-specific	Advantages	Disadvantages	Examples
Transcript profiling	Transcriptomics	cDNA, pyrosequencing	Y	Y	High results probability	Cost, complexity	Boer <i>et al.</i> (2004)
	Specific gene expression	qRT-PCR	Y	P	Low mass requirements	Among consumer-specificity	Sugiura <i>et al.</i> (2003)
Transcriptional/ Translation regulators	microRNA expression	qRT-PCR	Y	Y	Low mass requirements	Among consumer specificity	References within Scheible <i>et al.</i> (2011)
Protein profiling	Proteomics	2-D gel electrophoresis	N	Y	High results probability	Cost and sample preparation	Givskov <i>et al.</i> 1994
	Specific protein content	ELISA/Western blotting with use of specific antibodies	N	P	Ensures only one enzyme protein is measured	Potentially consumer specific	LaRoche <i>et al.</i> (1996)

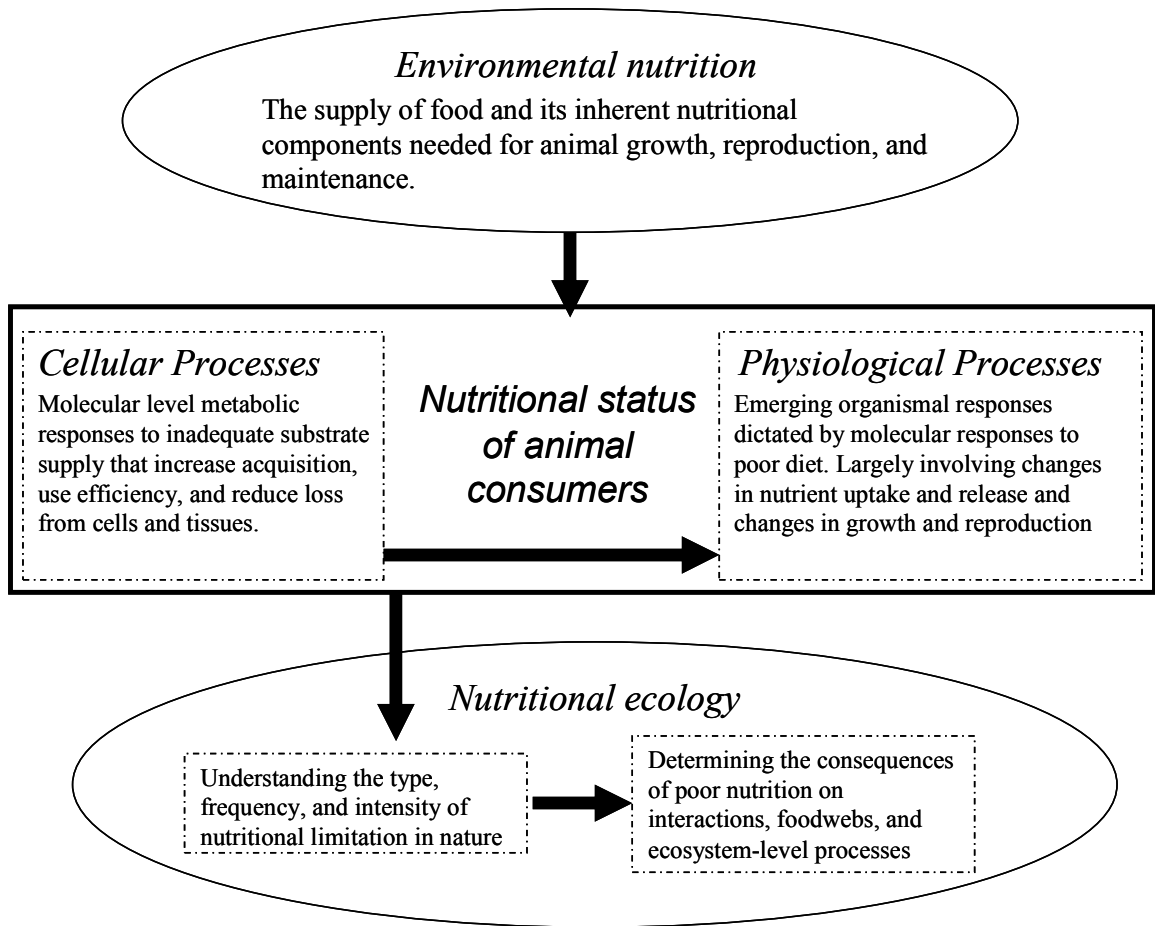
	Enzyme activity	Colorimetric Fluorometric Chemiluminescence	N	P	Cost effective, simple methodology	Need to isolate enzyme of interest	McCarthy <i>et al.</i> (2010) Wagner & Frost (2012)
Lipid Profiling	Lipidomics	ESI-MS	Y	Y	High results probability	Sample preparation	Han & Gross (2003)
	Steroid hormones, eicosanoids	Immunoassays, LC-MS, GC-MS, ESI-MS	Y	Y	High results probability	Sample preparation	Martin-Creuzburg <i>et al.</i> (2007), Han & Gross (2003)
Cellular membranes	Phospholipids	GC-MS	N	Y	High results probability	Sample isolation, consumer specificity	Van Mooy <i>et al.</i> (2009)
	Specific lipid content	GC-MS LC-MS	N	P	High results probability	Consumer specificity to changes	Guschina & Harwood (2009)
Metabolite composition	Metabolite profiling	<sup>1</sup> H-NMR GC-MS	N	Y	High results probability	Mass requirements, complexity	Boer <i>et al.</i> (2010)
	Specific metabolite content	HPLC	N	P	High results probability	Inference with gut contents	Tweeddale <i>et al.</i> 1998)

Biomolecular content	DNA:RNA ratios	Fluorescence determination	N	N	Mass requirements	Non-specific Baseline knowledge needed	Elser <i>et al.</i> (2000) Wagner <i>et al.</i> (1998)
	%Lipids, proteins, carbohydrates	Colorimetric determination	N	N	Ease of measurement	Inference with gut contents	Sterner <i>et al.</i> (1992)
Physiology	Elemental content	UV/Vis Spec	N	N	Ease of measurement	Inference with gut contents	Elser <i>et al.</i> (2001)
		Colorimetric determination					Wagner and Frost (2012)
	Growth rate	Length-weight regressions Indication of mass	N	N	Ease of measurement	Non-specific Inference with gut contents	Sterner <i>et al.</i> (1993) Frost and Elser (2002)
	Nutrient release rates	UV/Vis Spec Colorimetric determination	N	N	Ease of measurement	Inference with gut contents	Urabe <i>et al.</i> (1995)

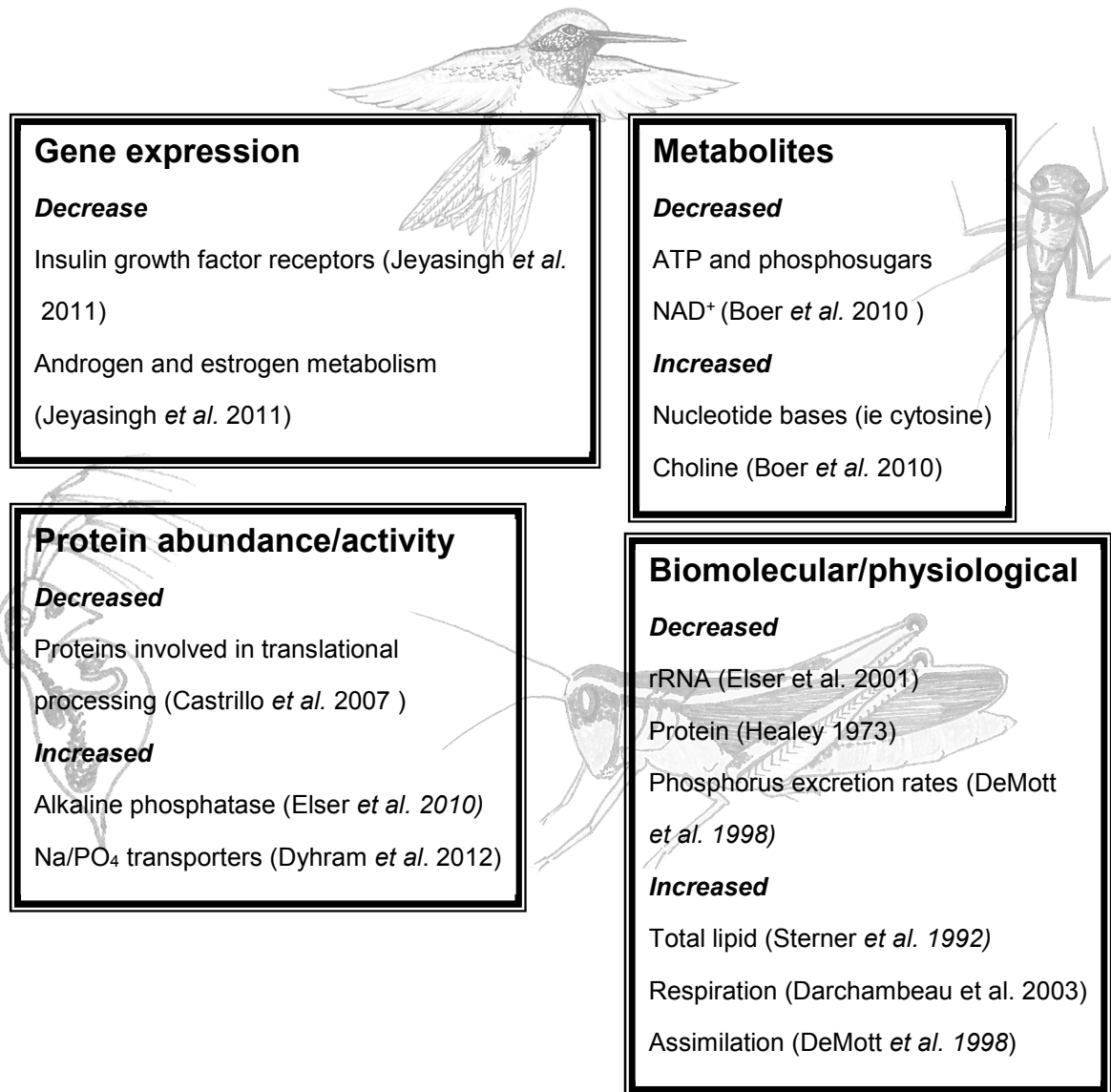
## **Figure Captions**

Figure 2.1: Linkages among the nutritional environment of consumers, internal cellular and physiological processes controlling animal nutrient use efficiency and the role of nutrition in ecology.

Figure 2.2: Putative nutritional profile of an animal consumer responding to acute dietary phosphorus stress. Listed are complementary molecular, biochemical, biomolecular, and physiological responses that dictate increase nutrient use efficiency. See supplementary information for more detailed information.



**Fig 2.1**



**Fig 2.2**

## 2.10 Supplementary

### Nutritional indicators and their uses in ecology

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## **Putative consumer nutritional profile under carbon, nitrogen, phosphorus, iron and biochemical limitation.**

While much of the insight on the nature and type of nutrient-specific changes in metabolism is derived from past work examining the nutrient responses of bacteria, yeast and plants, this information nonetheless can be used to illustrate how metabolic profiles of animal consumers should be altered by nutritional stresses. Here we summarize information on metabolic responses of different organisms to poor nutrition, using examples from animal consumers when available, to examine the biochemical and physiological profiles likely to be produced by different forms of nutritional limitation. Throughout, we illustrate how the aforementioned indicators can be integrated and used to demonstrate a particular form of nutritional limitation.

*Carbon-limitation.* Animal consumers acquire C and energy primarily through the ingestion and digestion of food-derived sugars, carbohydrates, proteins and fats. Post-digestive absorption and distribution of monosaccharides (including glucose), amino acids (AAs) and FAs provide tissues and cells with C and energy for metabolism. Low food quantity should thus lead to the up-regulation of genes and greater production of the proteins involved in the uptake of glucose, AAs, and FAs (Boer *et al.* 2003). In addition, short-term provision of energy for central metabolism (TCA cycle; Wu *et al.* 2004) would likely result from increased oxidation of FAs, gluconeogenesis and protein catabolism (Boer *et al.* 2003, Wu *et al.* 2004). As free sugars and other organic pools are consumed for energy, low food quantity should change the metabolite composition of consumers. For example, AMP:ATP ratios should increase due to energetic constraints on ATP production and an increased rate

of AMP production through the reduction of adenosine diphosphate (Hardie 2003). Additionally, concentrations of free AAs should increase as proteins are catabolized for energy and protein synthesis is limited by low energy supplies (Boer *et al.* 2010). Animal body (or tissue) content of protein and lipids should thus decrease reflecting the greater breakdown and reduced synthesis rates of these biochemical pools. One might also expect lower RNA:DNA ratios in C-limited animals due to reduced requirements for ribosomal RNA created by the energy-induced constraints on protein synthesis (Wagner *et al.* 1998, Boer *et al.* 2010).

*Nitrogen-limitation.* N-limitation should produce distinct metabolic effects and chemical profiles in animal consumers. At the genomic level, the expression of genes responsible for the production of AA transporters should increase (Boer *et al.* 2003, Hua *et al.* 2004), while genes involved in the uptake of glucose or FAs should decrease. There should be increased gene expression of autophagy pathways that are used to recycle unneeded proteins (Boer *et al.* 2003). Genes underlying lipid and carbohydrate storage should also be expressed more as the N-limited consumer stores, perhaps temporarily, excess and accumulating organic C (Hua *et al.* 2004). Gene expression of and subsequently enzyme activity in metabolic pathways that form ammonium (e.g., cleaving amide bonds) or glutamate from glutathione (Boer *et al.* 2003) should also increase to acquire more free AAs. The metabolite signature of N-limited consumers should be characterized by lower levels free AAs, especially ones leading to the formation of other AAs (e.g., glutamine which provides an ammonium group to arginine, histidine, and tryptophan; Boer *et al.* 2010). A further reduction in other AAs, particularly those containing multiple N atoms (i.e., arginine, histidine,

and lysine), would be expected in extremely N-limited consumers (Boer *et al.* 2010). Another metabolite intermediate involved in central metabolism,  $\alpha$ -ketoglutarate, should increase as this intermediate can mediate a transamination reaction that produces glutamate (Hua *et al.* 2004, Boer *et al.* 2010). One might also expect greater nucleotide catabolism, as occurs in ammonium-limited bacteria (Hua *et al.* 2004), to decrease protein synthesis and to increase N supply to other key metabolic pathways. The biomolecular content of N-limited consumers would likely be further characterized by increased lipid content, reduced protein content, and a slightly lower RNA:DNA ratio due to N constraints on ribosomal and protein production.

*Phosphorus-limitation.* Inadequate dietary P content should be accompanied by large changes in the acquisition, incorporation, and release of P. As a result, gene expression of P uptake and recycling systems should increase. For example, genes responsible for high affinity sodium phosphate transporters should show greater expression to increase P acquisition from the digestive tract (Sugiura *et al.* 2003). Other genes involved in phosphate uptake, utilization (e.g., PHO pathway) and recycling (e.g., adenosine kinase) should be up regulated to increase the potential for acquiring and retaining P (Wu *et al.* 2004). The metabolic signature of P-limited consumers would be characterized by lower amounts of ATP, phosphorylated sugars, and energy-rich molecules such as nicotinamide adenine dinucleotide (Boer *et al.* 2010). Guanosine, adenosine, and cytosine would all be expected to increase in P-limited consumers resulting from the release of P from the cleavage of RNA. Choline may also increase due to the recycling of phospholipids, which is an additional source of P for P-stressed organisms (Boer *et al.* 2010). At a biomolecular level, P-deficient

consumers should have lower protein content and decreased RNA:DNA ratios caused by reductions in protein synthesis and the reduced production of P-rich rRNA. An increase in lipid content should also be present in P-limited consumer reflecting the storage of excess C (Sterner *et al.* 1992).

*Iron limitation.* Beyond the major biochemical nutrients (C, N, and P), several minor elements also potentially constrain consumer growth and could exert differential effects on consumer body biochemistry. For example, Fe is known to limit primary producers in large areas of the open ocean (Martin and Fitzwater 1988) and may co-limit phytoplankton in some freshwater ecosystems (North *et al.* 2007). If the Fe content of algal-derived food fails to meet the minimum requirements by consumers, there should be corresponding changes in body biochemistry and reduced growth. Fe-limited consumers should increase Fe-specific transporters through increased gene expression and protein production (Georgatsou & Alexandraki 1994). Fe-limited animals should also contain less ferritin, a Fe-storage protein, and exhibit reduced hemoglobin levels (Hentze & Kuhn 1996). As heme should only be produced when Fe is available and its production is controlled, in part, by aminolevulinic acid synthase (Hentze & Kuhn 1996), this enzyme may also show decreased activity during periods of Fe limitation. The metabolic signature of Fe limited consumers may be similar to that of Fe-limited phytoplankton, which have been found to have greater content of succinate and citrate, both of which have the ability to chelate iron (Bölling and Fiehn 2005). Finally, the Fe-content of Fe-stressed consumers should decrease given that trace element content of metazoans is more variable and reflective of external supplies than other major nutrient constituents (Karimi & Folt 2006). This

approach to profiling and cataloguing complementary biochemical responses could be also used to determine limitation by other trace elements (e.g., zinc, copper, selenium, and manganese).

*Biochemical limitation.* Within aquatic ecosystems, FA limitation has been identified as a primary source of biochemical limitation for invertebrates that are consuming phytoplankton and cyanobacteria containing low levels of essential FAs (Müller-Navarra *et al.* 2000) and sterols (Von Elert *et al.* 2003). It thus seems likely that consumers can be limited or co-limited by these multiple nutritional components (Lukas *et al.* 2011; Sperfeld *et al.* 2012). Essential FAs or sterol limitation should decrease consumers' content of cholesterol and PUFAs. Such effects are likely to vary among animal species due to differences in their FA requirements and metabolic capabilities. For example, the FA content of species with a very low FA turnover (e.g., calanoid copepods, Scott *et al.* 2002), may respond weakly to fluctuations in dietary FAs. In contrast, FA content might be more useful as nutritional indicators in animals that have a higher FA turnover (e.g., daphnids) and replace their FAs within days (Taipale *et al.* 2011). For example, a dietary deficiency in  $\alpha$ -LA, a precursor to eicosatetraenoic acid (ETA, C<sub>20</sub>:4 $\omega$ 3; Wacker & Weithoff 2009), could lead to a depletion of  $\alpha$ -LA in such consumers. Limited levels of dietary PUFAs, required for planktonic crustaceans (due to slow synthesis; Von Elert 2002), also may either increase the enzymatic rates of elongation and desaturation (Schlechtriem *et al.* 2006) or deplete the body concentrations of long-chain PUFAs (Sperfeld & Wacker 2012). Additionally, activities of cyclooxygenases (COX) and lipoxygenases (LOX) in the metabolic pathway of eicosanoid synthesis (Heckmann *et al.* 2008) may be up-

regulated to maintain reproduction and immune function (Stanley-Samuels 1994). Concomitantly, non-structural FAs (mainly FAs without double bonds) would be stored in non-polar lipid pools or metabolized. In the case of sterol limitation, animal content of cholesterol may decrease while essential PUFAs would presumably increase. Reduced cholesterol may either decrease concentrations of steroid hormones or force animals to up-regulate the conversion into steroid hormones.

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## CHAPTER 3

### 3.1 PREFACE

**Title:** Responses of alkaline phosphatase activity in *Daphnia* to poor nutrition

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**Key words:** food quality, bioindicator, Cladocerans, ecological stoichiometry, enzyme activity

**Author contributions:** NDW and PCF conceived the project. NDW performed all the experiments and collected all the data. NDW and PCF analyzed the data wrote the paper.

## CHAPTER 3

### Responses of alkaline phosphatase activity in *Daphnia* to poor nutrition

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#### 3.2 Abstract

The use of biochemical and molecular indices of nutritional stress have recently been promoted for their potential ability to assess the *in situ* nutritional state of zooplankton. The development and application of these indicators should at least consider the cross reactivity with other nutritional stressors. We examined the potential usefulness of body alkaline phosphatase activity (APA) as an indicator of dietary phosphorus (P) stress in *Daphnia*. We measured growth rate, body P-content, and body APA of two species of *Daphnia* (*D. magna*, *D. pulex*) grown for different periods under diverse dietary conditions. We found P-poor food reduced daphnid growth rates and body P-content while body APA increased in both species. However, body APA increased in P-sufficient *D. magna* and *D. pulex* that were feeding on cyanobacterial compared to green algal food despite no differences in animal body P content. Body APA increased in *D. magna* fed P-poor food whether cyanobacterial or algal. Body APA also varied with age and other nutritional stresses (low food quantity, nitrogen-poor algae) in both daphnid species. Our results demonstrate that whole body homogenate APA in *Daphnia* is not singularly responsive to P-poor food, which will complicate or limit its future usefulness and application as an indicator of dietary P-stress in metazoans.

### 3.3 Introduction

The nutritional status of freshwater zooplankton is known to vary widely depending on the quantity and quality of algal food in their diet (Sterner and Elser 2002, Frost et al. 2005). The determination of a zooplankter's nutritional status usually has often been assessed with laboratory studies that measure changes in growth, reproduction, and/or body elemental composition to a changing diet (Sterner et al. 1993, Urabe et al. 1997, DeMott et al. 1998, Sterner and Schulz 1998). While these types of studies have provided important insight into consumer growth and life-history responses to nutrient poor food, they can be difficult to relate to field conditions because of differences found in the controlled laboratory environment. In the field, the assessment of zooplankton nutrition has often involved the simple comparison of consumer nutritional requirements (or body elemental composition) with that available in the putative food source (e.g., Andersen and Hessen 1991, Frost and Elser 2002, Frost et al. 2006). These field assessments can also be problematic as they either indirectly infer nutrition from collected food, which may or may not be the primary nutritional source of the animal, or involve time consuming laboratory experiments that measure zooplankton growth on field collected food (e.g., Elser et al. 2001). While both field and lab based approaches have been quite valuable, there is a need for alternative methods that determine the *in situ* nutritional status of zooplankton. One potential source of nutritional information could be derived from the examination of molecular, biochemical, and/or physiological response variables that are sensitive to diet. This indicator approach is premised on predictable and element-specific metabolic changes in animal bodies resulting from nutrient

limitation. Given our growing understanding of metabolic responses to nutrition (Clarke and Walsh 1993, McCarthy et al. 2010, Schwarzenberger et al. 2010, Jeysingh et al. 2011), this approach would appear to hold considerable promise of providing new methods to directly infer the nutritional state of zooplankton and other aquatic metazoans.

One potential indicator of nutritional stress that has received considerable study in diverse organisms is enzyme activity (Mayzaud and Poulet 1978, Hassett and Landry 1983, Clarke and Walsh 1993, Elser et al. 2010, McCarthy et al. 2010, Wojewodzic et al. 2010). For the detection of phosphorus (P)-limitation, the activity of alkaline phosphatase (AP) may be a suitable indicator given this enzyme's substrate promiscuity for phosphate monoesters. By cleaving phosphate from organically bound phosphate molecules, AP frees phosphate allowing for its transport and incorporation into animal tissues. Given this role in phosphorus acquisition, the animal's body AP activity (APA) has recently been shown to have promise as an indicator of P-deficiency in zooplankton (Elser et al. 2010, McCarthy et al. 2010, Wojewodzic et al. 2010). These studies found increased APA in homogenized animal bodies of both laboratory-held and field-caught *Daphnia* that were consuming P-limited algae. For example, McCarthy et al. (2010) found body APA in *Daphnia* linearly increased in response to increasing algal carbon to phosphorus (C:P) ratios. While APA is known to increase in the bodies of P-limited *Daphnia*, it remains unknown if these responses of body APA in *Daphnia* are P-specific or whether they are also affected by other nutritional stressors.

