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Gene expression under multiple stressors in Daphnia pulex

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by

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Gene expression under multiple stressors in *Daphnia pulex*

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. DECLARATION OF CO-AUTHORSHIP

I hereby certify that thesis contains materials from joint research under the supervision of Dr. Melania Cristescu (University of Windsor) and Dr. Norman Yan (York University and University of Windsor). Chapter 2 contains material from an article titled "An integrated multi-disciplinary approach for studying multiple stressors in freshwater ecosystems: *Daphnia* as a model organism" which has been published in *Integrative and Comparative Biology*. This article was co-authored by Altshuler I, Demiri B, Xu S, Constantin A, Yan N, and Cristescu ME.

Chapter 4 contains material from a manuscript titled "The evolutionary history of the sarco(endo)plasmic calcium ATPase". This manuscript was co-authored by Altshuler I, Vaillant JJ, Xu S, and Cristescu ME. In all chapters the main ideas, experimental design and data analysis were performed by the author. The contributions of co-authors were through guidance with field and laboratory work and revising manuscript drafts.

I certify that I have properly acknowledged the contribution of other researchers to my thesis and have obtained permission from each of the coauthors to include the above material in my thesis.

II. DECLARATION OF PREVIOUS PUBLICATION

This thesis includes two original papers that have been previously published/submitted for publication in peer-reviewed journal.

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ABSTRACT

Freshwater organisms are constantly under pressure from an array of stressors with complex affects. In this thesis I first review the interactive effects on *Daphnia pulex* of three prevalent anthropogenic stressors: climate change, calcium decline, and toxic metal exposure. Then, I examine gene expression levels of five *Daphnia* genes related to carapace building and calcium homeostasis to understand the effects and interaction of low calcium and predator presence. Finally, I use phylogenetic reconstruction to explore the evolutionary history of one of the tested genes, the Sarco(endo)plasmic Calcium ATPase. My results indicate that both stressors tested and their interaction affects the expression patterns of all the tested genes, often in surprising ways. The results from the phylogenetic reconstruction suggest that many ancient and recent gene duplication events have shaped the evolution of this gene.

DEDICATION

To my Mom, Grandmother, and Great-grandma

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CHAPTER I

GENERAL INTRODUCTION

1.1 Introduction

Globalization and the overharvesting of resources precipitated by explosive population growth have put intense pressures on ecosystems across the globe (Sala et al. 2000). This multitude of stressors includes acid deposition, toxic pollutant run off, climatic change, and invasive species. Furthermore, stressors in this thesis are defines as any environmental fluctuations that lower the fitness of an organism due to the requirement of the organism to expend energy to maintain homeostasis.

The complex interactions and effects of stressors are unpredictable. Single stressor studies often fail to predict the interactions of multiple stressors, as they can have surprising non-additive effects on populations and often on whole ecosystems (Christensen et al. 2006). Thus, it is critical to undertake studies that attempt to improve understanding of the effects of the combinations of stressors.

Organisms need to acclimatize and then adapt swiftly to this changing environment in order to survive. Organismal changes in response to stress that occur at the molecular level can be characterized based on their permanence, i.e. these changes can be temporary, semi-permanent, or permanent (Fig. 1). Organisms acclimate to a stressor with temporary molecular changes. These are physiological changes evoked by

the homeostatic regulatory response of the individual. The mechanisms leading to these physiological changes include changes in gene expression (Podrabsky and Somero, 2004) and translation control (Liu et al, 2012). These changes are temporary, i.e. they are reversible during the organism's lifetime if the stressor is removed, and they do not alter the inheritance of offspring. Semi-permanent molecular changes result from exposure to environmental fluctuations that are passed on through transgenerational epigenetic inheritance (Youndson and Whitelaw, 2008). The (F1) offspring inherit these changes, which include DNA methylation and histone modifications, even if the initial stress disappears (Youndson and Whitelaw, 2008; Ho and Burggren, 2009). This adaptive strategy may well provide information about future environmental risks that offspring might face (Agrawal, 1999). For example, in *Persicaria maculosa* (a perennial plant), offspring of individuals that were reared under low light allocated more resources to shoot growth compared to high light individuals (Sultan, 1996). Semi-permanent transgenerational inheritance does occur in animals. For example, female daphniids that are exposed to insect predators produce neonates (F1) with defensive structures (neck teeth) even if the predation stress is removed (Agrawal, 1999). In the (F2) offspring this phenomenon is still prevalent though the signal is diminished; moreover, the individuals whose grandmothers and mothers have been exposed to predation stress produced more prominent neck teeth compared to ones whose only mothers have been exposed (Agrawal, 1999).

Finally, the permanent molecular response to stress involves the fixation of advantageous mutation under selection. Most populations that maintain a relatively stable demography harbor significant genetic variation comprised of non-neutral mutations in the coding regions, the promoter, or involving variation in gene copy number. A permanent molecular response constitutes natural selection acting on mutations in the genome giving the organism a fitness advantage under a particular stressor(Chapin et al, 1993; Agrawal and Whitlock 2010).

In this thesis I report the temporary molecular changes involved in the acclimatization of an organism to two stressors. Specifically, I use the freshwater crustacean, *Daphnia*, as a model organism to understand the effects of low calcium stress and predator presence on gene expression.

In the second chapter I review the literature on the effects of multiple stressors on *Daphnia* and propose it as an ideal organism to study the effects of multiple stressors in aquatic ecosystems. Since it is particularly difficult to study the combined effect stressors, this approach requires a coordinated effort from multiple sub-disciplines of biology, and it requires a model organism such as *Daphnia*, an organism which has been widely adopted across biological subdisciplines. As a keystone species, *Daphnia* serves to link primary production with higher trophic levels. Additionally, it has an extremely 'ecoresponsive' genome (Colbourne et al., 2011) characterized by high mutation rates (Xu et al., 2012) and it has a flexible breeding system involving the transition between asexual and sexual phases (Hebert et al. 1993).To make the case for using *Daphnia* as a

model species, I review the joint and separate effects of natural and three anthropogenic stressors-climatic change, calcium decline, and metal contaminants - on daphniids. I propose that integrative approaches marrying various subfields of biology, specifically genetics, ecology, toxicology, and evolution can advance our understanding of the combined effects of stressors. I propose biologists should be measuring multiple responses at several levels of biological organization, from molecules to natural populations.

In my third chapter I consider the individual and combined effects of a natural and an anthropogenic stressor on the gene expression of *Daphnia pulex*. More specifically, I investigate the effects of calcium decline in combination with exposure to predator kairomones. Calcium, an essential element for all biota, has been drastically declining in soft water lakes of the Canadian Shield and other parts of eastern temperate North America and Northern Europe (Keller et al. 2001; Jeziorski et al. 2008). Because crustaceans, including *Daphnia*, have high calcium demands (Waervagen 2002), they are expected to be among the first species impacted by this sharp calcium decline. Here I propose that low calcium stress may interact synergistically with predation stress. Invertebrate predators, such as the larvae of the phantom midge, *Chaoborus,* release kairomones that trigger phenoplastic changes in *Daphnia*. These changes, which alter the phenotype but not the genotype, include increased stability of the carapace (Laforsch et al. 2004) and formation of neck teeth (Tollrian 1993), both of which reduce post-capture, ingestion efficiency. I consider whether the stress of calcium decline may

indirectly and negatively impact *Daphnia*'s survival since calcium may well be needed for the formation of the defensive structures. This chapter explores how gene transcription levels change with varying calcium concentrations while in the presence or absence of predator kairomones. The genes I selected are part of calcium homeostasis and carapace building pathways, and thus are logical candidates for altered gene expression by the selected stressors. Transcriptional response is often observed prior to phenotypic or population level changes, therefore, by looking at changes in gene transcription I hoped to detect early stress in organisms.

Finally, in my fourth chapter, I take a closer look at the evolutionary history of one of the candidate genes that I explored in chapter three, the sarco(endo)plasmic calcium ATPase (SERCA). This protein maintains calcium homeostasis in cells by actively pumping Ca^{2+} into the sarco(endo)plasmic reticulum (East et al. 2000). Although the molecular function of SERCA has been extensively explored, the evolutionary history of this group of proteins remains unknown outside the vertebrates. I use amino acid sequences from many organisms across the eukaryotic kingdom to conduct phylogenetic reconstructions. This approach allows the exploration of the evolutionary history of the SERCA related genes and facilitates the extension of future gene expression studies across other eukaryotic taxa. Understanding the evolutionary history of this gene family can greatly facilitate further gene expression and comparative studies since any recent gene duplication events could impact the interpretation of expression data.

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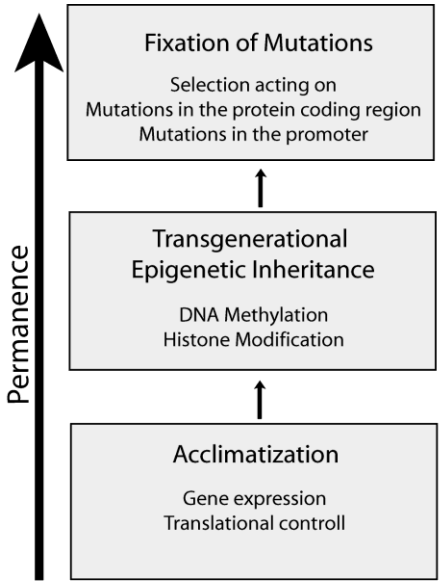

Figure 1.1: Schema representing the gradient of permanence of different ways that an organism responds to stress. Acclimatization is a temporary alteration to restore homeostasis and is not inherited by offspring. Transgenerational epigenetic inheritance is semi-permanent as it can span several generations. Fixation of advantageous mutations via natural selection constitutes a permanent response to a stressor.

CHAPTER II

AN INTEGRATED MULTI-DISCIPLINARY APPROACH FOR STUDYING MULTIPLE STRESSORS IN FRESHWATER ECOSYSTEMS: *DAPHNIA* AS A MODEL ORGANISM

2.1 Introduction

In the age of global climatic change, increased industrialization and over-exploitation of natural resources, many ecosystems, and the biodiversity they support, are at a tipping point from anthropogenic stress (Sala et al. 2000). The Millennium Ecosystem Assessment leaves no doubt that global ecosystems are in peril, but we still have far to go with the urgent task of understanding how these rapid environmental changes are going to impact ecosystem functioning and ecological services, as well as the future of our own species. While many studies have looked at the effects of individual stressors and their results have provided a solid foundation of our understanding of how specific stressors affect natural systems, few studies have considered their combined effects (Schindler 2001; Vinebrooke et al. 2004; Coors and De Meester 2008; Yan et al. 2008). There is an urgent need to understand the effects of multiple stressors, since individual stressors now commonly co-occur, often with unexpected and complex interactions (Christensen et al. 2006). The combinations of stressors could have: (1) additive effects—whereby the effect of the mix of stressors equals the sum of the effects of individual stressors, (2) synergistic effects—whereby the combined effect of two or more stressors is greater than the sum of the individual

stressors, or (3) antagonistic effects—whereby the combined effect of the stressors is less than the summed effects of individual stressors (Folt et al. 1999). Therefore, we cannot necessarily predict with confidence what the combined effects of multiple stressors will be simply by looking at the effects of single stressors (Christensen et al. 2006); the effects of multiple stressors must be specifically assessed.

Freshwater ecosystems are among the most stressed of global environments, bombarded with all classes of stressors, including changes in water chemistry (acidification, eutrophication, metal contaminants, and decline in calcium), changes in the physical environment (changes in land use, climatic change, and increase in UV radiation), the introduction of invasive species (zebra mussels, sea lamprey, and spiny water flea), and over-harvesting of resources (over-fishing). I argue that the most effective way to understand the combined effect of these stressors is to use a multi-disciplinary approach that integrates knowledge derived from ecological, physiological, toxicological, evolutionary, and genetics data, and by following ecological and biological responses at all levels of biological organization from molecules to natural populations and communities. One of the most important challenges that an integrative approach has to overcome is the transferability of data and ideas between disciplines and multiple biological levels and the application of the developed models beyond singular keystone species and ecosystems and across large sets of multiple stressors. There is a pressing need for selecting model organisms that play a critical role in maintaining the stability of the aquatic ecosystems, while also being both ecologically sensitive (responsive) and

geographically ubiquitous. I propose *Daphnia*, a freshwater microcrustacean, as one of the best model organisms for studying multiple stressors in limnetic ecosystems. *Daphnia* has been used as a model organism in studies of evolution, ecology, toxicology, and genomics (Colbourne et al. 2011). More recently, it has been suggested as a model species for ecological genomics, transcriptomics, and ecotoxicogenomics—fields that aim to understand how biotic and abiotic stressors, as well as evolutionary and life-history characteristics, affect gene expression and other biological and ecological changes (Watanabe et al. 2008; Orsini et al. 2011). Such studies can be used to understand how multiple stressors affect the organisms on a genetic and biochemical level. For example, microarray experiments can be used to screen the patterns of gene expression of thousands of genes in animals exposed to a particular stressor or to a combination of stressors. In addition, evolutionary and ecological studies have examined how *Daphnia* populations adapt to changing environmental conditions such as climatic warming (Mitchell and Lampert 2000; Van Doorslaer et al. 2010). This broad range of approaches can be used in integrative ways to shed light on the effects of multiple stressors on aquatic organisms.

