Consequences of Calcium Deficiency on Embryogenesis in a Keystone Freshwater Crustacean: Daphnia magna

by

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ABSTRACT

CONSEQUENCES OF CALCIUM DEFICIENCY ON EMBRYOGENESIS IN A KEYSTONE FRESHWATER CRUSTACEAN: DAPHNIA MAGNA

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Ambient calcium is declining in thousands of soft-water lake habitats as a consequence of unsustainable forestry practices, decreased atmospheric calcium deposition, and acidic deposition. The freshwater crustacean Daphnia magna possesses a high specific calcium content relative to other aquatic organisms, which makes it ideal for investigating the consequences of calcium decline. Adult and juvenile Daphnia have different tolerances to low ambient calcium as a result of their different life stage specific functions and calcium requirements. This study focuses on the consequences of calcium decline on Daphnia magna embryos and embryogenesis. An embryonic staging scheme was developed to pursue this focus. The major findings suggest that calcium is involved in developmental processes, and may have particular significance within the first 30hrs of embryonic development. Maternal calcium provisioning to embryos occurs exclusively during embryo formation, and females can adjust embryonic calcium provisioning (quantitatively) during times of ambient calcium shortage.
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CHAPTER 1 - GENERAL INTRODUCTION

The significance of calcium decline

Calcium is an essential element required for life and as such its presence is ubiquitous across the plant and animal kingdoms. Its functions range from structural reinforcement (i.e. fortifying bones, exoskeletons and plant cell walls) to molecular signalling (i.e. coordinating muscular contractions, neuronal cascades, and embryonic developmental events) (Webb and Miller, 2003; Webb, 1999). Calcium is an inorganic compound that must be exogenously acquired. Calcium uptake can either occur directly from the abiotic environment as dissolved calcium, or by ingestion of calcium containing organisms.

Since the industrial revolution the concentration of calcium in Canadian Shield soft-water lakes has been declining (Keller et al. 2001). This is in part attributable to the physical geography of the Boreal Shield, but also to anthropogenic activities that influence calcium cycling. The parent-material of this region (predominantly granite and felsic intrusive rock) is resistant to erosion and is subsequently characterized by shallow, acidic, nutrient poor soils (Baldwin et al. 2000). This creates an inherent vulnerability of the associated aquatic and terrestrial ecosystems to calcium deficiency (Baldwin et al. 2000; Lawrence and Huntington, 1999). All of which is further aggravated by processes, such as unsustainable forestry practices and acidic deposition, which remove and rapidly liberate calcium stores (respectively) from the ecosystem without an equivocal means of replenishment (Jezierski et al. 2008a). More colloquially, when calcium is removed it can take centuries for the calcium concentrations to recover in these environments. In some more extreme cases the calcium may be so depleted that the ecosystems may never
recover. Ambient calcium concentrations in south-central Canadian soft-water lakes have declined an average of 45% (ranging from 10-60%; Keller et al. 2001) with many of these lakes approaching calcium concentrations as low as 1 mg/L (Jeziorski et al. 2008a). This problem is not isolated to one country or ecozone and similar trends have been observed in soft-water lakes in regions with comparable physical geography (Alstad et al. 1999; Skjelkvåle et al. 1998).

**Daphnia magna as a model for investigating the consequences of calcium decline**

The consequences of calcium decline will be more severe for some organisms than others, particularly for those whose life history is intimately linked with abundant ambient calcium concentrations. Crustaceans are an example of one such taxon. Crustaceans undergo cyclic moult that require copious amounts of readily available dissolved calcium. Each moult requires that the new exoskeleton be formed and reinforced with calcium that is acquired through active uptake mechanisms (Jeziorski and Yan, 2006). This process is referred to as calcification. A populous freshwater crustacean genus native to the Boreal Shield is *Daphnia.*

*Daphnia* are model organisms for investigating the potential consequences of calcium decline on freshwater ecosystems for several reasons. First, they require large quantities of readily available environmental calcium to fortify their new carapace post-moult. Daphniids also molt frequently when compared to other crustaceans (Ebert, 1992; Halcrow, 1976), which requires that they more frequently build a new carapace and fortify it with calcium. Daphniids are incapable of extending the duration of calcification in response to decreased environmental calcium (Tan and Wang, 2009) and the calcium acquired through ingestion is insufficient for calcification and negligible when compared
to active uptake mechanisms (Tan and Wang, 2009). Furthermore they possess no known calcium storage mechanisms or organs (Cairns and Yan, 2009). These features mean that *Daphnia* have a limited ability to compensate for low ambient environmental calcium as they are unable to extend the duration of the calcification process, increase the amount of calcium they uptake through ingestion, and possess no known calcium storage structures. Given these conditions it is understandable that *Daphnia* require sufficient quantities of available environmental calcium in order to persist. Furthermore, *Daphnia* are considered freshwater keystone organisms (Altshuler *et al.* 2011), and keystone species have a disproportionately large influence on the population dynamics and structure of their habitat (Wagner, 2012). Zooplankton health can be used as a proximate measure for overall ecosystem health (Xu *et al.* 2001) and *Daphnia* are a type of zooplankton commonly examined (in combination with other zooplankton and abiotic factors) to investigate ecosystem health in this manner.

*Daphnia* are predominantly parthenogens (Yasuhiko *et al.* 2011; Figure A-1). This means that populations are comprised entirely of females who produce clutches of maternal genetic clones via asexual reproduction (Baker and Hebert, 1985). This strategy has made *Daphnia* very appealing to researchers as it supports the production of highly repeatable experimental designs that control for otherwise natural genetic variability within a population. Given this feature, it is not surprising that the *Daphnia pulex* genome has recently been published (Colbourne *et al.* 2011). When environmental conditions become less favourable, environmentally determined epigenetic modifications lead to the production of males (Yasuhiko *et al.* 2011) and sexual reproduction commences. This introduces genetic diversity into the population during adverse times, and produces robust
overwintering eggs (Schultz, 1977) which only begin to develop when conditions are once again favourable (Hairston, 1996). Finally, their ease of maintenance, phenotypic plasticity, and relatively quick life-cycle make *Daphnia* ideal for controlled laboratory studies. Given their ecological keystone status, ideal experimental qualities, and vital dependence on ambient calcium it is anticipated that by better understanding the consequences of calcium decline on *Daphnia* we may better project the consequences on entire freshwater ecosystems while targeting the evolutionary and physiological mechanisms responsible for vulnerability to calcium deficiency.

*Daphnia magna* was chosen as the species of investigation for my research because they provide all of the advantages formerly described for *Daphnia*, but also because they produce some of the largest clutches known in their genus. *Daphnia magna* can produce clutches of more than 100 embryos (Ebert, 2005), although it was more common for our lab strain that they produce in the 16-30 embryo range. Some would rightly argue that *Daphnia pulex* is a more ecologically relevant species for investigating calcium decline in soft-water lakes, as *D. pulex* are commonly found in soft water habitats (Ashforth and Yan, 2008). However, *D. pulex* frequently only produce clutches of 2 embryos. Since my research was investigating the consequences of calcium decline on embryos, *D. magna* was the better research candidate for my study as they provided a substantially larger quantity of embryos, at regular intervals.

**Calcium decline, *Daphnia magna* and embryogenesis**

Calcium requirements of crustaceans can vary not only among different species (Rukke, 2002a), but also among different populations (Rukke, 2002b) and life stages within a species (Hessen *et al.* 2000). The specific calcium requirement of juveniles is
typically higher than that of their adult counterpart (Porcella et al. 1969; Hessen et al. 2000). This means that the calcium required per mass dry weight is higher in juveniles than in adults. This difference is likely related to the different functional requirements of these life stages (i.e. juveniles invest substantial energy into growth and calcification, while adults have the added energy burden of reproduction). Despite this clear distinction between life-stage specific calcium requirements in Daphnia, no studies have further explored this phenomenon into the earliest life stage: the embryo. Calcium is presumably required for the orchestration of certain processes in D. magna embryogenesis (Whitaker, 2006); however, calcium’s function in Daphnia embryonic development, and its temporal and spatial distribution throughout embryogenesis, remains undocumented.

**Objectives and chapter description**

The main goal of my research was to explore the relationship between ambient environmental calcium concentrations and embryogenesis in Daphnia magna, while also exploring maternal provisioning of calcium to her embryos in calcium deficient conditions. Specifically, my major objectives were to localize calcium in Daphnia magna adult females and their embryos, to determine the effects of low environmental calcium on embryogenesis and to identify possible embryonic carryover effects on growth and reproduction that result from low ambient calcium concentrations. This will include: determining whether the embryonic calcium required for development is maternally provisioned, environmentally acquired, or both; exploring the potential consequences of environmental calcium deficiency on developmental schedule, embryo calcium content and embryo survival; exploring the persistence of any embryonic effects by conducting a
chronic exposure, multigenerational experiment monitoring *Daphnia* growth and brood size.