While APA is largely P-specific in both algae and bacteria (Kuenzler and Perras 1965, VanBogelen et al. 1996, Beardall et al. 2001), this element-specificity is less likely to widely apply to metazoans. For example, APA has roles not directly tied to P-acquisition in dipterans, where its activity increases during their development as they transition from larvae to pupae (Bourtzis et al. 1993, Eguchi 1995). This increase in APA in dipteran bodies results from an AP isozyme being cross linked with chitin and cleaving tyrosine phosphate, which assists with the sclerotization process (Psachoulia et al. 1989, Bourtzis et al. 1993). In fish, AP can be activated in mucous cells, where it provides a protective factor when the epidermis is wounded (Iger and Abraham 1990). In mammals, AP certainly has a role in phosphate metabolism but is also involved in bone formation, maturation of adipocytes, and probably cell differentiation (Bossi et al. 1993, Alia et al. 2005, Millan 2006). During bone formation, AP regulates the concentration of diphosphate ( $PP_i$ ), which at high concentrations can inhibit normal bone mineralization (Millan 2006). AP also can regulate the concentration of adenosine through hydrolysis of adenosine monophosphate in neuroblastoma cells (Millan 2006). It is unlikely that these additional roles of AP are directly inhibited or controlled by dietary P or extra-cellular phosphate concentrations. Indeed, the activity of certain AP isozymes in mammals can be inhibited by high concentrations of amino acids, such as L-arginine (Bossi et al. 1993, Millan 2006). Consequently, it is unclear whether AP and its activity will directly or specifically correlate with dietary P-deficiency in multicellular organisms. There is thus a need to assess how animal body APA responds to diverse nutritional

and environmental stresses that could confound its utility as an indicator of P-deficiency in future ecological studies.

Here, we assess the potential usefulness of APA as an element-specific indicator of dietary P-stress in a common freshwater invertebrate, *Daphnia*. We first grew two species of *Daphnia* (*D. magna*, and *D. pulex*) in the laboratory with algal food having a range of C:P ratios (~60 to ~1000 by mol) to examine the relationship between algal phosphorus and *Daphnia* body APA. After verifying P-sensitivity of body APA in our daphnid clones, we next examined whether body APA of these two daphnid species would vary in response to food type (i.e., green algae or cyanobacteria). We further measured the body APA of *D. magna* and *D. pulex* grown to different ages and under different nutritional constraints including food limitation, P-limitation, and nitrogen (N)- limitation. If *Daphnia* body APA is a specific indicator of dietary P-stress, then elevated body APA should only be found in animals grown in P-poor conditions and it should not increase in response to other nutritional stressors. However, if APA has functions other than acquiring phosphate in *Daphnia*, then we expect higher APA activity to result from other nutritional stressors besides P-limitation.

### **3.4 Methods**

*Algae and Daphnia culturing.* We grew two foods, *Scenedesmus obliquus*, and *Synechococcus leopoliensis* [Canadian Phycological Culture Centre strains 10 (purchased as *S. acutus*) and 102 respectively], in multiple semi-continuous culture jars that were diluted daily with growth media (Sterner et al. 1993) for *S. obliquus* or BG-11 media (Rippka et al. 1979) for *S. leopoliensis*. *S. obliquus* was grown with

differentially enriched media to produce P-rich, P-poor, and N-poor algal cells (Online resource, Table S3.1 for dilution and N & P concentrations used). *S. leopoliensis* was grown to produce P-rich and P-poor cells. Harvested cells were centrifuged (4066 g) for 15 min and resuspended in N and P-free COMBO media (Kilham et al. 1998). We analyzed the elemental composition of these concentrated food suspensions as described below and used these data to prepare food mixtures that contained the desired nominal food C:P ratio. After mixing, we saved samples of food mixtures for post-experiment determination of food C:P and C:N ratios. We determined the phosphorus content of cells on dried material after persulfate digestion using the molybdate-blue ascorbic acid colorimetric assay (APHA 1992). Carbon and nitrogen content of food mixes were also determined on dried cells using an Elemental Analyzer (Vario EL III, Elementar Incorporated, Mt Laurel NJ, USA). We used these foods to study how diet affects the body APA of two daphnids, *Daphnia magna* (Straus) and *Daphnia pulex* (Leydig). To produce experimental animals, we grew second generation clonal mothers in 400 ml of P-free COMBO media and ensured they were well-fed with P-rich *S. obliquus*. On the morning of each experiment, we collected <24 hr old neonates that were borne in the second to fifth brood from these well-fed mother *Daphnia*.

*General experimental design.* Experimental animals were always rinsed with N and P-free COMBO media 4 times to ensure all the brood mother food and COMBO media was removed. Experimental animals were grown in individual containers containing 20 ml (age 0-6 days) or 40 ml (age 7-14 days) of N-and P-free COMBO media. Except for when food quantity was being manipulated, *Daphnia*

were fed 4 mg C L<sup>-1</sup> (age 0-6 days) or 8 mg of C L<sup>-1</sup> (age 7-14 days) every other day. To verify that we produced the desired food quality effect on *Daphnia*, we measured body P content (n = 5) and mass specific growth rates (MSGR; n = 10) of animals from all food treatments. We saved 5 animals for body APA measurements from each food treatment by freezing individual animals at -80 °C as described below.

*Phosphorus gradient.* Previous studies have found increased body APA in *Daphnia* eating P-poor food (McCarthy et al. 2010, Wojewodzic et al. 2010). We further explored this relationship between body APA and food phosphorus content by using a more complete set of algal C:P ratios. We completed two consecutive growth experiments that used a range of algal C:P ratios (60 to 1000) for both *D. magna* and *D. pulex*. *Daphnia* were fed a total of 21 different food C:P ratios (Online resource, Table S3.2). For this experiment, *Daphnia* were grown for 6 days after which their responses (body APA, MSGR, and body %P) to P-poor food were measured.

*Type of food.* Once we established that body APA increased in our daphnid clones in response to P-poor algae, we examined whether body APA would respond differently to green algal and cyanobacterial foods. To do so, we grew *D. magna* and *D. pulex* with P-rich (C:P ratio ~ 150) and P-poor (C:P ratio ~ 700) *S. obliquus* (*S.o*) and *S. leopoliensis* (*S.l*) for 6 days. In addition to body %P and growth rate, we measured body %C (n = 3) and % N (n = 3) of these *Daphnia* for further verification that we produced the desired nutritional state in these animals.

*Element specificity of APA.* We also examined body APA responses of *Daphnia* to other types of non-P food nutritional deficiency in animals of different ages. *D. magna* and *D. pulex* were grown for 3, 6 and 14 days and fed one of four

different food conditions: high quantity of nutrient-rich food (GF; C:P ratio ~100, C:N ratio ~8), low quantity of nutrient-rich food (LF, *D. magna*: 0.75 mg C L<sup>-1</sup>, *D. pulex*: 0.5 mg C L<sup>-1</sup>), high quantities of phosphorus-limited food (PL, C:P ratio ~900, C:N ratio ~15) or high quantities of nitrogen-limited food (NL; C:P ratio ~100, C:N ratio ~25). In this design, the severity of food limitation increased with age as animals were not kept under constant food densities. The slightly higher food quantity provided to *D. magna* compared to our *D. pulex* was intended to partially compensate for differences in their mass. *Daphnia* were transferred to new N and P-free COMBO media on day 6 and 10 to reduce excessive accumulation of algae and waste products. In addition to daphnid body APA, MSGR, and body %P, we also measured body %C (n = 3) and %N (n = 3) after this experiment as further indication of the physiological effects of the LF and NL foods. Due to the small size of day 3 *D. pulex* animals, we pooled 2 animals for each replicate of APA, which was necessary to ensure adequate body mass for the analytical assay.

*Daphnia growth and body elemental composition.* We measured MSGR in all experiments to verify the desired nutritional state of animals was produced in each experiment. We determined the MSGR by measuring the mass gained over 6 days for animals consuming different foods. Neonate mass was determined by drying 3 replicates of 10 neonates in pre-weighed aluminum foil cups for 24 hr at 60°C and measuring dried mass with a microbalance. At the end of each experiment, 10 individual *Daphnia* were dried and weighed, which allowed MSGR to be calculated by:

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

Where B2 is the final weight, B1 is the average initial neonate weight, and time is number of days of *Daphnia* growth.

We used groups of 2-5 individual *Daphnia* to measure body %P content. We placed these animals into aluminum foil cups to dry at 60°C for 24 hr prior to the phosphorus analysis. Once dry, *Daphnia* were removed from aluminum cups and weighed on a microbalance. Body P content was determined on weighed samples after persulfate digestion using methods described above (APHA 1992). Body C and N content was determined by placing 3-10 individual *Daphnia* in preweighed tin cups, which were then dried for 24 hr at 60°C, weighed on a microbalance, and analyzed using an elemental analyzer.

*Alkaline phosphatase activity.* Before processing our experimental animals, we verified that 3-*O* Methylfluorescein phosphate (MFP) (Healey and Hendzel 1979) precisely and accurately measured body AP activity in *Daphnia* homogenates. We did so by measuring the AP kinetics of both *D. magna* and *D. pulex* grown for six days with P-rich (C:P ~ 150) and P-poor (C:P ~ 700) food conditions. This validation trial demonstrated the ability of the MFP method to measure body APA (see trial results presented in online resource Fig. S3.1). In addition to measuring enzyme kinetics, we also verified that daphnid body APA was not strongly affected by ingested algae. To do so, we grew *D. magna* under P-rich (C:P ~ 100) and P-poor (C:P ~700) food conditions for 6 days and switched their diet for 40 minutes prior to harvesting (See online resource for additional methods). Body APA was measured on these *D. magna* with different algal gut conditions and no significant differences were detected in

body APA irrespective of gut conditions (Online resource, Fig. S3.2). In all subsequent experiments (except where stated), alkaline phosphatase activity was measured on individual *Daphnia* bodies. Briefly, each daphnid was placed into a 1.5 ml centrifuge tube and frozen at  $-80^{\circ}\text{C}$  until AP activity was measured. Results from our initial trial also showed that freezing daphnid bodies at  $-80^{\circ}\text{C}$  for up to 12 weeks did not decrease body APA (Online resource, Fig. S3.3). APA was performed in a 0.1 M Tris-HCl pH 8.5 buffer without the addition of  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$  ions because we assumed these essential cofactors were present *in vivo* and would remain bound during homogenization, and other studies measuring *Daphnia* body APA have not included these cofactors. After removing tubes from the freezer, we added 0.1 ml (mark on centrifuge tube) of 0.1 mm glass beads and 200  $\mu\text{l}$  0.1 M Tris-HCl pH 8.5 buffer to each tube and homogenized *Daphnia* in a mechanical agitator (Bullet Blender 24, Next Advance) set on speed 8 for 3 minutes. Subsequently, we added an additional 600  $\mu\text{l}$  of Tris-HCl buffer to increase the final sample volume to 800  $\mu\text{l}$ . After thorough mixing, 100  $\mu\text{l}$  of each sample was pipetted in duplicate into a 96 well microplate. 100  $\mu\text{l}$  of 10  $\mu\text{M}$  MFP was added to each well to produce a final 5  $\mu\text{M}$  MFP concentration. These samples were read on a Bio-Tec instrument FL<sub>x</sub> 800 microplate fluorometer with the excitation 485 nm/20 nm and emission 528 nm/20 nm and sensitivity of 70. Sample measurement began as soon as they were inserted into the plate reader, time 0, 15, and 30 minutes after the initial reading. Samples were compared to standards of methylfluorescein (MF) in the range of 0.1 to 2  $\mu\text{M}$ , which was chosen because MF is formed when AP cleaves MFP. From differences in fluorescence after 15 or 30 minutes from the initial time (time 0), we calculated how

much MF was produced per hour. The amount of MF produced per hour was mass-standardized by dividing by average dry mass of animals collected from each experiment treatment combination.

*Statistical analysis.* All data was checked for normality by calculating the Shapiro-Wilk W values using PAST.exe (version 2.06; <http://folk.uio.no/ohammer/past/>). All data except for body APA was found to be normally distributed. After log transformation, body APA data was found to fit a normal distribution. All subsequent analysis of variance (ANOVA) and post hoc comparisons were completed using SAS version 9.1.

For the C:P range experiment, we first examined whether the relationships described between response variables and food C:P ratios differed between the two experimental runs. We calculated slopes of the relationships between MSGR, body %P, and APA against food C:P ratios for each of the two experiments and both species separated using SMATR (Falster et al. 2006). For both daphnid species, there were significant differences between the two experimental runs in the Y-intercept but no difference in the relationship's slope. Consequently, we used a 2-way mixed model ANOVA that accounted for variation attributed to the experiment run by adding this as a random factor. The main effects in this 2-way ANOVA were food C:P ratios and daphnid species identity. We interpreted a significant interactive effect within this ANOVA to mean that the effects of food C:P ratios on the response variable of interest differed between the two daphnid species.

We analyzed the effects of food type on daphnid body APA (and other response variables) using two separate 2-way ANOVAs. We determined the main

effects of food type or food C:P ratios and their interactive effects for *Daphnia magna* consuming both P-rich and P-poor *S. obliquus* and *S. leopoliensis*. We could not examine the interactive effect of species and food type with this experiment because *Daphnia pulex* did not survive when consuming P-poor *S. leopoliensis*. Consequently, we could only analyze the food type-species interactive effect between *D. magna* and *D. pulex* for animals eating P-rich *S. obliquus* and *S. leopoliensis*. We analyzed separately whether body APA responses in *D. magna* consuming P-rich and P-poor food depended upon the type of food (*S.o* vs. *S.l*). In both 2-way ANOVAs when a significant interaction term was found, a Tukey post hoc test was used to further determine what food treatment combinations were significantly different from each other ( $P < 0.05$ ).

To analyze the element specificity of APA, we first performed a 3-way ANOVA with the main effects of animal age, food type, and daphnid species for all dependent variables (body %C, body %N, body %P, mass, and body APA). As we found significant triple interactive effects for all dependent variables, we partitioned the data by animal age and performed a 2-way ANOVA on each age to more directly examine the effects of food type, daphnid species, and their interactions. These data were further separated by species before Tukey HSD (with the cumulative error set at  $P < 0.05$ ) tests were completed to determine differences among food types for each species.

### **3.5 Results**

*Effects of food C:P ratios on Daphnia body APA.* Decreasing algal P content had a negative effect on growth and body P-content but a positive effect on body

APA in both species of *Daphnia* (Figure 3.1). We found a significant interaction effect between food C:P ratios and daphnid species on animal MSGR ( $F_{(1,415)} = 5.00$ ,  $P = 0.03$ ), % body P ( $F_{(1,201)} = 4.37$ ,  $P = 0.04$ ), and body APA ( $F_{(1,205)} = 11.9$ ,  $P < 0.01$ ). As *D. magna* and *D. pulex* both increased body APA in response to increasing algal C:P ratios (Figure 3.1), these significant interaction terms indicate that the magnitude of response to increasing algal C:P ratio differed between the two species.

*Effects of food type (Scenedesmus vs. Synechococcus) on body APA.* Food type and daphnid species did not interactively affect body APA in animals consuming P-rich food ( $F_{(3,16)} = 0.01$ ,  $P = 0.93$ ). We did find a significant main effect of both treatments (food type;  $F_{(3,16)} = 8.94$ ,  $P = 0.009$  and species;  $F_{(3,16)} = 14.9$ ,  $P = 0.002$ ) with higher body APA in *D. pulex* and in daphnids consuming *S.l* (Figure 3.2). In contrast, there was a significant interaction between food type and daphnid species on animal MSGR ( $F_{(3,39)} = 5.41$ ,  $P = 0.03$ ; Figure 3.2). Although both daphnid species reduced their growth rate when consuming P-rich cyanobacteria, the magnitude of growth rate reduction in *D. magna* was relatively larger than that seen for *D. pulex*. For body P-content, there was only a daphnid species effect with *D. pulex* having higher body P-content than *D. magna* ( $F_{(3,15)} = 5.15$ ,  $P = 0.04$ ; Figure. 3.2). There was no significant interaction or main effects found between food type and species of daphnid eating P-rich food for either their body C-content or body N-content (data not shown).

We found no interactive effects between food C:P ratio and food type on body APA ( $F_{(3,15)} = 0.01$ ,  $P = 0.92$ ), % P ( $F_{(3,16)} = 0.13$ ,  $P = 0.73$ ), or MSGR ( $F_{(3,36)} = 3.55$ ,  $P = 0.07$ ) for *D. magna* (Figure 3.2). Irrespective of food type, *D. magna* had higher

body APA ( $F_{(3,15)} = 22.10, P = 0.0003$ ), less body P-content ( $F_{(3, 16)} = 48.93, P < 0.0001$ ) and slower growth rates ( $F_{(3,36)} = 394.82, P < 0.0001$ ) when grown on P-poor food. While *D. magna* grew slower when fed cyanobacteria compared to green algae ( $F_{(3,36)} = 111.7, P < 0.0001$ ), their body P content ( $F_{(3,16)} = 2.55, P = 0.13$ ) and body APA ( $F_{(3,15)} = 1.70, P = 0.21$ ) was not affected by food type irrespective of C:P ratio (Figure 3.2). P-limited cyanobacteria did not change the N-content in *D. magna* ( $F_{(3,8)} = 2.16, P = 0.18$ ). However, P-limited cyanobacteria did significantly reduce ( $F_{(3,8)} = 7.75, P = 0.02$ ) body %C in *D. magna* compared to their counterparts consuming green algae (43.6% versus 48.3% respectively).

*Element specificity of APA.* We found a triple interaction between day, species, and diet on *Daphnia* mass ( $F_{(23,215)} = 194, P < 0.01$ ), %C ( $F_{(23,48)} = 5.36, P < 0.01$ ), %N ( $F_{(23,48)} = 2.97, P = 0.02$ ), %P ( $F_{(23,94)} = 2.37, P = 0.04$ ) and body APA ( $F_{(23,95)} = 3.41, P < 0.01$ ). For each age, we found a significant interaction between diet and daphnid species on animal mass (Day 3,  $F_{(7,71)} = 5.07, P = 0.0031$ ; Day 6,  $F_{(7,72)} = 271.31, P < 0.0001$ ; Day 14,  $F_{(7,72)} = 274.27, P < 0.0001$ ; Figure 3.3). The nature of mass differences varied by animal, age, food and between species. While both species of *Daphnia* decreased their weight when consuming either low food quantity or different nutrient limited foods, the severity of food limitation increased with age particularly for *D. magna*. There were also relatively greater mass reductions for *D. magna* than *D. pulex* for a given diet type (Figure 3.3). In addition to these effects on mass, we verified the presence of the desired diet quality by examining the elemental composition of daphnids (Table 3.1). The change in the elemental composition varied by the limiting element and age of animal, with certain days having a significant

daphnid species-diet type interaction effect while other days, only the main effect was significant (Table 3.1). Generally, if a daphnid was fed low food quantity, N-poor or P-poor diet, its body C, N, and P-content decreased respectively.

In this experiment, body APA of *Daphnia* was affected by a significant interaction between diet type and daphnid species for all three age groups (Day 3,  $F_{(7,31)} = 4.77$ ,  $P = 0.006$ ; Day 6,  $F_{(7,32)} = 3.50$ ,  $P = 0.003$ ; Day 14,  $F_{(7,32)} = 15.97$ ,  $P < 0.0001$ ; Figure 3). Since food quantity did not remain constant between species and age, the precise relationship between food quantity and APA cannot be determined: our results nonetheless demonstrate that low food quantity conditions can increase body APA. After 3 days of growth, *D. pulex* had elevated body APA in all dietary stressed conditions whereas *D. magna* was not affected by diet. After 6 days, *D. pulex* body APA remained elevated in all dietary stressors while *D. magna* exhibited elevated body APA in only the P and N-limited diets but not low food quantity (Figure 3.3). After 14 days, both daphnid species had similar levels of body APA. However, on this day, *D. magna* elevated their body APA in response to all of the dietary stresses whereas *D. pulex* had elevated body APA in low food quantity and P-limited diets but N-limited fed animals decreased their activity to the same level of well-nourished animals.

### **3.6 Discussion**

We found that increased body APA is not a specific response to dietary P-stress in *Daphnia*. While body APA increased to dietary P-stress in both daphnid species tested, it also varied between the two species, food types (green algae versus cyanobacteria), among animals of different ages, and with different types of dietary

stress. The responses of daphnid body APA to nutritional stressors (low food and N-limitation) other than dietary P-stress are a particularly strong indication that APA cannot be used alone as a P-specific nutritional-stress indicator and must be accompanied by other P-related responses in future studies of animal P-stoichiometry.

The non-specificity of *Daphnia* body APA to dietary stressors has several potential explanations. For one, our enzyme assay (i.e., MFP) may also measure the activity of other enzymes other than AP. As the MFP assay estimates the rate that MF forms from MFP, other enzymes capable of cleaving a phosphoester bond could contribute to MF production and confound this index of APA.

There are several candidate enzymes that may be particularly problematic for the MFP assay. Na<sup>+</sup>/K<sup>+</sup> ATPases potentially interfere with our assay because they can cleave MFP (Fraser and McKenna 1998), and they have been previously shown to interfere with the measurement of APA (Ezawal et al. 1999). Tyrosine phosphatase and other phosphatases also cleave MFP (Sohn et al. 2004), and our assay could be measuring the activities of these enzymes in addition to AP. Alternatively, kinases seem less likely to react with MFP and other artificial substrates because they specifically cleave ATP (Manning et al. 2002). Acid phosphatases would not likely be important either because their pH optimum in bacteria is around pH 4-5 (Torriani 1960) while the pH in our samples was always held much higher (8.5).

Unfortunately, this type methodological problem (non-APA specificity of the assay) would hold true for all recently used methods to used to measure APA in daphnid bodies including colorimetric (McCarthy et al. 2010) and chemiluminescence (Elser et al. 2010, Wojewodzic et al. 2010) methods. Two potential methods that

would decrease substrate cross reactivity would either be to isolate and purify AP from whole body homogenate or to use a monoclonal antibody that specifically reacts against *Daphnia* AP (e.g., as Bossi et al. (1993) used for placental and intestinal AP of mammals to determine conformational changes in AP caused by point mutations). Purification of AP from the whole body homogenate may require a larger sample mass (e.g., multiples of daphnids per replicate), especially if APA is measured with dyes, which would potentially limit the application of APA as a biological indicator of P-stress in many field and experimental situations. Also, as with any purification, there is a potential for AP to be lost during processing, which would make this process potentially sensitive to the efficiency of extraction and purification. The use of an enzyme linked immunosorbent assay in which a secondary polyclonal antibody fluoresces only when an AP-specific monoclonal antibody reacts with AP from daphnids would eliminate cross reactivity and still permit a small sample mass and the use of a whole body homogenate. Unfortunately, it unclear whether AP antibodies currently available would react against *Daphnia* AP and monoclonal antibodies that specifically target *Daphnia* AP have yet to be developed.

Alternatively, the lack of AP specificity for dietary P-stress could reflect multiple and unknown roles of AP in *Daphnia* metabolism beyond P acquisition. For example, Jeyasingh et al. (2011) found *D. pulex* consuming P-poor food had less AP transcripts of one of the six identified isozymes (Colbourne et al. 2011) compared to *D. pulex* consuming P-rich food. It is thus possible that not all of the AP isozymes in the daphnid genome are used for dietary P acquisition. Besides P acquisition, AP assists in other metazoans with bone formation, adipocyte maturation, wound healing,

and the sclerotization process (Iger and Abraham 1990, Bourtzis et al. 1993, Alia et al. 2005, Millan 2006). Consequently, it seems unlikely that all the daphnid AP isozymes are only used for P acquisition and would show similar responses to low P content in the animal's diet.

*Daphnia* that consume cyanobacteria (*Synechococcus*) are known to experience growth limitation by sterols and essential fatty acids (Martin-Creuzburg et al. 2005, Wacker and Martin-Creuzburg 2007) with decreases in growth rate often observed even more substantial than that found in this study. Here we found that food type (*Scenedesmus* and *Synechococcus*) also affected the APA of *Daphnia* bodies. *Daphnia* eating P-rich cyanobacteria food had significantly higher APA compared to their counterparts eating P-rich green algae. However, when consuming P-poor food, there was no difference in body APA between *D. magna* consuming green algae or cyanobacteria. This appears partly related to the relatively high error estimated for P-poor, *S.o* fed daphnids in this experiment (Figure 2). However, it is possible that AP may be cleaving organic phosphoesters and increasing available supplies of organic carbon as found with bacterial ectoenzymes (Hoppe 2003), as *Daphnia* are known to have low C gross growth efficiency when fed cyanobacteria (DeMott 1998). Although the difference in body AP between daphnids consuming different food types is much smaller than the difference in body AP between P-rich and P-poor diets, this finding nonetheless indicates that differences in food type (e.g., among various algal and cyanobacterial taxa) could interfere with the use of this indicator to determine the dietary P-status of *Daphnia* and other metazoans.