Here, I review the ecology and evolutionary history of *Daphnia* that justifies its increased use in large-scale ecological-genomics studies and its potential use in understanding the effects of multiple stressors. I examine the effects of three widespread anthropogenic stressors: (1) climatic change, (2) calcium decline, and (3) metal contaminants in lakes. I consider these stressors in the context of their interacting

effects with each other and with other anthropogenic and natural stressors. Finally, I propose combining transcriptome profiling and mutation accumulation experiments as a novel approach to studying responses to multiple environmental stresses.

2.2 *Daphnia* as a model system for studying multiple stressors

Daphnia is a keystone species in the pelagic zone of most freshwater habitats (e.g., arctic and temperate lakes, lakes at high elevations, ephemeral ponds, ponds in sand-dunes) and provides a key link between primary production and higher trophic levels (Hebert 1978; Lampert 2006). Because *Daphnia*'s ecology, phylogeny, toxicology, and physiology is relatively well understood, access to its genome sequence (wFleabase.org) and other genetic tools such as genetic linkage map (Cristescu et al. 2006), cDNA libraries, microarrays allows for the study of environmental influences on gene functions in ways that are difficult in other model species (Eads et al. 2008; Colbourne et al. 2011). For example, the availability of genetic linkage maps and the transferability of crossing panels between laboratories can greatly advance the diagnosis of important ecological and environmental traits through quantitative trait locus (QTL) studies or the identification of heritable genotypes that affect gene expression using eQTL (expression QTL) approaches.

Compared to most other freshwater taxa, daphniids are highly sensitive to environmental perturbations (Schindler 1987) and they typically respond rapidly to stressors by altering their mode of reproduction (Hebert and Crease 1983), changing

their pattern of vertical migration within the water column (Stich and Lampert 1984; Dawidowicz 1992), and/or undergoing behavioral (Gerhardt et al. 2005) and phenoplastic changes (Tollrian 1993). These common natural and anthropogenic stressors include toxicants, bacterial infections, vertebrate and invertebrate predators and parasites, synthetic hormones, varying diets, UV radiation, hypoxia, acidity, salinity, and low ambient calcium levels (Colbourne et al. 2011). *Daphnia*'s extremely "ecoresponsive genome" (Colbourne et al. 2011; Tautz 2011) may account for the multiple habitat transitions among closely related species. For example, members of *Daphnia* species complexes (e.g., *Daphnia pulex*, *Daphnia galeata mendotae*, and *Daphnia longispina* species complexes) evolved ecologically relevant traits, enabling these closely related species to colonize habitats with distinct environmental conditions (Fryer 1991; Hebert 1995; Wellborn et al. 1996; Colbourne et al. 1997). The multiple lineages independently colonized and adapted to these freshwater habitats and are characterized by various degrees of reproductive isolation and significant intraspecific (among populations) genetic subdivision (Crease et al. 1997; Pfrender et al. 2000).

Daphnia typically reproduces by cyclical parthenogenesis; this strategy entails both clonal reproduction during optimal environmental conditions and sexual reproduction otherwise. The sexual phase is often triggered by environmental stresses such as crowding, cooling, or change in photoperiod, as well as by predation (but see Paland et al. 2005). *Daphnia*'sreproductive system allows us to maintain both lines of genetically identical individuals in the laboratory, as well as lines of genetically variant

clones; this allows us to understand if responses elicited from stressors do, or do not, have a genetic basis. *Daphnia* is an ideal system for studying multiple stressors because of its short generation time, well-studied ecology and evolutionary history, wide geographical distribution across many limnetic systems, high mutation and recombination rates, high sensitivity to changes in environmental conditions, unique cyclical parthenogenetic life history, and recent availability of many genomic tools.

2.3 Climatic change

It is now widely accepted that climatic change is impacting the structure, function, and biodiversity of freshwater ecosystems (Sala et al. 2000; Carvalho and Kirika 2003), both directly and indirectly. Here, I discuss the impacts associated with warming climate part of climatic change. Indirect interactions producing complex outcomes in populations in freshwater communities have been noted with food quality and availability, toxic contaminants, and UV radiation. The effects may be also direct, i.e., changes in ambient temperatures are likely to have a strong impact on the life-history parameters of many aquatic species. Moreover, under climatic change, temperature fluctuations may exceed the thermal tolerance limits of keystone species, such as *Daphnia*, and lead to cascading effects and trophic surges throughout the trophic web, thereby impacting the functioning of the freshwater ecosystem (Jeppesen et al. 2010). Therefore, studying how climatic change independently and in concert with

other stressors impacts the life history, ecology, physiology, and evolution of *Daphnia* should help us understand and predict major changes in freshwater ecosystems.

Temperature has great effects on the life history of *Daphnia*, inducing earlier emergence (Carvalho and Kirika 2003) and shorter lifespan (Bottrell 1974). In addition, as temperature increases, the filtering rate, metabolic rate, and demand for food also increase (Burn 1969). Even an increase in temperature of as little as 1.7°C during a short, but critical, seasonal period, can adversely affect *Daphnia* populations, and induce significant changes in entire food webs of lake systems (Wagner and Benndorf 2007). For example, for every rising degree of temperature, the predation on *Daphnia galeata* by the predatory cladoceran *Leptodora kindtii* and by planktivorous fish, started distinctly earlier in the season by 13.0 and 6.5 days, respectively, resulting in lower densities of *Daphnia* (Wagner and Benndorf 2007). Moreover, temperature changes can cause rapid microevolution of *Daphnia* populations and alter the community structure of freshwater habitats (Van Doorslaer et al. 2010). In a common garden experiment, *Daphnia* genetically adapted to increased temperature within one growing season (Van Doorslaer et al. 2010). Clones of *D. pulex* and *D. magna* were exposed for 6 months to different temperature treatments in large outdoor mesocosms that simulated small ponds. The clones experienced micro-evolutionary adaptation to higher temperature through increased size at maturity (Van Doorslaer et al. 2010). Clearly, daphniids may adapt quite rapidly to higher temperature. In addition, displacement of thermophobic by thermo-tolerant daphniid taxa are likely as the climate warms. For example, Lennon

et al. (2001) suggested that *Daphnia lumholtzi* may replace many native North American taxa as the climate warms since this species is tolerant of higher temperatures-outcompeting other *Daphnia* species above 25˚C. These findings suggest that it is important to incorporate micro-evolutionary responses of keystone species and colonization of non-native clones in models that aim to predict the effects of climatic change on populations, communities, and the dynamics of food webs (Van Doorslaer et al. 2010).

Climatic change may directly affect water quality by decreasing water availability, concentrating pollutants and contaminants, and increasing salinity (Schindler 2001). The sensitivity of organisms to chemical toxins and heavy metals in water varies with ambient temperature and tends to be toxin-specific. Climatic change affects the rate of uptake and detoxification of toxins and the sensitivity of organisms through changes in rates of metabolism and feeding (Cairns et al. 1975; Heugens et al. 2003). For example, in *Daphnia*, the rate of cadmium uptake significantly increases at higher temperatures, possibly because of elevated ventilation rates in response to increased metabolic rate and demand for oxygen (Cairns et al. 1975; Heugens et al. 2003); at the same time growth rate decreases (Heugens et al. 2006). These studies prove that metals are more toxic at higher temperatures because of increased bioaccumulation. Non-metallic toxins such as sodium dodecyl sulfate (SDS) also exhibit increased toxicity at high temperatures (Folt et al. 1999). However, once food is taken into consideration, the interaction of temperature and toxins dramatically changes.

While higher food levels appear to protect *Daphnia* from the toxic effects of cadmium (Heugens et al. 2006), SDS is more toxic at higher food concentrations (Folt et al. 1999).

Global warming has been also affects the exposure of freshwater organisms to UV radiation (Schindler et al. 1996; Yan et al. 1996) and the ability to repair the damage caused by UV (MacFadyen et al. 2004; Connelly et al. 2009). Elevated temperature in the environment may lead to decreased influx of water to lakes from streams and ground-water tables. In consequence, the supply of dissolved organic carbon to lakes decreases, and penetration of UV-B into lakes increases exponentially as dissolved organic carbon declines (Schindler et al. 1996). Furthermore, an increase in the ambient temperature induces changes at the molecular level that help *Daphnia* deal with damage from UV radiation. Zooplankton have two main mechanisms for repairing UV-induced DNA damage: the light-dependent photoenzymatic repair (PER) and light-independent nucleotide-excision repair (NER) (Rautio and Tartarotti 2010). MacFadyen et al. (2004) found that PER in *Daphnia pulicaria* is temperature dependent. The repair is less effective for UV-induced DNA damage at lower temperatures, owing to decreased efficiency of the enzyme. Although, UV-induced DNA damage was observed to be higher at elevated temperatures, the efficiency of the repair mechanism also increased; thus, the net UV-induced DNA damage at lower temperature is still more serious compared to that occurring at higher temperatures (MacFadyen et al. 2004). In contrast, Connelly et al. (2009) found that survival as well as the repair of DNA was higher at lower temperatures in four *Daphnia* species exposed to UV radiation. While several studies

have reported the effects of UV radiation on *Daphnia*, very few studies focused on the interactive effects of UV and temperature on this important keystone species (Rautio and Tartarotti 2010). Furthermore, *Daphnia* inhabits lakes with varying temperatures and UV exposures, ranging from clear-water, oligotrophic arctic lakes with high permeability of UV to canopy-covered eutrophic ponds (Hessen 1999). This combination of a broad ecological setting provides the opportunity for investigating the interactive effects of UV and temperature.

2.4 Decline in calcium

Soft-water lakes on the Boreal Shield and other parts of eastern North America and Northern Europe are currently experiencing a trend of rapid decline of calcium (Keller et al. 2001; Jeziorski et al. 2008). In lakes of Ontario, Canada, for example, the levels of calcium have dropped by 13% since the mid-1980s (Jeziorski et al. 2008). Calcium decline has likely emerged from a combination of at least three unrelated anthropogenic stressors: multiple cycles of forest re-growth after logging in the watershed, historical and ongoing atmospheric deposition of acid (Watmough 2003; Jeziorski et al. 2008), and reduced runoff as climate changes (Yao et al. 2011). Since crustaceans have high demands for calcium, they are expected to be very sensitive and among the first taxa impacted by this sharp decline in calcium levels (Cairns and Yan 2009). Indeed, daphniids have a significantly higher demand for calcium, averaging 2.38% Ca by dry weight, compared to 0.23% Ca in non-daphniid planktonic cladocerans

and copepods (Waervagen 2002; Jeziorski and Yan 2006). In laboratory experiments where animals are well-fed, calcium concentrations of 1.5 mg/l and below have a negative impact on the animals' reproductive capabilities, and animals die below 0.5mg/l Ca^{2+} (Ashforth and Yan 2008). However, under natural conditions of ambient food, the animals do not survive at calcium concentrations below 1.4 mg/l (Cairns 2011, personal communication). This likely explains the observation that the probability of observing daphniids significantly drops in lakes with calcium concentrations below 1.5–2.0 mg/l Ca^{2+} (Jeziorski et al 2008). Unfortunately, about 35% of the lakes in Ontario are already below this threshold (Jeziorski et al. 2008).

In the context of multiple stressors, several anthropogenic and natural stressors could have complex interactions with declining calcium levels. For example, animals experiencing limited calcium supplies have been proven to be more prone to UV-induced DNA damage, suffering reduced survival in low-Ca/high UV treatments (Hessen and Rukke 2000a). In addition, declining calcium levels may certainly interact synergistically with heavy metal contaminants in freshwater habitats. Water hardness is well known to protect *Daphnia* and other animals from toxic effects of metal contaminants. This protection has been mainly attributed to Ca^{2+} concentrations and to a lesser extent Mg^{+2} (Borgmann et al. 2005; Kozlova et al. 2009). For example, increased aqueous and body calcium in *Daphnia* directly decreases the efficiency of the uptake and assimilation efficiency of zinc and cadmium (Tan and Wang 2008). Calcium acts as a competitive inhibitor of toxic metals by binding more efficiently to sites on the "biotic

ligand", i.e. sites of cation uptake that can be treated as analogous to chemical ligands, than do other metal cations (Paquin et al. 2000; Santore et al. 2002). Increasing calcium concentrations in the medium drastically diminishes the toxic effects of cadmium (Clifford and McGreer 2010), zinc (Clifford and McGreer 2009), copper (De Schamphelaere and Janssen 2002), and nickel (Kozlova et al. 2009). Consequently, calcium offers protection for *Daphnia* and other freshwater organisms from heavy metal contaminants (Table 1). The biota of soft-water lakes are already more susceptible to metal toxicity than are hard-water organisms, and with decreasing calcium levels, the exposure of animals to toxic levels of metal contaminants may well increase.

In addition to these physical and chemical anthropogenic stressors, *Daphnia* may become more prone to biotic stressors with declining concentration of ambient calcium. The animals could be more susceptibility to invertebrate predators such as the phantom midge, *Chaoborus*. The midge is a gape-limited predator that targets juvenile *Daphnia* because of their small size (Pastorok 1981). *Daphnia* have evolved ways that reduce predation by triggering phenotypically plastic changes in juveniles in response to kairomones released by the *Chaoborus*. These changes include formation of neck teeth (Tollrian 1993) and an increased stability of the carapace (Laforsch et al. 2004). Since calcium minerals form a major part of the carapace (Hessen and Rukke 2000b), declining ambient calcium levels could lead to reduced formation of neck teeth and to loss of rigidity of the carapace, making the animals more vulnerable to predation by *Chaoborus*. Clearly, declining calcium concentrations may well have drastic effects on the survival

and reproduction of *Daphnia* and other crustaceans. However, interacting effects with other anthropogenic and natural stressors seem to have a cascade of complex effects that are as yet not well understood.