Chapter 2 addresses the issue of localizing calcium in embryo and adult *Daphnia magna*. Specimens of *Daphnia magna* were cryosectioned and stained using the calcium marker Alizarin Red-S (ARS). Although adult *Daphnia* morphology is fairly well understood, no studies have attempted to visually localize whole body calcium in adult *Daphnia magna*. Information regarding daphniid embryonic morphology is far less available. An account of embryonic development is given in Chapter 2, and presented in the form of an embryonic staging scheme, to complement the results found in the ARS staining trials. Although its interpretive power is limited on its own, generally localizing calcium in adults and embryos provides the proper grounds to begin exploring calcium’s functions in *D. magna*, and it is an essential step in targeting mechanisms that might be affected by calcium decline. Chapter 3 addresses the effects of low ambient calcium on embryogenesis and its potential carryover effects to later life stages and subsequent generations. A combination of radioactive tracer experiments using $^{45}$CaCl and flame atomic absorption spectrophotometry (AAS) were used to investigate the source of calcium required for embryogenesis, and maternal capacity to adjust embryo calcium provisions in response to calcium limited conditions. One can conceptually liken the idea of maternal calcium provisioning to her embryos with lecithotrophy, in that the mother provisions all necessary calcium to her embryos during the formation of the embryo, and does not supplement this calcium with additional calcium transfer after the embryo is formed (i.e. matrotrophy). These concepts will be used in this context throughout the text. A series of pharmacological, time-lapse experiments are used to determine the
consequences of low calcium environments on embryo developmental schedule and embryo survival. Developmental progress was analysed using the staging scheme developed and presented in Chapter 2. Finally a multigenerational experiment was conducted to explore the potential long term consequences of calcium deficiency during embryogenesis, on growth and reproduction.

The following chapters will outline these objectives in more detail, while highlighting assumptions and unknowns that surround the issue of calcium deficiency and *Daphnia magna* embryogenesis.
CHAPTER 2

A general morphological description of *Daphnia magna* calcium distribution in adults and embryos: the development of an embryonic staging scheme for use in understanding calcium’s role in daphniid embryogenesis.

ABSTRACT

A developmental staging scheme was created in order to investigate the effects of low environmental calcium on developmental schedule and general developmental processes. *Daphnia magna* embryos and adults were cryosectioned and stained using the calcium marker Alizarin Red-S to develop a general sense of calcium’s distribution. Calcium was localized in the peripheral structures of the embryos (thoracic appendages, cephalic region and rostrum, the developing carapace) as well as in the embryonic gut tube and ventral neuroectoderm. Calcium was localized in the gut epithelium of adults, and in unknown cells that were either stellate, or round and may have a muscular support function (structural and nutritional respectively: a supposition partially based on consistent co-localization with muscle). Calcium was localized in the carapace of embryos, but not in the carapace of adults (a known calcareous structure). This might be a consequence of physical barriers existing in adults that do not yet exist in embryos and may also be related to methodological constraints. Despite minor shortcomings, the methodology developed in this study, and general localization of calcium, are a good foundation from which future studies can investigate the mechanistic consequences of calcium decline on *Daphnia magna.*
INTRODUCTION

Daphnia (Crustacea, Cladocera) have been adopted as model organisms by multiple fields of biological research, and the species within this single genus have been extremely useful in bettering our understanding of fundamentally disparate questions. The attractiveness of Daphnia as biological tools/ambassadors begins with their parthenogenic asexual reproductive strategy (which produces populations that are genetically identical; Barker and Hebert, 1986), and general ease of laboratory culture, but extends into much more complex realms. Daphnia are capable of extraordinary epigenetic modifications that manifest as measurable phenotypic changes (e.g. production of helmet structures and neck-teeth in the presence of predator kairomones; Harris et al. 2012). This feature alone makes them valuable for gaining insights into the mechanisms that underlie environmentally induced polyphenisms. It is not surprising, given this unique opportunity for investigating epigenetic effects, that the Daphnia pulex genome was recently sequenced (Colbourne et al. 2011). Daphnia have also attracted much attention from: ecotoxicologist looking to investigate the consequences of different pharmaceuticals and persistent biological chemicals on zooplankton health and fitness (Kast-Hutcheson et al. 2001); ecologists who use these keystone species (along with other biotic and abiotic parameters) to determine the health of aquatic ecosystems (Xu et al. 2001); and evolutionary biologists who use information gathered from Daphnia to further our understanding of evolutionary events, and elucidate Crustacea’s phylogenetic position within the Arthropods (Costa et al. 2007; Ungerer, Eriksson and Stollewerk, 2011).
It is well established that calcium is declining in soft-water lakes of the Canadian-Shield and in parts of Scandinavia (Keller et al. 2001; Skjelkvåle et al. 1998). It is also well established that a decrease in environmental calcium concentrations can have serious fitness consequences in freshwater crustaceans (Alstad et al. 1999; Hessen et al. 2000; Ashforth and Yan, 2008; Jeziorski et al. 2008a). One species of crustacean that has received a large amount of attention is *Daphnia magna* (Hessen et al. 2000; He and Wang, 2009; Tan and Wang, 2008). This is due to the fact that daphniids demonstrate disproportionately high specific calcium requirements when compared to other freshwater crustacean zooplankton (Yan et al. 1989). Very little is known about the fitness, and developmental consequences of calcium deficiency in *Daphnia* embryos relative to what is known about adults and juveniles. And even less is known about the specific pathways and mechanisms in *Daphnia* that are affected by low environmental calcium concentrations. A basic understanding of *Daphnia* morphology and development is essential for understanding of the consequences of calcium decline on this organism. Therefore the purpose of this Chapter is to update and complement existing information on *Daphnia* morphology and development using microscopy.

The major objectives of Chapter 2 were to: 1. Develop a discursive account of *Daphnia magna* embryogenesis, and develop a staging scheme from this information; and 2. To localize calcium in adult and embryo *Daphnia magna* using the calcium stain Alizarin Red-S.

**General Daphnia magna embryogenesis**

*Daphnia magna* produce two types of eggs. There are diploid, asexual clones that can develop into either males or females (environmentally determined). And there are
haploid sexual eggs that, once fertilized, produce overwintering eggs (i.e. ephippia). These ephippia are so robust that they can remain viable in lake sediment for over a century (Hairston, 1996). Because they are so impressive, the ultrastructural features of *Daphnia magna* overwintering embryos have been examined and described using different modern stress testing and imaging techniques (Kawasaki *et al.* 2004). *Daphnia magna* sexual embryos have received little attention by comparison. However, based on their phylogeny and what is known about close relatives of *Daphnia* a few general assumptions can be made about the basics of their developmental strategy.

All arthropods are protostomes. This means that the blastopore invagination is destined to become the stomodeum, while the point of archenteron fusion will become the anus. All protostomes also undergo triploblastic embryogenesis, which means that their development features the same 3 primary germ layers as deuterostomes: endoderm, mesoderm, and ectoderm. Early embryogenesis in protostomes generally features spiral cleavage patterns that result in a mosaic embryo. That is to say, at a very early age, irreversible cell destinies are established due to variable distribution of cytoplasmic determinants (a consequence of spiral cleavage). Therefore, should the cells of a young morula or blastocyst be damaged or altered, development may be completely aborted.

**Developing a staging scheme for *Daphnia magna***

Live specimen time-lapse photography was used to develop a thorough description of *Daphnia magna* embryogenesis and an embryonic staging scheme was developed from this information. Developmental staging schemes are valuable because they allow for preliminary investigation of the consequences of a stressor or treatment on embryogenesis. This allows the researcher to identify specific times and stages of interest.
during development (with respect to the treatment they are employing on the embryos) and helps to target specific processes and mechanisms that may be affected by the experimental treatment. A number of studies in ecotoxicology and evolutionary developmental biology have created developmental staging schemes for *Daphnia magna* embryogenesis (Kast-Hutcheson *et al.* 2001; Ungerer *et al.* 2011; Wang *et al.* 2011). However, the descriptions and the frameworks they generated (although valuable and ideal for their studies) are either descriptively limited on their own, or intensely focused to cater to their specific purposes. This reduces the suitability of these staging schemes for other research questions. The staging scheme herein is intended to operate as an intermediate on the continuum of descriptive specificity of *Daphnia magna* embryogenesis. It was developed to be autonomous of any specific research questions or goals and is intended to serve as a visually interpretable description of developmental processes that can be exapted for multiple research questions.

**Localizing calcium via cryosectioning and Alizarin Red-S staining**

*Daphnia magna* adults and embryos were fixed and embedded for cryosectioning in order to more accurately localize calcium in their tissues. Cryosectioning was chosen with the intention of developing a staining methodology for *Daphnia magna* that facilitated the maximum number of post-sectioning processing options. Specifically, along with other standard immunohistochemical (IHC) techniques, cryosectioning allows for in situ hybridization (ISH) of the samples. ISH is used to localize DNA and RNA sequences using fluorescent anti-sense probes of the desired genes. This technique could be used to better our understanding of the physiological pathways affected by calcium decline by targeting the mechanisms involved in calcium uptake. Although there was not
enough time to investigate these mechanisms as a part of the present study, the methodology developed could be used in future studies intended to determine the genetic underpinnings of *Daphnia* calcium uptake pathways. The brightfield stain Alizarin Red-S was used to visualize general calcium distribution in adults and embryos. Alizarin Red–S and its efficacy in visualizing calcium in *Daphnia magna* will be addressed in the discussion, along with the advantages and disadvantages of the developed cryosectioning methodology. Calcein-AM was also used in an attempt to visualize calcium; however, the protocol used proved to yield unsuccessful results which were uninformative. The technique is included in the Appendix as a reference for future experiments (See Appendix, page 6).