While we found that body APA of *Daphnia* increased in animals fed high food C:P ratios, the nature of this increase differed between experimental runs in our study. This is perplexing given the similarities between our subsequent, multiple experimental runs: same species, same clonal lineage, constant maternal nutrition of *Daphnia*, nearly identical room temperature, same type of media, same quantity, and similar ranges of C:P ratios of experimental algal foods. Consequently, it appears that even small (heretofore unidentified) differences among experimental runs can alter *Daphnia* body APA. One likely explanation for this result is the physiological state and age of the algal culture varied among runs because we found similar between run variability in both species of *Daphnia*. However, it's unclear why *S. obliquus* cultures would vary among runs and why this would affect daphnid body APA. Nonetheless, the presence of between-run experimental variability is further indication that the relatively simple application of body APA measurements to diagnose *Daphnia* P-nutrition should be undertaken with caution.

While we document here increases in daphnid body APA with increasing food C:P ratios, Wojewodiz et al. (2010) found a unimodal response with *D. magna* exhibiting greatest body AP activity when fed intermediate C:P ratios of 300 to 400. One possible reason for the differences between the two studies is Wojewodiz et al. (2010) fed *Daphnia* P-rich green algae for 40 minutes prior to the end of their experiment to reduce the potential confounding effects of algal-derived APA in the animal's gut. McCarthy et al. (2010) found that daphnids APA remained elevated under P limitation even when fed heat denatured algae (no gut derived APA). These divergent response curves seem unlikely to result from algal derived APA as we

found no significant changes in *D. magna* body APA irrespective of gut conditions. This implies APA responses to food C:P ratios may be daphnid species and/or clonal-specific as previously documented for *Daphnia* responses to low food P content (Seidendorf et al. 2010). The effects of short-term acclimation to food quality and inter-specific variability in body APA responses to P-poor food nonetheless need further study.

Body AP activity has recently been advocated as a novel indicator of physiological P-stress in *Daphnia* (Elser et al. 2010, McCarthy et al. 2010, Wojewodzic et al. 2010). A biochemical indicator of dietary P-stress in zooplankton (and other animals) would have considerable utility for future stoichiometric studies of foodwebs examining the frequency, duration, and severity of animal P-limitation and its effects on ecosystems. However, this utility depends upon the nutritional indicator being element-specific (i.e., responds only to P-limitation), generally applicable to multiple and diverse taxa (i.e., responds similarly among species), and relatively easy to measure. We found that whole body homogenate AP activity is not P-specific and varies (to some extent) between two closely related species. Although measuring AP activity can be relatively simple and inexpensive with MFP or other substrate-based enzyme activity methods, these assays on whole body homogenate are potentially complicated by cross reactivity of enzymes and substrates. Consequently, further development and validation of this approach is needed before its wider application to studies of nutritional stress in animals.

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### 3.9 Table and Figures

**Table 3.1:** Body elemental composition of *Daphnia* when fed different food qualities and quantities for 3, 6, and 14 days. 2-Way ANOVA showing main effects of diet (D), animal species (Sp), and their interaction (D\*Sp). Different letters indicate species separated post-hoc Tukey tests with a cumulative error of P <0.05.

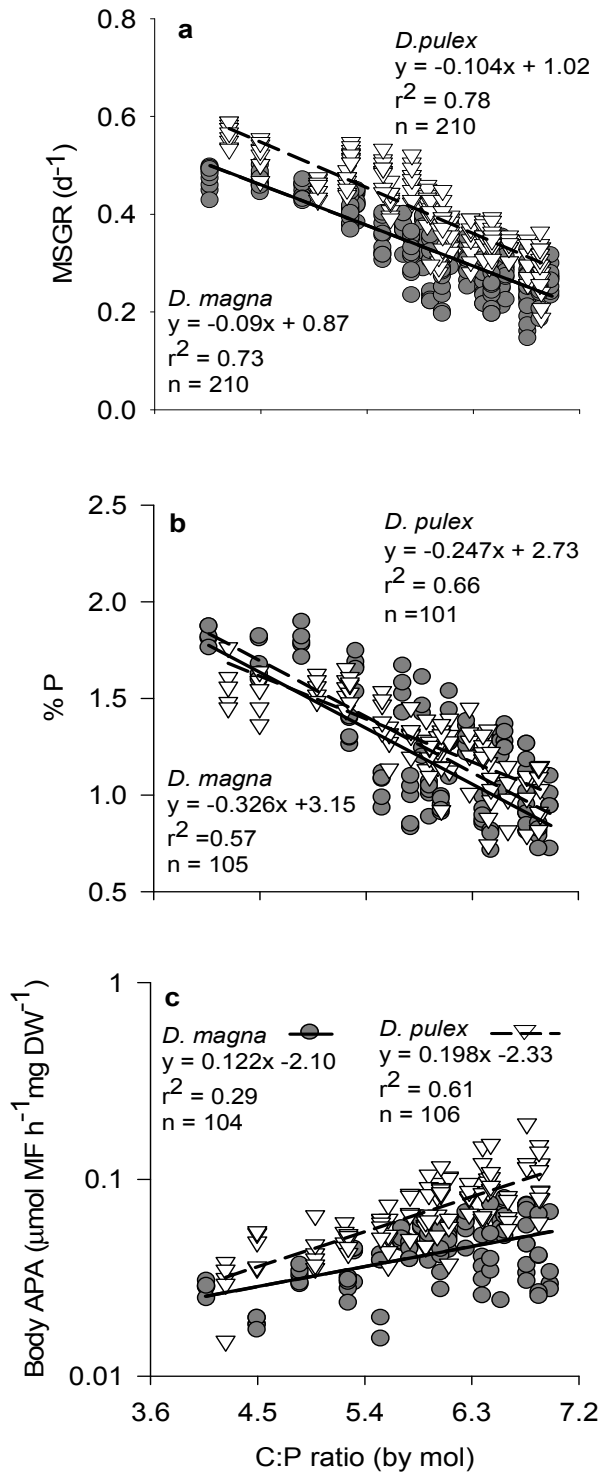
		%C			%N			%P		
Day		3	6	14	3	6	14	3	6	14
<i>2-Way</i>	Diet F	20.3	104	86.3	15.8	22.2	82.8	6.74	48.1	72.7
<i>ANOVA</i>	P value	<0.001	<0.001	<0.001	<b>&lt;0.001</b>	<0.001	<0.001	<b>0.001</b>	<0.001	<0.001
<i>Results</i>	Species F	99.9	37.6	49.1	29.8	6.14	23.5	8.27	5.55	139
	P value	<0.001	<0.001	<0.001	<b>&lt;0.001</b>	0.02	<0.001	<b>0.007</b>	0.02	<0.001
	D*Sp F	5.67	24.2	9.69	2.28	4.46	3.17	0.54	3.91	7.66
	P value	<b>0.008</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.12	<b>0.02</b>	<b>0.05</b>	0.66	<b>0.02</b>	<b>&lt;0.001</b>
Species	Diet									
<i>D. magna</i>	GF	45.7 <sup>ab</sup>	47.3 <sup>a</sup>	49.4 <sup>a</sup>	8.50	9.14 <sup>a</sup>	9.86 <sup>a</sup>	1.62	1.47 <sup>a</sup>	1.35 <sup>a</sup>
	LF	41.4 <sup>b</sup>	38.2 <sup>b</sup>	42.7 <sup>b</sup>	9.13	8.57 <sup>b</sup>	8.56 <sup>b</sup>	1.73	2.10 <sup>b</sup>	1.26 <sup>a</sup>
	PL	43.5 <sup>c</sup>	47.4 <sup>a</sup>	49.3 <sup>a</sup>	7.37	8.20 <sup>b</sup>	8.28 <sup>bc</sup>	1.40	1.19 <sup>a</sup>	0.77 <sup>b</sup>
	NL	44.4 <sup>ac</sup>	46.8 <sup>a</sup>	49.7 <sup>a</sup>	7.07	7.72 <sup>c</sup>	7.83 <sup>c</sup>	1.76	1.47 <sup>a</sup>	1.19 <sup>c</sup>
<i>D. pulex</i>	GF	49.6 <sup>a</sup>	50.5 <sup>a</sup>	51.0 <sup>a</sup>	9.46	8.85 <sup>ab</sup>	9.83 <sup>a</sup>	1.69	1.53 <sup>a</sup>	1.60 <sup>ab</sup>
	LF	46.5 <sup>b</sup>	44.0 <sup>b</sup>	47.1 <sup>b</sup>	9.40	9.54 <sup>b</sup>	9.16 <sup>b</sup>	1.97	1.74 <sup>b</sup>	1.43 <sup>b</sup>
	PL	47.4 <sup>ab</sup>	45.7 <sup>bc</sup>	50.0 <sup>a</sup>	9.01	8.60 <sup>ab</sup>	8.81 <sup>b</sup>	1.63	1.06 <sup>c</sup>	1.12 <sup>c</sup>
	NL	45.5 <sup>b</sup>	47.6 <sup>c</sup>	50.7 <sup>a</sup>	8.18	7.87 <sup>a</sup>	8.32 <sup>c</sup>	1.96	1.47 <sup>a</sup>	1.63 <sup>d</sup>

## Figure Captions

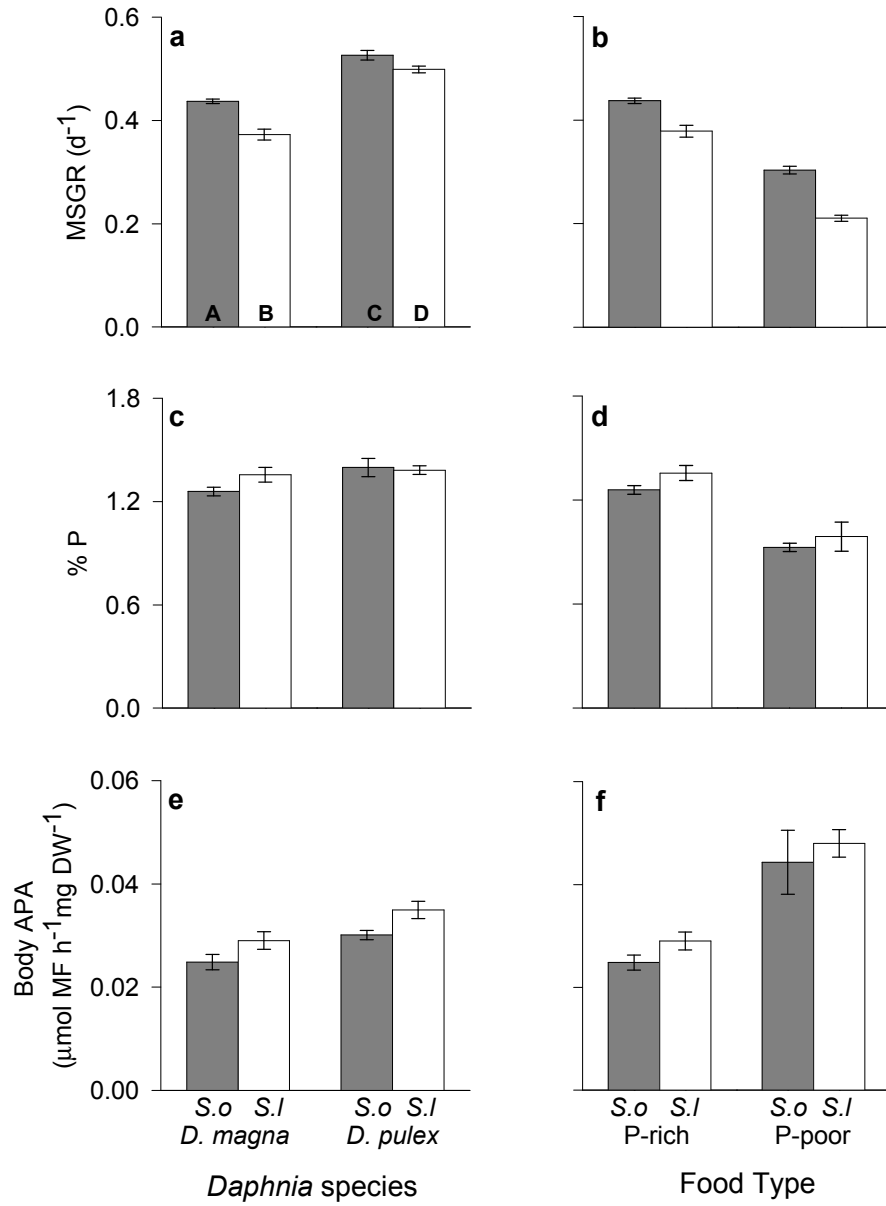
**Figure 3.1:** Responses to increasing algal C:P ratios (natural log transformed) of *D. magna* (circles, solid line) and *D. pulex* (triangles, dashed line): a) daily mass specific growth rate, MSGR, b) body P content (%) and c) body  $\log_{10}$  APA measured as methylfluorescein (MF)

**Figure 3.2:** (a) Daily MSGR, (c) body P content, and (e) body APA of *Daphnia magna* and *D. pulex* eating P-rich *S. obliquus* (S.o., grey bars) or *S. leopoliensis* (S.l., white bars), mean  $\pm$ SE. (b) Daily MSGR, (d) body P content and (f) body APA of *D. magna* eating P-rich or P-poor *S. obliquus* (S.o., grey bars) or *S. leopoliensis* (S.l., white bars), mean  $\pm$ SE. Letters indicate significant differences from each other at  $p < 0.05$  using Tukey post hoc test. APA data was  $\log_{10}$  transformed for statistics to meet assumptions of normality

**Figure 3.3:** Mass (mg dry weight, left column) and body APA ( $\mu\text{mol MF h}^{-1} \text{ mg mass}^{-1}$ , right column) of *D. magna* (grey bars) and *D. pulex* (white bars) on (a, b) day 3, (c, d) day 6 and (e, f) day 14 when consuming 4 food treatments: high food quantity and quality algae (GF), low food quantity and high quality algae (LF), high food quantity P-limited algae (PL) and high food quantity N-limited algae (NL), mean  $\pm$ SE. Letters indicate significance of  $p < 0.05$  within one species using Tukey post hoc test. APA was log transformed for statistics to meet assumption of normality.



**Fig. 3.1**



**Fig. 3.2**

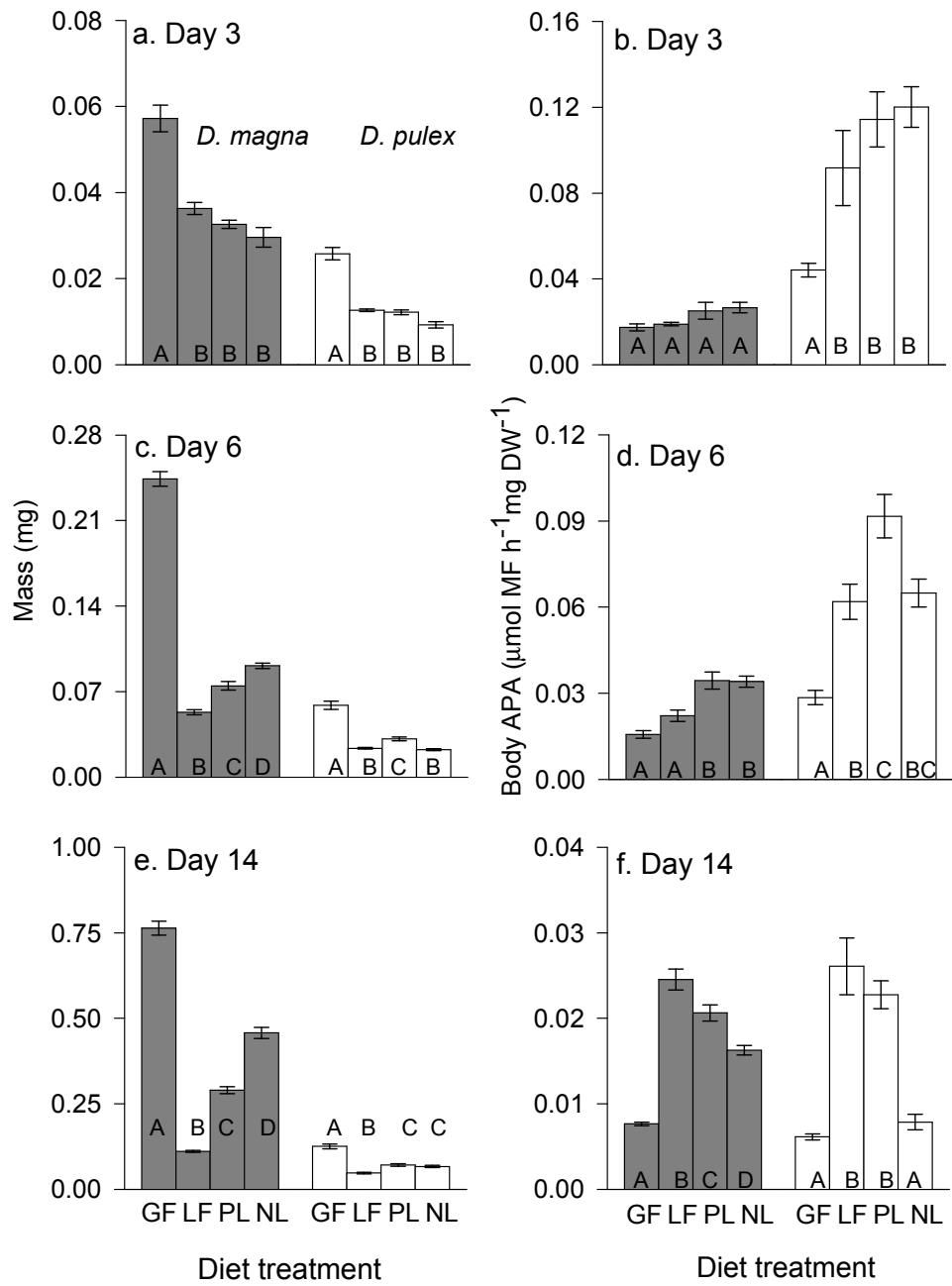


Fig 3.3

### 3.10 Supplementary

#### Responses of alkaline phosphatase activity in *Daphnia* to poor nutrition

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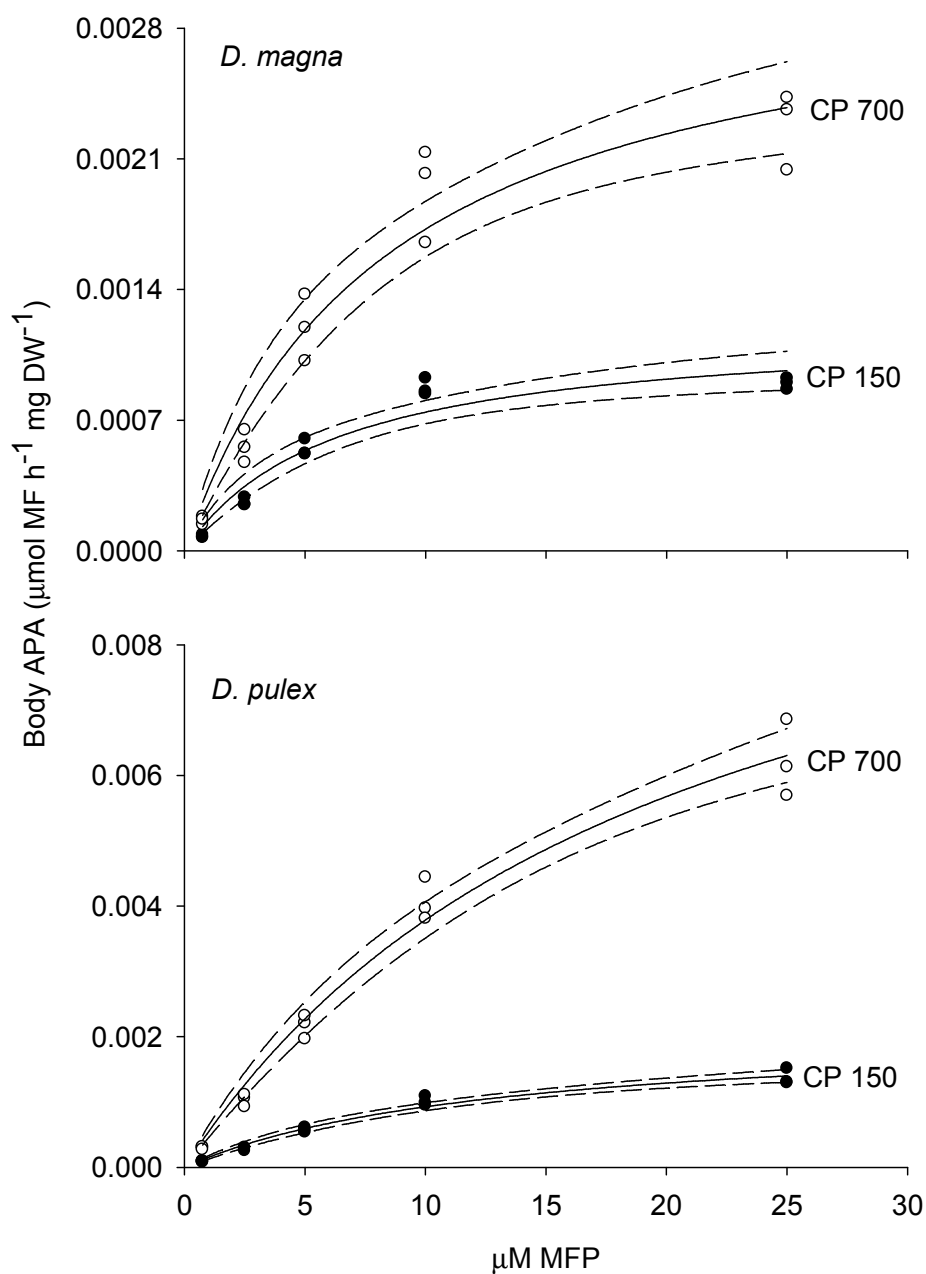
†contact information of corresponding author: [nicolegoulding@trentu.ca](mailto:nicolegoulding@trentu.ca) 705-748-1011 ext. 6465

**Reference:** *Published in Oecologia* (2012) 170:1-10

## Additional Experiments

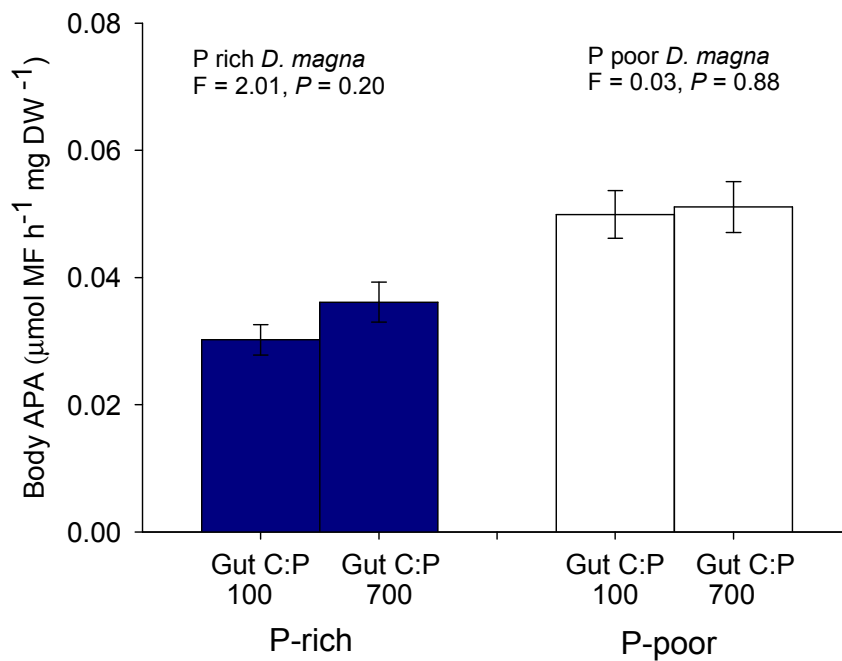
*Determination of substrate concentration for APA.* As measurement of *Daphnia* body APA using MFP have not been previously reported, we completed an initial experiment ensure that MFP was added in excess. To do this, we grew *D. magna* and *D. pulex* for 6 days on P-rich (C:P ratio ~150) and P-poor (C:P ratio ~700) *S. obliquus* algal food. To ensure animals were not food limited, we fed 4 mg C L<sup>-1</sup> of food to all animals every other day. At the end of the experiment, 10 *Daphnia* were dried to determine the average weight of each treatment. Three additional animals of each food treatment were placed in a 1.5 ml centrifuge tube to which 0.1 mm glass beads (to the 0.1 ml mark on the tube) and 200 µl 0.1 M Tris-HCl buffer pH 8.5 were added. Centrifuge tubes were then placed in to a bullet blender (Next advance) and set on speed 8 for 3 minutes of homogenization. After this first homogenization, we added 600 µl of Tris-HCl buffer to increase the final sample volume to 800 µl. This homogenate (100 µl) was then pipetted into 5 microwells, along with 100 µl MFP ranging in concentration from 1.5 µM to 50 µM. This dilution produced a range of final MFP concentrations (0.75 -25 µM). Samples were placed into a microplate fluorometer (Bio-Tec instrument FL<sub>x</sub> 800), which measured the fluorescence from excitation wavelength of 485 nm/20 nm and emission wavelength of 528 nm/20 nm and sensitivity set to 70 once every 90 seconds for 10 minutes. The rate of MF formation was calculated by converting the fluorescence values for the first 540 seconds with *D. magna* and 450 seconds with *D. pulex* in to µM of MF using a standard curve of MF. The rate of MF formation was then standardized to µmol of MF per hour per milligram of animal dry weight and plotted against the final

substrate concentration in  $\mu\text{M}$ . Plotting  $\mu\text{mol}$  of MF per hour per milligram of dry weight against the final substrate concentration shows that APA adheres to Michaelis-Menten kinetics (Fig. S1), although under these conditions the maximum velocity was not obtained for *D. pulex*. However,  $V_{\text{max}}$  was obtained for *D. manga* and the resulting Michaelis-Menten constant was  $6.2 \mu\text{M}$  for C:P 150 and  $8.4 \mu\text{M}$  for C:P 700. Using this data, we choose  $5 \mu\text{M}$  of MFP as the substrate concentration to be used in future APA measurements because it would not saturate the fluorometer over 30 minutes with the sensitivity set to 70. We also choose  $5 \mu\text{M}$  of MFP because this was concentration was commonly used to determine APA in phytoplankton (Healey and Hendzel 1979).