2.5 Metal stressors

Pollution by heavy metal contaminants in aquatic ecosystems, many attributed to the mining industry, is an ongoing concern around the world. For several decades, ecotoxicologists have intensively studied the adverse biological impact of excess metal elements such as copper and cadmium on various aquatic organisms (Heugens et al. 2001). *Daphnia* have been an important model system for investigating the adverse impact that metal toxins exert on freshwater ecosystems. Metal contaminants usually have negative effects on individual development, population growth rate, longevity, and reproduction. In the past decade, the rapid development of genomic technologies, such as microarrays, has revolutionized the field of ecotoxicology by revealing how metal contaminants can affect gene expression in many aquatic taxa (Neumann and Galvez 2002). In this genomic era, *Daphnia* is an important model system for crustaceans and freshwater organisms due to the availability of its whole genome sequence and microarrays (Colbourne et al. 2011). The numerous ecotoxicogenomic studies employing *Daphnia* as a model have made three types of contributions. First, characteristic profiles of gene expression have been established for various metal contaminants including copper, cadmium, zinc, and nickel (Poynton et al. 2007; Vandegehuchte et al. 2010;

Poynton et al. 2011). Second, these studies have facilitated the development of novel biomarkers to monitor and identify the level and types of metal contaminants in aquatic ecosystems (Connon et al. 2003; Poynton et al. 2008; Garcia-Reyero et al. 2009). Third, because *Daphnia* have a large number of genes (i.e., 36% of the minimal set of ~30,907 genes) that have no detectable homologues in other sequenced animal lineages (Colbourne et al. 2011), a growing catalogue of novel genes involved in a response to acute metal stress have the potential to be discovered. For example, three new genes coding for the cadmium detoxification protein metallothionein have been identified in *D. pulex* (Shaw et al. 2007).

2.6 Approaches to the study of multiple stressors

Despite these substantial advances, it remains a grand challenge for ecotoxicologists to link changes in genetic expression to adverse outcomes at the individual and population level (Snell et al. 2003; Snape et al. 2004; Fedorenkova et al. 2010). Because of the well-understood ecology of *Daphnia*, we have the potential of using the information of metal-induced molecular changes to predict ecological outcomes on populations. For example, Connon et al. (2008) performed a microarray study to investigate the changes in gene expression of *Daphnia magna* exposed to cadmium in relation to the pattern of population growth. In light of a lowered population growth rate observed in their experiment, these authors examined the detrimental effects of changes in gene expression on cellular processes related to

growth, moulting, and metabolism, thereby establishing a logical link between measured responses of gene to population-level changes. However, this kind of work has been infrequent, and a great amount of gene-expression data and endpoint toxicological tests are still needed to establish meaningful correlations between changes in gene expression and population levels (Fedorenkova et al. 2010), and eventually to responses by entire communities and ecosystems (Whitham et al. 2006). Compared to other model organisms, *Daphnia* has a few unique advantages. *Daphnia* is widely distributed in aquatic habitats with different ecological conditions (lake versus pond, contaminated versus uncontaminated habitats). The ecology of *Daphnia* in these habitats is also well studied. With the extraordinary ease of laboratory maintenance, it is possible to perform common garden experiments on populations of *Daphnia* with a particular genotype and directly examine the link between gene expression and population-level changes in these environmentally relevant scenarios.

However, to better understand the long-term impact of metal contaminants and multiple stressors on aquatic ecosystems, it is necessary to quantify induced heritable genetic changes, such as mutations and transcriptional changes, to better predict the acclimation, evolution, adaptation and the future of biota in stressed environments. Mutations provide the raw material upon which natural selection can work and are thus essential for the adaptation of populations to new environments. On the other hand, environmental stressors, such as heavy metals, may stimulate increased mutation rates which could be damaging to the integrity of the genome. To date, few efforts have been

devoted to relating temperature changes, decreased level of calcium, and metal stress to changes in mutational and transcriptional processes. I suggest that combining the classic mutation accumulation (MA) experiments with controlled exposure to multiple stressors in *Daphnia* would provide insights into this largely unexplored field. The classic MA experiments consist of multiple isogenic lines derived from the same asexual ancestor or a common highly inbred base population (Halligan and Keightley 2009). Each of these MA lines is propagated in a benign, unchallenging environment and is bottlenecked at each generation (e.g., a single, randomly picked individual for clonal or hermaphroditic species). This results in an extremely low effective population size for each MA line, thus reducing the efficiency of natural selection to a minimum and allowing the majority of mutations, except those having extreme effects such as lethality, to accumulate over time in a neutral manner. Numerous MA studies have been performed and have provided insights into the rate and the spectra of spontaneous mutations for several model species in non stressful environments (Denver et al. 2000, 2004; Lynch et al. 2008; Keightley et al. 2009), whereas little work has been conducted on the transcriptional changes in MA lines (Rifkin et al. 2005). Because of the uniform original genetic background for all the MA lines, it is easy to compare the mutational and transcriptional changes among the MA lines exposed to normal and challenging environments (e.g., combinations of anthropogenic stressors). Analyses of the genomes and transcriptomes of treated and untreated MA lines using the next generation of sequencing technologies allows the investigation of fitness and gene-expression

changes, as well as the spectrum of genomic mutations deleterious to overall fitness. The results arising from such studies can have important evolutionary and ecotoxicological implications, allowing us to understand if multiple stressors cause unique molecular stress responses compared to a single stressor, and to clarify the genetic basis of the organism's response to single and multiple stressors.

Although aquatic organisms now commonly face multiple environmental stressors such as decline of calcium, rise of temperature, and metal toxins, few data are available for understanding the combined effects of multiple stressors. This is largely because the effects of single stressors are already difficult to characterize from the angles of physiology, gene expression, and ecology. However, with the growing understanding of effects of single stressors, I envision a better understanding of how aquatic organisms deal with multiple stressors. I can attempt to understand the effects of multiple stressors by integrating diverse fields of biology (Fig. 1). First ecotoxicogenomic and ecological genomic studies aim to integrate gene-expression data with ecological knowledge to reveal how organisms alter their gene expression in response to environmental and anthropogenic stressors. For example, gene transcription and translation may be altered in animals exposed to metals (Shaw et al. 2007), predator-born kairomones (Schwarzenberg et al. 2009), toxic chemicals (Watanabe et al. 2007), hypoxia (Zeis et al. 2009), change in temperature (Schwerin et al. 2009), microcystin-producing cyanobacteria (Schwarzenberg et

al. 2009; Miyakawa et al. 2010), and hormone derivatives that trigger the production of males (Eads et al. 2008). The results from these studies constitute baseline data that would become more useful in the context of mutation-accumulation experiments in which treatment with single/multiple stressors are used to examine how mutation rates and transcriptome profiles are altered in a stressful environment. Secondly, integration of genetic and toxicological work can indicate if biotic ligand models would need to be developed for multiple *Daphnia* genotypes or species to be effective at predicting environmental risk and toxicity of contaminants (Janssen et al. 2000). In addition, we can predict if it is metals that are regulating the recovery of daphniids from historical stress, such as in heavily contaminated lakes near the Sudbury, Ontario, smelters region (Valois et al. 2010) by combining ecological surveys with this ecotoxicogenomical approach. Lastly, because *Daphnia*'s dormant eggs are preserved in sediments; historic generations can be hatched via methods of resurrection ecology (Kerfoot and Weider 2004). Therefore, we can use this paleolimnological approach in combination with genomic and phylogenetic techniques to illuminate how animals dealt with historic environmental challenges (Eads et al. 2008; Orsini et al. 2011), providing perhaps excellent clues to the future of these keystone pelagic herbivores.

2.7 References

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Table 2.1: Fold of increase (max EC50/min EC50) in toxicity of heavy metal attributable to high and low concentrations of ambient calcium

Metal Tested	Range in concentration of Ca^{2+} (mg/L)	Metal EC50 concentrations range (free ion activity)	Times increase in toxicity of the metal from high to low [Ca^{2+}]	Source
Cadmium	1.2 – 64.4	0.1 – 1.05 µM	10.5	Clifford and McGeer 2010
Zinc	1.6 – 59.7	1.25 – 21 µM	18	Clifford and McGeer 2009
Copper	10 – 160.3	8.6 – 48.7 nM	5.6	De Schamphelaere and Janssen 2002
Nickel	0.8 – 58.8	3 – 62.5 µM	20.8	Kozlova et al. 2009

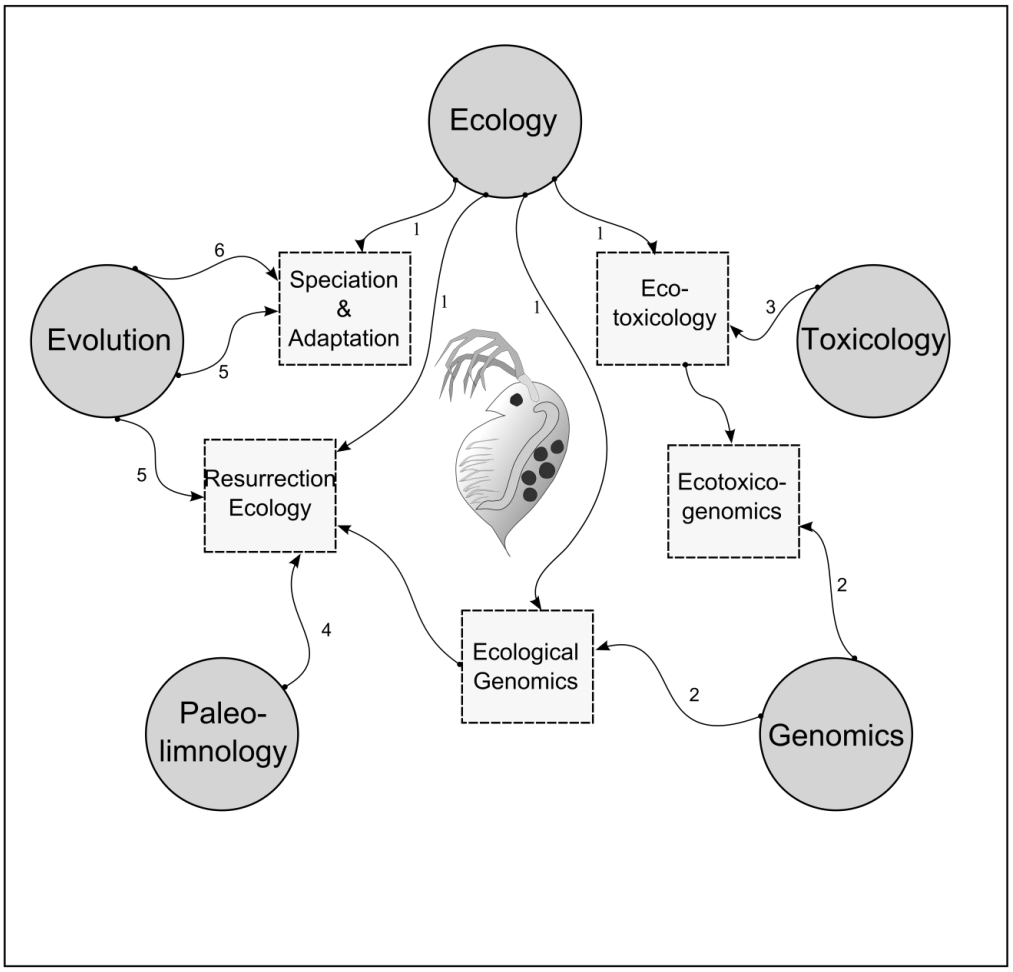

Figure 2.1: Integrative approaches to studying multiple stressors using *Daphnia* as a model organism. The arrows connect primary fields of study (in circles) to form interdisciplinary fields (in squares). The numbers on the arrows indicate the tools, techniques or the knowledge that can be used to integrate multiple data sets: (1) ecological surveys/knowledge, (2) genomic tools (microarrays, sequencing), (3) toxicity tests, (4) retrieval of diapausing eggs from sedimentary cores, (5) evolutionary theories/knowledge, and (6) phylogenetic reconstruction techniques

CHAPTER III

GENE EXPRESSION PROFILES OF *DAPHNIA PULEX* IN THE PRESENCE AND ABSENCE OF PREDATOR KAIROMONES OVER A RANGE OF CALCIUM CONCENTRATIONS

3.1 Introduction

The central dogma of molecular biology asserts that the flow of information stored in the genome, travels through transcription into mRNA molecules, and follows the translation into peptides, eventually forming proteins. The composition of proteins reflects on the phenotype of the individual and the information therein cascades to population responses. However, there are many known epigenetic changes, that represent exceptions to the central dogma and involve modification in the genomes that result in phenotypic changes buy do not involve heritable nucleotide changes such as methylation of DNA, transcriptional control, transcriptional silencing via siRNA, and miRNA (Danchin et al. 2011). Still, the flow of information from the genome to the mRNA is still a major part of organismal response to environmental cues. Thus, we can reliably use gene expression levels to gauge how organisms respond to stressors and, potentially, the health of ecosystems.

The ability of an organism to quickly and effectively respond to stressors is essential for its survival, growth and reproduction. This is especially important in freshwater organisms, such as daphniids that are exposed to a large variety of natural and anthropogenic stressors. *Daphnia* have evolved an “ecoresponsive genome” to effectively deal with these environmental challenges (Colbourne et al. 2011). The large

genome contains over 30,000 genes, compared to ~13,000 genes in *Drosophila* (Colbourne et al. 2011; Adams et al. 2000). Many of the *Daphnia* genes are uncharacterized, though are believed to be involved with its response to environmental stressors as these genes appear to be up-regulated only in the presence of the particular stressors (Colbourne et al. 2011). For example, daphniids respond to hypoxia by up regulating their haemoglobin levels via several haemoglobin coding genes (Kimura et al. 1999; Colbourne et al. 2011). Thus, *Daphnia's* 'ecoresponsive genome' and our extensive knowledge of its ecology, makes it an ideal organism for studying the interaction between genes and the environment.