**MATERIALS AND METHODS**

**Animal husbandry**

*Daphnia magna* *Culture Maintenance*

A clone of *Daphnia magna* was acquired from the Environmental Science Department at the University of Guelph. The animals were kept at the Hagen Aqualab. They were maintained at 21°C (± 2°C), with a photoperiod of 16:8 hour (light:dark) under cool full spectrum lighting (940 lumen, 15 W). Animals were fed a ration of ~4.7x10^5 cells/mL *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* (total feed concentration = 9.375x10^5 cells/mL) on Monday, Wednesday and Friday. Animals were kept in soft COMBO medium (Kilham et al. 1998). The *Daphnia* were kept in 1600mL volumes of COMBO medium at population densities of 40-45 females/1600 mL culture. Water was replaced three times per week via reverse filtration, and cultures were
discarded after a 6 week lifespan or until reproduction had declined. In order to propagate new cultures, 40 juveniles from pre-existing cultures were collected and reared as formerly described. All glassware and equipment was routinely sterilized in an autoclave. Materials that could not withstand autoclaving temperatures were cleaned in a dilute bleach solution and rinsed using distilled water.

*Algae Culture Maintenance*

Algae cultures of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* were grown in heat sterilized COMBO medium. All culturing flasks were sealed from the environment using parafilm, and were oxygenated using standard air bubblers in combination with sterilized nalgene air filters (0.22 µm) and tubing. Cell counts of the algae cultures were conducted weekly (using a hemocytometer) to monitor culture growth. Ethanol and flame sterilized pipettes were used for culture sampling and any open culture flask was surrounded with 3-4 portable Bunsen burners to create an upward air current and prevent culture contamination. Cultures were grown to densities of 30x10^6 cells/mL at which point they were stored at 4°C until they were fed to *Daphnia magna cultures*. Algae stored longer than 4 months were discarded.

*Embryon isolation technique*

Embryo isolation was performed in a shallow petri dish (~10mL capacity). Gravid females were immobilized using the negative pressure created by a 0.5ml plastic disposable transfer pipette (females were held dorsally to minimize or eliminate any physical damage). A second transfer pipette was used to create a gentle stream of water. When the immobilized female contracted her post-abdomen, the abdominal process was lifted and a stream of water was directed into her exposed brood chamber. The water
stream was sufficient to liberate the embryos from the brood chamber. Embryos were then immediately isolated from females as swimming behaviours can cause mechanical damage to the exposed embryos. Females were then returned to their cultures with little to no physical harm (Figure A-2).

**Time-lapse procedure and manual monitoring**

Time-lapse imaging was used to monitor developmental changes in the embryos. After embryos were isolated they were transferred individually into a 24-well plate. Due to physical limitations regarding microscope stage mobility and range, only 16 wells of a 24 well plate were usable during automated embryonic development monitoring. The 4 leftmost and 4 rightmost wells were excluded to accommodate. Isolated embryos were distributed into 1 of the 16 available wells. The embryo containing well plate was then mounted and fixed to the microscope stage and sealed to prevent evaporation during the 72 hour experimental duration. The embryo locations were mapped using NIS-Elements Advanced Research Software. Images were taken every hour for 72 hours, or until the embryos had reached stage 5 (at which point the embryos become motile and move from their mapped position, making it impossible to capture images beyond this point). These data were used in combination with manual embryo monitoring techniques to develop an embryonic staging scheme for *Daphnia magna*. Manual embryo monitoring was conducted to complement time-lapse imaging experiments as embryo development was only observable up to the entrance into stage 5 using time-lapse imaging.

**Cryosectioning**

Adult and juvenile specimens were fixed in 4% paraformaldehyde, 1x PBS (made from Phosphate Buffered Saline 10x Solution, BP399-500, Fisher BioReagents®)
overnight at 4°C. They were then rinsed 3x (equal volume) in 1xPBS and stored in 30% sucrose solution at 4°C overnight. They were then transferred to a 1:1, 30% sucrose:OCT (optimal cutting medium: Shandon CRYOMATRIX™ Thermo Scientific) and stored overnight at 4°C. Finally they were transferred to 100% OCT and stored at 4°C overnight. Using dry ice and a pool of sub-zero cooled ethanol in a petri dish, samples were embedded in cryoblocks in fresh OCT and were stored at -80°C until they were cut. Samples were cut in 25-50µm sections and mounted onto 0.5% Gelatin, 0.05% Chromium (III) Potassium Sulfate Dodecahydrate coated slides (See Appendix, page 3).

**Alizarin Red-S staining and slide preparation**

An aqueous Alizarin Red-S (ARS) solution (2%, pH 5) was prepared and mechanically mixed to promote dissolution of the compound. Specimen bearing slides were completely dried in a dust protected environment and then, using Coplin jars, immersed in the Alizarin Red-S solution for 3-5 minutes (the duration of this step was flexible as the main objective of this work was to localize calcium using ARS and results were not influenced by ARS signal strength). Slides were then gently rinsed by placing them in 1xPBS solution (which was refreshed when the solution was saturated with excess Alizarin Red-S) and lifting the slides intermittently, until the excess gelatine and Alizarin Red-S had been completely removed. Excess PBS was removed using Kim wipes© and air exposure. Slides were mounted in a glycerol DABCO Tris-HCl buffered mounting medium (pH 8.5) and sealed using a fluorescently neutral clear nail polish (See Appendix, page 4). Excess mounting medium was carefully removed using Kim wipes© and ethanol. Slides were then visualized using NIS-Elements AR software and a Nikon-Ti inverted microscope.
RESULTS

*Daphnia magna* embryonic staging scheme

*See Figures 1.1 and 1.2.*

The following description of embryogenesis refers to development at 21°C (± 2°C). Embryogenesis was divided into 7 visually discernable stages (0-6), and their related features with the final stage (stage 6) signifying maturation into a juvenile. Stage 0 embryos (~0-15 hours) possess essentially no visible distinguishing characteristics. However, cleavage and blastulation must occur during this stage (regardless of whether or not it can be visualized using low magnification, whole organism, bright-field imaging). Embryos are spherical to ellipsoidal in shape and have large central fat droplets and many surrounding yolk granules. The embryo is contained within two protective envelopes: the inner vitelline membrane and the outer chorion (Figure 1.2; Obreshkove and Fraser, 1940). In stage 1 (15-25 hours) gastrulation and morphogenesis occur. The earliest landmarks of this stage are the cephalic invaginations which appear as small indentations along the sides of the embryo. When these invaginations occur, the embryo seems to organize itself into two regions: the lighter, motile, developing peripheral cells (ventral), and the inner, darker undifferentiated cells (dorsal). The posterior invagination and the lateral carapace ‘buds’ are two other distinguishing features that arise during this stage. Hatching of the chorion is the landmark event that signifies entrance into stage 2 (25-35 hours). This means that this outer membrane is shed from the developing embryo. Antennal and maxillary appendage rudiments are distinct and forming. Thoracic appendage partitioning is evident. The posterior invagination gives way to a distinct furrow that runs bilaterally, separating the left and right hemispheres sagittally. The
embryo is now able to grow in size as it is no longer spatially limited by the rigid chorion. Organogenesis begins in stage 2. During stage 3 (35-40 hours) the shape of the embryo begins to change from the round precursor to something more fusiform and more sophisticated organ structures develop. The cephalic features become more recognizable in stage 3 (i.e. the early eye spots and early neural/brain organization). The darker patch of yolk granules and fat droplets that was once rather uniform and circular seems to follow the changing shape of the embryo with an out-pocketing developing in the cephalic region. The early post-abdomen begins to protrude in the posterior region of the embryo. The carapace becomes visually more robust and prominent. Bifurcation of the secondary antennae becomes clear, and the inner developmental envelope (the vitelline membrane) is still present and visible. Near the end of stage 3, the carapace buds begin a rapid extension posteriorly. The stage completes with the completion of this rapid carapace extension. Within 3-4 hours the post-abdomen is entirely enclosed in the carapace and the posterior (which was just characterized by a protruding post-abdomen) is now rounded and enclosed. During stage 4 (40-50 hours) the thoracic appendages and the secondary antennae continue to become more sophisticated and clearly demonstrate their segmentation. Setae are now visible on the antennae and appendages. The embryo begins to vigorously move and twitch; however, this movement is restricted to the confines of the vitelline membrane. Organogenesis is well underway, and the cephalic region (rostrum/labrum) become much more prominent. The organism appears ovoid, and is now recognizable as the precursor to the familiar juvenile appearance. Entrance into stage 5 (45-55 hours) is signified by the hatching of the second embryonic membrane: the vitelline membrane. The secondary antennae are now extended and motile, as are the
thoracic appendages. Neuronal tissue seems to be specializing and investing into the ommatidia of the compound eye. Dorsally the ostium is maturing and beating. The carapace fully encloses the embryo in the bivalve fashion characteristic of these crustaceans. The caudal spine is still curved and follows the contours of the posterior of the embryo. The gut does not appear to be fully developed, or is veiled by an opaque wall of maternally provision yolk granules. At stage 6 (55+ hours) the embryo is considered a juvenile. Completion of the transition into the juvenile stage is marked by several features. First none of the maternally provisioned yolk remains. The gut is completely formed. The compound eye is a unified collection of ommatidia rather than a partitioned developing structure. The caudal spine has extended and the post-abdomen is protected by a sophisticated, armoured, chitinous post-abdominal claw. The antennal setae are fully extended.