**Figure S3.1:** Body APA ( $\mu\text{mol of MF h}^{-1}\text{mg DW}^{-1}$ ) enzyme kinetics for *Daphnia magna* (top) and *Daphnia pulex* (bottom) grown under P-rich (CP 150) and P-poor (CP 700) conditions. Non linear Michaelis-Menten line of best fit is added with 95 % CI (dashed lines).

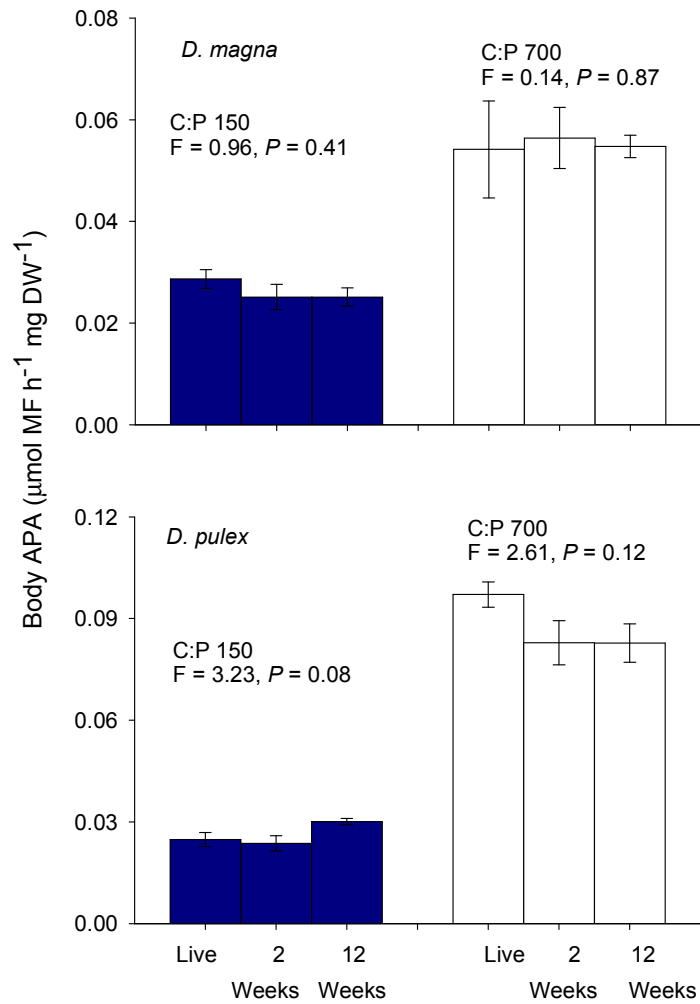
*Influence of algal APA in Daphnia gut.* Phosphorus limited algae and bacteria are known to produce AP thus, ingested algae potentially influence the APA of *Daphnia* bodies. We analyzed the potential influence of the consumed algae on the body APA of *D. magna* following a similar procedure as Wojewodzic et al (2010). We grew *D. magna* under both P-rich (C:P ~ 100) and P-poor (C:P ~ 700) food provided for 6 days at a concentration of 4 mg C L<sup>-1</sup> and with replenishment every other day. After 6 days, we transferred *D. magna* (n = 5) to 20 ml of N and P free COMBO media and fed them either C:P ~ 100 (low APA algae) or C:P ~ 700 (high APA algae) for 40 minutes as described by Wojewodzic et al (2010). After 40 minutes, *Daphnia* were removed, rinsed in N and P free COMBO media, placed in a 1.5ml centrifuge tube, and their whole body homogenate APA was measured as described above. An additional 10 *Daphnia* were grown as described above and dried for 24 hr before weighing to obtain the average animal dry mass in each treatment. Our results from this experiment (Fig. S2) indicate that algal switching and thus gut-derived algal APA does not affect the body APA in *D. magna*. There was no significant difference between animals with diets switched for 40 minutes from animals who did not have their gut content changed.



**Figure S3.2:** Influence of diet switch on body AP activity of *D. magna*. Shown are data from *D. magna* grown on P-rich algae (C:P ~100; blue bars) and on P-poor algae (C:P ~ 700; white bars). Gut C:P ratios indicate the C:P ratios of food provided for the 40 minutes prior to animal harvesting. Error bars are  $\pm 1$  SE. All body APA data was log transformed prior to one-way ANOVA (each animal type separately) to meet assumptions of normality.

*Degradation of Daphnia APA.* We wanted to ensure that our preservation techniques would not degrade our samples both short term and long term. Using the same *Daphnia* for the enzyme kinetics we grew additional *Daphnia* from both P-rich and P-poor treatments and placed them in 1.5 ml centrifuge tube in the -80 °C freezer. AP activity was performed as described above on a subsample (n = 5) of these *Daphnia* after 2 and 12 weeks of being in the freezer. Analyzing these samples weeks

apart allow us to see any degradation or differences in extraction efficiencies. We performed a one way ANOVA on log transformed APA data to see if there were differences due to degradation or extraction efficiencies. There was no significant degradation over the 12 weeks in both P-rich and P-limited *D. magna* and *D. pulex* (Figure S3). This also implies the extraction efficiencies remained constant as APA did not change each time the daphnids were measured.



**Figure S3.3:** AP activity during storage of *D. magna* (top) and *D. pulex* (bottom) consuming P-rich (C:P 150; blue bars) and P-poor (C:P 700; white bars) algae. APA on live daphnids and up to 12 weeks in a -80 °C freezer. Error bars are  $\pm 1$  SE. APA data was log transformed for one way ANOVA to meet assumptions of normality.

**Table S3.1:** Dilutions and concentration of nutrients added to the algal and cyanobacterial cultures

	Nutrient status	Dilution ( rate day <sup>-1</sup> )	μM of P added	μM of N added
<i>S. obliquus</i>	N & P rich	0.55	70	1050
<i>S. obliquus</i>	P limited	0.2	7	1050
<i>S. obliquus</i>	N limited	0.15	70	420
<i>S. leopoliensis</i>	P rich	0.3	42	3000
<i>S. leopoliensis</i>	P limited	0.15	14	3000

**Table S3.2:** Average elemental composition of *S. obliquus*  $\pm$  SD used for *D. magna* and *D. pulex* CP range experiments.

Species	Run	C:P (by mol)	C:N (by mol)	N:P (by mol)
<i>D. magna</i>	1	58.6 $\pm$ 2.0	5.5 $\pm$ 0.02	11.5 $\pm$ 0.35
<i>D. magna</i>	1	129 $\pm$ 0.3	9.5 $\pm$ 0.20	14.7 $\pm$ 0.28
<i>D. magna</i>	1	203 $\pm$ 6.3	11.1 $\pm$ 0.44	19.8 $\pm$ 0.15
<i>D. magna</i>	1	302 $\pm$ 15	11.7 $\pm$ 1.2	28.2 $\pm$ 1.3
<i>D. magna</i>	1	357 $\pm$ 23	12.6 $\pm$ 0.43	30.9 $\pm$ 3.1
<i>D. magna</i>	1	449 $\pm$ 53	13.1 $\pm$ 1.4	37.2 $\pm$ 0.4
<i>D. magna</i>	1	520 $\pm$ 61	13.8 $\pm$ 0.2	40.7 $\pm$ 4.2
<i>D. magna</i>	1	696 $\pm$ 32	12.8 $\pm$ 1.4	59.5 $\pm$ 8.7
<i>D. magna</i>	1	719 $\pm$ 14	13.6 $\pm$ 1.5	57.9 $\pm$ 7.8
<i>D. magna</i>	1	865 $\pm$ 44	14.1 $\pm$ 1.2	66.8 $\pm$ 2.0
<i>D. magna</i>	1	1050 $\pm$ 14	13.8 $\pm$ 2.4	84.3 $\pm$ 14
<i>D. pulex</i>	1	146 $\pm$ 28	8.3 $\pm$ 0.11	19.0 $\pm$ 3.9
<i>D. pulex</i>	1	187 $\pm$ 4.5	11.2 $\pm$ 0.28	18.0 $\pm$ 0.01
<i>D. pulex</i>	1	270 $\pm$ 18	12.0 $\pm$ 0.47	24.4 $\pm$ 0.71
<i>D. pulex</i>	1	369 $\pm$ 15	11.0 $\pm$ 1.0	36.5 $\pm$ 4.7
<i>D. pulex</i>	1	407 $\pm$ 11	10.9 $\pm$ 0.54	40.6 $\pm$ 0.84
<i>D. pulex</i>	1	449 $\pm$ 6.5	12.0 $\pm$ 0.16	40.4 $\pm$ 0.06
<i>D. pulex</i>	1	532 $\pm$ 7	12.4 $\pm$ 0.65	46.6 $\pm$ 1.9
<i>D. pulex</i>	1	624 $\pm$ 33	12.7 $\pm$ 0.39	53.2 $\pm$ 1.3
<i>D. pulex</i>	1	737 $\pm$ 39	13.7 $\pm$ 0.50	58.4 $\pm$ 1.0
<i>D. pulex</i>	1	968 $\pm$ 85	12.4 $\pm$ 0.30	84.9 $\pm$ 9.4
<i>D. pulex</i>	2	68.8 $\pm$ 2.3	5.8 $\pm$ 0.03	12.9 $\pm$ 0.38
<i>D. magna &amp; pulex</i>	2	89.7 $\pm$ 2.0	6.6 $\pm$ 0.15	14.7 $\pm$ 0.65

<i>D. magna &amp; pulex</i>	2	193 ± 11	9.3 ± 1.2	22.6 ± 1.6
<i>D. magna &amp; pulex</i>	2	254 ± 2.9	11.1 ± 0.39	24.9 ± 1.2
<i>D. magna &amp; pulex</i>	2	323 ± 24	11.4 ± 1.0	31.1 ± 5.3
<i>D. magna &amp; pulex</i>	2	379 ± 41	12.1 ± 0.94	34.2 ± 6.7
<i>D. magna &amp; pulex</i>	2	420 ± 56	13.0 ± 0.48	35.1 ± 6.1
<i>D. magna &amp; pulex</i>	2	596 ± 62	14.2 ± 1.2	46.0 ± 9.0
<i>D. magna &amp; pulex</i>	2	638 ± 74	13.6 ± 2.6	53.0 ± 18
<i>D. magna &amp; pulex</i>	2	865 ± 19	12.8 ± 0.22	73.5 ± 3.0
<i>D. magna &amp; pulex</i>	2	958 ± 49	12.6 ± 0.54	82.0 ± 0.72

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## CHAPTER 4

### 4.1 PREFACE

**Title:** Metabolomic differentiation of nutritional stress in an aquatic invertebrate

**Authors:** Nicole D. Wagner<sup>1†</sup>, Brian P. Lankadurai<sup>2</sup>, Myrna J. Simpson<sup>2</sup>, Andre J. Simpson<sup>2</sup> and Paul C. Frost<sup>3</sup>

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**Reference:** *Published in* Physiological and Biochemical Zoology (2015) 88:43-52

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**Keywords:** ecological stoichiometry, food quality, metabolite profiling, nutritional ecology, nutritional indicator

**Author Contributions:** NDW, PCF and MJS conceived the project. NDW performed all the experiments and the gross biochemical data. BPL, MJS ran and analyzed the metabolomics NDW and PCF wrote the manuscript with contributions from all authors.

## CHAPTER 4

### **Metabolomic differentiation of nutritional stress in an aquatic invertebrate**

*Published in Physiological and Biochemical Zoology (2015) 88:43-52*

#### **4.2 Abstract**

Poor diet quality frequently constrains the growth and reproduction of primary consumers, altering their population dynamics, interactions in food webs, and contributions to ecosystem services such as nutrient cycling. The identification and measurement of an animal's nutritional state is thus central to studying the connections between diet and animal ecology. Here we show how the nutritional state of a freshwater invertebrate, *Daphnia magna*, can be determined by analyzing its endogenous metabolites using <sup>1</sup>H NMR-based metabolomics. With a multivariate analysis, we observed the differentiation of the metabolite composition of animals grown under control conditions (good food and no environmental stress), raised on different diets (low quantity, nitrogen-limited, and phosphorus-limited), and exposed to two common environmental stressors (bacterial infection and salt stress). We identified eighteen metabolites that were significantly different between control animals and at least one limiting food type or environmental stressor. The unique metabolite responses of animals caused by inadequate nutrition and environmental stress are reflective of dramatic and distinctive effects that each stressor has on animal metabolism. Our results suggest dietary-specific induced changes in metabolite composition of animal consumers hold considerable promise as indicators of nutritional stress and will be invaluable to future studies of animal nutrition.

### 4.3 Introduction

Poor food quality slows growth, limits reproduction, and reduces survival of consumers (Brett & Müller-Navarra 1997; Sterner and Schulz 1998; Sterner and Elser 2002; Frost *et al.* 2005). The effects of food quality on animal life-history traits and underlying physiological processes, in turn, alter the growth of populations, interspecific dynamics within foodwebs, and the structure and function of ecosystems (Sterner and Elser 2002). Identifying and assessing an animal's nutritional state is central to understanding these links between nutrition and animal ecology (Wagner *et al.* 2013). Despite this, the determination of the nutritional state of primary consumers generally relies upon non-specific methods that involve the measurement of growth and reproductive responses to dietary stress (e.g., Brett and Müller-Navarra 1997; Sterner and Schulz 1998; McCarthy *et al.* 2011). In this study we examined a new approach to assessing an animal's nutritional state by testing the hypothesis that its metabolome (defined as the set of low molecular weight metabolites within cells) is responsive to nutrient and environmental stressors.

Physiological responses to poor food quality by animal consumers are controlled by a complex interaction of cellular and molecular mechanisms underlying gross metabolic adjustments (Frost *et al.* 2005; Jeysingh *et al.* 2011). In plants, the regulation of growth processes by nutrients is tightly connected to changes in the absolute and relative concentrations of metabolites in cells and tissues (Meyer *et al.* 2007; Stitt *et al.* 2010; Rivas-Ubach *et al.* 2012). For animals, one might simplistically expect that poor nutrition would reduce the supply of limiting substrates to cells, decrease their intracellular concentrations, and directly limit

growth (Boer *et al.* 2010). Conversely, non-limiting substrates may accumulate in cells as their supply exceeds the reduced demand in slower growing animals (Boer *et al.* 2010). Changes to the supply of and demand for metabolic substrates may be accompanied by metabolic adjustments (e.g., induction of different metabolic pathways) that would compensate for the depletion of growth limiting and the build-up of non-limiting metabolites (Boer *et al.* 2010). Taken together, these metabolite responses may provide considerable insight into the regulation and responses of organismal metabolism to poor nutrition and environmental stress (Bölling and Fiehn 2005; Meyer *et al.* 2007; Boer *et al.* 2010; Warren 2011).

An emerging method to study molecular-level responses in organisms is metabolomics, which catalogues systemic changes in the metabolome of cells and organisms to external stressors. The metabolome is defined as the set of low molecular weight metabolites within cells, tissues or biofluids of an organism at a particular physiological or developmental stage (Fiehn 2001; Hollywood *et al.* 2006). The rapid and relatively large responses of metabolite composition to external stressors provide a distinct advantage to this approach over transcriptomics or proteomics, which can exhibit slower and less dramatic responses to external stress (Hollywood *et al.* 2006). Consequently, metabolomic approaches are being applied in medicine, pharmacology and toxicology (Whitfield *et al.* 2004; Robertson 2005; Simpson and McKelvie 2009) to detect and understand organism responses to chemical exposure, disease, and genetic modifications. Despite its advantages and growing use in complementary fields, there have been few studies of the metabolome in ecology and its potential value to the study of nutritional ecology remains largely

unappreciated (Peñuelas and Sardan 2009; Rivas-Ubach *et al.* 2012; Lankadurai *et al.* 2013).

Here we studied the metabolomic responses of an aquatic invertebrate, *Daphnia magna*, to multiple dietary and environmental stressors. Specifically, we examined responses in the metabolite composition of *Daphnia magna* grown under good nutrition (high food quantity and quality), low food quantity, low food nitrogen (N) content, low food phosphorus (P) content, salt stress, and after exposure to infective spores of a pathogenic bacteria. We generally expected the metabolite composition of *Daphnia* to reflect the metabolic pathways that are being utilized for each specific nutrient limitation and environmental stress. These changes should be strongest for essential food components (e.g., essential amino acids) and less so for non-essential nutrients, which *Daphnia* could compensate by altering underlying metabolic pathways to generate more of the limiting component. Regardless, unique metabolite responses to different nutritional stressors would signify that this method can provide useful indicators of the past dietary quality consumed by animals. To date, only five metabolomics studies have studied *D. magna* (Taylor *et al.* 2009; Taylor *et al.* 2010; Vandenbrouck *et al.* 2010; Poynton *et al.* 2012; Nagato *et al.* 2013) and these have focused strictly on contaminant exposure. Nutritional variation has not yet been studied using this sensitive and informative technique. As such, there is great potential to advance the understanding the role of nutrition on the metabolome of *D. magna* and potential impacts to its ecology.

#### 4.4 Methods

*Algae and Daphnia Culturing.* We grew *Scenedesmus obliquus* (Canadian Phycological Culture Centre strain 10, purchased as *S. acutus*), in multiple semi-continuous culture jars diluted and refreshed daily with differentially enriched media (Sternner *et al.* 1993) to produce P-rich, P-poor and N-poor algal cells (See Table S4.1 for dilution and N & P concentrations used). Harvested cells were centrifuged (4066 g) for 15 min and the resulting pellet was resuspended in N and P free COMBO media (Kilham *et al.* 1998). We determined the P content of concentrated algal cells after persulfate digestion of dried subsamples using the molybdate-blue ascorbic acid colorimetric assay (American Public Health Association 1992). Carbon (C) and N content of the concentrated algal suspensions were also determined on dried cells using an Elemental Analyzer (Vario EL III, Elementar Incorporated, Mt Laurel NJ, USA). Using the elemental composition of the concentrated algal suspensions, we mixed these foods to generate nominal C:P and C:N ratios.

We used these prepared diets to study the metabolomic responses of *Daphnia magna* (Straus) to different nutritional and environmental stresses. Animals for our experiments were born to *D. magna* (second generation, clonal sisters) who had been held in groups of 10 animals in 400 ml of P free COMBO media and fed with high food quality and quantity *S. obliquus*. On the morning of each experiment, we collected neonates (<24 hrs old) and placed them in P and nitrogen (N)-free and food-free COMBO until they were allocated (in <1hr) to an experimental treatment.

*Experimental design.* *Daphnia* neonates were rinsed 4 times in N and P free COMBO media to ensure no brood mother food or media remained before

transferring them into an experimental container. Animals were grown in groups of 20 in glass jars containing 400 ml of N and P free COMBO for 6 days. During this period (on days 0 and 3), they were fed 4 mg of carbon per liter (C/L) of either good food (GF; C:P ~150, C:N ~8), P-limited ( PL; C:P ~ 900, C:N ~15), N-limited (NL; C:P ~100, C:N ~25), or low food (LF; same quality as GF but only 1.5 mg C/L).

We also grew daphnids that were exposed to two types of environmental stressors, high salt concentrations and bacterial infection. These animals were also fed 4 mg C/L of GF on days 0 and 3 and otherwise treated as other experimental animals. For the high NaCl treatment (Na), we exposed animals to salt at a concentration of 34 mM. We choose this high but sub-lethal NaCl concentration to ensure exposed animals only exhibited reduced growth but not elevated mortality. For the bacterial exposure treatment (BAC), *Daphnia* were exposed to spores of an endoparasitic bacterium, *Pasteuria ramosa* (Ebert *et al.* 1996). Spores were obtained from frozen, infected *Daphnia* that were homogenized with a pestle. We determined the density of spores in the infected *Daphnia* homogenate by counting 4 replicate aliquots using a hemocytometer. We then exposed groups of 20 animals to 3750 spores per ml of COMBO media (1.5 million spores per jar) for the duration of the growth experiment, which is a relatively high spore dose that causes infection of a high percentage (>90%) of exposed *Daphnia* (Regoes *et al.* 2003).

After six days of growth, animals were collected for metabolomic analysis, growth rate measurements, elemental analysis (body C, N, and P content; See table S4.2 for number of replicates), and gross biochemical profiling (total lipids, total protein, DNA content, and RNA content; See table S4.2 for number of replicates).

The number of *Daphnia* in each <sup>1</sup>H NMR replicate differed among treatments to yield approximately 10 mg of dry mass per sample. For <sup>1</sup>H NMR analysis (n varied with treatment; between 4-10; See table S4.2 for number of replicates) we placed enough wet mass to approximate 10 mg dry mass into a 1.5 ml centrifuge tube. After this, we removed all the N and P-free COMBO media and immediately froze animals with liquid nitrogen. For biochemical analysis, animals were also placed in a 1.5 ml centrifuge tube with the COMBO removed and placed in liquid nitrogen immediately (n = 5). Animals were subsequently lyophilized and placed in a -80 °C freezer until further chemical analysis. For growth rate (n varied with food treatment; See table S4.2 for number of replicates) and elemental analysis (n =5), one jar consisting of 20 animals was harvested for each replicate and animals were desiccated for 48 h prior to processing. Due to the sample mass required for the metabolomic analysis, we grew multiple sets of the same treatment over several separate experimental durations and ensured their growth rate (n = 4-5 per duration) did not vary by more than 10%.

*Preservation and extraction of metabolites.* Lyophilized *D. magna* were homogenized in a 1.5 mL centrifuge tube using a 5 mm wide stainless steel spatula, then extracted using 0.75 mL of a 0.2 M monobasic sodium phosphate buffer solution (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a preservative (Brown *et al.* 2009). Buffer solution was made with D<sub>2</sub>O (99.9% purity, Cambridge Isotope Laboratories) adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D<sub>2</sub>O, Cambridge Isotope Laboratories Inc), and contained 10 mg/L of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard (Brown *et al.* 2008, Brown *et al.* 2009).

Each group of pooled *daphnids* were extracted and analyzed separately by  $^1\text{H}$  NMR. Each sample was combined with buffer solution, vortexed for 30 seconds, then sonicated for 15 minutes to aid with the extraction and centrifuged ( $\sim 15,000$  g) for 20 minutes and the supernatant was transferred into a new 1.5 mL centrifuge tube. The centrifugation procedure was repeated once more to remove any remaining particulate material. Samples were then transferred into 5 mm High Throughput<sup>plus</sup> NMR tubes (Norell Inc.; NJ, USA) for  $^1\text{H}$  NMR analysis. We also note that the modified Bligh and Dyer extraction described by Wu *et al.* (2008) was also attempted during preliminary studies (data not shown). However, the buffer extraction was found to be superior in terms of metabolite yield and extraction efficiency for polar metabolites and was therefore the method used to study shifts in the *D. magna* metabolome.