Daphnia species are negatively impacted by the declining calcium levels in soft water lakes across the Canadian Shield and northern Europe (Jeziorski et al., 2008). Calcium concentrations under 1.5 mg/l lower *Daphnia*'s rate of reproduction (Ashforth and Yan 2008), whilst below 0.5 mg/l of Ca^{2+} the animals die. *Daphnia* have much higher calcium content when compared to other freshwater zooplankton (Waerwagen et al, 2002). The animal's sensitivity to low calcium is due to loss of calcium though molting and excretion, thus requiring them to constantly replenish their calcium stores (He and Wang, 2009). Calcium uptake from softwater environments is energetically costly, especially at low ambient concentrations. For example, under high food concentrations *Daphnia* have higher calcium content (He and Wang, 2009), potentially because more energy can be allocated to active uptake of Ca^{2+}, thus we can expect that the high

energy requirements under low Ca^{2+} will lower the ability of daphniids to effectively respond to other natural and anthropogenic stressors.

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In addition to low calcium, *Daphnia* populations in the Canadian Shield are also exposed to many natural stressors such as invertebrate predators. The larval stage of the phantom midge, *Chaoborus* (Diptera) represents a chief predator that often controls *Daphnia* populations (Kurek et al., 2010). The larvae release kairomones which induce phenoplastic traits in *Daphnia*. In juvenile daphniids these changes include hardening of the carapace (Laforsch et al., 2008) and formation of neck spines (neck teeth) (Riessen and Trevett-Smith, 2009). In adult *Daphnia* these include changes in the reproductive strategy to produce smaller number of eggs but of a larger size, even if kairomone exposure was brief and preceded egg deposition (Imai et al., 2009). Since these responses have an energetic cost, the animals have evolved to only manifest them in the presence of the predators (Riessen and Trevett-Smith, 2009; Tollrian and Leese, 2010). This way, the same genotype can produce two distinct phenotypes depending on the environmental cues. The two stressors- low calcium and presence of *Chaoborus*- are intrinsically associated since calcium is a major part of *Daphnia*'s carapace and can determine the size and growth rate of the animals (Hessen and Rukke, 2000) and the inducible changes greatly alter the carapace structure and increase the size of the individual (Riessen and Trevett-Smith, 2009) thus likely increasing the calcium demand.

Low environmental calcium may interfere with the production of these traits in the presence of the predatory *Chaoborus,* but this possibility has not been explored.

Several genes are known to be differentially expressed in the presence of *Chaoborus* kairomones. Genes involved in the endocrine system, such as growth factors, and morphogenic genes, such as Hox genes, are up-regulated in juveniles exposed to kairomones (Miyakawa et al., 2010). In addition, ribosomal genes are also up-regulated, these are involved in providing the ribosomal RNA necessary for building the Ribosomes, the organells involved in catalyzing protein synthesis. However, cyclophylin which is involved in protein folding is down-regulated (Schwarzenberg et al., 2009; Tollrian and Leese, 2010). Unfortunately, detailed gene expression under calcium limitation are sparse and have not been conducted outside of cultured cell lines (Deyama et al., 1999) and plants (Snowden et al., 1995). Moreover, little is known about the interaction between low calcium stress and other stressors. Thus, I use an *a priori* candidate gene approach to test the effects of low calcium on gene expression patterns in *Daphnia pulex* in the presence and absence of *Chaoborus* kairomones. Since calcium is integral to the formation of the carapace, candidate genes include two genes coding for carapace cuticle proteins and three genes coding for proteins involved in calcium homeostasis. I use presence and absence of predator kairomones as an additional stressor to examine any non-additive effects of the two stressors.

3.2 Methods

Individual *Daphnia* were reared in the presence and absence of *Chaoborus* kairomones over a gradient of four calcium concentrations. I extracted total RNA, reverse transcribed it and quantified the expression of five genes related to carapace building and calcium homeostasis, using three endogenous genes as controls. Statistical analyses were then preformed to identify significant gene expression differences between the treatments.

Exposure conditions

The experiment was conducted using *Daphnia pulex* since this species has known calcium thresholds (Ashforth and Yan, 2008) and its genome has been sequenced (Colbourne et al., 2011). The particular lineage used in this experiment was isolated from Sherborne Road Pond near Dorset, Ontario, Canada (Riessen and Trevett-Smith, 2009) and is known to have induced phenoplastic changes in the presence of predators. Around 20 parthenogenetic individuals derived from one progenitor mother were exposed to each treatment. The eight treatments included four calcium concentrations (0.5, 1.5, 2.5, and 5.0 mg/l of Ca^{2+}) in the presence (+) or absence (-) of *Chaoborus* kairomones. These calcium levels were chosen for ecologically relevant reasons. The animals die below 0.5 mg/l Ca^{2+} and experience decreased reproductive ability below 1.5 mg/l Ca^{2+} (Ashforth and Yan, 2008). The original FLAMES medium contained 2.5 mg Ca^{2+}/l and was modeled after two lakes in the

Canadien Shield, Red Chalk and Blue Chalk, which are pristine and supports a stable multi-species assemblage of *Daphnia* (Celis-Salgado et al., 2008; Yan et al. 2008), and 5.0 mg Ca^{2+}/l was chosen as a concentration under which the animals are assumed to not be limited in calcium. The softwater FLAMES medium (Celis-Salgado et al., 2008) with adjusted calcium concentrations was used as the experimental medium. The *Chaoborus* kairomones were introduced into the experimental media by culturing fourth-instar *Chaoborus americanus* (with neonate daphniids supplemented as food) at a density of 20/L for 24 hours prior to use. All treatment media were filtered through a 1.2-*μ*m Whatman filter to ensure possible bacterial and all algae contamination was removed. The animals were kept in a Conviron E7/2 temperature-controlled chamber at 20°C under a 16:8 light:dark cycle. Daphniids were fed daily *at libitum* a 4:1 ratio of *Pseudokirchneriella subcapitata : Scenedesmus obliquus*. The algae was cultured in Bold's Basal Medium (Bold 1949, Bischoff and Bold 1963) and harvested during the log phase. *Daphnia* neonates (>24h) were separated into the eight experimental treatments- four calcium concentration in presence (+) or absence (-) of *Chaoborus* kairomones. The animals were reared to maturity (15 days) in the appropriate experimental mediums, sampled and stored in ~1.0 ml of RNAlater at -80˚C for RNA extraction and q-PCR. To ensure most stable expression levels, the sampled animals were in inter molt stage, this was determined by the presence of eggs in the brood pouch. Three biological

replicates were collected from each treatment.

Target and reference gene selection

I selected genes that code for proteins involved in calcium homeostasis and building the carapace. Five genes (Table 3.1) were selected for the study; *ICP1* and *ICP2* (Insect Cuticle Protein 1 and 2), *Calb32* (Calbindin 32), *SERCA* (Sarcoplasmic Calcium ATPase), and *IP3R* (Inositor triphosphate receptor). Three common housekeeping genes were used for the study (Table 3.1); *GAPDH* (gyceraldehyde 3-phosthate dehydrogenase), *Stx16* (Syntaxin 16), and *CAPON* (C-terminal pdz ligand of neuronal nitric oxide synthase) (Spanier et al., 2010). Expression of the housekeeping genes was screened under several treatments to ensure constant expression.

RNA extraction and RT-qPCR

The RNA was extracted by following the RNA extraction and purification protocol described by Lopez and Bohuski (2007). A total of 3-4 adult *Daphnia* were used per biological sample to ensure enough RNA yield. The RNA was purified with the RNeasy Mini Kit (QIAGEN), and treated with RNase-free DNAse I (QIAGEN) treatment for 15 min to ensure no DNA contamination in the samples. The purified RNA was eluded in 30 µl of RNAse-free water. The concentration and purity of the RNA samples was checked with the NanoVue spectrophotometer (GE Healthcare Technologies) using the 260/280 nm absorbance ratio, and all samples were found to be of high RNA purity

(2.01-2.02 ratio). For cDNA synthesis, 40 ng of total RNA was reverse-transcribed with Sensiscript Reverse Transcription Kit (QIAGEN) using the Oligo (dT) Primer (50 µM) from Ambion. The qPCR reactions were performed in triplicate using the Power SYBR Green PCR Master Mix (Applied Biosystems) using 2 µl of cDNA, 0.4 µl (10 µM) of each primer, 10 µl of the 2x Power SYBR Green PCR Master Mix, and water to bring the final volume to 20 µl. I ran both negative control reactions with no cDNA as well as no-RT control reactions to ensure no DNA contamination. The qPCR reactions were run on the Applied Biosystems 7500 SDS Real-Time PCR System according to the manufacturer's instruction. The thermal cycling program included an initial 10-minute denaturation at 95°C and 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Data Analysis

The geometric mean of the three housekeeping genes (*GAPDH*, *CAPON*, and *Stx16*) was used to calculate the normalization factor (n), to normalize the target genes and acquire the ΔCT (threshold cycle) values (Livak and Schmittgen, 2001). The high calcium (5.0 mg/l) in the absence of *Chaoborus* (-) treatment was used as the calibrator for the fold change calculations via the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and $\log_2$ transformed. A 2-way ANOVA was used to test whether calcium, predator kairomones, or the interaction of the two stressors governed the expression of each target gene. A 1-way ANOVA was used to determine if there were significant changes in gene expression across calcium concentrations in the kairomone and non-kairomone

treated animals. Tukey test was used on significant 1-way ANOVA results to test which particular calcium treatments had a significant effect on expression. A T-test was used to test for significant difference in gene expression between the kairomone and non-kairomone treatments at each calcium concentration. Significance was assessed at $p \leq 0.05$.

3.3 Results

At least one of the two stressors, limited calcium or presence of kairomones, affected the expression of the candidate genes. Moreover, the interaction of the two stressors also had an effect of the level of gene transcription in all genes. No general pattern of up or down regulation was observed that governed all the genes, meaning the expression pattern was unique to individual genes.

Insect cuticle proteins (*ICP1 and ICP2*)

The expression of both genes coding for insect cuticle proteins was altered by at least one of the two stressors. The decrease in calcium did not change the expression of *ICP1* in absence of kairomones (Fig. 3.1; Table 3.4). However, in the kairomone treatment, the decrease in calcium (5.0-0.5 mg/l) increased the expression of *ICP1* 7.3 fold ± 2.1SEM ($p = 0.007$) (Fig. 3.1; Table 3.5). In addition, kairomone exposure affected *ICP1* differently depending on the calcium concentration. At low calcium concentrations (0.5 and 1.5 mg/l) kairomone exposure up-regulated the expression of *ICP1* ($p = 0.041$,

0.033). At the higher concentrations (2.5 and 5.0 mg/l), kairomone exposure did not have an effect on gene expression of *IPC1* (Fig. 3.1; Table 3.3). Thus, the combination of low calcium and kairomones affected the expression of *ICP1* ($p = 0.0005$; Table 3.2). The expression of *ICP2* was altered by the interaction of calcium and kairomones ($p = 0.0003$; Table 3.2). The kairomone exposure up-regulated the expression of *ICP2* 39 fold ± 18SEM ($p = 0.035$) at 2.5 mg/l Ca^{2+}. At 5.0 mg/l, however, the kairomone exposure down-regulated *ICP2* 62 fold ± 16SEM ($p = 0.031$; Fig 3.1; Table 3.3).

Sarcoplamic membrane proteins- Sarco(endo)plamic reticulum calcium ATPase and Inositol triphosphate receptor (*SERCA* and *IP3R)*

The expression of the two genes (*SERCA* and *IP3R)* coding for sarcoplasmic membrane proteins, was effected by the interactions of calcium and kairomones ($p = 0.036, 0.0008$; Table 3.2). In the non-kairomone treated animals, the low calcium (0.5 mg/l) exposure increased the expression of *IP3R* 5 fold ± 1.0 SEM ($p = 0.0001$) compared to the highest calcium concentration (Fig. 3.1; Table 3.4). In the kairomone treated animals, however, calcium did not affect the expression of *IP3R* (Fig. 3.1; Table 3.3). Calcium and the interaction between the two stressors had a significant effect on the expression of *SERCA* ($p = 0.36$; Table 3.2). While in the absence of kairomones (Table 3.4) calcium concentration did not influence the expression of SERCA, in the kairomone treatment, the decrease in calcium (5.0-0.5 mg/l) increased the expression of *SERCA* 3.3 fold ± 0.2SEM ($p = 0.018$) (Fig. 3.1; Table 3.5). Moreover, kairomone exposure decreased

the expression of SERCA 2.2 fold ± 0.2 SEM (p = 0.001) at the highest calcium concentration (Fig. 3.1; Table 3.3).