**Alizarin Red-S: Embryos**

Embryos treated with Alizarin Red-S consistently showed staining in the thoracic appendages, ventral cephalic region, ventral neuroectoderm, gut tube, and the carapace. This staining seemed to be in peripheral tissues surrounding the body (with the exception of the gut tube). They consistently lacked staining in their inner cell region which seemed to be predominantly undifferentiated (Figures 1.3, 1.4, 1.5).

**Alizain Red S-Adults**

Adult female *Daphnia magna* demonstrated consistent Alizarin Red-S staining in the stellate cells (near the carapace), thoracic cells (large and small), and ovaries. Staining was present in varying degrees in the gut epithelium. Control images demonstrate cases of natural red pigmentation in regions of hemolymph pooling (at the epipodites and
dorsally along the anterior/posterior axis), and in the ommatidia of the compound eye. Finally no staining was observed in the carapace (a known calcareous structure) (Table 1.1).
FIGURES AND TABLES

Developmental Schedule

**Figure 1.1:** Time-lapse demonstration. Letters correspond to the chronological progression through the times series. Numbers represent hours post set up.
Figure 1.2: Embryonic staging scheme. The top row represents stages 0-3 from left to right. The bottom row represents stages 4-6 from left to right. Scale bars in order are: 85 µm, 85 µm, 85 µm, 85 µm, 100 µm, 150 µm. This refers to development at 21°C (± 2°C). **Stage 0:** Embryos possess essentially no visible distinguishing characteristics. They are ellipsoidal and have large central fat droplets (FD) and many surrounding yolk granules. Cleavage and blastulation occur. **Stage 1:** Gastrulation and morphogenesis occur. The earliest landmarks of this stage are the cephalic invaginations (CI*). The posterior invagination (PI), and the lateral carapace ‘buds’ (Cp) are other features visible during this stage. **Stage 2:** The chorion hatches (EC). The posterior invagination gives way to a distinct furrow (Pf) that runs bilaterally, separating the left and right hemispheres sagittally. The secondary antennae (aII) are developing and the thoracic appendages (Tha) are clearly segmented. **Stage 3:** Organogenesis begins. The cephalic features become more recognizable (i.e. the early eye spots (eE) and early neural/brain organization (eB)). The darker patch of yolk granules and fat droplets develops an outpocketing in the cephalic region. The early post-abdomen (ePd) begins to protrude in the posterior region of the embryo. The carapace (Cp) becomes visually more robust and prominent. Bifurcation of the secondary antennae is becoming clear, and the inner developmental envelope (the vitelline membrane) is still present and visible. Stage 3 culminates with rapid carapace extension. **Stage 4:** the post-abdomen is entirely enclosed in the carapace and the posterior (which was just characterized by a protruding post-abdomen) is now rounded and enclosed. The thoracic appendages (Tha) and the antennae (aII) continue to become more sophisticated and clearly demonstrate their segmentation. Setae
are now visible on the antennae and appendages (S). **Stage 5:** the vitelline membrane hatches. The secondary antennae (aII) are now extended and motile, as are the thoracic appendages. Neuronal tissue seems to be specializing and investing into the compound eye (Es). Dorsally the ostium (Os) is beating. The carapace fully encloses the embryo in the bivalve fashion characteristic of these crustaceans. The caudal spine is still curved and follows the contours of the posterior of the embryo. **Stage 6:** the embryo is considered a juvenile. The gut is formed. The compound eye is a unified structure. The caudal spine has extended. The post-abdomen is protected by a chitinous post-abdominal claw. Antennal setae are extended. Maternal yolk provision have been completely consumed.

**Alizarin Red-S**

*Embryos*

**Figure 1.3:** Image 1 demonstrates stage 0 control embryos. Image 2 demonstrates stage 3-4 embryos. Image 3 demonstrates a partial sagittal section of a late stage (4-5) embryo. The cut was
likely oblique which resulted in a complete anterior section of tissue and an incomplete posterior section of tissue. Image 4 demonstrates stage 5 embryos. Images 2-4 are stained with ARS.

Figure 1.4: Image 1 demonstrates a stage 1 control embryo. Image 2 demonstrates an early stage 3 control embryo. Image 3 demonstrates two different orientations of stage 3 Alizarin Red-S stained embryos. The upper left embryo is a sagittal section, and the lower right embryo provides a ventral view through the thoracic appendages. The midline (ML) is stained red and bordered by unstained cells on either side. This is a qualitatively similar pattern to the ventral neuroectoderm (Ungerer et al. 2011). Image 4 demonstrates two ventrally oriented embryos (right and center) that have had the thoracic appendages and ventral neuroectoderm sheared away exposing a mass of unstained cells. The embryo on the left is a sagittal section that demonstrates the peripheral staining of the antennae, thoracic appendages and carapace.
Figure 1.5: Demonstrates late stage control (images 1, 5 and 6) and Alizarin Red-S stained (images 2, 3 and 4) embryos.
Adults

Figure 1.6: Demonstration of serial cryosections of an adult female *Daphnia magna*. Thickness ~ 25 µm. Scale bar ~ 100 µm. General adult *Daphnia magna* morphology is well documented and resources describing this are readily available (Ebert, 1992). Features of interest are the: compound eye (CE); skeletal muscle (SkM); primary antenna/antennule (aI); mandibles (Mdb); carapace (Cp); midgut (Mg); hepatic caecum/diverticulum (Cae); brood chamber (Bc); thoracic appendages (Tha).
Table 1.1: Illustrating the staining results of Alizarin Red-S on cryosections of *Daphnia magna*. 
(++) signifies strong, consistent positive staining; (+) signifies positive and mostly consistent 
staining; (+ -) signifies neutrality to the stain (possible natural red pigmentation of the structure 
which limits the effectiveness of Alizarin Red-S on these structures); (-) signifies no staining; (-*) 
negative result for staining in a known calcium bearing structure.

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**Figure 1.7**: Featuring stellate cells. These cells are associated with the dorsal carapace. Red boxes outline magnification fields. Images 1-3 are stained with Alizarin Red-S and images 2 and 3 are magnifications of the cells in image 1. Images 4 and 5 are control images of qualitatively similar carapace (Cp). Dorsal (D), posterior (P), anterior (A), ventral (V).
Thoracic cells

Figure 1.8: Featuring thoracic cells (ThaC) often entangled in the skeletal muscle fibres of the thoracic appendages. Image 1 is a control. Image 2 is treated with ARS. Using a polarizing filter these images highlight the unstained muscle fibre in high contrast to the stained thoracic cells. Images 3 and 4 are stained with ARS and image 5 is a control demonstrating a similar cluster of muscle and cells. Images 6, 7, and 8, also demonstrate the clusters of thoracic cells within the muscle tissue. Images 9-13 demonstrate ThaC at high magnification. When the thoracic cells (ThaC) are obscured by other tissues or are in a different focal plane/depth, the staining can appear to be unspecific. (*) denotes clearly outlined cells that become obscured (**) when the focal plane is adjusted. Thoracic appendages (Tha), skeletal muscle (SkM), embryo (EM), dorsal (D), ventral (V), anterior (A), posterior (P).
**Figure 1.9:** Featuring thoracic cells (ThaC). Although I’ve termed these “thoracic cells” they are also present along the ventral side of the post-abdomen. Image 1 is a control and images 2 and 3 are stained with Alizarin Red-S. Image 3 demonstrates a long string of faintly stained thoracic cells (below the line). The same linear type arrangement is visible in image 1 (control).
Gut epithelium

Figure 1.10: Images 1, 3, 4 and 5 demonstrate the staining of the ciliated epithelium of the gut. Image 1 demonstrates the staining of the cuboidal/columnar epithelium (colEp) of the gut and its apical ciliated brush border (Cbb). Also visible is the basal squamous epithelial layer (sqEp). The sagittal section (Image 2) demonstrates consistent pink staining of the colEp of the gut. Image 2 also nicely presents the granular (gr) ovary tissue and the virtually unstained skeletal muscle (SkM). Images 3-5 demonstrate colEp staining variation. In some cases the ciliated columnar epithelium is darkly stained (as in image 3); however, some epithelium is much more lightly stained (as in image 4). Also (as seen in image 3 and 5) the adjacent skeletal muscle appears to have some pink pigmentation. Image 5 may be demonstrating pigmentation of an adjacent skeletal muscle fibre or ‘vasculature’ (Vas). Images 6-9 feature
control columnar epithelium of the gut. Gut lumen (gl) in all images is lined with a string of columnar epithelial cells. It seems that the clarity of the colEp can be obscured by the angle of the section/cut.
Compound Eye

Figure 1.11: The left-most 6 images demonstrate the consistency and saturation of staining in the compound eye (CE) of treated adult females, with one high magnification demonstration of the ommatidia of the CE. Images 7-10 are control images of the compound eye. They demonstrate the gradient of natural red pigmentation seen in the unstained CE from light to dark (images 7-10 respectively). Also visible are: the gut caecum (Cae), the ‘brain’ (Br), optic nerve (ON) and nerve fibres (NF) leading to the nuchal organ (kidney type structure/homologue).
Large Thoracic Cells

Figure 1.12: Featuring the large thoracic cells (LThaC). These cells are consistently found at the base of thoracic appendages (1, 2, 5) and antennae (1, 3, 4), but can also be found in the more distal thoracic tissues (3). Images 6-8 feature natural pink pigmentation that resembles the LThaCs (images 6 and 8), and pigmentation that is likely due to dorsal vasculature which directs hemolymph to the heart (image 7).
**Granular ovaries**