*$^1\text{H}$  NMR methods.*  $^1\text{H}$  NMR spectra of *D. magna* extracts were acquired with a BrukerAvance III 500 MHz spectrometer using a  $^1\text{H}$ - $^{19}\text{F}$ - $^{15}\text{N}$ - $^{13}\text{C}$  5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded Z gradient.  $^1\text{H}$  NMR measurements were performed using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression, 128 scans, a recycle delay of 3 s, and 16 K time domain points (Simpson and Brown 2005). Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift ( $\delta$ ) of 0.00 ppm.

*Gross biochemical analysis.* Total lipid content was analyzed by weighing lyophilized *Daphnia* and homogenizing using a motorized pestle in 2:1

chloroform:methanol (v/v). Extraction followed the micro sulfophosphanillan (SPV) method as developed by Gardner *et al.* (1985) and further validated by Lu *et al.* (2008). Standards were prepared by dissolving cholesterol in 2:1 chloroform:methanol (v/v).

Total protein content was analyzed as described by Nandakumar *et al.* (2003), with several modifications. *Daphnia* were homogenized with a motorized pestle in 100  $\mu$ l 30% trichloroacetic acid (TCA), and incubated at 4 °C for 40 minutes, then centrifuged at 12000 RPM for 10 minutes. After removing the supernant the extracted TCA pellet was dissolved in 100  $\mu$ l of 0.2M NaOH. An additional 400  $\mu$ l of 10mM TRIS-HCl pH 7 buffer was added, and protein content was determined using the Bradford assay (Bradford 1976) with bovine serum albumin standards.

Total nucleic acid content (DNA and RNA) was analyzed as described by Gorokhova and Kyle (2002) with few modifications. *Daphnia* were homogenized with the use of a mechanical agitator (Bullet Blender 24, Next Advance) set on speed 8 for 3 minutes with the addition of 0.1 ml (mark on centrifuge tube) of 0.1 mm glass beads and 200  $\mu$ l TE buffer. Subsequently, we added an additional 800  $\mu$ l of TE buffer to increase the final sample volume to 1000  $\mu$ l. Samples were placed into a microplate reader (Bio-Tec instrument FL<sub>x</sub> 800) with the excitation 485 nm/20 nm and emission 528 nm/20 nm and sensitivity of 75.

*Growth rate and elemental composition.* To assess growth rate, we first collected 3 samples of 10 neonates, dried them in a desiccator for 48 hr, and then determined the average initial mass of the *Daphnia* with a microbalance. After 6 days

of growth, we dried *Daphnia* collected from replicate jars (n = 4-5) in a desiccator for 48 hr and determined the mass gained over six days using the following equation:

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

Where B2 is the final average mass per *Daphnia*, B1 is the average initial neonate mass, and time is number of days of *Daphnia* growth.

Depending on the mass of *Daphnia* we removed 3-5 individuals from a jar replicate to determine the P-content using the sodium molybdate method (American Public Health Association 1992). Body C and N content was determined by placing 3-10 dried *Daphnia* from each replicate jar and placed into a tin cup and analyzed with the Elementar CN analyzer (Vario EL).

*Data analysis.* <sup>1</sup>H NMR spectra were analyzed between 0.5 and 10 ppm and divided into buckets 0.02 ppm wide, for a total of 475 buckets using AMIX 3.8.4 (BrukerBioSpin, Rheinstetten, Germany; See Figure S1 for pipeline schematic) statistics tool (Bundy *et al.* 2004; Lankadurai *et al.* 2011a). The region of 4.70–4.85 ppm was excluded to eliminate small residual H<sub>2</sub>O/HOD signals. The integration mode was set at the sum of intensities and the spectra were scaled to total intensity. This created a matrix in which each row represents a *D. magna* sample and each column contains the integrated area of the original spectral intensities contained within each bucket region. Individual principal component analysis (PCA) scores plots were constructed to compare the metabolic response of the control and *D. magna* subjected to dietary and environmental stressors. Levene's test was used to test for variance homogeneity among the PCA scores, which were found to have

equal variances at  $\alpha = 0.05$  (Brown and Forsythe 1974). A t-test (two-tailed, equal variances) was also performed on the PCA scores to determine if there was a significant difference between the scores of *D. magna* from both the control and experimental classes at  $\alpha = 0.05$  (Boroujerdi *et al.* 2009). The scores from the PCA scores plots were then imported into Microsoft Excel (version 12.0.6504, Microsoft Corporation, Redmond, WA) and were averaged per class and re-plotted with their associated standard errors. Corresponding PCA loadings plots, which show the relative weight for each bucket, were also acquired for the average PCA scores plot to identify the metabolites that were contributing to the separation between the scores of the control and *D. magna* subjected to both dietary and environmental stress (Bundy *et al.* 2002; Brown *et al.* 2009; Brown *et al.* 2010).

Difference class  $^1\text{H}$  NMR spectra were constructed to identify metabolites that had significantly increased or decreased relative to the control (Ekman *et al.* 2008; Lankadurai *et al.* 2011b). The buckets generated by AMIX 3.8.4 statistics tool, which represents the binned  $^1\text{H}$  NMR spectra of *D. magna* extracts were then imported into Microsoft Excel. Levene's test was used to test for variance homogeneity among the buckets, which were found to have equal variances at  $\alpha = 0.05$  (Brown and Forsythe 1974). A t-test (two-tailed, equal variances) was then performed comparing the buckets of the controls with that of the experimental class to identify the buckets that were statistically different at  $\alpha = 0.05$ . Average  $^1\text{H}$  NMR spectra were obtained by averaging the buckets of each experimental and control treatments separately. Difference class  $^1\text{H}$  NMR spectra were then obtained by subtracting the buckets of the average controls from that of the average experimental treatment. The buckets

representing the peaks of metabolites that weren't statistically significant from the controls were then replaced with a zero resulting in a t-test filtered  $^1\text{H}$  NMR difference spectra (Ekman *et al.* 2008). The buckets were then imported into Origin 7 (version 7.0383, OriginLab Corporation, Northampton, MA) to plot the difference  $^1\text{H}$  NMR spectra. The percent changes in the intensity of metabolite peaks of *D. magna* subjected to dietary and environmental stress relative to the control *D. magna* were obtained by dividing the buckets that pertain to the metabolites in the experimental class by the corresponding buckets in the control for each day. The metabolite peaks were identified using a database of the  $^1\text{H}$  NMR spectra of a series of standard metabolites and metabolite derivatives that we identified in *D. magna*.

The gross biochemical data were analyzed using analysis of variance (ANOVA) in SAS v. 9.1. Pairwise contrasts were used to examine differences between the GF control and the nutrient and environmental stress treatments with  $\alpha$  set at a cumulative error rate of 0.05.

#### **4.5 Results**

The prescribed diets and external stressors caused physiological changes within the *Daphnia* as shown by both traditional (Fig. 4.1) and novel indicators (Fig. 4.2, Table 4.1) of nutrient status. Both diet and external stressors caused significant changes in mass-specific growth rate ( $F_{5,95} = 182.9$ ,  $P < 0.001$ ) and the body C ( $F_{5,26} = 81.94$ ,  $P < 0.001$ ), N ( $F_{5,26} = 79.6$ ,  $P < 0.001$ ), P ( $F_{5,25} = 156.5$ ,  $P < 0.001$ ), lipid ( $F_{5,24} = 40.8$ ,  $P < 0.001$ ), protein ( $F_{5,24} = 10.9$ ,  $P < 0.001$ ) content and RNA:DNA ratio ( $F_{5,36} = 49.5$ ,  $P < 0.001$ ). *Daphnia* that were limited by an element (C, N, or P) had less of that element within their body and the corresponding major biochemical pool was also

decreased (e.g., under N-limitation, body N decreased as well as % protein; Fig. 1). Besides exhibiting a lower growth rate, salt stressed *Daphnia* significantly increased their body C- and N-content and decreased their body P and RNA content. There were few and relatively small changes in the biochemical composition of *Daphnia* exposed to pathogenic bacterial spores (Fig. 4.1).

The metabolite composition of *Daphnia* varied among animals raised under these different nutritional and environmental stressors. In our multivariate analysis of animal responses to each stressor individually, we found significant separation between the PCA scores of control animals and *D. magna* experiencing all forms of dietary and environmental stress ( $P < 6 \times 10^{-6}$  for all except BAC animals  $P = 0.03$ ; Supplementary Material, Fig. S4.2A to S4.2D). When analyzed altogether, the metabolite composition of *Daphnia* clearly differed among animals grown under the various types of nutritional or environmental stress (Fig. 4.2).

Using the PCA loadings plots (Figs. 4.2B and 4.2C) and the t-test filtered  $^1\text{H}$  NMR difference spectra (Supplementary Material, Fig. 4.S3), we identified several amino acids and sugars, two messenger molecules, two nucleotide derivatives (variation of the nucleotide forms), and a choline derivative that were all significantly affected by at least one stressor (Table 4.1). Due to the overlapping resonances within the  $\delta$  3.40 to 4.00 ppm region of the  $^1\text{H}$  NMR spectra (Figs. 4.2B, 4.2C and S4.3), many peaks could not be definitively assigned to individual metabolites. Nonetheless, metabolite composition within this region was significantly affected by both dietary and environmental stressors (Figs. 4.2B, 4.2C and S4.3).

Within these data, we found free amino acid concentrations were strongly affected by nutrition and environmental stress. Both essential (methionine, valine, threonine, leucine, lysine, histidine, phenylalanine and tryptophan in invertebrates; Cowey & Forster 1971; Müller-Navarra 2008) and non-essential amino acids (arginine, glycine, glutamate, alanine) changed in stressed *Daphnia* relative to the controls (Table 4.1). While P-limited *Daphnia* did not show a clear trend for increasing or decreasing their free amino acid content, N-limited animals decreased and low food quantity animals increased their free amino acid content. Salt stressed *Daphnia* significantly decreased the content of all free amino acids while little response was observed in animals exposed to pathogenic bacterial spores (Table 4.1).

We also found sugars and other intermediates of metabolism changed in response to nutritional and environmental stressors. Low food quantity significantly decreased some metabolites (succinate, glucose/maltose, ATP derivatives and GDP derivatives) but increased others (betaine, the choline derivative, and lactate/threonine; Table 4.1). Dietary N and P stress also resulted in significant responses of metabolites but ones that were different from each other and that of low food quantity (Table 4.1). Salt stress reduced succinate and the choline derivative but increased glucose/maltose and betaine in *Daphnia* relative to controls, while animals exposed to spores showed no change in these metabolites (Table 4.1).

#### **4.6 Discussion**

We found the metabolome and the biochemical pools of *Daphnia* changed in a stress specific manner to the diet and environmental stressors tested. In general when consumers were limited by a particular food element, it was decreased in associated

metabolite and biochemical pools. Although environmental stressors changed the metabolite and biochemical pools, these body chemistry changes were distinct from that created by nutritionally derived stress. Our data suggests metabolite composition and biochemical pool content change with stress in a specific manner, which may allow for the optimization and development of nutritional profiles and help advance the field of nutritional ecology.

We found that changes to the metabolite composition of *Daphnia* that likely reflected the supply and demand for metabolites in a given food treatment. Specifically, growth limitation by a particular nutrient appeared to increase demand for metabolites containing that limiting nutrient and subsequently altered the animal's metabolite composition. For example, *Daphnia* grown on low food quantity had greater relative amounts of free amino acids in their bodies. This result probably reflects the intracellular production of AA from protein breakdown, to provide energy for maintenance through glucogenic pathways in which amino acids are converted into glucose. We also found evidence that consumers grown under N limitation decreased the relative amount of some, but not all, free amino acids in their bodies. In particular, amino acids with more than one nitrogen side group (arginine or lysine) significantly decreased in N-limited consumers. This effect has been observed in yeast and is likely a mechanism to increase N use efficiency (Boer *et al.* 2010).

Growth limitation was also associated with changes to the total body content of several biochemical classes. For example, RNA:DNA ratios decreased in animals grown under all forms of nutrient limitation and experiencing salt stress. This would be consistent with less demand for ribosomal RNA (Elser *et al.* 2000) due to slower

growth rates and lower rates of protein synthesis created by all forms of stress. With the exception of low food quantities, we also found stress and slower growth associated with increased total lipid content, which is an indicator of greater energy storage. Such short-term energy storage would likely increase growth rates in animals if and when nutrient limitation was alleviated. While our results show that both the metabolite and biochemical pools are responsive to nutritional and environmental stress, future work could focus on how molecular and physiological processes coordinate changes in these pools (biomolecules and metabolites) in response to different external stress. Such work would increase our understanding of the mechanisms organisms employed to maintain their internal homeostatic state in the face of external stress.

While environmental stress caused changes within the metabolome and biochemical pools, these changes did not strongly link to the supply and/or demand for a given metabolite/biochemical pool as observed for responses to poor diet. Under high salt conditions, we found metabolomic responses of *Daphnia* differed from those previously documented for other organisms exposed to saline stress. While organisms including other crustaceans (McNamara *et al.* 2004), plants (Sanchez *et al.* 2008), bacteria (Kol *et al.* 2010), and spiders (Foucreau *et al.* 2012) have been found to increase free amino acid concentrations to balance the solute concentrations in their cells with increases in extracellular solute concentrations, our study found *Daphnia* decreased most metabolites including many of the AA. It is unclear whether contrasting metabolic responses by daphnids to high salt conditions reflects the

experimental conditions used here or are unique to this important freshwater herbivore.

One explanation for the lack of AA response to salt stress is that *Daphnia* use other mechanisms to deal with the solute imbalances. We found that salt-stressed *Daphnia* significantly increased their carbon content that didn't correspond to changes in their protein or lipid content. While this result may have resulted from increased carbohydrate content (e.g., starches) in these stressed animals, it seems more likely that the higher C content was due to unidentified C-rich metabolites. We found increases of betaine (a known osmoprotectant), but increases in this one pool alone likely does not account for the overall increase of 12% C-content of salt-stressed animal. *Daphnia* may have likely increased other C-rich metabolites that were not identified by our NMR-metabolome approach. Increasing other C-rich molecules could account for the differences in body C-content and would osmotically balance the solute concentrations within the cells. Testing this last hypothesis will require a more targeted metabolomic approach in which individual metabolites can be separated and quantitatively measured.

We also found relatively limited metabolomic changes in *Daphnia* exposed to a highly pathogenic bacterium. This likely reflects the limited virulence and minimal disease effects of the bacterial pathogen during the early stages of infection (Ebert *et al.* 1996). In other words, the metabolite composition of animals was not found to respond strongly to bacterial disease during its asymptomatic stages. Nonetheless, the individual PCA scores plot did show a significant separation in the daphnid metabolome to BAC exposure along PC2, which indicates there are some changes,

albeit unidentified, in the metabolome of early stage infected *Daphnia*. Later disease stages may produce even more profound metabolite changes in the host organism and should be examined in future studies. For example, virulent infections were found to alter both the amino acid and sugar metabolism of two vascular plants (Ward *et al.* 2010). We conclude that the limited metabolite responses of invertebrates to infectious disease found here are likely stage-specific and should not limit further work in this area.

Our results show clear separation in the metabolite composition of an aquatic invertebrate exposed to different dietary and environmental stress. These results indicate that a metabolomic approach could provide new tools in identifying the nutritional status of consumers. Currently, the nutritional status of consumers is largely inferred from measurements of growth rate and body elemental content responses to poor diet. Although both approaches are useful, the same response (e.g., slow growth rates) results from different forms of nutrient limitation and from environmental stress (e.g., high salinity). Such non-specific responses reduce our ability to precisely identify the proximate limiting agent and thus hinder the advancement of nutritional ecology (Wagner *et al.* 2013). While non-specificity would be problematic for interpreting the responses of individual metabolites, the combined responses of multiple metabolites would likely be distinctive among different forms of stress and provide strong evidence of a particular form of nutrient-limitation (Wagner *et al.* 2013). This is a considerable advantage to using a metabolomic approach, whether it targets specific indicator metabolites or characterizes the entire metabolome. It is important to note that we employed a non-

targeted NMR-based metabolomics approach. However, based on the knowledge gained through this study, more targeted approaches using molecule-specific analyses are now possible and will enable future growth in this area of research.

Nutritional profiling, as described here, will require considerable validation before it can be applied in nutritional ecology. Shotgun approaches such as used here are an excellent starting place to identify potential nutrient specific metabolites. Once fewer nutrient specific metabolites have been identified and validated by ensuring their uniqueness to confounding variables including ontogeny, species differences, and other environmental conditions (e.g., temperature), these nutrient-specific indicators would then be used to develop a known database to classify unknown animals, through the use of classification and multidimensional statistical tools (Frost et al. 2014) After this validation process, nutritional profiling could provide *in situ* assessments of nutritional limitation in animal consumers, which would be especially valuable to the studies of nutrition and ecology (Wagner *et al.* 2013). For example, the prevalence and type of nutritional limitation could be precisely tracked through time and space in animal populations (Wagner *et al.* 2013). Indicator profiles could also be used to study animal responses to other types of nutritional (e.g., amino acid or lipid limitation) or environmental stresses (e.g., high temperature or low oxygen). Finally, this approach could be used to study interactions among multiple forms of nutritional limitation. Such work would help determine whether one form of limitation prevails over others or whether the co-limitation by multiple elements is manifested in natural populations of different animal taxa.

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#### 4.9 Tables and Figures

**Table 4.1.** Percent (%) change in metabolites of *Daphnia* grown under low food (LF), low nitrogen(LN), low phosphorus (LP), high salt(HNa) and exposed to infective spores (BAC)compared with the control *Daphnia*. The percent changes that were significantly different (P<0.05) from the control (based on a t-test of control vs. stressed) are denoted in green (positive) and red (negative).

Metabolite	Abv	<sup>1</sup> H NMR Chemical shift (ppm)	Class/ Function	LF	LP	LN	HNa	BAC
Alanine	ALA	1.47	AA	27±8	42±14	-2±6	-41±5	-17±7
Arginine	ARG	1.91	AA	14±1	4±2	-3±1	-15±2	-2±0.9
Glutamate	GLU	2.35	AA	-4±4	-6±4	-10±4	-11±4	-1±4
Glycine	GLY	3.55	AA	15±5	26±4	18±4	6±6	-5±4
Leucine	LEU	0.95	AA	9±3	5±3	-5±2	-22±2	-2±2
Lysine	LYS	3.01	AA	4±2	-5±2	-7±2	-14±3	-0.8±2
Methionine	MET	2.13	AA	13±3	11±3	-2±2	-18±2	-3±2
Phenylalanine	PHE	7.31	AA	10±3	5±4	-7±2	-26±3	-5±3
Tryptophan	TRY	7.71	AA	11±5	6±5	-4±4	-24±4	-3±4

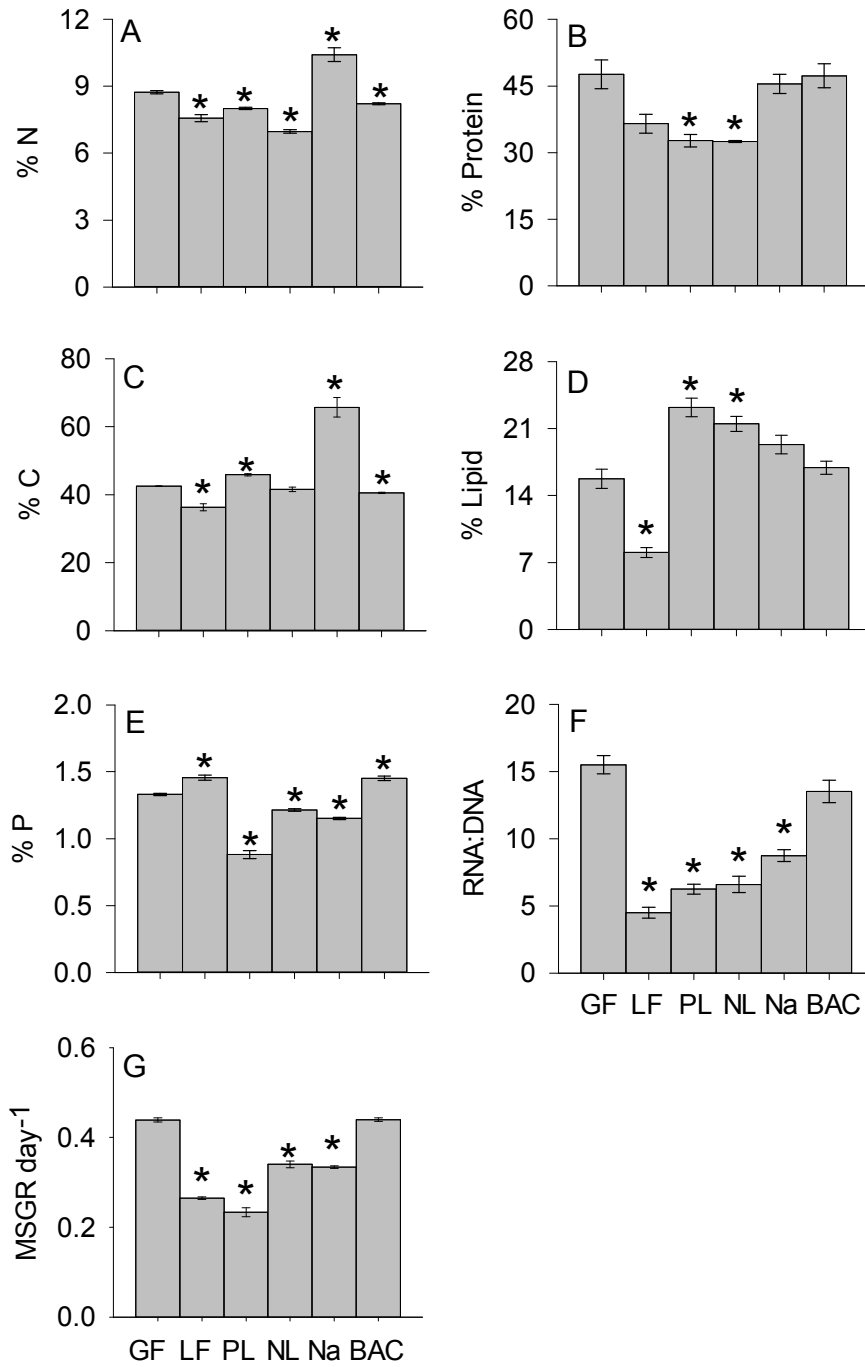
Valine	VAL	1.03	AA	8±4	10±3	-5±2	-21±3	-4±3
ATP-derivative	ATP	8.19	Energy Met	-39±5	-60±8	-44±7	-4±6	-5±6
Choline derivative (GPC)	CHO	3.21	Energy Met	16±4	-35±3	-26±3	-19±4	-8±3
GDP-derivative	GDP	8.13	Energy Met	-91±7	-84±7	-58±7	19±9	3±6
Succinate	SUC	2.39	Energy Met	-6±1	-12±2	-17±1	-12±2	-2±2
Glucose/Maltose	GLUC	5.23	Sugars	-67±7	30±14	109±11	163±25	6±6
Lactate/Threonine	LAC	1.33	Sugars/AA	15±5	72±23	51±11	-4±4	4±3
<i>scyllo</i> -Inositol	SCY	3.35	Sugar alc	-11±5	-18±5	17±5	-0.6±6	-6±6
Betaine	BET	3.25	Osmolyte	4±2	4±2	16±2	22±3	2±2

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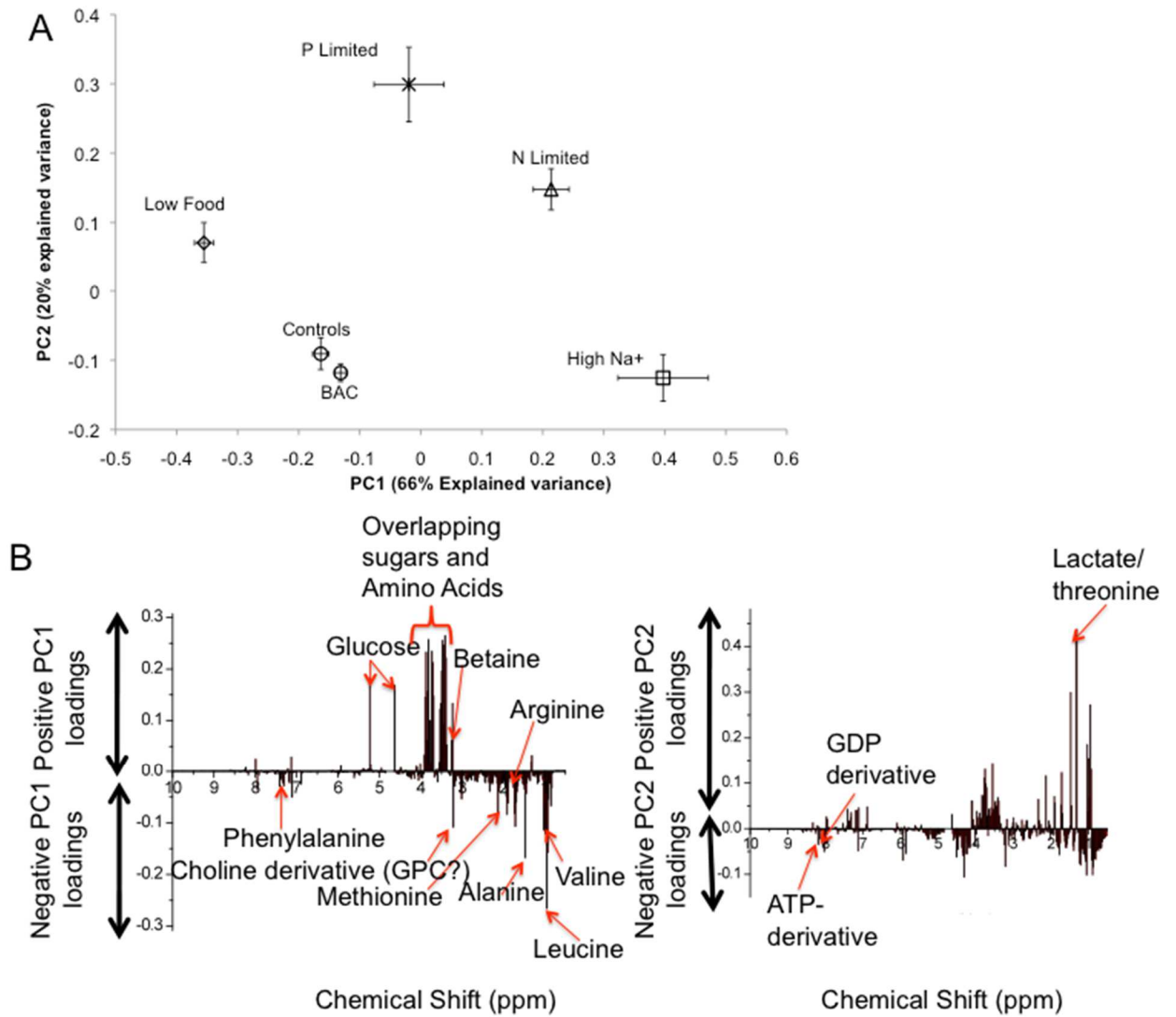
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**Figure 4.1:** Gross biochemical analysis of *D. magna* grown for 6 days under good food (GF), low food (LF), P-limited (PL), N-limited (NL), high salt (Na) and exposed to spores (BAC). (A) % C (B) % lipid (C) % N (D) % protein (E) % P (F) RNA:DNA (G) mass specific growth rate per day (MSGR day<sup>-1</sup>). Error bars are  $\pm$ SE, with \* being significantly different from GF controls with a cumulative  $\alpha$  0.05.