Calcium binding protein *(Calb32)*

Calcium concentration, kairomone exposure and their interaction affected the expression of *Calb32* (Table 3.2). The calcium decrease did not affect the expression of *Calb32* in the non-kairomone treatment (Table 3.4). However, in the kairomone treated animals, calcium decline (5.0-0.5 mg/l) increased the expression of *Calb32* 4.1 fold ± 0.3 SEM (p = 0.0021) (Fig. 3.1; Table 3.5). Furthermore, kairomone exposure did not affect *Calb32* expression at the two lowest calcium concentrations, however, it up-regulated *Calb32* 3.2 folds ± 0.4 SEM (p = 0.0438) at the intermediate calcium concentration (2.5 mg/l) (Fig 3; Table 3.3) and down-regulated *Calb32* 1.9 fold ± 0.3 SEM (p = 0.048) at highest concentration (5.0 mg/l) (Fig 3; Table 3.3).

3.4 Discussion

Insect Cuticle Proteins (ICPs)

The carapace (cuticle) of insects and crustaceans is made up of interlinked chitin filaments and cuticle proteins (Andersen et al., 1995). In addition, calcium is a major component of crustacean carapace (Willis, 1999). The cuticle can range from hard to soft with varying degrees of plasticity (Andersen et al., 1995). These properties are governed by the type of protein expressed and amount of sclerotization (Willis, 1999;

Andersen 1995). Different developmental stages and tissue types have unique cuticle properties thus requiring the expression of specific cuticular proteins (Charles, 2010). Cuticle proteins belong to multigene families with sequences ranging from only a few amino acid substitution differences to having no common features at all (Andersen et al.,1995). Multiple cuticle proteins are present in any organism and are differentially expressed depending on the tissue type, development, and molt stage (Charles, 2010). *Daphnia* are no exception. They have a multitude of insect cuticle proteins (ICP) (p.o.; www.fleabase.com). Many ICPs have a conserved R&R motif (Willis, 1999), this motif appears to be present in *ICP2*'s protein product, but absent in *ICP1*'s (p.o., sequence from JGI *Daphnia* database).

The expression of the two *ICP* genes in this study is governed by ecological cues – i.e. calcium, kairomones, and the interactions of the two (Fig. 3.1), though their expression patterns are different. The expression of *ICP2* is distinctive and non-linear, in the non-kairomone treatment it was down-regulated only at 2.5 mg/l Ca^{2+}. This non linear expression pattern is not unique; other stressors have similar effects on the expression of cuticular protein genes (Soetaert et al., 2007). The exposure to 50 µg/l of cadmium compared to a control increased the expression of several cuticular proteins but decreased their expression at 100 µg/l of cadmium. Since many *Daphnia* genes are turned on only during specific environmental challenges (Colbourne et al., 2011), this differential expression of cuticular protein genes may be the organism's response to a particular combination of stressors. For example, decreasing calcium concentration

increases the expression of *ICP1*, but only in kairomone exposed animals. Thus, *ICP1* is highly expressed in animals that have a higher demand for calcium, but lower availability of it.

Molting related genes, including cuticle proteins, are often differentially expressed under stress. Exposure to fenarimol, a chlorinated fungicide, delays growth and down-regulates the expression of genes involved in the molting cycle of daphniids (Soetaert et al. 2006), specifically genes coding for proteolytic enzymes involved in breakdown of exoskeleton, and several cuticular proteins (Soetaert et al. 2006). Similarly, fenoxycarb, an endocrine disruptive insecticide, down-regulates the expression of a cuticle protein in a concentration dependent manner. This molecular response manifests itself in reduced reproduction rates, triggered male production, and phenotypic abnormalities such as underdeveloped second antennae and curved carapace spines (Oda et al., 2005; Kim et al., 2011). This differential expression is not exclusive to organic contaminants - metals also effect the expression of cuticular proteins. For example, nickel exposure up-regulates genes involved in carapace formation (Vandenbrouck et al., 2009). Cuticular proteins are integral to the molting cycle, thus making them prime candidates for markers of disturbed growth and reproduction.

Sarcoplamic membrane proteins (*SERCA* and *IP3R)*

Both SERCA and IP3R play important roles in cell calcium signaling pathways (Berridge et al., 2000). Cells tightly regulate their cytoplasmic calcium concentrations since Ca^{2+} ions are used in a multitude of concentration dependent processes. For example, during signal transduction in a resting cell various pumps and exchangers keep calcium concentrations low- one of the main pumps being SERCA. SERCA uses active transport to pump Ca^{2+} into the sarcoplasmic reticulum for calcium storage (Wuytack et al., 2002). On the other hand, the IP3R is a calcium channel that releases Ca^{2+} from the sarcoplasmic reticulum to propagate calcium signaling (Berridge et al., 2000). The role of IP3R is to generate local and global calcium signals that regulate a multitude of cell processes such as transcription and motility (Foskett et al., 2007). Decrease in calcium concentrations increases the expression of both genes. However, *SERCA*'s expression was up-regulated at lower calcium concentrations in kairomone exposed animals only and the expression of *IP3R* was up-regulated at lower calcium concentrations, only in non-kairomone treated animals.

Since both proteins are involved in a host of signaling pathways and processes, we cannot pinpoint which one is driving the up or down-regulation of the gene. However, just the simple correlation between stressors and gene expression could be used as a biomarker to monitor natural populations of *Daphnia* for these two stressors. Using gene expression as a biomarker for ecotoxicity monitoring has been previously proposed (Steinber et al., 2008; Ha and Choi, 2009). For example, the expression of hemoglobin genes is up-regulated in animals exposed to toxic chemicals and hypoxia,

and has been correlated with decrease in reproduction of the animals (Ha and Choi, 2009). Unfortunately, biomonitoring has predominantly focused on toxicant stress effects, as opposed to other anthropogenic and natural stressors. Thus, expression of *SERCA* and *IP3R* could be used in concert to monitor *Daphnia* for stress relating to low calcium and *Chaoborus* presence.

(*Calb32*) Calbindin32

Calbindin is a calcium binding protein (CBP) part of the EF-hand homolog family containing six calcium binding domains (Reifegerste et al., 1993). It acts as a weak calcium buffer (Schwaller et al., 2002) and is expressed in vertebrates in renal and intestinal epithelia as well as the nervous system (Reifegerste et al., 1993). One of its major functions is vitamin-D dependant absorption of calcium in birds and mammals (Reifegerste et al., 1993; Bouillon et al, 2003). In anthropoids its function is less understood, though a *Drosophila* calbindin gene has been characterized and dubbed as Calbindin-32 (*cbp*) for its molecular weight. Lower calcium exposure (0.5-2.5 mg/l) up-regulated the calbindin homolog gene (*Calb32*) in kairomone exposed *Daphnia*. *Chaoborus* kairomones potentially increase *Daphnia*'s demand for calcium because of the involved carapace alterations (Riessen and Trevett-Smith, 2009). If calbindin plays a similar roll in crustaceans as it does in mammals and birds, then the increase in its expression under low calcium conditions and increased calcium demand is intuitive. My results provide some support that the function of calbindin in anthropoids, or at least in

crustaceans, may also be related to calcium absorption. Thus, *Daphnia* may produce more of the CBPs involved in absorption of calcium in response to low ambient calcium concentrations and increased calcium demand.

Connecting gene expression and higher response levels

Observing the biochemical, physiological, and population level changes in concert with gene expression is vital in drawing connections between genes and the environment; two studies highlight this point. A study by Soetaert et al. (2007) made the connection between physiological and molecular responses. High levels of cadmium exposure decreased the growth rates and reduced energy stores of *D. magna* neonates, this physiological response was correlated with increased expression of genes coding for digestion enzymes. In addition, the exposure caused reduction in mitochondrial electron transport activity, which is a proxy for O_2 consumption; this was reflected by the down-regulation of genes involved with oxygen transport (Soetaert et al., 2007).

If we consider population level changes, cadmium exposure decreases population growth rate (PGR) of *D. magna*. This decrease in PGR is mirrored by changes in gene expression- specifically the majority of the genes affected are part of cellular processes such as growth, molting, and stress response (Connon et al., 2008). Though there does not appear to be a direct pattern of regulation, some of the genes involved in the molting cycle, were up-regulated while others were down-regulated (Connon et al.,

2008). Predictably, however, the exposure up-regulated genes involved in a general stress response such as ones that code for heat shock protein (Connon et al., 2008).

In addition to looking at the relationship between the stressor and gene expression responses, it is important to differentiate between an initial response and an acclamatory one. Multigenerational studies elucidate how the animals acclimatize to continuous stress exposure as opposed to an acute one (Vandegehuchte et al., 2010a). Zinc exposure on daphniids over three generations reduced the reproduction rate in the first two generations but not in the third. This was supposedly due to an acclamatory effect and was reflected by fluctuating gene expression between the generations (Vandegehuchte et al., 2010a). The third generation of the exposed daphniids had a smaller number of differentially expressed genes (Vandegehuchte et al., 2010a), presumably since the animals acclimated to the stress and fine-tuned their molecular response.

3.5 Conclusion

Daphnia show complex gene expression patterns when exposed to calcium and predator kairomones. While not all genes responded to the individual stressors, all five were affected by their interactions. This lends support to a model of non-additive responses to multiple stressors. Through this study only looked at two stressors and their interactions, I predict additional stressors would further complicate the expression patterns. Thus, for further applications of gene expression data, such as gene

characterization and biomarker selection it is important to first test the expression of genes under variety of stressors and their combinations. Furthermore, studies using animals that have been exposed to the stress for several generations would be more informative and likely simplify results since the organisms would be acclimated to the stress and their molecular response fine-tuned.

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Table 3.1: Primers and genes used for qPCR cDNA amplification

Gene symbol	Gene name	Accession number	Primer Sequences (5'-3')	Source
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase	DappuDraft_302823	TGGGATGAGTCACTGGCATAC GAAAGGAACGACCAACAACAAAC	Spanier et al., 2010)
CAPON	C-terminal pdz ligand of neuronal nitric oxide synthase	DappuDraft_100564	TAACGAGTCGGGAGGAAGTG GCTGGACTTGAGCCAGTATCTC	(Spanier et al., 2010)
Stx16	Syntaxin 16	DappuDraft_194044	CACATTGGTCGTCCTTAGTCTTG TGCTATACGTTACGCTTGTCCTTAC	(Spanier et al., 2010)
IP3R	Inositor triphosphate receptor	DappuDraft_227934	TGCGAATGAATCCGCCATTGTCAC AACTGCATCCTTTGGTTCAAGCCG	This thesis
SERCA	Sarco(endo)plasmic reticulum ATPase	DappuDraft_65262	TCAAGGGAGTTCAATGTGCCCTCT AAGGTGGCATGACCAGCATAGACT	This thesis
Calb32	Calbindin-32	DappuDraft_299840	ATTCAGTGCCTTCGATGTAGCCGT CAATTTCATGCGACAGTTCCGCGA	This thesis
ICP1	Insect Cuticle Protein 1	DappuDraft_326396	ATGGCGTTGTCCTCTCCATCAACT TCAAGTCTGCGAGCATCTTGACGA	This thesis
ICP2	Insect Cuticle protein 2	DappuDraft_60650	TTCGTCAGCGCGATAGGAAACGAT ATTCGCCTGGGAAGTTAAGGACAA	This thesis

Table 3.2: Two Factor ANOVA testing the effect of calcium, kairomones or their interaction on the expression of *Calb32, ICP1, ICP2, IP3R,* and *SERCA*. Log transformed normalized fold change values were used. Significant p-values indicating the treatment which had significant effect on the gene expression are in bold.

Gene	Source of Variation	df	P-value
ICP1	Calcium	3	0.07978
	Kairomones	1	0.95942
	Calcium X Kairomones	3	**0.00056**
ICP2	Calcium	3	**0.01103**
	Kairomones	1	0.62712
	Calcium X Kairomones	3	**0.00035**
IP3R	Calcium	3	**0.00015**
	Kairomones	1	**0.0171**
	Calcium X Kairomones	3	**0.00083**
SERCA	Calcium	3	**0.03628**
	Kairomones	1	0.14975
	Calcium X Kairomones	3	**0.0362**
Calb32	Calcium	3	**0.00214**
	Kairomones	1	**0.02389**
	Calcium X Kairomones	3	**0.00584**

Table 3.3: T-test comparison of means between kairomone and non-kairomone treatments at each calcium concentration for the expression of *Calb32, ICP1, ICP2, IP3R,* and *SERCA*. P-values indicating significant effect of kairomones on gene expression are in bold.

Gene	Calcium(mg/L)	P-value
ICP1	0.5	**0.041017**
	1.5	**0.033617**
	2.5	0.198516
	5	0.089593
ICP2	0.5	0.734952
	1.5	0.713778
	2.5	**0.035489**
	5	**0.031743**
IP3R	0.5	0.482712
	1.5	0.276827
	2.5	0.392626
	5	**0.002894**
SERCA	0.5	0.833013
	1.5	0.273803
	2.5	0.139337
	5	**0.001833**
Calb32	0.5	0.489227
	1.5	0.069096
	2.5	**0.038873**
	5	**0.048935**

Table 3.4: One way ANOVA testing if calcium had a significant effect on the gene expression in the presence or absence of kairomones. Bold p-values indicate significant effect of calcium on the expression of the gene.

Gene	Kairomone presence	Pr (>F)
IPC1	Without	0.1746
	With	**0.007101**
IPC2	Without	**0.004695**
	With	**0.01656**
IP3R	Without	**0.00008**
	With	0.1885
SERCA	Without	0.9653
	With	**0.00322**
Calb32	Without	0.1482
	With	**0.001549**

Table 3.5: Tukey Multiple Comparison Test to test which specific calcium treatments significantly affected gene expression. Bold p-values indicate significant difference in expression between two calcium treatments.