*Figure 1.13:* Featuring granular (gr) ovary (Ov) tissue and large adjacent cells (LC). Images 1 and 2 are stained with Alizarin Red-S and image 3 is a control.
**Figure 1.14:** From the description and images of *Daphnia magna* carapace provided by Halcrow (1976), I made this general schematic diagram of daphniid carapace. The black squares represent Ca$^{2+}$/Na$^+$ transporters. These are positioned in the apical and basal membranes of the membranous layer to transport calcium. The green triangles represent the H$^+$ proton pumps located at the basal membrane of the membranous layer. These maintain electrochemical gradients favourable to the required directional transport of calcium. The periwinkle circles represent vesicular transport to the distal regions of the carapace. The inner and outer membranous layers contain large mitochondria and microtubules for energy and structural support respectively (Halcrow, 1976; Skinner, 1992; Roer and Dillaman, 1984). Magnification $\approx x20,000$. 
DISCUSSION

*Daphnia magna* embryonic staging scheme

The developmental staging scheme thoroughly describes seven visually distinguishable stages of *Daphnia magna* embryogenesis (stages 0-6) as they occur at 21°C (± 2°C). Although previous studies have also put forth staging schemes for *D. magna* embryogenesis, these schemes either tended toward gross simplicity (Wang *et al.* 2011; Sobral *et al.* 2001) or gross complexity (Ungerer *et al.* 2011). The staging scheme I have developed in the present study was intended to operate as an intermediate placeholder along the continuum of descriptive complexity. This means that it is not overly simple or complex (a circumstance that offers little practical or interpretive power to the staging scheme beyond the scope of the study questions and applications). It is anticipated that the intermediate descriptive nature of staging scheme developed in this study can effectively be applied to multiple research questions. In chapter 3 of my thesis I demonstrate how this staging scheme can be used to investigate research questions, and how the results can be interpreted.

**Alizarin Red-S: Embryos**

In order to determine which embryonic tissues and cells contain calcium, cryosectioned embryos were stained using Alizarin Red-S. Embryos treated with Alizarin Red-S consistently showed staining in: 1. thoracic appendages, 2. ventral cephalic region, 3. ventral neuroectoderm, 4. gut tube, and 5. carapace. This staining seemed to be in peripheral tissues surrounding the body (with the exception of the gut tube). Controls did not demonstrate any pigmentation in these regions. They consistently lacked staining in their inner cell region which seemed to be predominantly undifferentiated. This may be a
demonstration of calcium’s strong presence in all animal pole cells, and lack of presence in the vegetal cells. The presence of calcium in the animal pole has been seen in both Zebrafish and Xenopus embryogenesis, which suggests that Alizarin Red-S is specifically binding to tissues in the animal pole cells of *Daphnia magna* (Webb and Miller, 2003). Pan-embryonic calcium signalling patterns (i.e. waves of signalling that carry over the entire embryo) have been described in many developmental models during the earliest stages of morphogenesis, and calcium signalling is likely a key component of epiboly, involution, convergence and extension events at this time in *Daphnia magna*, and throughout development (Webb and Miller, 2003; Witaker 2006). The major staining regions (which are numbered above) will be described individually in more detail along with some potential implications of this staining. However these functional implications are speculative and require further investigation.

*Thoracic Staining*

The thoracic appendages of *Daphnia* are important for three reasons: they are the site of the gill epithelium and osmoregulation (Kikuchi, 1983); they create a ventilatory and feeding current for the filter feeders (Pirow *et al*. 1999b); and they act as supplementary locomotory structures (although the post-abdomen and secondary antennae are the primary locomotory structures). The strong presence of calcium in this developing tissue could mean that calcium is required for the proper development of these vital appendages (Figure 1.3-2, 1.3-4; Figure 1.4-3; Figure 1-5-2). More specifically it may be involved in segmentation of this thoracic/abdominal region. In Zebrafish (*Danio rerio*) development, calcium alternates among: intercellular pan- embryonic signalling, intercellular localized signalling, and highly localized intracellular waves of
signalling (Webb and Miller, 2003). During organogenesis and proper segmentation, calcium reverts to a very localized, intracellular signalling modality in the abdominal segments of the organism (Webb and Miller, 2003). It might be possible that in *Daphnia magna* organogenesis, calcium is operating as an orchestrator of thoracic development in a similar intracellular localized signalling manner which could account for its appearance in these tissues.

*Ventral cephalic and ventral neuroectoderm staining*

The ventral-cephalic region is the major sensory and neurological processing center of *Daphnia*. Structures such as the compound eye and ocellus, primary antennae, nuchal organ, optic nerve, and brain, are all found in this region. It has been demonstrated in numerous bodies of literature that calcium signalling is largely involved in the development of homologous sensory processing structures in *Xenopus*, Zebrafish and chick embryos (Webb and Miller, 2003). These structures truly become sophisticated between stages 4 and 5 in *Daphnia* but their organization begins as early as stage 3 (Figure 1.4-3, 1.4-4). It is possible that during these stages localized intracellular, or localized intercellular calcium signalling would occur since this is the time of more specific morphogenesis and organogenesis. During this time calcium signalling reverts back to localized and specific communication patterns (Webb and Miller, 2003).

Consistent staining was also observed in putative ventral neuroectoderm of the embryonic daphniids (Figure 1.4-3). This tissue was outlined thoroughly in a recent study exploring the role of neuroblasts in *Daphnia magna* neurogenesis (Ungerer *et al.* 2011). Calcium is used as a signalling molecule in developing neurons of the neural plate in other developmental model organisms (Whitaker, 2006). Therefore, calcium’s very
localized and specific presence in this tissue may suggest that calcium is involved in events such as ventral neural induction and neurolation in *Daphnia magna* and overall neurogenesis. Future studies should use live calcium imaging techniques to explore this possibility.

*Gut tube staining*

Although the gut tube is often visually obscured by the presence of vegetal cells in the foreground, when the gut is captured in cross section or sagittal section it is clearly and uniformly stained with Alizarin Red-S (Figure 1.3-3). There is little evidence in the literature suggesting that calcium is extensively involved in gut development of other developmental model organisms. Therefore it is important to entertain alternative possibilities regarding calcium’s presence in the embryonic gut of *Daphnia magna*, given that there is little support for this in other developmental models. The gut in *Daphnia magna* is surrounded by muscle tissue; and different vertebrates have been shown to require calcium for the orchestration of specific stages in muscle development (Webb and Miller, 2003). Although this is a possible explanation it is not highly likely as the staining seems to be specific to the gut epithelium (Figure 1.3-3; Figure 1.5-3). It might be possible that this staining is related to the maturation and functioning of calcium uptake structures that exist at the level of the gut epithelium (Zanotto and Wheatly, 2003). It is possible that calcium is being sequestered in these tissues as a result of the onset of calcium uptake mechanism maturation. This proposition could be explored by using in situ hybridization to localized calcium-transport protein transcription in the gut of the developing embryo to determine whether these proteins are active during development.
Carapace

The carapace structure of *Daphnia magna* is unique when compared to larger aquatic crustaceans and terrestrial crustaceans in that it is much thinner, less fortified and does not require pore canals (Figure 1.14). Also because of the frequency with which they moult, daphniids do not exhibit a dramatic partitioning of the new and old cuticle (Halcrow, 1976). The moulting process is much more gradual and continuous in daphniids and small crustaceans than that of their larger cousins (Halcrow, 1976). Also due to their small size, they do not require pore canals to allow nutrients to flow to the less permeable outer integument layers. A simple schematic of *Daphnia magna* integument structure is included for reference (Figure 1.14). Similar to the gut tissue, the membranous layer of the cuticle possesses calcium transport proteins. These channels are needed in order to sequester calcium in the membranous layers. This calcium is then transported using vesicles to the outermost cuticle layers (Halcrow, 1976). Presumably by stage 6 the embryo carapace structure is completely developed. However, prior to stage 6, it is difficult to determine how developed the carapace integument layers are. The consistent staining in embryonic carapace could be a reflection of the calcium uptake that occurs in the membranous layers of the integument. An alternative explanation for this staining is that the epicuticle and procuticle have yet to mature and fortify. This might alleviate potential structural constraints that limit ARS from staining carapace structures as is seen in adults (ex. Figure 1.7-4; Figure 1.12). Since the structure of the embryonic carapace is unknown it is difficult to draw further conclusions. This will be discussed more in comparison to the adult carapace (page 45).
**Alizarin Red-S: Adults**

Adult female *Daphnia magna* were fixed and cryosectioned and stained using Alizarin Red-S to localize calcium. Adult females demonstrated consistent Alizarin Red-S staining in stellate cells (near the carapace; Figure 1.7), thoracic cells (large and small; Figure 1.8, 1.9 and 1.12), and ovaries (Figure 1.13). Staining was present in varying degrees in the gut epithelium (Figure 1.10). Control images demonstrate cases of natural red pigmentation in regions of hemolymph pooling (at the gill epithelia/epipodites at the base of the 2nd-5th thoracic appendages {Ebert, 2005} and dorsally along the anterior/posterior axis; Figure 1.12-6, 1.12-7, 1.12-8), and around the ommatidia of the compound eye (Figure 1.11-7, 1.11-8, 1.11-9, 1.11-10). Finally no staining was observed in the carapace (a known calcareous structure; demonstrated in numerous figures such as Figure 1.12-2). These major findings will be discussed individually along with their implications.