**Figure 4.2:** (A) Principal component analysis (PCA) scores plot of mean <sup>1</sup>H NMR spectra of *D. magna* tissue extracts. The mean scores and standard error of animals grown under good food conditions (controls), low food quantity, N-limited food, P-limited food, High Na<sup>+</sup> and bacterial exposure treatment (BAC) are shown on the PCA scores plot. The mean scores (with associated standard error) were obtained by averaging the scores of each *Daphnia* treatment (n = 5). PCA loadings plot for (B) PC1 and (C) PC2 showing the metabolites that were major contributors to the separation observed in the mean PCA scores plot. The abscissa refers to the <sup>1</sup>H NMR chemical shifts (ppm) in the loadings plots.



**Fig. 4.1**



**Fig. 4.2**

## 4.10 Supplementary

### Metabolomic analysis of nutritional stress in an aquatic invertebrate

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and Paul C. Frost<sup>3</sup>

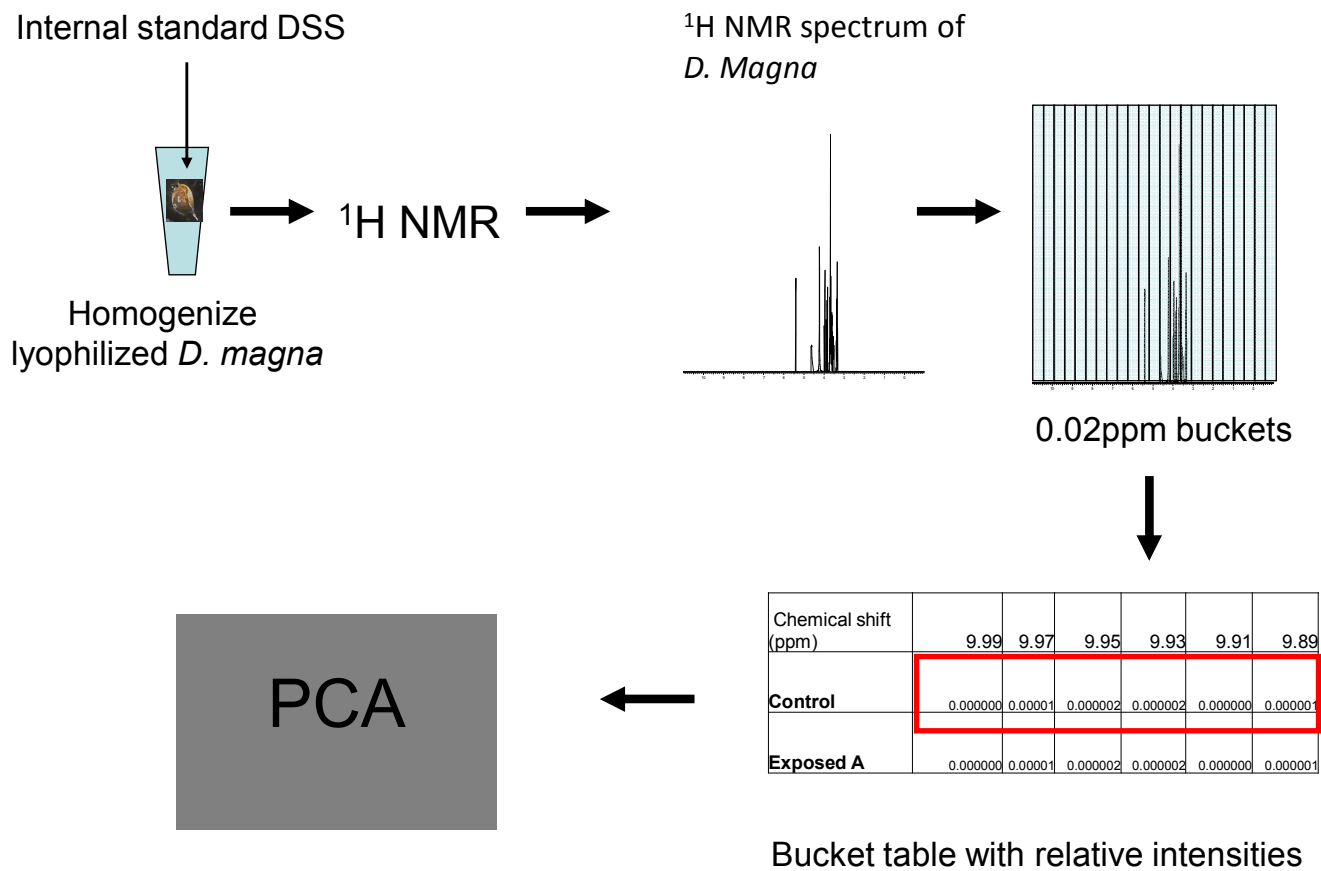
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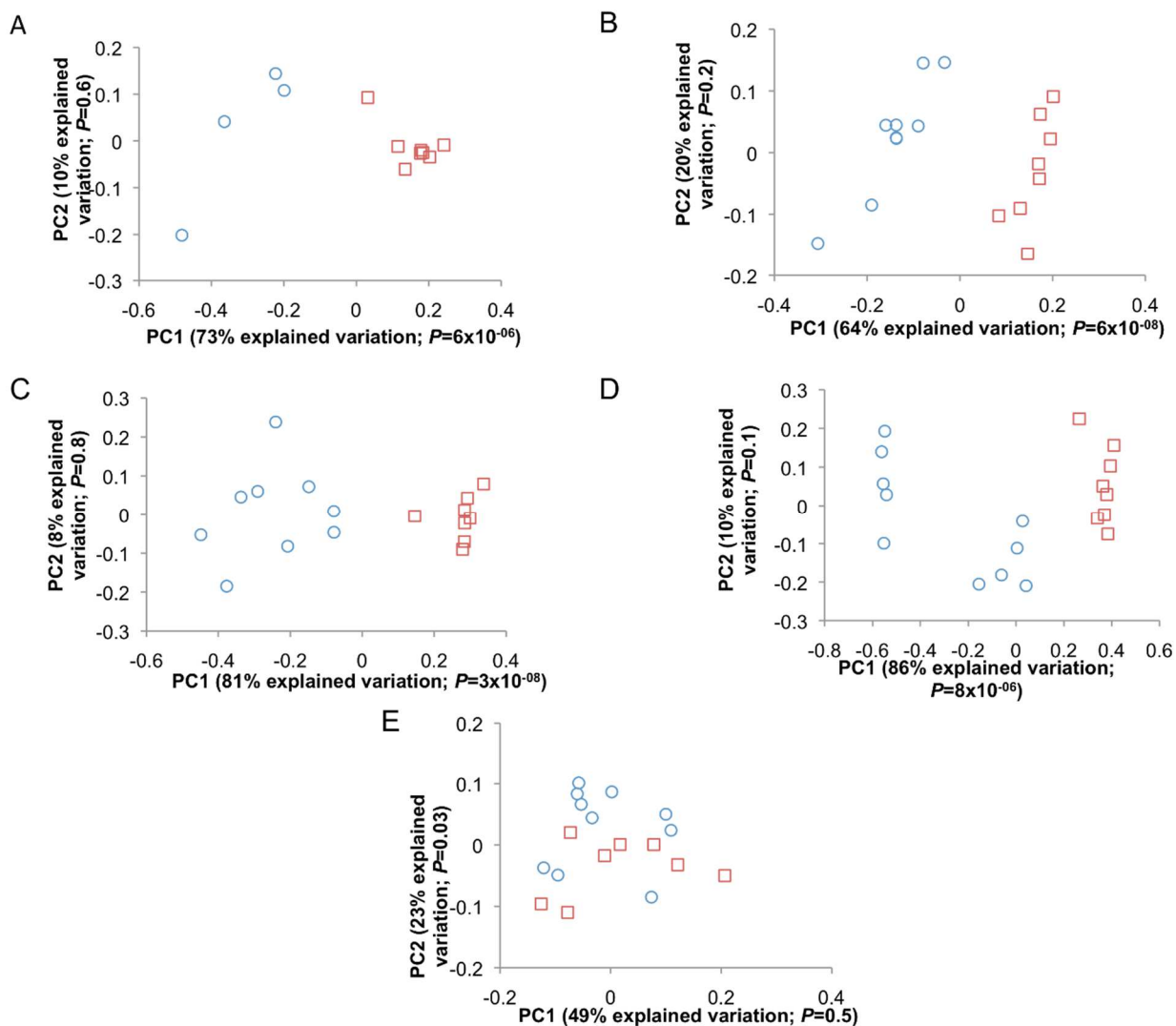
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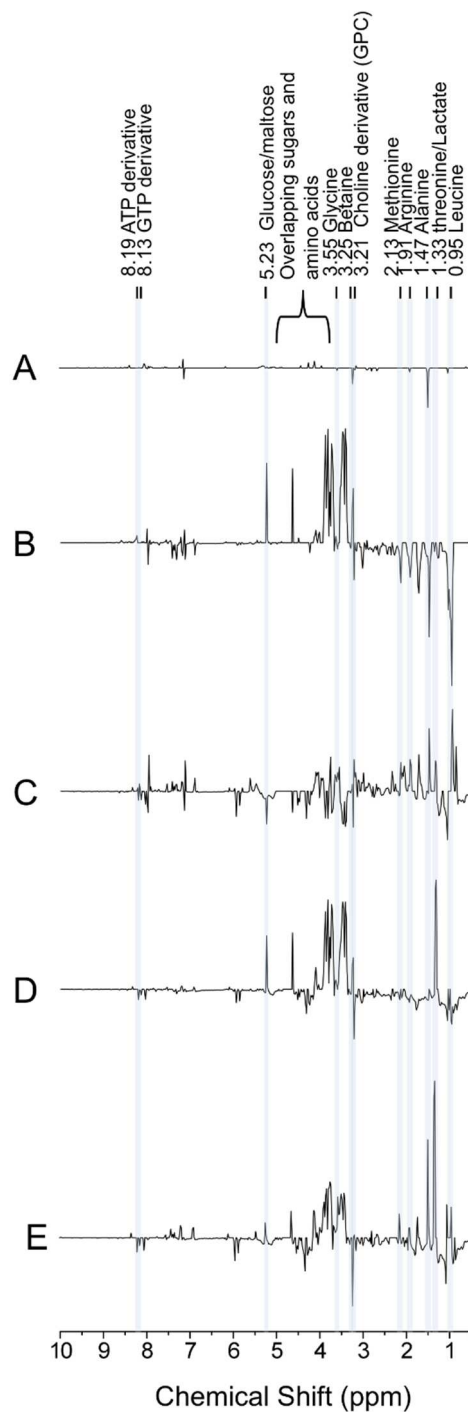
**Reference:** *Published in* Physiological and Biochemical Zoology (2015) 88:43-52



**Figure S4.1:** Pipeline schematic of methods used to obtain PCA results



**Figure S4.2.** PCA scores plots of PC1 (first PCA component) versus PC2 (second PCA component) for  $^1\text{H}$  NMR spectra of *Daphnia magna* tissue extracts showing the separation of control *Daphnia* ( ) from nutritionally and environmentally stressed *Daphnia* (○). *Daphnia* were subjected to conditions of (A) low phosphorus, (B) low food, (C) low nitrogen, (D) high NaCl, and (E) exposed to spores from the bacterium *Pasteuria ramosa*. The *P*-values were obtained from t-tests that compared the scores of the control *Daphnia* to the nutritionally and environmentally stressed *Daphnia* for each component.



**Figure S4.3.** T-test filtered <sup>1</sup>H NMR difference spectra of *D. magna* tissue extracts obtained by subtracting the mean buckets of the control *Daphnia* from the mean buckets for each nutritional and environmental treatment and retaining the buckets that were statistically different from the controls at  $\alpha = 0.05$ . (A) Exposed to spores from the bacterium *Pasteuria ramosa*, (B) high NaCl, (C) low food, (D) low nitrogen, and (E) low phosphorus.

**Table S4.1:** Dilutions and concentration of nutrients added to the algal *S. obliquus* cultures

Nutrient status	Dilution ( rate day-1)	$\mu\text{M}$ of P ( $\text{NaHPO}_4$ ) added	$\mu\text{M}$ of N ( $\text{NaNO}_3$ ) added
N & P rich	0.55	70	1050
P limited	0.2	7	1050
N limited	0.15	70	420

**Table S4.2:** Number of Replicates (Reps) per response variable for each of the following diet treatments; high food quality and quantity (GF), phosphorus limited (PL), low food quantity (LF), nitrogen limited (NL) high salt (Na) and exposed to spores (BAC). 20 *Daphnia* were grown per jar with response variables requiring less than 20 animals (%P, %C, %N, %Lipid, %Protein, RNA:DNA) were sub-sampled from 1 jar per replicate.

Diet	<sup>1</sup> H NMR		MSGR		%P		%C %N		%Lipid		%Protein		RNA:DNA	
	# Jars/ Rep	# Reps	# Jars/ Rep	# Reps	# Daphnia /Rep	# Reps	# Daphnia /Rep	# Reps	# Daphnia /Rep	# Reps	# Daphnia/ Rep	# Reps	# Daphnia/ Rep	# Reps
<b>GF</b>	3	8	1	50	3	5	5	5	4	5	4	5	1	7
<b>PL</b>	6	10	1	14	5	5	7	5	10	5	10	5	1	7
<b>LF</b>	5	4	1	19	5	5	7	5	10	5	10	5	1	6
<b>NL</b>	5	9	1	9	4	5	7	5	10	5	10	5	1	7
<b>Na</b>	7	10	1	9	5	5	7	5	10	5	10	5	1	10
<b>BAC</b>	3	10	1	8	3	5	5	5	4	5	4	5	1	8

## CHAPTER 5

### 5.1 PREFACE

**Title:** Effects of food quality on free amino acid metabolism in *Daphnia*

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**Keywords:** Nutritional profile, Bio-indicator, ecological stoichiometry, food quality, *Daphnia*,

**Author Contributions:** NDW, ZY, and PCF designed the study, NDW and ZY ran the experiments. NDW and ABS developed the amino acid method. NDW and PCF analyzed the results and wrote the manuscript with contributions from all authors.

## Chapter 5

### Effects of food quality on free amino acid metabolism in two aquatic zooplankters

#### 5.2 Abstract

Animals frequently face nutritional limitation caused by imbalances between the nutrients they require and that in their food sources. Nutritional imbalances alter life-history traits such as reducing growth and reproduction rates. While these changes in life-history traits are governed by molecular and biochemical processes, the effects of nutritional imbalances on metabolic processes remain poorly studied. One biochemical aspect of metabolism that may be impacted by poor nutrition is the free amino acids (FAA) composition of body tissues as the dietary supply and metabolic demand for these metabolites will likely change with diet. We examined FAA responses in animal bodies to different levels of limitation by three nutritional stressors (phosphorus and nitrogen limited and low food quantity) in two cladoceran zooplankton species (*Daphnia magna* and *Daphnia pulex*). We extracted and quantified 14 different FAAs from lyophilized *Daphnia* through separation with the HPLC. Similar responses in body FAA composition between the two species of *Daphnia* grown under different types of nutritional limitation were found. Generally, low food quantity and low food P content increased body FAA content in both species, whereas some FAAs decreased or increased when animals were grown under N limitation. Discriminant analysis revealed models for each species of *Daphnia* performed extremely well with a high predictive ability to classify unknown consumers into diet treatments. Changes in FAA illustrate the strong effects of food quality on biochemical properties of invertebrate animals. When combined with other

nutritional responses, variable body FAA composition, as described here, may provide an important source of information about animal nutrition in laboratory and in nature.

### 5.3 Introduction

Animal consumers frequently face nutritional limitation in both aquatic and terrestrial environments (Raubenheimer et al., 2009; Sterner and Elser, 2002). These imbalances are created by plasticity in the nutrient composition of autotrophs and less flexibility in their consumers (Frost et al., 2005; Sterner and Elser, 2002). When nutrient stressed, animal consumers often exhibit reduced growth and reproductive rates (Frost et al., 2005), increased metabolic rates (Darchambeau *et al.* 2003; McFeeters & Frost 2011), and increased assimilation efficiencies for the limiting nutrient (DeMott et al., 1998). These physiological responses to poor food quality can alter consumer population dynamics (Andersen et al., 2004), create transgenerational effects (Frost et al., 2010), and result in relatively rapid evolution of animal life-history traits (Gorokhova et al., 2002; Yamamichi et al., 2015).

The nutritional status of animal consumers has been inferred through calculations of threshold elemental ratios or measured with growth bioassays in laboratory settings (Wagner et al., 2013). While these approaches have provided many insights into nutritional physiology of different organisms, there remains much to be learned about cellular and molecular responses to poor food quality in many ecologically important animal taxa. Molecular responses happen at various levels of organization within the animal including in the transcriptome (Jeyasingh et al., 2011; Roy Chowdhury et al., 2015) and metabolome (Boer et al., 2010; Wagner et al., 2015). Nutrient specific metabolomic responses have been identified in yeast and are consistent with metabolic changes needed to maintain internal homeostasis (Boer et al., 2010). Given the responsive nature of the metabolome to environmental stressors including nutrition (Boer et al.,

2010; Wagner et al., 2015), we examined the free amino acid (FAA) metabolite profile in an aquatic invertebrate, *Daphnia*. Our hypothesis was that FAA profiles are affected by the presence and type of nutritional stress, due to differences in the supply of amino acids (AAs) from food and the demand for those AAs within the animal consumer.

FAAs are metabolites that may be especially affected in animal bodies by nutrition as they are involved with regulating central metabolism and, specifically, the tricarboxylic acid cycle (TCA; Salway 2004). After protein digestion in the upper alimentary canal, FAA are transported to tissues and cells, where they are either: 1) incorporated into proteins during translation, 2) converted to alternative amino acids through various metabolic pathways or 3) deaminated and degraded into acetyl co-A either for ATP synthesis or storage as lipids (Salway, 2004). The fate of digested amino acids depends on the supply to and demand for the FAA of the organism (Boer et al., 2010), which should vary with the type of nutritional limitation experienced by an animal consumer. During fast growth many, if not most, FAA are used for the synthesis of proteins involved in the production of new biomass. In contrast, slow growth caused by P-limitation may increase FAA pool sizes due to decreased rates of protein synthesis (Boer et al., 2010; Wagner et al., 2015). Under these conditions, FAA can also be converted into lipids to prevent the buildup of free substrates and reduce substrate inhibition of central metabolism (Salway, 2004). Limitation by nitrogen (N) specifically may result in decreased cellular FAA pool sizes, even during slow growth, because the supply of essential AAs and non-essential AAs may not match that needed to fully support protein production and other metabolic requirements. Under low food conditions, FAA pools may increase due to protein catabolism to fuel ATP synthesis (Wagner et al.,

2015). These increases in pool size may not include essential amino acids, which should continue to be utilized for protein synthesis. Due to these differences, body FAA profiles in animal consumers would plausibly differ among these types of nutritional limitation. If so, the profiles of FAA may be a useful indicator of the nutritional status of consumers (sensu Wagner *et al.* 2013).

Here we examined responses of animal body FAA content to nutritional stressors between two daphnid species (*D. magna* and *D. pulex*) and assessed their potential usefulness as a nutritional indicator. We did so by growing two species of a cladoceran zooplankter, *D. magna* and *D. pulex*, on different qualities and quantities of algae and measuring their body FAA content, growth rates, and elemental composition. We expected differences in the FAA profiles in animal bodies between good food conditions and the nutritionally deficient diets. We also expected differences in the body FAA profiles between the two species as differences in growth (Wagner and Frost, 2012) and respiration (McFeeters & Frost 2011) have been observed.

#### **5.4 Methods and Analysis**

*Algae and Daphnia Culturing.* We varied the elemental composition of *Scenedesmus obliquus* (Canadian Phycological Culture Centre strain 10, purchased as *S. acutus*) that was grown in multiple semi-continuous culture jars and diluted daily with differentially enriched media (Sterner *et al.*, 1993) to produced P-rich, P-poor and N-poor algal cells. The P content of concentrated algal cells was determined after persulfate digestion of dried subsamples using the molybdate-blue ascorbic acid colorimetric assay (APHA, 1992). Carbon and nitrogen content of the concentrated algal suspensions were also determined on dried cells using a CN Analyzer (Vario EL III, Elementar

Incorporated, Mt Laurel NJ, USA). Using the elemental composition of the concentrated algal suspensions, foods were mixed to generate diets with different nominal C: P and C: N ratios, which were verified with subsequent elemental analysis.

*Experimental design.* We used these diets to study the amino acid responses of *Daphnia magna* and *Daphnia pulex*. Animals used in these experiments were born to second generation, clonal sisters who were held in groups of 10 animals in 400 ml of P free COMBO media (Kilham et al., 1998) and fed high food quality and quantity of *S. obliquus*. On the morning of each experiment, neonates were collected (<24 hrs old) and placed in nutrient-free and food-free COMBO for a short period (<1 hours) until they were allocated to an experimental treatment (Wagner and Frost, 2012; Wagner et al., 2015).

*Experimental design.* We placed ten *Daphnia* neonates, after rinsing with N- and P-media, into separate glass jar that each contained 400 ml N and P free COMBO. We fed experimental animals their assigned food type every other day over the six day experiment. To explore the effects of the intensity of nutritional limitation, we used four levels of food quantity, C:N ratio, and C:P ratio (See Table S5.1 for actual C:N:P ratios of diets and food quantity levels).