Gene	Kairomone presence	Compared Ca treatments (ml/l)	P
ICP1	With	0.5-1.5	0.824782
		0.5-2.5	0.084403
		0.5-5.0	**0.007459**
		1.5-2.5	0.274036
		1.5-5.0	**0.023194**
		2.5-5.0	0.346328
ICP2	Without	0.5-1.5	0.731389
		0.5-2.5	0.991412
		0.5-5.0	**0.008319**
		1.5-2.5	0.5726
		1.5-5.0	**0.033527**
		2.5-5.0	**0.005818**
	With	0.5-1.5	0.907306
		0.5-2.5	**0.01798**
		0.5-5.0	0.912659
		1.5-2.5	**0.044962**
		1.5-5.0	0.999999
		2.5-5.0	**0.044021**
IP3R	Without	0.5-1.5	0.957506
		0.5-2.5	0.159892
		0.5-5.0	**0.000165**
		1.5-2.5	0.079737
		1.5-5.0	**0.000108**

		2.5-5.0	**0.001719**
SERCA	With	0.5-1.5	0.695701
		0.5-2.5	0.084664
		0.5-5.0	**0.018772**
		1.5-2.5	**0.018151**
		1.5-5.0	**0.004525**
		2.5-5.0	0.709479
Calb32	With	0.5-1.5	0.987568
		0.5-2.5	0.770484
		0.5-5.0	**0.002182**
		1.5-2.5	0.913971
		1.5-5.0	**0.00217**
		2.5-5.0	**0.006974**

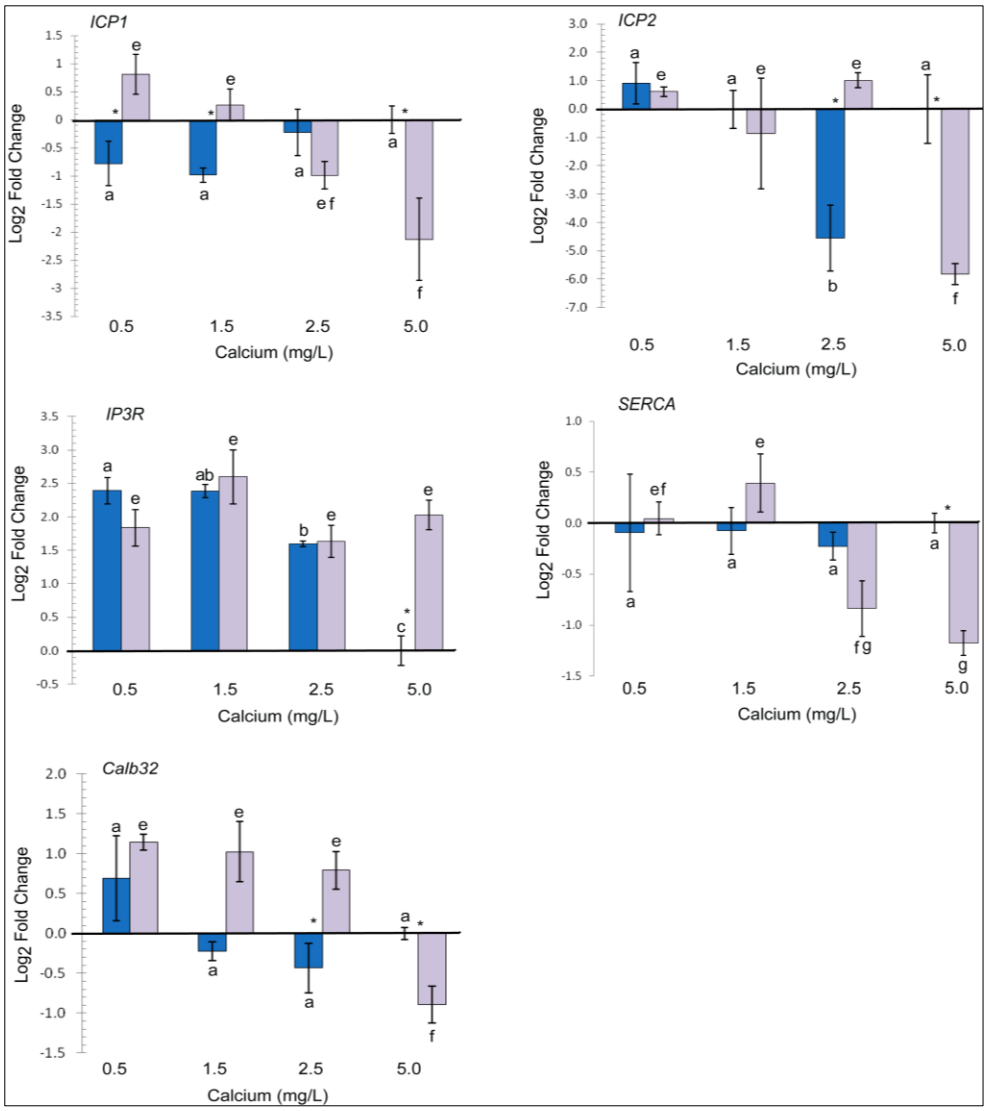

Figure 3.1: Gene expression in $\log_2$ scale. Gene indicated in top right corner. Dark bars are non-kairomone treatments. Light bars are kairomone treatments. Y-axis is $\log_2$ (fold change) with non-kairomone 5.0 mg/l Ca^{2+} treatment as the calibrator. X-axis corresponds to calcium concentrations. Letters represent statistically similar gene expression across calcium treatments. Stars represent statistically different expression between the kairomone and the non-kairomone treatments. Error bars are based standard error of the mean (SEM).

CHAPTER IV

THE EVOLUTIONARY HISTORY OF THE SARCO(ENDO)PLASMIC RETICULUM

4.1 Introduction

A clear understanding of the evolutionary history of gene families is essential for studying their function, expression and the evolutionary forces responsible for their diversification. Many evolutionary events such as gene duplication are important drivers of the expansion of gene families but can also confound functional genomics and gene expression studies that focus on orthologous genes. Consequently, a solid phylogeny of the genes of interest, especially multiple-copy genes, is needed before performing gene expression or comparative studies. The availability of many sequenced genomes greatly facilitates the investigation of the evolutionary history of many environmentally relevant gene families, such as the P-type II ATPases. This family of cation transporters plays a key role in the adaptation of organisms to variable environments, including variation in cation concentrations due to their shared specificities for Ca^{2+}, K^{+} and Na^{+} (Corradi and Sanders, 2006). Although the nomenclature of this gene family has been revisited, it is generally accepted that P-type II ATPases include five closely related sub-families (SERCA, PMCA, NK/HK, ENA, and ACU) (Benito et al., 2004; Corradi and Sanders, 2006). This study focuses on investigating the key evolutionary events that have led to the extensive diversification of sarco(endo)plasmic calcium ATPases (SERCA) across the major domains of eukaryotes.

Scarco(endo)plasmic Reticulum Calcium-ATPase (SERCA) is a key player in calcium signalling (Berridge et al., 2003), which is involved in many aspects of cellular function (Clapham, 2007), including transcription (Flavell and Greenberg, 2008), cell motility (Qi et al., 2007), apoptosis, exocytosis, and signal transduction (Kudla et al., 2010). For example, during calcium-mediated signal transduction, the depolarization of the cell membrane in active cells causes an extensive influx of calcium into the cytoplasm. However, this influx of calcium needs to be reversed for proper cellular function (Clapham, 2007). To reduce cytoplasmic Ca^{2+} concentrations, SERCA uses ATP to actively pump calcium into the sarco(endo)plasmic reticulum for storage (Wuytack et al., 2002; Berridge et al., 2003). The essential cellular function of SERCA makes it an interesting target for evolutionary studies as it is ubiquitous and indispensable across eukaryotic taxa.

Given the importance of the SERCA proteins to both cellular and organismal physiology, changes in the function, location, and expression of SERCA constitute significant evolutionary events. Previous genetic studies revealed that several gene duplication events occurred in the evolution of the SERCA. Three genes are present in vertebrates (ATP2A1-3), coding for three SERCA isoforms, SERCA 1-3 (Wuytack et al., 2002), while only one gene has been described in invertebrates, with the exception of the human parasitic blood fluke, *Schistosoma mansoni* (Talla et al., 1998; Wuytack et al., 2002). Interestingly, each of the vertebrate genes undergoes alternative splicing, resulting in ten SERCA proteins: SERCA 1a/b, SERCA 2a/b and SERCA 3a/b/c/d/e/f

(Martin et al., 2002; Hovnanian, 2008). These isoforms and their splice variants have tissue specific expression patterns. For example, SERCA 1a is expressed in fast twitch muscles of adults and SERCA 1b in neonates (Brandl et al., 1987). SERCA 2a is expressed primarily in cardiac and slow-twitch skeletal muscles, whereas its splice variant, SERCA 2b, is expressed in almost all non-muscle cells and is often considered the house keeping variant (Hovnanian, 2008). Furthermore, SERCAs 3 and 2b are found in a wide range of cells including lymphocytes, epithelial, endothelial, and mast cells, as well as Purkinje neurons of the cerebellum (Wuytack et al., 2002; East, 2000). The efficiency of the pump varies among the isoforms with SERCA 1a/b having a higher turnover rate than SERCA 2b and a higher affinity for calcium than SERCA 3 (East, 2000; Lytton et al., 1992). Between the two SERCA 2 isoforms, SERCA 2b (1042aa) has a 2-fold higher calcium binding ability but a 2-fold lower turnover rate compared to the shorter variant SERCA 2a (997aa) (East, 2000; Verboomen et al., 1992;Campbell et al., 1991; Wuytack et al., 2002). The single SERCA gene in invertebrates also undergoes alternative splicing and tissue specific expression, similar to the vertebrate SERCA 2 (Escalante and Sastre, 1993; Chen et al., 2002; Fan et al., 2007; Mandal et al., 2009). The pattern of splicing has been demonstrated in invertebrates such as *Artemia franciscana* and *Caenorhabditis elegans* (Escalante and Sastre, 1993; Wuytack et al., 2002). Based on the observation that SERCA2b is the housekeeping variant, the single SERCA protein in invertebrates has been suggested to be most closely related to the vertebrate SERCA 2 (Wuytack et al., 2002).

The P-type ATPases have a complex history of gene duplication events. For example, *Arabidopsis* has about 46 P-type ATPase genes with multiple isoforms in each family while humans have about 36 genes. Several hypotheses have been proposed to explain the evolution of this complex gene family. Firstly, multiple isoforms could have evolved to suit specific cell types, and would have tissue specific regulation (Baxter et al., 2003). Secondly, the different isoforms could be adapted to optimally function under a variety of conditions and stressors (e.g., toxic cations, Mäser et al., 2001), allowing the organism to inhabit a large variety of habitats and niches. Lastly, distinct from previous views that all genes are functional under certain conditions, it has been suggested that a fraction of isoforms could be functionally redundant duplicates (Baxter et al., 2003). These alternative hypotheses that are consistent with the theoretical predictions of the evolutionary fate of gene duplicates (Innan and Kondrashov, 2010) are difficult to distinguish. To address this issue, an initial step is to characterize the historical gene duplication events that have occurred during the evolution of such gene families. SERCA is the most well characterized P-type ATPase, having an X-ray crystallography structure and used as an archetype for homology modeling of other P-type ATPases (Kühlbrandt, 2004). Despite the extensive knowledge and interest in its structure, function, and expression, little is known about SERCA's evolutionary history. Here, I use protein sequences and phylogenetic approaches to examine the relationship among SERCA homologues across eukaryotic taxa. Specifically, I assess the role of gene duplication in the evolution of vertebrate SERCA isoforms and test previous hypotheses regarding

phylogenetic relationships of three vertebrate SERCA isoforms with invertebrates in a Eukaryotic phylogenetic context. Furthermore, I explore the protein-based eukaryotic phylogeny of SERCA to examine various likely gene duplication events in other phylogenetic lineages and their evolutionary implications.

4.2 Methods

Sequence retrieval

A total of 81 SERCA amino acid sequences of vertebrate, invertebrate, plant, fungi, and other unicellular eukaryotes such as protists and ciliated protozoans were retrieved from Genbank, Uniprot, Ensmble, and JGI (Table 4.1). I conducted the searches using the key terms "Sarcoplasmic/endoplasmic calcium ATPase", "SR Ca^{2+} ATPase" and "SERCA". In addition, I used BLAST searches based on annotated vertebrate and invertebrate sequences. If multiple splice variants of the protein were reported, the canonical sequence was used.

Sequence alignment and phylogenetic analyses

I performed the amino acid sequence alignment using ClustalW (Thompson et al. 1994) with a Gap Opening Penalty of 10 and Gap Extension Penalty of 0.2. The highly variable 3' terminal ends of the sequences were removed from the alignment to avoid ambiguity. Minor adjustments were made manually to the alignment. Phylogenetic analyses were performed using neighbour-joining (NJ) and Bayesian inference

approaches, in MEGA (Tamura et al., 2007) and MrBayes (Ronquist et al., 2003) respectively. Members of other P-type II ATPases sub-families were used as outgroups (Table 4.1) in all phylogenetic reconstructions. Outgroup sequences include the Plasma Membrane Ca^{2+} ATPase (*ATP2B1*), Secretory Pathway Ca^{2+} ATPase (*ATP2C1* and *ATP2C2*), Na^+/K^+-transporting ATPase (*ATP4A1*), K^+-transporting ATPase (*ATP4A*), as well as the fungal specific Na^+/K^+ ATPase (*ACU1*) and Na^+ transport ATPase (*ENA1*) (Axelsen and Palmgren, 1998). I conducted the neighbour-joining tree using pairwise deletion and the Jones-Taylor-Thornton (JTT) (Jones et al., 1992) amino acid model. Bootstrap analyses were based on 1000 replicates. The Bayesian inference tree was constructed with the WAG model of amino acid substitution (Whelan and Goldman, 2001), which was selected as the most appropriate model because of its significantly greater contribution to the posterior probability distribution than eight other fixed rate models. This model can handle a large number of sequences and is applicable to a wide range of protein families, but retains the advantages of a maximum-likelihood approach and accounts for multiple substitutions at the same site (Whelan and Goldman, 2001).Three independent runs of 4 Markov chains were conducted for 1,000,000 generations with a sampling frequency of 10 and the first 25% of sampled trees discarded as burn-in.