**Stellate cells**

The stellate cells are filopodus cells that were consistently in close proximity to the carapace (Figure 1.7). These cells could be remnants of several different cellular arrangements in *Daphnia magna*. Based on size alone (~50-100 µm) it is unlikely that these cells are a dissociated part of the carapace (as demonstrated in Figure 1.14) because they are much larger than the observed gut epithelia in the organism (~5-20 µm) and they appear in discontinuous patches (Figure 1.7-2, 1.7-3). It is also unlikely that these cells are carapace nutrient support cells, given the presence and function of the central haemocoel of the integument (Halcrow, 1976; Figure 1.14). These cells might have been involved in anchoring carapace to skeletal muscle arrangements. Their filamentous
appearance would suggest a structural rigidity due to either microtubules, actin microfilaments or intermediate filaments (all of which have demonstrated functional requirements of calcium: O’Brien et al. 1997; Sasaki et al. 1987; Makowski and Ramsby, 1998, respectively). In order to determine their inner cell components, a counter stain technique targeting microtubules and filamentous cells structures should be employed on thinner plastic sections of these regions. Also, thicker sections of *Daphnia magna* may preserve muscular attachments better and could be used to determine the relationship of the stellate cells to muscle.

**Thoracic Cells**

The most common, consistent and abundant staining observed in *Daphnia magna* was in the thoracic cells. These cells were either quite large (50-100 µm) or relatively small (20-50 µm). However in both cases the cells were large when compared to epithelial cells of the gut (5-20 µm). The large thoracic cells often appeared in bundles/aggregations at the base of the thoracic appendages and seemed to disrupt the otherwise continuous stretch of thoracic muscle fibres extending into the appendages (Figure 1.12). The small cells would sometimes arrange in chains along a muscle fibre (Figure 1.8, 1.9) but otherwise appeared rather irregularly throughout the muscle tissues and thoracic region (Figure 1.8). The small thoracic cells were also present in large quantities in the muscular region of the post-abdomen. More specifically they appeared to closely follow the arrangements of muscle tissue that connect the distal tip of the post-abdomen to the central midgut (Figure 1.9). These two different cells could serve two different functions. First the large aggregations of cells in the thoracic appendages suggest that they may be involved in osmoregulation (Kikuchi, 1983). The respiratory
epithelia of Daphnia are located in the proximal segments of the thoracopods. As these cells are involved in ion transport (Pirow et al. 1999a, Kikuchi, 1983, Péqueux, 1995) it is likely that they would contain calcium (bound or free). The smaller cells may serve a muscular support role as they seem to run more closely with muscle fibres than the large thoracic cells (Figures 1.8 and 1.9). However, unlike the stellate cells, the thoracic cells demonstrate rather unassuming, round-ovoid shapes. They also seem to possess (at high magnification) spherical vesicles that could be nutrient transport vesicles (Figure 1.8, images 10-13).

Ovaries

The granular ovaries consistently stained positive for calcium. There were also large cells adjacent to the ovaries that seemed to possess a large, central nucleus (Figure 1.13-1). These cells are most likely immature ova (Allen and Banta, 1929; Ebert, 2005). As calcium is critical for development (Webb and Miller, 2003), and the cellular divisions that precede fertilization or ovum maturation, it is not surprising that this tissue stained positive for calcium.

Gut Epithelium

The gut epithelium demonstrated moderately strong positive staining for calcium. Although the staining was not as consistent or prominent as in the thoracic cells and ovaries, it still merits attention as there was no staining in the control gut epithelium. In many cases it has been demonstrated that crustaceans receive very little of their required calcium from uptake at the level of the gut epithelium (Roer and Dillaman 1985); however, they do receive some calcium from these mechanisms which may partially contribute to the staining of the gut. It has also been demonstrated that in crustaceans
with more sophisticated digestive tracts, calcium that is recovered from the carapace during premolt phase is often stored in the cardiac stomach epithelium as calcareous gastroliths (Zanotto and Wheatly, 2003). This calcium is then stored until it is needed for reinforcement of the new carapace, at which time it is solubilized and transported to the epicuticle and exocuticle (Zanotto and Wheatly, 2003). Although no storage organ or gastroliths have been identified in Daphnia it is possible that the gut epithelial cells still retain some ancestrally founded role in calcium reclamation. Since calcium storage and mobilization are time sensitive processes the variability of the staining of this region could be related to the time at which the females were fixed for processing. The molt cycle in *Daphnia magna* is a gradual process, and absolute calcium quantities required for calcification are low in *Daphnia* when compared to other crustaceans. Based on these parameters it is possible that there is no need for such long term calcium storage structures as gastroliths in *Daphnia magna* (under calcium conditions that satisfy their calcium requirements). Perhaps instead calcium is stored more temporarily through complexing with proteinaceous calcium modulators until calcium needs to be mobilized again. It would be interesting to pursue this notion using live-animal calcium visualization techniques, or by exploring the localities of different calcium protein modulator homologues in *Daphnia*.

**Compound Eye**

The compound eye and associated nervous structures were particularly intriguing. Although the controls demonstrated various degrees of natural red pigmentation in the ommatidia (Figure 1.11), the Alizarin Red-S treated samples consistently had a more vibrant appearance. However, given the resolution of the stain, and lack of counter-stain,
it is difficult to confirm or discredit staining in this region. Also, the nervous tissue in the treated samples demonstrated varying amounts of staining, which was also inconsistent. The compound eye in *Daphnia magna* contains 22 ommatidia (Macagno *et al.* 1973). It has been long known that *Daphnia* possess some means of colour vision. Each ommatidium possesses 8 photoreceptors that fall into one of the four following spectral classes: 348, 434, 525, and 608 nm (UV through to yellow) (Smith and Macagno, 1990). Given that these ommatidia possess no known photoreceptors in the red long-wave end of the EM spectrum, perhaps the red pigmentation observed in the eye related to the minimal absorption of red light. Given these features, it might be advantageous to use a non-red brightfield stain, or fluorescent stain to eliminate the issue of natural pigmentation interfering with the stain.

Calcium is a signal transduction molecule in neuronal cascades. Calcium has been found in the developing optic structures of other crustaceans and vertebrates (Zanotto and Wheatly, 2003). This suggests that it is possible that calcium is localizing in the optic region of *Daphnia magna*, and future studies should not disregard this possibility.

**Carapace**

The two most intriguing results regarding the carapace are that the adult carapace did not stain positive for calcium using ARS and while the embryonic carapace (or carapace precursor) did stain positive for calcium. The lack of positive staining in the adult carapace was unexpected. However upon further investigation into the structural nature of the carapace and reference to similar studies investigating the affinity of Alizarin Red-S to carapace structures, this result was less mysterious.
There are several possible reasons that can explain why calcium did not stain positive in the carapace. First, calcium might be structurally inaccessible to the stain based on the composition of the daphniid carapace. Although the overall structure of the daphniid carapace is different from the classic carapace of crustaceans (Figure 1.14), the physiological and molecular components of the carapace are likely consistent with other crustaceans. Calcium takes different forms in the different cuticular layers of crustaceans. In the epicuticle, calcium deposits as spherulitic calcite islands amidst the lipid protein matrix (Roer and Dillaman, 1984). Where in the exo and endo-cuticle (collectively the procuticle in Daphnia) calcium deposits as calcite crystal aggregates interspersed within the lamellae of the chitin/protein matrix (Roer and Dillaman, 1984). From a structural accessibility perspective, the calcite deposits might have limited accessibility due to the binding/deposition pattern of the calcium in the different carapace matrices, thereby either reducing ARS affinity for the calcite or reducing ARS ability to contact the calcite.

Second, there may be constraints on ARS affinity for calcium due to the histochemical procedures employed. Often amorphous calcium deposits are highly soluble at low pH values, and can be removed during histochemical procedures (McGee-Russell, 1958). This could have contributed to the lack of calcium staining in the carapace; however, it is unlikely as the measured pH values of the 1xPBS and aqueous ARS used in the staining process were 7.3 and 5 respectively. Also the fine structures of the carapace may not have been visible if the sections were cut too thick for the inner carapace structures and structural features to be penetrated by an aqueous stain or even visualized at all.
Limited staining may also be caused by the time of female fixation. A study exploring the staining efficacy of Alizarin Red-S (and many other stains) on carapace structures in *Menippe rumphii* demonstrated that at the premoult and postmoult stages Alizarin Red-S was not visible in the epicuticle or the endocuticle, with only limited staining in more proximal integument tissues (Babu et al. 1985). Perhaps the time of female fixation during the molt cycle is also important for visualizing calcium in the carapace structures (the epi- and procuticle, or the epidermis/intermembranous tissue) in *D. magna*. This is another possible reason why calcium was not observed in the carapace.

Ultimately, the lack of stain in the carapace may be attributable to a number of factors: issues regarding calcium deposit shape, physiological accessibility of calcium to the stain (how it’s bound to the carapace), structural accessibility of carapace calcium (sections may be too thick), temporally dependent calcium sequestration mechanisms, and possibly suboptimal technical procedural constraints (pH dissolving stored calcium).