After six days of growth, animals were collected for amino acid analysis (n = 5), mass specific growth rate (MSGR; n = 5) and elemental analysis (body %C, %N, and %P content; n = 5). For the amino acid analysis, *Daphnia* were placed into a 1.5 ml centrifuge tube and immediately frozen in liquid nitrogen (n = 5; 1-2 jars of *Daphnia* were used per replicate). Animals were subsequently lyophilized and placed in a -80 °C freezer until analysis of their body FAA content. For MSGR and body elemental

composition, one jar (10 animals) was collected for each replicate and placed in the drying oven for 12 hr at 60°C before being analyzed.

*Amino acid analysis.* Lyophilized *Daphnia* were homogenized with a motorized pestle in 100 µl 0.4M borate buffer (pH 10.5) for 1 minute. Following homogenization, samples were centrifuged for 8 min at 12000 g and the supernatant was transferred to a new 1.5 ml centrifuge tube. This procedure was repeated an additional 2 times to ensure all FAAs were extracted. An additional 200 µl of borate buffer was added to make the final volume 500 µl. Following extraction, samples were filtered through a 0.2 µm polycarbonate filter into a HPLC vial and then loaded into an HPLC (Waters, Milford, MA, USA).

*Amino acid derivatization.* Amino acids were derivatized as described by Pereira *et al.* (2008). Briefly, 250 mg *o*-Phthaldialdehyde (OPA) was dissolved in 1.5 ml of ethanol with 200 µl of 2-mercaptoethanol and topped up to 10 ml with 0.4 M borate buffer (pH 10.5). The reagent solution was left to settle for 90 minutes, stored in dark glass vial at 4°C and freshly prepared every 9 days. The derivatization procedure was performed in the sample injection loop according to the following sequence: 10 µL of buffered sample mixture was injected into the sample loop followed by 10 µL of OPA. During the derivatization period of 3 min, the flow is maintained at 0 mL/min to keep the reagent into the loop and promote derivatization. Then, the loop contents (20 µL) was forced to enter into the column by changing the mobile phase flow to 1 mL/min.

*HPLC conditions.* Amino acids were separated with HPLC using a Waters (Milford, MA, USA) liquid chromatograph controlled by the Empower Pro Software and equipped with an auto-injector (Waters 2695, separations module) and a multi λ

Fluorescence detector (Waters 2475). Chromatographic analysis was performed using a 3.9 mm x 150 mm Nova-Pack RP-C18 column, with a particle size of 4  $\mu\text{m}$ , purchased from Waters (Milford, MA, USA). The HPLC conditions followed Pereira *et al.* (2008): 1) mobile phase A: 1% of tetrahydrofuran, 8% methanol and 91% phosphate buffer (10 mM, pH 6.24) and 2) mobile phase B: 80% methanol and 20% phosphate buffer (10 mM, pH 6.43). The flow rate was set at 1 ml/min and the column maintained at 35°C for a total elution time of 80 min following the gradient as described in Table S5.2 with the last 13 min being column regeneration (Pereira et al., 2008). The eluted OPA derivatives were detected by monitoring their fluorescence at 335 nm excitation and 440 nm emission wavelengths.

*Calculations.* Amino acid concentrations were based on the fluorescence of known concentrations of a multi-amino acid standard consisting of 1 mM of each alanine, serine, threonine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid, asparagine, and arginine. A standard curve was created by injecting different amounts of this multi-amino acid standard, which resulted in linear curves with r-squares greater than 0.99 for all amino acids. Standard curves were completed every time we made a new OPA dye.

*Growth rate and elemental composition.* To assess *Daphnia* growth rate responses to food quality, 3 samples of 10 neonates were collected and dried overnight at 60°C to determine the average initial mass of the *Daphnia* with a microbalance. After 6 days of growth, differently nourished daphnids were collected from replicate jars and dried overnight at 60°C to calculate the MSGR determined by the equation;  $\text{MSGR} = [\ln(B2) - \ln(B1)]/\text{Time}$ . Where B2 is the final average mass per *Daphnia*, B1 is the average initial

neonate mass, and time is number of days of *Daphnia* growth. Body P-content was determined by drying replicate jars and weighing the *Daphnia* prior to a persulfate autoclave digestion (APHA, 1992). Replicate jars were also saved for C:N analysis using an Elementar analyzer (Vario EL).

*Statistical analysis.* Growth rate and body elemental composition were analyzed using either a linear or logarithmic regression analysis (SAS 9.3) to determine whether food quality effects resulted from each of the prescribed diets. To examine whether the amino acid responses differed between the two daphnid species, we analyzed whether the ordinary least squares linear regressions had a common slope using the program SMATR (Falster et al., 2006; Warton et al., 2006). If a common slope was found, we concluded that the daphnid species were not different in their responses to nutritional limitation.

We next examined how the FAA profiles separated in multidimensional space in both species of *Daphnia*. To do this, we used a non-parametric nearest neighbor (kNN) model in SAS 9.3. As kNN and discriminate analysis use discrete variables to classify unknown samples, we included all data on animals consuming high food quality and quantity (HQQ) (n =15; C:P 100, C:N 8, and highest food quantity) and removed the first intermediate diet (C:P 300, C:N 10 and 2 mgC/L; *D. magna*, 0.5mgC/L *D. pulex*) from the model, leaving the two most nutrient poor diets within each nutrient gradient. To select the amino acid entered into the model, we performed a stepwise discriminate analysis with forward selection of  $p = 0.001$ . The resulting amino acids were then entered into the kNN model with a  $k = 3$ . We chose a  $k$  of 3 as given by the general rule of the square root of the number of food treatments (e.g.  $\sqrt{7}$  rounded to nearest whole number;

Duda *et al.* 2001). The overall misclassification rate was determined using the cross-validated (leave one out) kNN.

## 5.5 Results

Animals fed different food quality and quantity exhibited reduced growth rates and altered elemental composition (Table 5.1). We found significant linear/ logarithmic relationships between MSGR and low food N, P, and food quantity in both species of daphnids ( $p < 0.0001$ ; Table 5.1). There were also negative relationships between food C:N and C:P ratios and the body content of *Daphnia* of the same limiting nutrient (N and P respectively; Table 5.1). In contrast, we found a positive relationship between the algal food quantity and body C content in both species of *Daphnia* (Table 5.1).

Body FAA profiles in both species of *Daphnia* also exhibited strong responses to nutrient limitation (Fig 5.1; Table 5.2). In general, food quantity and P limitation caused both species of *Daphnia* to increase concentrations of FAA in their whole body homogenates, N limitation reduced concentrations of some body FAAs and increased others in both *Daphnia* species (Table 5.2). Even though body FAA content responded in the same way (positive or negative slope) to increasing dietary limitation, we found significant differences in these slopes for most free amino acids between two zooplankton species (Fig 5.1A; Table 5.2). For most FAAs, there was a steeper response slope between body content and food quantity and P-limitation in *D. pulex* compared to *D. magna*. There were also differences in responses to N-limitation but these were not consistent among species (Table 5.2).

We further examined amino acid profiles in multidimensional space to see if there would be significant separation associated with diet in *D. magna* and *D. pulex* (Fig 5.2).

For *D. magna*, the body FAAs that caused the most statistical separation among diets were asparagine, aspartic acid, arginine, threonine, and tyrosine, whereas an almost entirely different set (alanine, isoleucine, methionine, serine and threonine) of body FAA created the separation in diets for *D. pulex*. These FAAs were entered into the nonparametric discriminant analysis (kNN) with a Wilks' lambda of  $F_{138} = 37.6$  for *D. magna* and  $F_{138} = 47.36$  for *D. pulex* with a  $p < 0.001$  for both models. For both species of *Daphnia*, eigenvalues for the first 3 canonical axis had a p-value  $< 0.0001$  (*D. magna* Canonical axis 1  $F_{30} = 37.6$ , Canonical axis 2  $F_{20} = 24.3$ , Canonical axis 3  $F_{20} = 12.08$ ; *D. pulex*  $F_{30} = 47.36$ , Canonical axis 2  $F_{20} = 30.68$ , Canonical axis 3  $F_{10} = 16.34$ ) with the first two axes explaining 91% and 84% of the total variation in *D. magna* and *D. pulex* respectively (Fig 5.2). The resulting kNN model performed extremely well as the cross-validated model had an overall misclassification rate of 2% for *D. magna* due to a misclassification between the HQQ diets and the low food quantity diets (1 mg C/L; Table 5.3). For *D. pulex*, the overall misclassification rate was 7% due to a low frequency of misclassification between of HQQ and C:N 15 and between low food quantities and C:P gradients (Table 5.4).

## 5.6 Discussion

Our hypothesis that the FAA content was affected by nutritional stress was supported as we found the concentrations of many FAA responded in a dose dependent manner with increasing dietary limitation in *Daphnia*. Generally body FAA composition was altered by the supply and demand of the limiting nutrient provided by the food resource. While similar trends in body FAA profiles were found between the two daphnid species, the body FAA profiles varied in magnitude for each nutritional stress. We found

excellent separation of body FAA in multidimensional space between diets for both *Daphnia* species and after further examination of confounding variables, we advocate the use of metabolic indicators to determine the nutritional status of animal consumers in field conditions.

Our results are consistent with the concept that diet modulates body FAA composition by altering the supply and demand for AA within *Daphnia*. The clearest example of this in our study was found in animals grown under P-limitation. *Daphnia* have relatively high P- requirements due to their fast growth rate, which is associated with their high content of P-rich ribosomal RNA (rRNA; Elser *et al.* 1996, 2000). Low P food should thus limit growth rates of *Daphnia* and create a surplus of non-P metabolites (such as FAA) needed for biomass production (Wagner *et al.* 2015). We found strong linear negative relationships between the majority of FAAs and the P-content within the food, with more FAAs being present in body tissues of animals consuming low P diets. This linear negative relationship between food P content and body FAA concentrations strongly indicates that daphnid growth was not directly limited by FAAs under these conditions. Elevated food C:P ratios and low P-intake would be more likely to create a low demand for FAA relative to the high supply of FAA provided in the animal's diet.

We also found evidence of FAA supply and demand interactions in N-limited *Daphnia*. These animals presumably received a low supply rate of AA from their consumption of N-poor algae. Given that the transcriptome (Miller *et al.*, 2010), metabolome (Bölling and Fiehn, 2005) and the fatty acid profiles (James *et al.*, 2011) of algae are altered under N deprivation, we speculate the total amino acid composition (free and proteins) are also altered under N deprivation. Changes to the algal total AA profiles

may explain why some FAA are decreased in *Daphnia* bodies (Val, Leu, Thr; Table 3) whereas others accumulate under N-deprived conditions (Met, Phe, Trp; Table 3). We suspect growth is limited by essential amino acids (Met, Phe, Trp, Val, Leu, Thr, His; Cowey & Forster 1971; Müller-Navarra 2008) that decreased with increasing N-limitation. To test this further, AAs could be supplemented to the *Daphnia* grown under different N-diets by adding these metabolites to the water (Koch et al., 2011; Wacker and Martin-Creuzburg, 2012). Assuming daphnids have access to dissolved AAs, this approach permits identification of the particular AA that limits growth. It would be particularly interesting to couple this supplementation approach to measurements of body FAA to see if animal metabolite composition changes in response to altered dissolved supplies.

Decreases in food quantity is well known to produce starvation (i.e., severe energy limitation) in consumers. When energy demands are not met by the dietary supply of carbohydrates, lipids, and proteins in food-quantity limited animals, other nutrient pools can be catabolized and utilized as energy sources. The breakdown of lipids occurs through beta oxidation and ultimately forms acetyl CoA, which feeds into the TCA cycle to continue the generation of energy for the organism. Similarly proteins can be catabolized and the resulting FAAs can be converted to energy after deamination through the TCA cycle (Salway, 2004). Under food quantity limitation, we found increased body content of many essential and nonessential amino acids in both daphnid species. This pattern of the accumulation of FAA under starvation conditions have also been observed in fish (Gillis and Ballantyne, 1996). We speculate the increase in FAA in food-quantity limited animals may be a form of temporary storage. The storage may prime animals for

the reinitiating of growth upon the consumption of more food by ensuring protein synthesis will not be limited by AAs.

We found diet induced differences in the body FAA content to vary between the two species of *Daphnia*. While these two species are morphologically similar and both found in lentic habitats (e.g., lakes and ponds), they are estimated to have diverged around two million years ago (Lehman et al., 1995). This long period of genetic separation provides ample time for the evolution of unique metabolic adaptations to nutrient-stress. These two species of *Daphnia* differ in their physiological responses (i.e growth rates and elemental composition) to dietary P-deficiency (Seidendorf *et al.* 2010; McFeeters & Frost 2011; Wagner & Frost 2012; Hood & Sterner 2014). These past studies have shown *D. pulex* grows faster and has a higher body P-content than *D. magna* when grown on P-rich algae (Wagner and Frost 2012; Hood and Sterner 2011). Our results show that dietary limitation also leads to contrasting biochemical responses between these two taxa. For most of the FAA and diet limitations, *D. pulex* exhibited a steeper response slope compared to *D. magna*. The steeper slope seen with lower food P content may be because *D. pulex* stores more FAA under nutrient deprived conditions, which allows them to rapidly reinitiate growth once food conditions change. It is less clear why *D. magna* would not employ this temporary storage mechanism, but perhaps has an unknown fitness cost. Future studies should examine links between growth rate, elemental and biochemical composition to FAA and other metabolites among zooplankton species to help reveal how metabolic functions relate to life history traits.

Despite differences in FAA profiles of two daphnid species, both were found to respond strongly and uniquely in their body FAA profiles to all three forms of limitation.

These responses produced considerable statistical separation among diet treatments in multivariate space. This type of statistical differentiation holds considerable utility for the determination of the nutritional status of field caught consumers. To assess the nutrient status of consumers, one would require species level identification and a database of metabolic responses to nutritional limitation (Frost et al., 2014). The advantages of HPLC as used here to measure FAA is its low mass requirement (0.5 mg) and relative simplicity, compared to other techniques such as proton NMR metabolomics (Wagner et al., 2015). FAA profiles may need to be coupled with other indicators of nutritional stress to more accurately characterize the type and intensity of nutritional limitation of consumers *in situ* (Frost et al., 2014). The addition of other nutritional indicators that respond in a monotonic nature would enhance the ability of the nutritional profile to distinguish among dietary stressors. With the addition of other intermediate metabolites, it would be easiest to examine the metabolome of nutrient stressed consumers in a targeted metabolomics approach using an HPLC for metabolite separation and a triple quadrupole mass spectrometer for metabolite identification (Wagner et al., 2015; Yuan et al., 2012).

Future use of metabolite composition to infer the nutritional state of an animal consumer will first require wide-scale testing of the robustness of the approach. Aquatic ecosystems can vary in ambient temperature, light, pH, dissolved ions (i.e., salinity), and presence of predators, all of which have well-known effects on zooplankton metabolism (Lampert and Sommer, 2007). Environmental conditions may alter nutrient-specific responses in aquatic consumers, which would greatly constrain the application of this approach (Frost et al., 2014). For example, low water temperatures may reduce body

FAA responses to poor food quality because the demand for nutrients scales with temperature (Persson et al., 2011). If so, conclusions about an animal's past diet based on its metabolite composition would only be valid for conditions that closely match that in the original laboratory setting. Metabolic responses to poor nutrition could also vary within an organism depending on its age or development stage, duration of exposure to the nutrient poor food, or the intensity of limitation (Wagner and Frost, 2012; Wagner et al., 2013). It is clear that there is a need to examine the sensitivity of nutritional profiles in zooplankton as many of these environment-diet interactions have yet to be considered.

We describe the body FAA profiles of two daphnid species and their responses to poor food quality and found many body FAA pools respond monotonically to diet. These body FAA responses varied between species appear related to differences in nutrient demands and utilization. Nonetheless, these adjustments to consumer metabolism with dietary limitation and the use of multivariate classification statistics have the potential to be useful the field of nutritional ecology for studies at scales from individual organisms to whole ecosystems.

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## 5.8 References

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## 5.9 Tables and Figures

**Table 5.1:** Regression of mass specific growth rate (MSGR d<sup>-1</sup>), and body composition (%C, %N, %P) of daphnid species (*D. magna*, and *D. pulex*) grown over a range of food quantity, phosphorus limited and nitrogen limited green algae. Bold indicates a significant regression (P < 0.05). All regressions are linear with the exception of \* indicates a logarithmic regression.

			MSGR d <sup>-1</sup>	%C	%N	%P
Food Limitation	<i>D. magna</i>	Slope	<b>0.586*</b>	<b>2.13*</b>	0.016	<b>-0.048</b>
		R2	<b>0.975</b>	<b>0.845</b>	0.024	<b>0.206</b>
		P-value	<b>P&lt;0.0001</b>	<b>0.038</b>	0.635	<b>0.044</b>
	<i>D. pulex</i>	Slope	<b>0.956*</b>	<b>2.52*</b>	<b>0.339</b>	<b>-0.056</b>
		R2	<b>0.897</b>	<b>0.868</b>	<b>0.501</b>	<b>0.41</b>
		P-value	<b>P&lt;0.0001</b>	<b>P&lt;0.0001</b>	<b>0.01</b>	<b>0.004</b>
Phosphorus Limitation	<i>D. magna</i>	Slope	<b>-4.0 E-04</b>	<b>-5.8E-03</b>	<b>-2.5E-04</b>	<b>-5.0E-04</b>
		R2	<b>0.832</b>	<b>0.691</b>	<b>0.640</b>	<b>0.738</b>
		P-value	<b>P&lt;0.0001</b>	<b>0.001</b>	<b>0.002</b>	<b>P&lt;0.0001</b>
	<i>D. pulex</i>	Slope	<b>-4.0 E-04</b>	<b>-6.8E-03</b>	<b>-1.8E-03</b>	<b>-6.0E-04</b>
		R2	<b>0.918</b>	<b>0.915</b>	<b>0.748</b>	<b>0.752</b>
		P-value	<b>P&lt;0.0001</b>	<b>P&lt;0.0001</b>	<b>P&lt;0.0001</b>	<b>P&lt;0.0001</b>
Nitrogen Limitation	<i>D. magna</i>	Slope	<b>-1.7E-02</b>	-0.011	<b>-0.140</b>	2.3E-3
		R2	<b>0.953</b>	0.010	<b>0.957</b>	0.076
		P-value	<b>P&lt;0.0001</b>	0.752	<b>P&lt;0.0001</b>	0.284
	<i>D. pulex</i>	Slope	<b>-6.5E-03</b>	<b>-0.054</b>	<b>-0.056</b>	-2.8E-3
		R2	<b>0.927</b>	<b>0.389</b>	<b>0.632</b>	0.199
		P-value	<b>P&lt;0.0001</b>	<b>0.030</b>	<b>0.002</b>	0.055

**Table 5.2:** Comparison of linear regression and slopes from each amino acid between two daphnid species (*D. magna*, and *D. pulex*) grown over a range of food quantity, phosphorus limited and nitrogen limited green algae. Bold indicates a significant regression ( $P < 0.05$ ), where both species have significant regression test for common slope using SMATR was used.

			Aspartic acid	Glutamic acid	Asparagine	Serine	Threonine	Alanine	Arginine	
Food Limitation	<i>D. magna</i>	Slope	<b>-1.82E-01</b>	<b>-1.81E-01</b>	<b>-1.79E-01</b>	<b>-7.44E-02</b>	2.09E-02	-5.34E-02	-2.4E-01	
		R2	<b>0.548</b>	<b>0.424</b>	<b>0.704</b>	<b>0.363</b>	0.003	0.037	0.093	
		P-value	<b>&lt;0.0001</b>	<b>0.002</b>	<b>&lt;0.0001</b>	<b>0.005</b>	0.817	0.415	0.191	
	<i>D. pulex</i>	Slope	-1.96E-01	-3.85E-01	-2.00E-01	-2.02E-01	-2.48E-01	<b>-4.99E-01</b>	<b>-2.06E+00</b>	
		R2	0.18	0.182	0.054	0.041	0.051	<b>0.208</b>	<b>0.503</b>	
		P-value	0.057	0.061	0.325	0.389	0.339	<b>0.043</b>	<b>&lt;0.0001</b>	
	Common slope									
	Phosphorus Limitation	<i>D. magna</i>	Slope	<b>1.26E-03</b>	<b>3.59E-03</b>	<b>1.76E-03</b>	<b>2.08E-03</b>	<b>3.09E-03</b>	<b>2.96E-03</b>	-4.56E-05
			R2	<b>0.782</b>	<b>0.816</b>	<b>0.884</b>	<b>0.856</b>	<b>0.856</b>	<b>0.757</b>	0
P-value			<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.966	
<i>D. pulex</i>		Slope	<b>2.31E-03</b>	<b>1.92E-03</b>	<b>4.91E-03</b>	<b>5.26E-03</b>	<b>6.07E-03</b>	<b>4.98E-03</b>	<b>9.26E-03</b>	
		R2	<b>0.885</b>	<b>0.727</b>	<b>0.883</b>	<b>0.867</b>	<b>0.888</b>	<b>0.872</b>	<b>0.789</b>	
		P-value	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	
Common slope		<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>			
Nitrogen Limitation		<i>D. magna</i>	Slope	<b>2.42E-02</b>	<b>2.10E-02</b>	<b>1.36E-02</b>	<b>1.34E-02</b>	<b>-6.29E-02</b>	-1.52E-02	<b>-2.09E-01</b>
			R2	<b>0.336</b>	<b>0.201</b>	<b>0.294</b>	<b>0.327</b>	<b>0.686</b>	0.095	<b>0.787</b>
	P-value		<b>0.007</b>	<b>0.047</b>	<b>0.014</b>	<b>0.008</b>	<b>&lt;0.0001</b>	0.187	<b>&lt;0.0001</b>	
	<i>D. pulex</i>	Slope	-8.93E-03	-1.90E-05	3.44E-03	-5.22E-03	<b>-3.52E-02</b>	-6.11E-03	<b>-3.07E-02</b>	
		R2	0.102	0	0.036	0.059	<b>0.891</b>	0.092	<b>0.399</b>	
		P-value	0.169	0.997	0.420	0.304	<b>&lt;0.0001</b>	0.195	<b>0.003</b>	
	Common slope						<b>No</b>		<b>No</b>	

			Tyrosine	Methionine	Valine	Tryptophan	Phenylalanine	Isoleucine	Leucine	
Food Limitation	<i>D. magna</i>	Slope	<b>-2.13E-01</b>	<b>-1.89E-01</b>	<b>-2.03E-01</b>	<b>-1.84E-01</b>	<b>-2.57E-01</b>	<b>-2.05E-01</b>	<b>-3.94E-01</b>	
		R2	<b>0.458</b>	<b>0.62</b>	<b>0.294</b>	<b>0.643</b>	<b>0.353</b>	<b>0.350</b>	<b>0.337</b>	
		P-value	<b>0.001</b>	<b>&lt;0.0001</b>	<b>0.013</b>	<b>&lt;0.0001</b>	<b>0.006</b>	<b>0.006</b>	<b>0.007</b>	
	<i>D. pulex</i>	Slope	<b>-9.78E-01</b>	<b>-7.52E-01</b>	<b>-1.09E+00</b>	-3.47E-01	<b>-1.50E+00</b>	<b>-1.16E+00</b>	<b>-2.53E+00</b>	
		R2	<b>0.422</b>	<b>0.414</b>	<b>0.613</b>	0.103	<b>0.534</b>	<b>0.645</b>	<b>0.635</b>	
		P-value	<b>0.002</b>	<b>0.002</b>	<b>&lt;0.0001</b>	0.167	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	
	Common slope		<b>No</b>	<b>No</b>	<b>No</b>		<b>No</b>	<b>No</b>	<b>No</b>	
	Phosphorus Limitation	<i>D. magna</i>	Slope	<b>2.93E-03</b>	<b>3.88E-03</b>	<b>3.60E-03</b>	<b>-6.80E-04</b>	9.24E-05	<b>4.55E-03</b>	<b>5.01E-03</b>
			R2	<b>0.684</b>	<b>0.881</b>	<b>0.859</b>	<b>0.344</b>	0.002	<b>0.878</b>	<b>0.835</b>
P-value			<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.007</b>	0.862	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	
<i>D. pulex</i>		Slope	<b>7.07E-03</b>	<b>5.80E-03</b>	<b>4.00E-03</b>	<b>1.34E-03</b>	<b>5.75E-03</b>	<b>2.61E-03</b>	1.25E-03	
		R2	<b>0.858</b>	<b>0.885</b>	<b>0.806</b>	<b>0.895</b>	<b>0.782</b>	<b>0.703</b>	0.140	
		P-value	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.105	
Common slope		<b>No</b>	<b>No</b>	<b>Yes</b>	<b>No</b>		<b>No</b>			
Nitrogen Limitation		<i>D. magna</i>	Slope	<b>5.63E-02</b>	1.87E-03	<b>-3.17E-02</b>	<b>1.16E-02</b>	<b>5.84E-02</b>	<b>-3.23E-02</b>	<b>-6.71E-02</b>
			R2	<b>0.792</b>	0.006	<b>0.328</b>	<b>0.236</b>	<b>0.626</b>	<b>0.378</b>	<b>0.444</b>
	P-value		<b>&lt;0.0001</b>	0.736	<b>0.008</b>	<b>0.03</b>	<b>&lt;0.0001</b>	<b>0.004</b>	<b>0.001</b>	
	<i>D. pulex</i>	Slope	<b>3.16E-02</b>	<b>2.20E-02</b>	-7.00E-04	3.32E-02	<b>8.21E-02</b>	<b>-4.33E-02</b>	<b>-9.46E-02</b>	
		R2	<b>0.398</b>	<b>0.285</b>	0	0.016	<b>0.563</b>	<b>0.694</b>	<b>0.797</b>	
		P-value	<b>0.003</b>	<b>0.015</b>	0.926	0.591	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	
	Common slope		<b>No</b>				<b>No</b>	<b>Yes</b>	<b>Yes</b>	

**Table 5.3:** Leave one out cross validation of nonparametric discriminant analysis (kNN) for *D. magna*.

From Treatment	Number of Observations classified into treatment							Percent correctly classified
	HQQ	C:N15	C:N24	C:P500	C:P800	1mg C/L	0.5mg C/L	
HQQ	13	0	0	0	0	2	0	87
C:N15	0	5	0	0	0	0	0	100
C:N24	0	0	5	0	0	0	0	100
C:P500	0	0	0	5	0	0	0	100
C:800	0	0	0	0	5	0	0	100
1 mg C/L	0	0	0	0	0	5	0	100
0.5 mg C/L	0	0	0	0	0	0	5	100

**Table 5.4:** Leave one out cross validation of nonparametric discriminant analysis (kNN) for *D. pulex*.

From Treatment	Number of Observations classified into treatment							Percent correctly classified
	HQQ	C:N15	C:N24	C:P500	C:P800	0.25mg C/L	0.15mg C/L	
HQQ	14	1	0	0	0	0	0	93
C:N15	0	5	0	0	0	0	0	100
C:N24	0	0	5	0	0	0	0	100
C:P500	0	0	0	4	1	0	0	80
C:800	0	0	0	0	5	0	0	100
0.25mg C/L	0	0	0	0	0	5	0	100
0.15mg C/L	0	0	0	0	0	1	4	80

**Figure captions:**

**Figure 5.1:** Example of linear relationship between diet and FAA content. 1a. Example of different slopes for the relationship between  $\mu\text{g}$  of asparagine per dry mass and C:P content of the algae. Open circles are *D. pulex* closed circles *D. magna*. 1b. Example of similar slopes for the relationship between  $\mu\text{g}$  of valine per dry mass and C:P content of the algae. Open circles are *D. pulex* closed circles *D. magna*.