4.3 Results and Discussion

Overall phylogenetic pattern

The SERCA alignment consisted of 81 sequences (61 unique taxa) spanning 1575 amino acids and contained 220 conserved and 818 parsimony informative sites. Both the BI and NJ analyses returned highly congruent tree topologies. Two major monophyletic clades were consistently recovered. The first group contained clades A, B and C, which consist of metazoan, fungal, and plant sequences, respectively (Fig. 4.1). The second group contained clade D that encompasses plant and protist sequences (Fig. 4.1). Within clade A, the chordates were monophyletic and contained two reciprocally monophyletic clades corresponding to vertebrates and tunicates.

SERCA gene duplication and evolution

Within metazoans, the SERCA sequences of the chordates formed a well-supported monophyletic group that includes two sister clades, corresponding to the vertebrates and tunicates. In vertebrates, each of the three SERCA isoforms (i.e. SERCA1-3; coded by *ATP2A1-3*, respectively) formed highly supported monophyletic groups. This pattern indicates that these three SERCA isoforms arose through two rounds of gene (or whole genome) duplication events after divergence from the tunicates but before the separation of fishes and tetrapods. Furthermore, *ATP2A1* and *2* are sister groups and likely arose from a more recent duplication event than the one leading to *ATP2A3*. My findings do not support the previous suggestion that SERCA2 is the most ancestral of the three isoforms (Wuytack et al., 2002). Furthermore, tunicates

have undergone several independent and relatively recent gene duplication events (Fig. 4.1).

My study reveals that invertebrates also have experienced several instances of gene duplication events leading to multiple *SERCA* paralogs. Previous studies identified that the parasite trematode *Schistosoma mansoni*has two SERCA proteins that are coded by the genes *SMA1* and *SMA2* (Talla et al., 1998). Based on my analysis, the two *S. mansoni* calcium ATPases group together, despite the low bootstrap support and high sequence divergence (29.5%). This suggests that *SMA1* and *2* are likely paralogs that arose from an ancient gene duplication event. Furthermore, *Helobdella robusta* also possesses three *SERCA* genes, dubbed here as SERCA A, B and C. While *SERCA A* and *B* group with other annelid sequences *SERCAC* is highly divergent and basal to the animal clade. Thus, *SERCA C* likely arose due to an ancient gene duplication event, while *SERCA A* and *B* are the result of relatively recent gene duplication event. This hypothesis is consistent with findings of several other gene duplications within the *H. robusta* genome, across a variety of unrelated gene families (Kourakis and Martindale, 2001; Cristescu et al., 2008; Cho et al., 2010).

Plants are particularly reliant on calcium signaling to control many of vital physiological processes and stress responses (Reddy et al., 2011; Conde et al., 2011). Because plants use the same ion to respond to different stimuli, the temporal and spatial patterns of the signals must vary (Kudla et al., 2010). Thus, the regulation of proteins involved in calcium signaling must be fine tuned in order to induce a stimulus

specific response (Sanders et al., 2002). While *Arabidopsis* is known to have four Endoplasmic Ca^{2+} ATPases (coded by genes *ECA 1-4*; Kabala and Klobus, 2005), most plants have only three (Baxter et al., 2003). Based on my analysis, the plant sequences formed two monophyletic clades, Plant1 and Plant2 (Fig 1.). Plant1 consists of the *ECA 1*, *2*, and *4* genes and is sister to the clade formed by apicomplexan sequences. However, Plant2, which contains the *ECA 3* genes, is sister group to metazoans. The presence of two divergent clades of *SERCA* genes in plants indicates a possible ancient duplication in the common ancestor of plants, animals, and fungi, followed by the loss of one of the duplicates in the lineage leading to animals and fungi. Interestingly, the *ECA 3* encoded protein serves to pump calcium into both the Golgi and endoplasmic reticulum (Mills et al., 2008). However, animals and fungi have a separate set of proteins (SPCA) that pump calcium into the Golgi that appear to be absent in plants (Mills et al., 2008). The altered function and localization of the *ECA 3* encoded protein to the Golgi may have played a role in its evolution and maintenance in plant genomes and warrants further investigation. The evolution of complex calcium signaling in plants was likely facilitated by duplication of Ca^{2+} ATPase genes which diverged in their patterns of regulation and localization.

Gene duplication events are an essential part of the evolutionary process as they generate novel gene functions and families. The initial increased dosage of gene products resulting from a gene duplication event may be beneficial or detrimental to the organism. The function of the new gene will be retained through stabilizing selection if

the increased dosage is beneficial or lost through purifying selection if it is detrimental (Innan and Kondrashov, 2010). However, if increased dosage has no effect, the gene is no longer under selective pressure and is free to accumulate mutations. Therefore, the duplicated gene can either become a pseudogene, or gain novel function through changes in the protein structure or expression pattern (Kondrashov et al., 2002; Colbourne et al., 2011). Duplicated genes may also gain novel function by translocation into different regulatory regions. Such events can drastically alter the location, timing, and conditions of their expression. It appears that duplicated *SERCA* genes gain novel functions; this is especially apparent in the three vertebrate *SERCA* genes that exhibit tissue specific expression patterns likely resulting from divergence in regulation of these genes following the duplication events. Evidence of both ancient and recent gene duplication events in many taxa demonstrates the capacity of *SERCA* genes to multiply and retain functional significance.

From gene tree to species tree: Paraphyly of Crustacea

If the major ancient gene duplication events are ignored, the overall phylogenetic pattern recovered was consistent with that of the combined protein data of α-tubulin, β-tubulin, actin, and elongation factor 1–alpha (Baldauf et al., 2000). Moreover, the recovered phylogeny provides valuable information into the evolution path of crustaceans. The phylogenetic relationships among arthropod taxa, especially those within Pancrustacea remain unclear in many phylogenetic studies (Jenner, 2010).

Here, the monoplyly of the Pancrustacea SERCA proteins was highly supported. The SERCAs of hexapods formed a monophyletic group. However, crustaceans appear to be paraphyletic; *Panulirus argus*, *Procambarus clarkii*, and *Porcellio scaber* are sister to the hexapods, and not to the clade formed by the branchiopods *Daphnia pulex* and *Artemia franciscana*. This observation is consistent with other molecular and morphological based studies that support the monophyly of Pancrustacea, including all members Crustacea and Hexapoda. (Harzsch, 2002; Sinakevitch et al., 2003; Regier et al., 2005; Mallatt and Giribet, 2006; von Reumont et al., 2009; Edgecombe, 2010). To date there is no consensus regarding the placement of Hexapoda within the paraphyletic crustacean group. The proposed sister clades include Branchiopoda (Regier et al., 2005; Mallatt and Giribet, 2006), Malacostraca (Harzsch, 2002; Sinakevitch et al., 2003), and Copepoda (von Reumont et al., 2009). The SERCA protein-based phylogenetic analyses suggest Malacostraca as the sister group to Hexapoda. Future detailed studies utilizing a combination of morphological and molecular data are still necessary to elucidate the phylogenetic relationships within Pancrustacea. In addition, several other monophyletic groups were recovered across invertebrates, including arthropods, nematodes, molluscs, and annelids.

An ecological perspective

Understanding the evolutionary history of SERCA proteins is particularly interesting in light of a drastic decline of calcium in both aquatic (Keller et al., 2001;

Jeziorski et al., 2008) and terrestrial (Likens et al., 1998; Watmough and Dillon, 2003) ecosystems. The depletion of calcium is affecting a broad range of organisms such as plants, zooplankton, and birds (Cairns and Yan, 2009; Pabianand Brittingham, 2011) that use calcium not only in cell signaling and regulation of proteins, but also as a major structural element. For example, a reduction of calcium in plants would be expected to reduce the structural integrity of woody tissues and thus increase susceptibility to structural damage, as well as reduce their capacity to repair this damage, defend against pathogens and herbivory, and respond to a number of abiotic stressors (see McLaughlin and Wimmer 1999; Reddy et al. 2011). However, little is known about the capacity of organisms to adapt to low calcium levels or the broad ecological impact of the severe calcium decline. The investigation of the evolutionary history of essential genes that regulate calcium levels can facilitate future studies that explore gene expression and function as well as broader studies that aim to bridge the gap between genetics and ecology.

Conclusion

Overall, the phylogenetic analyses reveal several recent and ancient gene duplication events across different taxonomic levels during the evolution of *SERCA* genes. Notably, gene duplication events have resulted in proteins with new function and expression patterns in plants and vertebrates. My results have refined the

understanding of the complex evolutionary history of this gene family and will greatly facilitate gene expression and comparative studies that focus on *SERCA* genes.

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Table 4.1: List of protein sequences used for phylogenetic analyses

Species	Common name	Paralogs	Accession number	Evidence
Acyrthosiphon pisum	pea aphid		GenBank ID: XP_001943129	EST
Anolis carolinensis	lizard	*ATP2A1*	Ensembl ID: ENSACAP00000005618	Ensemble genebuild prediction
		ATP2A2	Ensembl ID: ENSACAP00000015925	Ensemble genebuild prediction
Anopheles gambiae	African malaria mosquito		Uniprot ID: Q7PPA5	Inferred from homology
Apis mellifera	honey bee		GenBankID: XP_393851	EST
Arabidopsis thaliana	mouse-ear cress	*ECA1*	Uniprot ID: P92939	Protein level
		ECA2	Uniprot ID: O23087	Transcript level
		ECA3	Uniprot ID: Q9SY55	Transcript level
		ECA4	Uniprot ID: Q9XES1	Transcript level
Artemia franciscana	brine shrimp		Uniprot ID: P35316	Transcript level
Aspergillus niger	fungi		Uniprot ID: A2RBD0	Inferred from homology
Babesia bovis	apicomplexan		Uniprot ID: A7AUB0	Inferred from homology
Bombyx mori	domestic silkworm		Uniprot ID: C7AQP4	Transcript level
Brugia malayi	nematode		Uniprot ID: A8PF97	Inferred from homology
Caenorhabditis briggsae	nematode		Uniprot ID: A8XSD9	Inferred from homology
Caenorhabditis elegans	nematode		Uniprot ID: Q9XTG6	Transcript level
Capitella teleta	polychaete worm		JGI Protein ID: 165811 *	EST
Ciona intestinalis	sea squirt	A	JGI Protein ID: 207981 *	EST
		B	JGI Protein ID: 295594 *	EST
Ciona savignyi	sea squirt	A	Uniprot ID: Q75UU1	Transcript level
		B	Ensembl ID: ENSCSAVP00000016616	EST
Cryptococcus neoformans	yeast		Uniprot ID: Q5KCV6	Inferred from homology
Cryptosporidium hominis	apicomplexan		Uniprot ID: Q5CNZ6	Inferred from homology
Culex quinquefasciatus	mosquito		Uniprot ID: B0XEW6	Inferred from homology
Danio rerio	zebrafish	*ATP2A1*	Uniprot ID: Q642Z0	Transcript level
		ATP2A2	Uniprot ID: Q7ZW18	Transcript level
Daphnia pulex	water flea		JGI Protein ID: 219234 *	EST
Drosophila grimshawi	fruit fly		Uniprot ID: GH20185	Inferred from homology
Drosophila melanogaster	fruit fly		Uniprot ID: P22700	Protein level
Drosophila pseudoobscura	fruit fly		Uniprot ID: Q292Q0	Inferred from homology
Gallus gallus	chicken	*ATP2A1*	Uniprot ID: P13585	Transcript level

		ATP2A2	Uniprot ID: Q03669	Transcript level
		ATP2A3	Uniprot ID: Q9YGL9	Transcript level
Halocynthia roretzi	tunicate		Uniprot ID: Q8IAC0	Transcript level
Helobdella robusta	leech	A	JGI Protein ID: 156619 *	EST
		B	JGI Protein ID: 106126 *	EST
		C	JGI Protein ID: 185062 *	EST
Homo sapiens	human	*ATP2A1*	Uniprot ID: O14983	Protein level
		ATP2A2	Uniprot ID: P16615	Protein level
		ATP2A3	Uniprot ID: Q93084	Protein level
Laccaria bicolor	Mushroom		Uniprot ID: B0D3J7	Inferred from homology
Lottia gigantea	owl limpet		JGI Protein ID: 181748 *	EST
Lumbricus rubellus	humus earthworm		Uniprot ID: D0EXD4	Transcript level
Makaira nigricans	Blue marlin		Uniprot ID: P70083	Transcript level
Molgula tectiformis	Tunicate	A	Uniprot ID: B5MGP1	Transcript level
		B	Uniprot ID: B5MGP3	Transcript level
Monosiga brevicollis	choanoflagellate		JGI Protein ID: 7291 *	EST
Mus musculus	mouse	*ATP2A1*	Uniprot ID: Q8R429	Transcript level
		ATP2A2	Uniprot ID: O55143	Protein level
		ATP2A3	Uniprot ID: Q64518	Transcript level
Nasonia vitripennis	jewel wasp		GenBank ID: XP_001603571	EST
Nematostella vectensis	sea anemone		JGI Protein ID: 160437 *	EST
Oryza sativa japonica	rice	*ECA3*	Uniprot ID: Q10DF1	Inferred from homology
		ECA4	Uniprot ID: Q8H8W1	Inferred from homology
Panulirus argus	Caribbean spiny lobster		Uniprot ID: Q49LV5	Transcript level
Paramecium tetraurelia			Uniprot ID: Q9N9D8	Transcript level
Pinctada fucata	pearl oyster		Uniprot ID: B2KKR1	Transcript level
Placopecten magellanicus	sea scallop		Uniprot ID: O77070	Transcript level
Plasmodium berghei	parasitic protist		Uniprot ID: Q4Z579	Inferred from homology
Plasmodium falciparum	parasitic protist		Uniprot ID: Q5R2K7	Inferred from homology
Plasmodium yoelii	parasitic protist		Uniprot ID: Q27764	Inferred from homology
Populus trichocarpa	poplar tree	*ECA1*	Uniprot ID: B9HPP7	Inferred from homology
		ECA2	Uniprot ID: B9I912	Inferred from homology
		ECA3	Uniprot ID: B9IBQ1	Inferred from homology