Another curious result regarding carapace staining was the inconsistency in staining between adults and embryos. The embryos demonstrated consistent and prominent positive staining in the carapace epithelium, where the adults had little if any staining in the carapace. This might be a consequence of the juvenile and adult fortification processes that structurally change the carapace. These changes in the carapace may reduce ARS accessibility to the sites of calcite deposit. It is possible that either the young embryo has not yet undergone the fortification process (rendering the epicuticle and procuticle extremely permeable), or perhaps the epicuticle and procuticle are not even yet established.
The chorion is also a chitinous and calcareous matrix used to protect the asexual embryos. Although it is a known carrier of calcium, calcium was also not detected in the chorion of stage 0 and 1 embryos (e.g. in Figure 1.3-2). It is possible that this membrane is damaged and lost in histochemical procedure; however, this is unlikely as there appears to be remnants of the chorion (Figure 1.3-2) as well as the less fortified vitelline membrane present in preparations (Figure 1.3-4). As the chorion is produced by the adult female, and is likened to the carapace structurally, it seems fitting that it should not bear positive results for calcium, similar to the adult carapace. This further supports the notion that the embryonic carapace staining may be a result of the nascent condition of the tissue its permeability, while the lack of staining in the adult carapace (and chorion) is a result of structural fortification interfering with the efficacy of ARS for binding calcium. Regardless, it would be interesting to investigate the cause of this life stage specific difference in carapace staining more closely by processing the carapace epithelium of embryos and adults in thinner sections and at higher magnification.

Alizarin Red-S: Technique

The reliability of Alizarin Red-S (sodium alizarin sulphonate) binding affinity to mineral calcium has been examined in parallel with other calcium markers and it has proven to be a consistent and informative compound for localizing calcium. The anthraquinone salt can be prepared in acidic, basic, alcoholic and aqueous solutions, all of which will yield slightly different results (McGee-Russell, 1958). In this experiment a 2% Alizarin Red-S aqueous solution (pH 5) was used to stain tissue samples. This method has been suggested as one of the best to visualize whole body calcium (McGee Russell, 1958). An aqueous solution was also used because strong acidic and basic techniques had
proven to be unnecessarily aggressive on cryosectioned *Daphnia* preparations (previous unpublished work), and because the structural properties of the salt begin to change and produce a suboptimal working medium for the employed cryosectioning techniques when pH is adjusted (i.e. at higher pH Alizarin Red-S becomes thixotropic and viscous).

One major limitation of Alizarin Red-S is the inability to demonstrate the relative quantities of calcium in different regions on a small scale. Although it is an ideal general broadcast stain, it lacks the temporal and quantitative specificity of other techniques (such as aequorin luminosity imaging). Alizarin Red-S is inclined to complex with both free calcium and certain bound forms of calcium so it is impossible to draw information regarding calcium’s specific functions and mobilization from these images alone. But by comparing this information with what is already known regarding calcium’s functions in other developmental models, we can begin to target potential processes of interest in *Daphnia magna*.

What can be gathered from the present work is that calcium is likely present in the pinkish cells/regions. Future studies should first confirm this localization pattern using additional calcium stains, and by conducting negative control experiments (i.e. that sequester or dissolve calcium from the tissue entirely, or fully saturate ARS with calcium), rather than just presenting untreated controls. Once this localization is confirmed, the specific calcium bearing regions should be visualized using techniques that highlight calcium mobilization. This will provide insight into the patterns of calcium signalling and the potential functions of this signalling. Some examples of calcium mobilization visualization techniques are aequorin imaging, and GFP and calmodulin
fluorescent labels. These procedures (although fundamentally different) can be conducted on live organisms and visualized using video or rapid time-lapse techniques.

**Cryosectioning: Technique**

Cryosectioning is an extremely versatile imaging technique because cryosectioned samples are receptive to both standard immunohistochemical techniques (which typically highlight proteins and minerals) and in-situ hybridization techniques (which use labelled complementary DNA and RNA transcripts to highlight genetic sequence expression). This versatility can make cryosectioning a valuable technique. However this histochemical versatility comes with a mechanical cost. The polysaccharide cryomatrix used to embed the tissue is relatively soft (when measured next to paraffin and plastic as embedding media). In many cases this is sufficient to maintain and preserve tissue structure. But in the case of *Daphnia magna*, tissue structure and orientation was compromised by cryosectioning. This is likely due to the large regions of open space in the *Daphnia magna* body plan. Additionally, due to the fundamental composition of the carapace (Skinner et al. 1992) the chitinous exoskeleton of *Daphnia* is rather flexible, despite being fortified with calcium. It seems that rather than fracture or cut smoothly, the flexibility of the carapace leads to the distortion of the carapace in the sections. Based on the flexibility of the carapace, the body plan of Daphnia, and the relatively soft nature of the cryomatrix, I would advise future researchers to use a more formidable embedding matrix when sectioning and imaging *Daphnia magna* if they have no future intentions of pursuing in-situ hybridizations. However, with a good fundamental knowledge of *Daphnia* morphology, it is rather painless to navigate through the structurally
compromised samples of tissue and collect valuable information about the organs and tissues therein.
CHAPTER 3

Limiting environmental calcium and its effects on *Daphnia magna* embryonic calcium content, embryonic development, maternal calcium provisioning, and growth and brood size over two generations.

ABSTRACT

Freshwater crustaceans in Canadian Shield and Scandinavian soft-water habitats are currently faced with the threat of decreasing ambient calcium concentrations. Little is known regarding the nature of calcium’s relationships with embryogenesis in *Daphnia magna*. Calcium radiotracer (\(^{45}\text{CaCl}\)) experiments and Flame AAS were used to determine whether the calcium found in embryos during stage 0–stage 4 of development is maternally provisioned. The results also suggested that females are capable of reducing their calcium loss to reproductive processes (i.e. embryo calcium content), when environmental calcium concentrations are low; however, the mechanisms they use to accomplish this are unknown. Furthermore, embryos that were formed by mothers in low ambient calcium environments experienced an accelerated progression through stages 1 and 2 of development. Multigenerational experiments investigating the carryover effects of embryonic calcium deficiency suggest that there is little to no effect of low calcium on growth and brood size over two generations.
Introduction

Soft-water lakes occur in high frequency in the Canadian Shield (Keller et al. 2001) and in Scandanavia (Skjelkvåle et al. 1998). Recent studies have shown that ambient calcium concentration is declining in soft-water lakes (Keller et al. 2001; Skjelkvåle et al. 1998). This is because the calcium poor, erosion resistant physical geography of these particular habitats is unable to compensate for anthropogenic activities that remove and pre-maturely liberate calcium stored in the ecosystem (Baldwin, Desloges and Baird, 2000; Jeziorski and Yan, 2008a). Unsustainable forestry practices (that continuously remove stored nutrients from the soil) and acidic deposition (which lowers the pH of water and reduces its propensity to retain minerals and sustain life) are two major causes of this decline (Lawrence and Huntington, 1999). Because the calcium is removed at rates that exceed the habitat’s natural capacity to replenish, the terrestrial and aquatic biota of these environments have to cope with a dramatically depleted calcium condition. Calcium is an extremely important mineral in biological systems. It operates as both a signalling molecule and a chaperone of structural support ubiquitously across living phyla. Calcium decline has occurred so rapidly that many organisms are unable to adapt, thus resulting in severe ecological consequences which manifest as shifts in ecosystem composition. This shift is a reflection of the different capacities of the affected species to cope with reduced environmental calcium at the physiological level. As it is not feasible to investigate the consequences of environmental change on all organisms in an ecosystem, zooplankton health and composition are commonly used as proximate measures to interpret ecosystem responses to change (Xu et al. 2001). Yan et al. 1989, demonstrated that different zooplankton species consistently
possess different specific-amounts of trace metals. This study also indicated that *Daphnia* contain a higher quantity of calcium when compared to the other investigated zooplankton. *Daphnia*’s specific-calcium content averaged nearly 10x higher than the succeeding measured zooplankton value, and nearly 60x higher than the lowest measured zooplankton value: both of which were of fellow crustaceans. Crustaceans require large quantities of ambient calcium in order to properly calcify their carapace (Zanotto and Wheatly, 2003) and ingestion of calcium alone is not sufficient to meet the mineral demand of calcification (Tan and Wang, 2009). *Daphnia* exhibit a shorter intermolt phase during calcification than other, more heavily calcified crustaceans (Halcrow, 1976) and *Daphnia magna* have cuticular elaboration vesicles at the apical surface of their epidermis throughout all stages of calcification (Halcrow, 1976). This suggests that the epidermis is continually working to generate the next carapace, and not entering a state of decreased activity (as seen in fiddler crabs and other crustaceans) (Halcrow, 1976; Green and Neff, 1972). These features might contribute to the relatively high specific calcium content of *Daphnia* even when compared to other crustaceans (Yan et al. 1989), and may be contributors to their effectiveness as models for investigating the consequences of calcium decline.

*Daphnia* demonstrate remarkable phenotypic plasticity (Harris, Bartlett and Lloyd, 2012). Environmentally triggered epigenetic modifications can drastically alter their physical morphology and determine sex (Yasuhiko et al. 2011). *Daphnia* are predominantly parthenogenic and reproduce asexually to create clutches of maternal clones. However when induced by the appropriate environmental cues, *Daphnia* undergo epigenetic modifications that produce the male phenotype, and cause females to produce
haploid eggs. Once these eggs are fertilized they form an overwintering ephippium that can withstand harsh environmental conditions and remain in diapause for decades until environmentally triggered to resume development (Hairston, 1996; Cáceres, 1998). Because they are so strong, the ultrastructural features of *Daphnia magna* overwintering embryos have been examined and described using modern imaging techniques (Kawasaki et al. 2004). One recent study also examined their usefulness as a paleolimnological indicator of ecosystem calcium composition (Jeziorski et al. 2007). However, these eggs proved to be poor calcium indicators as mothers actively resorb most of the calcium from the ephippia before deposition (Jeziorski et al. 2007).