**Figure 5.2:** Free amino acid discriminate analysis of day 6 *Daphnia*. 2A. *D. magna* eating HQQ (●) C:P 500(▲) C:P 800(Δ) C:N 15(■) C:N 20(□) 1mgC/L(◆) 0.5mgC/L(◇) with 95% confidence intervals. 2B *D. pulex* eating HQQ (●) C:P 500(▲) C:P 800(Δ) C:N 15(■) C:N 20(□) 0.25mgC/L(◆) 0.15mgC/L(◇) with 95% confidence intervals.

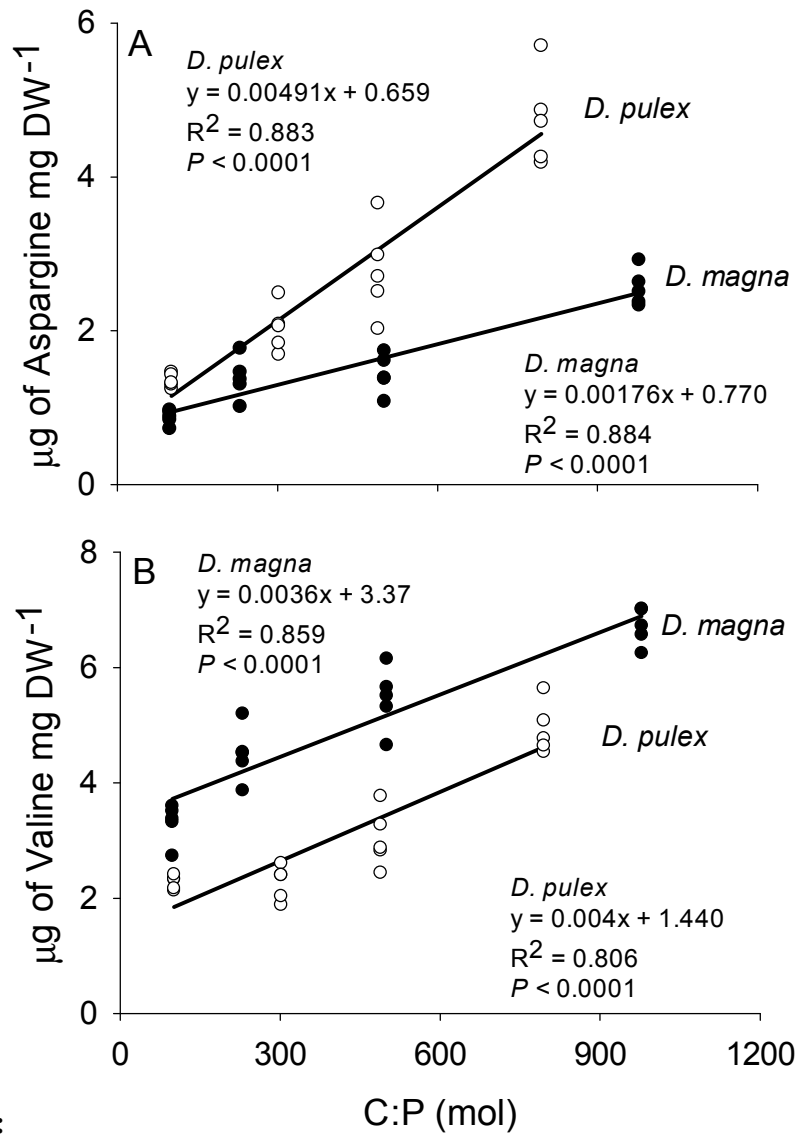
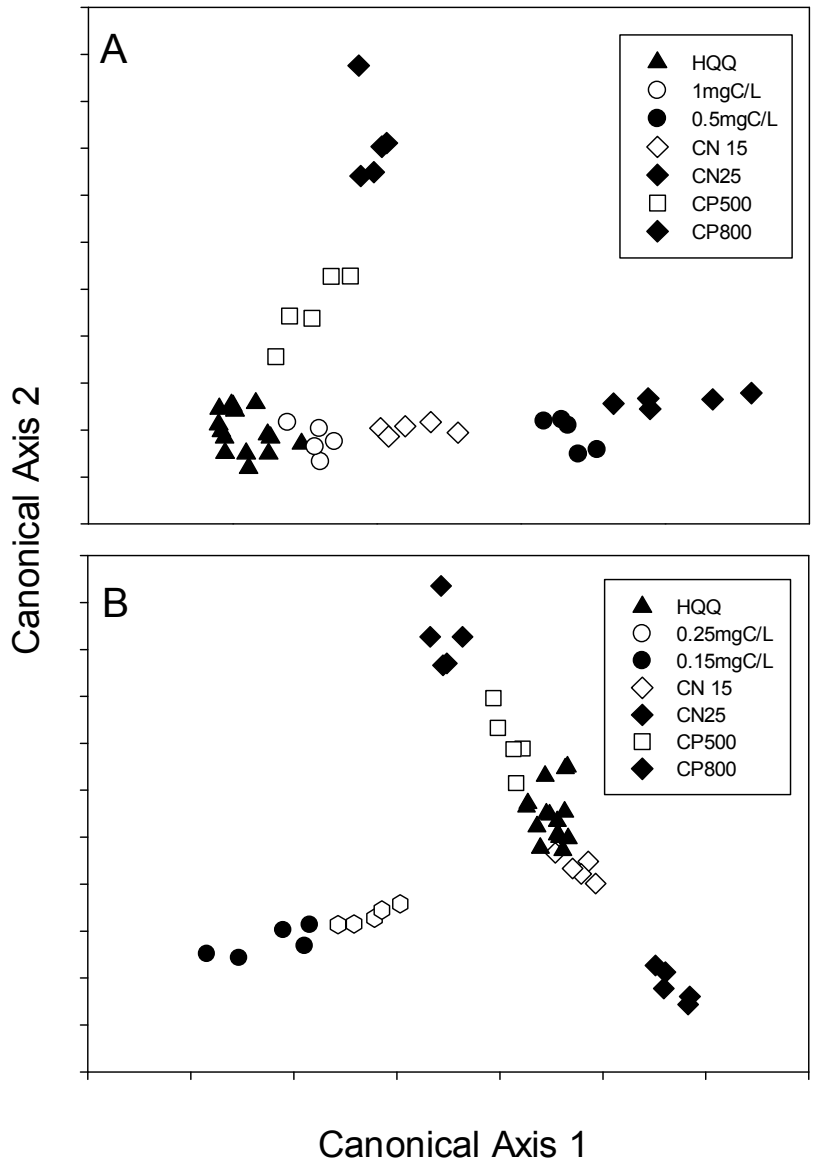


Fig 1:



**Fig 2:**

## 5.10 Supplementary

### Effects of food quality on free amino acid metabolism in two aquatic zooplankters

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**Table S5.1:** Elution Gradient used to separate free amino acids

Time (min)	Flow rate	% Mobile Phase A
0	1 ml/min	100
6	1 ml/min	100
17	1 ml/min	85
25	1 ml/min	80
33	1 ml/min	70
45	1 ml/min	60
61	1 ml/min	20
67	1 ml/min	0
70	1 ml/min	0
71	1 ml/min	100
80	1 ml/min	100

**Table S5.2** Carbon, phosphorus, and nitrogen diet ranges for both *D. magna* and *D. pulex*

Range	Units	<i>D. magna</i>	<i>D. pulex</i>
Food quantity range	mg C/L	4	2
		2	0.5
		1	0.25
		0.5	0.15
Phosphorus quality range	C:P (mol)	98	100
		230	300
		500	490
		980	800
Nitrogen quality range	C:N (mol)	6	6
		10	10
		16	16
		25	30

## CHAPTER 6

### General Discussion

As consumers frequently encounter nutritional limitation in aquatic ecosystems, it is imperative to accurately assess their nutritional status. However current methods for assessing consumer nutritional state usually rely on inferences that are generally imprecise, not specific to a particular type of nutrient, and are not applicable to individual animals *in situ*. These current methods to assess the nutrient status of consumers (e.g. elemental threshold ratios, and growth bioassays) have difficulty or cannot distinguish between co-varying nutritional components. For example, a change in the P-content of algal food sources is often accompanied by changes in other essential nutritional components (e.g., sterols; Boersma 2000). This covariation creates uncertainty about which food component is the primary limiting nutrient (Boersma 2000) and clouds easy interpretation of experimental and field results. Given the inability of current techniques to assess the nutritional status of consumers, this dissertation focused on novel techniques to investigate nutrition in ecology.

In my initial research of the nutrition literature in other fields of biology, it became evident that the treatment of nutrition in the field of ecology was far behind other fields such as nutrigenomics. Nutrigenomics examines the effects of nutrients (many micronutrients or secondary metabolites) on gene expression and metabolomics of mainly humans and livestock (van Ommen and Stierum 2002). This field of research specifically attempts to answer questions such as: What are the biomarkers for nutrient -x? What genes are differentially expressed when nutrient -x is consumed? and How does genetic

polymorphism affect nutrient metabolism? While these questions are very similar to the kinds of questions nutritional ecologists are currently asking (Jeysingh et al 2011; Frisch et al. 2014), the methods applied to these questions by ecologists are unlikely to give a nutrient-specific answer. With modern molecular tools, macronutrient-specific genes (Boer et al. 2003) and metabolites (Boer et al. 2010) have been identified in yeast. Similarly, many nutrient responses in metabolism from plants (Bölling and Fiehn 2005), bacteria (Hua et al. 2004) and humans (O'Sullivan et al. 2011) have been identified. Altogether, the promising results of metabolic changes caused by nutrient stress seen in other fields of biology suggested these approaches would have value for the study of aquatic invertebrates. Here I attempted to adapt the methods to examine the nutrient metabolism in *Daphnia*.

This dissertation represents a first attempt of nutritional profiling in the aquatic consumer *Daphnia*. After examining other scientific fields of nutrition, it became apparent biomarkers for nutritional stress could be identified (Boer et al. 2003, Boer et al. 2010). This ultimately led to the concept of the nutritional indicator, which in later studies became the nutritional profile. The nutritional profile represents a set of metabolic changes that when examined would indicate if the consumer is experiencing nutritional stress and what type of stress (Frost et al. 2014). The nutritional profile could potentially be used to answer ecological questions, such as how often are consumers co-limited? Or are population crashes caused by low food quantity or low quality? As answers to these questions are central in understanding biogeochemical cycles and other ecological processes, the ability to identify limiting nutrients with increased accuracy would revolutionize the field of nutritional ecology. My dissertation examined the nutrient

specificity of a P indicator (alkaline phosphatase activity), as well as the nutritional profile of the whole metabolome, and free amino acids. The next few paragraphs I discuss the main findings of each chapter.

## **6.1 Summary of experimental chapters**

My first study examined changes in alkaline phosphatase activity (APA) in response to multiple elemental limitation. This enzyme is responsible for cleaving phosphate mono-esters from organic molecules thus allowing for phosphate to be transported from the gut, through the gut lining, and into cells. Initial studies performed on *Daphnia* displayed promising results with increases of APA when daphnids were P-limited (McCarthy et al. 2010, Wojewodzic et al. 2010, Elser et al. 2010) However, none of these studies examined how APA changed under other forms of elemental limitation, thus the question still remained whether APA was a nutrient-specific indicator of P-status. By examining APA at different ages and under different types of nutritional stress, we found AP activity was not a suitable indicator of P-limitation as it varied between ages, species, and increased under C-, N-, and P-limitation. As whole body homogenate levels of AP proved to be a nonspecific indicator of nutrient limitation, I decided to examine changes in consumer metabolism on the whole metabolome level.

In the third chapter, I examined the metabolomic response to multiple forms of nutritional stress in *Daphnia magna*. As I wanted to ensure the metabolomic response was nutrient specific in natural environments, the metabolites from two common environmental stressors (bacterial infection, and salt stress) were also examined. I found unique metabolic profiles in response to both nutritional and environmental stressors. Our results confirmed previous findings suggesting that metabolites change in response to the supply

of nutrients from the diet with respect to demand for those nutrients within the animal (Boer et al. 2010), providing a nutrient-specific indicator of environmental and dietary stress. While powerful, metabolomic profiling can be costly and time consuming. Consequently, I next focused on a subset of the metabolome (amino acids) to streamline our nutritional profiling process making it easier for use in ecology.

As most of the identified metabolites from our metabolomic study were free amino acids (FAA), I decided to further examine FAA metabolism in response to various intensities of nutritional limitation and also between species. To examine free amino acid metabolism, I grew two species of *Daphnia* (*D. magna*, and *D. pulex*) and fed them different levels of limitation by three nutritional stressors (low phosphorus, low nitrogen and low food quantity). After extracting and analyzing the FAA with an HPLC, we found in general daphnids fed P-limited, and low food quantities of algae increased their FAA body content, whereas N-limited daphnids had increases in some FAA and decreases in others. These changes in FAA body content were caused by changes between the supply of amino acids and the demand of the amino acids within the animal's body. I also found differences in FAA metabolism between the two species examined with *D. pulex* accumulating more FAA when fed P-limited and low food quantities. Thus, amino acids as examined here appears to be a successful indicator of nutritional limitation in *Daphnia*, albeit a species-specific indicator.

## **6.2 Significance and future directions**

The main contribution of this research is that it provides the initial step in the development of the nutritional phenotype. I show here how this phenotype can be used as a bioindicator profile by providing evidence that a particular form or intensity of dietary

stress can produce metabolic changes in *Daphnia*. While I do not claim to have fully developed the nutritional profiling approach, I have provided the framework needed to develop this important tool in future research. Nutritional indicators could be identified based on the physiological responses (i.e., changes in metabolic pathways) of animal consumers to nutrient limitation (DeMott *et al.* 1998, Frost *et al.* 2005, Mutch *et al.* 2005). Specifically the molecular and biochemical pathways involved in nutrient uptake, incorporation and mobilization would represent a potential source of novel indicators (van Ommen and Stierum 2002). During the initial phase, the identification of nutritional indicators would clearly benefit from emerging information rich fields (e.g., transcriptomics, proteomics, metabolomics) due to their ability to profile systematic changes in consumer metabolism in response to nutrient deprivation (van Ommen and Stierum 2002). Profiling or descriptive shotgun studies of consumer nutrition stress can capture whole-scale responses of organisms to different nutritional stresses (Tanzer *et al.* 2003). Characterization of such system-wide responses has permitted the isolation and use of specific indicators that are intimately connected to different nutrients of interest in studies of plants and microbes.

Response variables could be generated from all ‘omic scales including transcriptomics (Boer *et al.* 2003, Jeyasingh *et al.* 2011), proteomics (Kolkman *et al.* 2006) and metabolomics (Boer *et al.* 2010, Wagner *et al.* 2015). By adapting conceptual and analytical approaches from nutritional science we could improve the ability to assess dietary limitation in zooplankton consumers in aquatic ecosystems. Nutrigenomics uses ‘omics (e.g., transcriptomic and metabolomics, van Ommen & Stierum 2002) to study how metabolic pathways in humans and other organisms relate to different diets and

lifestyle choices (van Ommen & Stierum 2002). These ‘omic approaches have identified hundreds to thousands of molecular responses that are nutrient-specific responses and provide insight into regulatory control and physiological effects (Boer et al. 2010). In general, the transcriptome is a rich source of information on an organism’s nutritional state as seen in studies of model organisms including *Escherichia coli* (Hua et al. 2004), *Saccharomyces cerevisiae* (Boer et al. 2003; Wu et al. 2004), *Arabidopsis thaliana* (Morcuende et al. 2007) and *Daphnia pulex* (Jeyasingh et al. 2011, Roy Chowdhury et al. 2015). For example, a comprehensive study of yeast (*S. cerevisiae*), found that 484 genes responded to low supplies of glucose, nitrogen, phosphorus and sulfur in a nutrient specific manner (Boer et al. 2003). Many of these nutrient-responsive genes are involved in regulating nutrient metabolism by affecting nutrient fluxes and pools (e.g., increases in amino acid transporters), and mobilizing stored nutrients mainly through increased catabolism (Boer et al. 2003). Similar results have been found in proteomic studies of yeast where nitrogen-limitation increased the relative abundance of proteins responsible for nitrogen acquisition (Kolkman et al.2006). Glucose-limited yeast increase the abundance of proteins that generate alternative carbon sources through beta lipid oxidation and oxidative phosphorylation pathways. Metabolomic studies of nutrient-stressed organisms have also identified an array of nutrient-specific responses. For example, the metabolite composition in yeast changes strongly and uniquely in response to glucose-, N- and P-limitation (Boer et al. 2010). Specifically, pyruvate, glutamine and adenosine triphosphate were found to be nutrient-specific growth limiting metabolites in glucose-, N- and P-limited yeast, respectively (Boer et al. 2010), which means they each decreased substantially in cells experiencing each type of nutrient limitation. In humans,

metabolomic analyses have been used to discriminate between participants consuming diets rich in fruits and vegetables compared with diets rich in red meat and low in fruit and vegetables (O'Sullivan 2011). Similar nutrient-specific metabolomic responses has also been displayed in *Daphnia magna*, with complete separation in multivariate space between both nutritional (low food, phosphorus and nitrogen limited) and environmental stress (high salt media) treatments (Wagner et al. 2015). If systematically applied to individuals experiencing different forms of stress, 'omic characterization of animals, has the potential to greatly expand our knowledge of the molecular biology of nutritional metabolism and, while doing so, provide a thorough depiction of nutritional phenotypes. While large quantities of data are produced by '-omic' approaches, data reduction techniques would be employed to identify unique responses to each nutritional stressor. Data reduction techniques, including multivariate statistics (principle components analysis), multivariate classification models (discriminant analysis, nearest neighbour) and non-linear black box modeling techniques (artificial neural network), are typically employed to identify unique stress indicators (Lancashire *et al.* 2009). For example, identification of nutrient-specific changes from system wide responses could follow the approach described by Boer *et al.* (2003, 2010), who used cluster analysis to identify unique gene expression responses to specific forms of nutrient limitation. Another example includes discriminating between low and high meat based diets in humans (O'Sullivan et al. 2011). The metabolites responsible for discriminating between diets included glycine, phenylacetylglutamine, and actetoacetate were significantly higher in low meat diets, whereas concentrations of trimethylamine *N*-oxide (TMAO), *O*-acetylcarnitine, and *nm*-dimethylglycine were present in higher concentrations in high

meat, low fruit diets diets (O'Sullivan *et al.* 2011). Once 'omic data have been reduced through multivariate statistics, a more limited sets of response variables have been identified, more cost effective and quantitative approaches can be developed. For example, targeted and quantitative gene expression, of lower cost, can be developed using data generated by transcriptomics (Wagner *et al.* 2013). Specifically, sodium phosphate co-transporter genes have been linked to P-limitation in rainbow trout and provides strong evidence of this form of nutrient limitation in fish growing in aquaculture (Sugiura *et al.* 2003). Another example includes the increased gene expression of digestive proteases in response to cyanobacterial food sources (Schwarzenberger *et al.* 2010) and to increase the expression of genes underlying the eicosanoid synthesis pathway in response to higher fatty acid content of their diet (Schlotz *et al.* 2012). Once a putative nutritional profile is generated from reduced omic data they profile would need to be validated before being applied to field conditions.

Future use of metabolic composition to infer the nutritional state of an animal consumer will first require wide-scale testing of the robustness of the approach. Aquatic ecosystems can vary in ambient temperature, light, pH, dissolved ions (i.e., salinity), and presence of predators, all of which have well-known effects on zooplankton metabolism (Lampert and Sommer 2007). Environmental conditions may alter nutrient-specific responses in aquatic consumers, which would greatly constrain the application of this approach (Frost *et al.* 2014). For example, low water temperatures may reduce body metabolic responses to poor food quality because the demand for nutrients scales with temperature (Persson *et al.* 2011). If so, conclusions about an animal's past diet based on its metabolite composition would only be valid for conditions that closely match that in

the original laboratory setting. Metabolic responses to poor nutrition could also vary within an organism depending on its age or development stage, duration of exposure to the nutrient poor food, or the intensity of limitation (Wagner and Frost 2012; Wagner *et al.* 2013). It is clear that that there is a need to examine the sensitivity of nutritional profiles in zooplankton to all of these important environmental variables. Although this task appears daunting, I believe the nutritional profile will be robust to confounding variables. For example I would expect under changes in temperature metabolism would be altered, however the nutritional signal should still be apparent, just the magnitude of the response variables may be different. The nutritional profile could possibly adapt a ratio approach where the response variable of interest is compared to static response which would generate a ratio that could be used to determine if the consumer is nutritionally stressed. This approach is similar to current approaches within ecological stoichiometry as defined at the threshold elemental ratio (Frost *et al.* 2006), and is also similar to the house keeping gene standardization approaches used in molecular biology.

Nutritional profiling, as described here, holds considerable promise in assessing the health of animal consumers in aquatic ecosystems. Current efforts to understand the consequences of external nutrient loading on aquatic foodwebs, and particularly consumer communities, are constrained by inadequate methods to assess the *in situ* nutritional state of organisms. Other aspects of global change include changing weather patterns, atmospheric deposition of nutrients, increased areas of agricultural and urban land use and industrial (or mining) pollution. All of these human-related drivers can alter the balance of elements entering into lakes and have potentially strong effects on the nutrition of plankton communities (Schade *et al.* 2005). The development and testing of

nutritional profiling will add to our current abilities to detect, understand and mitigate these negative effects of human development. Consequently, this dissertation provided the framework for moving emerging, highly powerful molecular techniques from laboratories into the hands of working environmental scientists to better understand nutrient cycling in aquatic ecosystems.

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