Porcellio scaber	woodlouse		Uniprot ID: Q8I897	Transcript level
Procambarus clarkii	red swamp crayfish		Uniprot ID: O17315	Transcript level
Rana clamitans	green frog	*ATP2A1*	Uniprot ID: Q9DDB9	Transcript level
Ricinus communis	castor oil plant	*ECA3*	Uniprot ID: B9R6Y5	
Schistosoma mansoni	blood fluke	*SMA1*	Uniprot ID: Q27779	Transcript level
		SMA2	Uniprot ID: O96527	Transcript level
Strongylocentrotus purpuratus	purple sea urchin		Uniprot ID: Q308S5	Transcript level
Tetrahymena thermophila	ciliate protozoa		Uniprot ID: Q22BT1	Inferred from homology
Toxoplasma gondii	apicomplexan		Uniprot ID: Q5IH90	Transcript level
Tribolium castaneum	red flour beetle		GenBank ID: XP_966783	EST
Trichoplax adhaerens	placozoan		Uniprot ID: B3S4L8	Inferred from homology
Uncinocarpus reesii	fungi		Uniprot ID: C4JXS5	Inferred from homology
Ustilago maydis	fungi		Uniprot ID: Q7Z8J8	Inferred from homology
Vitis vinifera	grape	*ECA2*	Uniprot ID: F6I6G6	Inferred from homology
Xenopus laevis	African clawed frog	*ATP2A1*	Uniprot ID: Q7ZXY6	Transcript level
		ATP2A2	Uniprot ID: A2RV57	Transcript level
		ATP2A3	Uniprot ID: Q0V9S4	Transcript level
Outgroups				
Homo sapiens	human	*ATP2B1*	Uniprot ID: P20020	Protein level
Homo sapiens	human	*ATP2C1*	Uniprot ID: P98194	Protein level
Homo sapiens	human	*ATP2C2*	Uniprot ID: O75185	Protein level
Homo sapiens	human	*ATP1A1*	Uniprot ID: P05023	Protein level
Homo sapiens	human	*ATP4A*	Uniprot ID: P20648	Transcript level
Ustilago maydis	fungi	*ACU1*	Uniprot ID: Q703G5	Inferred from homology
Saccharomyces cerevisiae	fungi	*ENA1*	Uniprot ID: P13587	Protein level

* All JGI Protein ID sequences were taken from their respective genome portal of the organisms

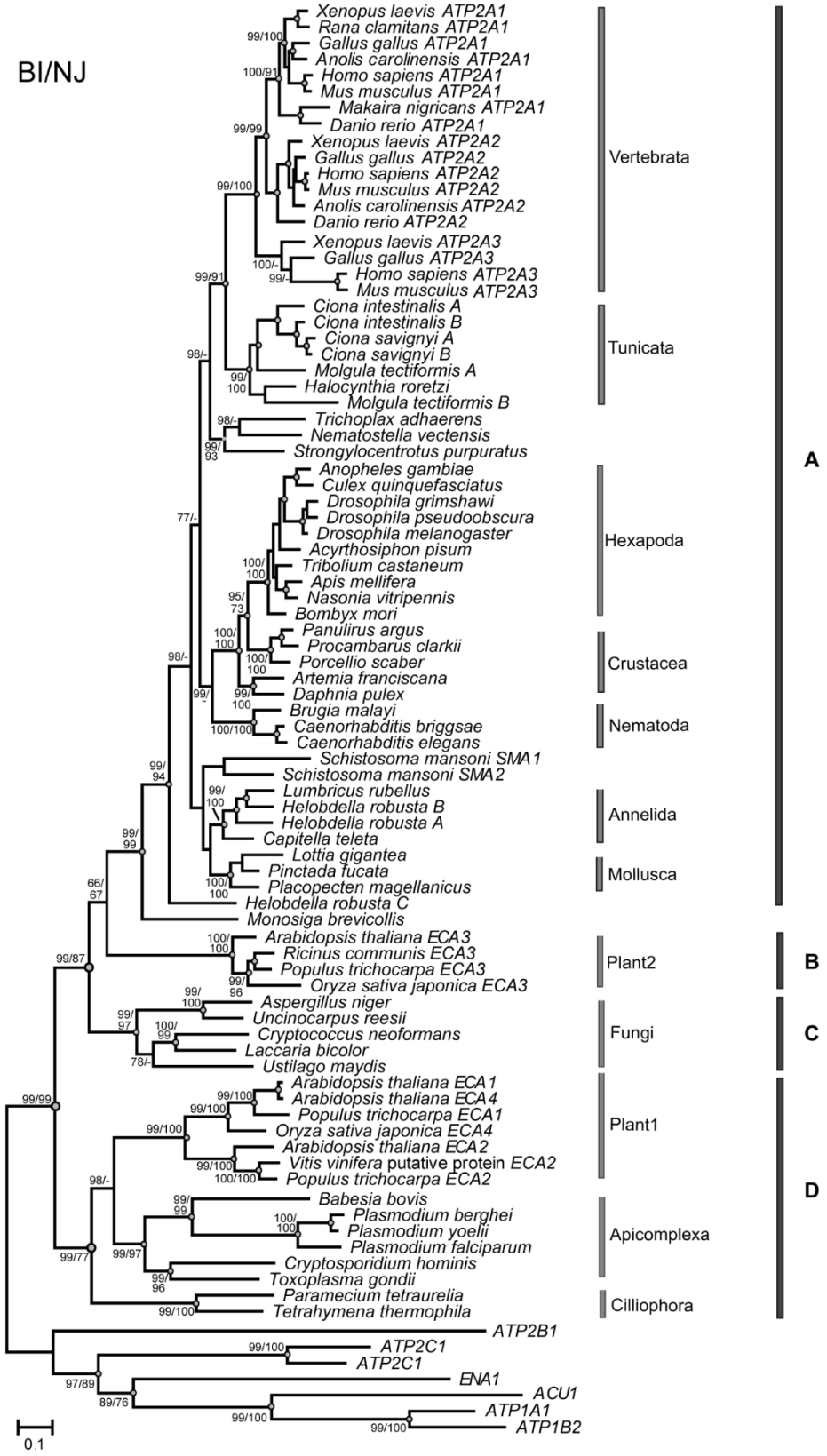
BI/NJ
Xenopus laevis ATP2A1
Rana clamitans ATP2A1
Gallus gallus ATP2A1
Anolis carolinensis ATP2A1
Homo sapiens ATP2A1
Mus musculus ATP2A1
Makaira nigricans ATP2A1
Danio rerio ATP2A1
Xenopus laevis ATP2A2
Gallus gallus ATP2A2
Homo sapiens ATP2A2
Mus musculus ATP2A2
Anolis carolinensis ATP2A2
Danio rerio ATP2A2
Xenopus laevis ATP2A3
Gallus gallus ATP2A3
Homo sapiens ATP2A3
Mus musculus ATP2A3
Vertebrata
Ciona intestinalis A
Ciona intestinalis B
Ciona savignyi A
Ciona savignyi B
Molgula tectiformis A
Halocynthia roretzi
Molgula tectiformis B
Tunicata
Trichoplax adhaerens
Nematostella vectensis
Strongylocentrotus purpuratus
Anopheles gambiae
Culex quinquefasciatus
Drosophila grimshawi
Drosophila pseudoobscura
Drosophila melanogaster
Acyrthosiphon pisum
Tribolium castaneum
Apis mellifera
Nasonia vitripennis
Bombyx mori
Hexapoda
Panulirus argus
Procambarus clarkii
Porcellio scaber
Artemia franciscana
Daphnia pulex
Crustacea
Brugia malayi
Caenorhabditis briggsae
Caenorhabditis elegans
Nematoda
Schistosoma mansoni SMA1
Schistosoma mansoni SMA2
Lumbricus rubellus
Helobdella robusta B
Helobdella robusta A
Capitella teleta
Annelida
Lottia gigantea
Pinctada fucata
Placopecten magellanicus
Mollusca
A
Helobdella robusta C
Monosiga brevicollis
Arabidopsis thaliana ECA3
Ricinus communis ECA3
Populus trichocarpa ECA3
Oryza sativa japonica ECA3
Plant2
B
Aspergillus niger
Uncinocarpus reesii
Cryptococcus neoformans
Laccaria bicolor
Ustilago maydis
Fungi
C
Arabidopsis thaliana ECA1
Arabidopsis thaliana ECA4
Populus trichocarpa ECA1
Oryza sativa japonica ECA4
Arabidopsis thaliana ECA2
Vitis vinifera putative protein ECA2
Populus trichocarpa ECA2
Plant1
Babesia bovis
Plasmodium berghei
Plasmodium yoelii
Plasmodium falciparum
Cryptosporidium hominis
Toxoplasma gondii
Apicomplexa
Paramecium tetraurelia
Tetrahymena thermophila
Cilliophora
D
ATP2B1
ATP2C1
ATP2C1
ENA1
ACU1
ATP1A1
ATP1B2
0.1

Figure 4.1: Phylogenetic tree of SERCA amino acid sequences from 57 taxa using Bayesian inference. The numbers at the nodes indicate posterior probabilities/bootstrap supports. The nodes highlighted with grey circles represent consensus NJ and BI analyses with bootstrap support higher than 70%.

CHAPTER V

GENERAL DISCUSSION

5.1 General discussion

Understanding the effects and interactions of anthropogenic stressors with natural ones on native biota is critical to setting guidelines for protecting ecosystem function (Oughton, 2007). In the literature review chapter I stressed the need to study combinations of stressors by demonstrating how their interactions sometimes have non-additive effects on *Daphnia*. In particular, I focused on three key anthropogenic stressors- calcium decline, climatic change, and metal toxicants, and their interactions with other anthropogenic and natural stressors such as predation, UV light, and food availability. These interactions produce unexpected effects on *Daphnia* that could not have been predicted based on single stressor studies. In addition, I demonstrated how integrating diverse biological fields such as ecology and molecular genetics could be a useful approach in studying these interactions.

Gene expression profiling is a powerful tool with the potential to tease out the complex interactions of multiple stressors on organismal function. In chapter 3, I used gene expression levels to experimentally demonstrate the non-additive effects of low calcium and predator presence on several *Daphnia* genes. The animals responded to declining calcium concentrations differently when exposed simultaneously to predator kairomones. All genes altered their expression patterns in response to the interaction of the two stressors. In addition, in most genes, there was only an expression change with varying calcium concentration in either the kairomone or the non-kairomone treated

animals. Precisely, *ICP1*, *SERCA*, and *CaIb32* only altered in expression with varying calcium concentrations in the kairomone treated animal. Conversely, *IP3R* only change in expression with varying calcium concentrations in the non-kairomone treated animals. *ICP2* was the only gene that was differentially expressed with varying calcium concentrations in both the kairomone and non-kairomone treatment.

Finally, I took the first steps of expanding this study to other organisms by exploring the evolutionary history of one of the targeted genes, the sarco(endo)plasmic reticulum calcium ATPase (SERCA). I constructed a phylogeny based on amino acid sequences of *SERCA* from vertebrate, invertebrate and plant taxa to investigate any gene duplication events within this gene. This is an essential step since we need to be aware of any duplication events that may complicate expression studies, and make sure we are considering ortholog genes between species. *SERCA* genes have a history of ancient and recent gene duplication events. These events, range from taxon specific duplication, as with the leech, to kingdom level, as with plants.

The link between gene expression levels and environmental function is still in its infancy. On the whole, many studies that look at the effects of toxicants and other stressors on gene expression (Shaw et al., 2007; Soetaert et al., 2007; Schwarzenberger et al., 2009). However, the relationship between stress and expression in natural populations still needs to be explored. Thus, there are several avenues that could stem from the current study. First, exploring the expression of more genes, via microarray (Shaw et al. 2007) and RNAseq (Schirmer et al., 2010) technologies would identify co-

regulated genes. Based on this information, ecologically relevant molecular pathways could be built (Eads et al., 2008). These pathways could help us understand howorganisms react to environmental cues on a molecular level. In addition, gene expression studies of natural populations need to be preformed to validate the laboratory results. These studies would gage the natural variation in expression patterns across individuals and habitats. Lastly, since the amount of the transcript does not always predict levels of proteins (Schwanhausser et al., 2011), we need to perform proteomic studies to link mRNA levels to the amount of the final protein product.

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