It is likely that in times of calcium shortage different physiological mechanisms would be competing for sufficient calcium. Ecdysis is arguably the most calcium heavy task undertaken by *Daphnia* and up to 90% of an individual’s calcium can be lost in a single moult (Alstad et al. 1999). Previous research has suggested that daphniids possess no known calcium storage structure (Porcella et al. 1969), and are unable to compensate for decreased environmental calcium by increasing food intake or extending the duration of the calcification phase of their moult cycle (Tan and Wang, 2009). The organism must then recover this loss via active uptake of readily available ambient calcium and can only significantly reduce the effects of this loss by adjusting their calcium efflux mechanisms (Tan and Wang, 2009; He and Wang, 2009).

Age specific differences in calcium requirements have been documented in *Daphnia* (Porcella et al. 1969; Hessen et al. 2000). Although the adults require more total calcium due to a larger body size, juveniles have a higher specific calcium demand than adults, likely due to the increased frequency of moultng during juvenile growth. This
makes them more responsive and vulnerable to decreases in environmental calcium. Despite this understanding that life stage can result in different tolerances to decreased environmental calcium, no studies have investigated how calcium decline might influence embryogenesis.

Calcium is an orchestrator of major events in embryonic development across a number of taxa (epiboly and involution communication, cell adhesion, and the development of complex tissues that comprise the brain, eyes, and muscle; Webb and Miller, 2003; Whitaker, 2006). However its specific functions in *Daphnia* development have not been investigated. Consequently, very little is known about the potential impact of a reduced calcium environment on *Daphnia magna* embryonic development. The primary objective of my research was to test the effects of reduced environmental calcium on *Daphnia magna* embryogenesis. In order to accomplish this it was necessary that I develop an embryonic staging scheme to outline the major developmental landmarks found in normal *Daphnia magna* embryogenesis (Chapter 2). More specifically the major goals of this research were to determine how changes in environmental calcium influence embryogenesis and embryo survival, while also determining the source of calcium required for embryogenesis (i.e. is it maternally provisioned, environmentally acquired, or both?). If maternal provisioning could be confirmed, another objective was to determine whether mothers could adjust calcium efflux by reducing loss of calcium to reproduction (i.e. reduce the amount of calcium invested in embryos when environmental calcium concentrations were limiting). And finally, this study aimed to determine whether reduced environmental calcium during
embryogenesis resulted in carryover effects on growth and brood size over multiple generations.

**Hypotheses and Predictions**

The source of calcium required for embryonic development has never been explicitly examined. It is known that *Daphnia magna* have an open brood chamber, meaning their embryos are in direct contact with the external environment. Females generate a distinct, separate ventilation pattern for their broods (Seidl *et al.* 2002), which suggests selective permeability of the embryonic membranes for gas diffusion. Obreshkove and Fraser (1940) proposed that *Daphnia magna* receive all nutrients required for development from their mothers, but this was never experimentally tested. It is unknown at what time during development embryos begin to directly interact with the external environment (and external calcium), but it is believed that osmoregulation begins after the hatching of the vitelline embryonic membrane (Charmantier and Charmantier-Daures, 2001). With these facts in mind, I anticipated that the calcium required for development comes from the mother and that embryos begin interacting with the external calcium environment once the vitelline membrane has ruptured.

The development and physiology of aquatic invertebrates is largely influenced by their surrounding environment, and often their metabolism, and rates of activity and development are directly affected by parameters such as temperature and dissolved O$_2$ (Yu *et al.* 2013). Given that *Daphnia* are aquatic invertebrates whose metabolic and physiological rates of activity may be largely affected by environmental parameters, it is expected that embryos formed by mothers in calcium poor conditions will show developmental schedule deviations when compared to control groups due to the decrease
in available calcium for calcium dependent embryonic processes. I also expected that after rupture of the vitelline membrane, embryos begin directly interacting with the ambient environment. I believe that at the time of vitelline membrane rupture, embryos in calcium poor environments will begin to demonstrate signs of stress due to the onset of exposure to suboptimal calcium conditions. Also, since daphniids exercise exceptional phenotypic plasticity in so many other life facets, it is expected that mothers are capable of modifying their embryonic calcium provisioning and will provision less calcium into reproduction if environmental calcium concentrations are low.

Finally numerous studies have explored the consequences of embryonic stress on later life stages and life history traits (McCormick and Gagliano, 2009) and suggest that there are later life consequences to embryonic stress. It is anticipated that embryos subject to calcium deficiency (whether onset occurs during embryo formation or at the initiation of osmoregulation) will demonstrate a reduced growth rate that will summate in a reduced reproductive rate when compared to controls.

In order to address these hypotheses I used several techniques. First manual and time-lapse monitoring of embryogenesis in different environmental calcium conditions was conducted to monitor embryo survival and developmental schedule deviations. A series of radioactive calcium (\(^{45}\text{CaCl}\)) uptake experiments were conducted to determine the source of calcium required for embryogenesis. Flame AAS was used to measure the calcium content of embryos reared in different calcium environments to investigate the plasticity of maternal calcium efflux through her embryos. Finally, a 2 generation study was conducted to explore the embryonic carryover effects of calcium deficiency on juvenile and adult growth and survival.
MATERIALS AND METHODS

In all text: High calcium= 25mg/L, medium/normal calcium= 10mg/L, and low calcium= 5 mg/L, and no calcium= 0mg/L unless otherwise stated. The term normal is used for 10mg/L calcium because it is the standard/baseline concentration of ambient calcium of the chosen media (COMBO) for Daphnia culture maintenance (Kilham et al. 1998).

Animal husbandry

Daphnia magna Culture Maintenance

Refer to Chapter 2, page 13.

Calcium Adjusted Culture Maintenance

Animals were reared as described in Chapter 2 (page 13) with the following amendments. COMBO medium preparation was adjusted to achieve final calcium concentrations of 5mg/mL (low), 10mg/L (medium) and 25mg/L (high). This was accomplished by either adding more or less CaCl$_2$·2H$_2$O stock medium. As it has been demonstrated that Daphnia magna do not uptake significant calcium from their food (Tan and Wang, 2009) algae culture maintenance was not adjusted. Before feeding algae to calcium adjusted Daphnia cultures, algae were condensed via centrifugation and rinsed in calcium-free COMBO medium 3x before being re-suspended in the calcium appropriate medium. After rinsing and re-suspension the algae was fed to calcium adjusted cultures in the same concentration as the standard culturing procedure.
Embryo isolation technique

Embryo isolation was performed in a shallow petri dish (~ 10mL capacity). Gravid females were immobilized using the negative pressure created by a 0.5ml plastic disposable transfer pipette (females were held dorsally to minimize or eliminate any physical damage). A second transfer pipette was used to create a gentle stream of water. When the immobilized female contracted her post-abdomen, the abdominal process was lifted and a stream of water was directed into her exposed brood chamber (Figure A-2). The water stream was enough to liberate the embryos from the brood chamber. Embryos were then immediately isolated from females as swimming behaviours can cause mechanical damage to the exposed embryos. Females were returned to their cultures after with little to no physical harm.

Influence of environmental calcium on embryogenesis and embryo survival

Time-lapse and manual embryo monitoring data was collected to determine the effects of environmental calcium concentration on embryogenesis, specifically to determine the effects on developmental schedule and survival. Developmental schedule was the chosen parameter because it highlighted the times during development that were most affected by environmental calcium concentration. The results gathered from these experiments may help to pinpoint time sensitive processes and mechanisms that could be affected by environmental calcium concentration. In order to determine whether embryo developmental schedule was affected by the embryos’ direct interaction with ambient environmental calcium during embryogenesis, embryos formed by mothers in a common
~10mg/L calcium environment were isolated from their mothers and placed in a 0mg/L, 5mg/L or 25mg/L calcium environment. Their progress through embryogenesis in vitro was monitored then monitored hourly for 72 hours. In order to determine whether embryo developmental schedule was affected by changes experienced by the mothers as a consequence of different ambient environmental calcium concentrations, embryos were collected from mothers that were kept in either a 5mg/L or 25mg/L calcium environment\(^1\), and were individually redistributed into a calcium environment that matched the environment in which they were formed. Their progress through embryogenesis in vitro was monitored hourly for 72 hours or until they moved from the field of view at stage 5.

Developmental schedule was analyzed using the staging scheme developed through the work presented in Chapter 2 (Table 2.1). Data are presented as both cumulative time (sum of progress in hours) and relative time (absolute time in hours spent in a given stage). The chosen in vitro ‘environments’ used in both the time-lapse imaging and manual monitoring were 24 well plates (~2-3mL volume capacity per well).

*Manual embryo monitoring*

Manual embryo monitoring was conducted to determine the effects of environmental calcium concentration on embryogenesis (developmental schedule) and embryo survival. Manual embryo monitoring was also conducted to complement time-lapse imaging experiments as embryo development was only observable up to the

\(^1\) Mothers and juveniles cannot survive and reproduce in a 0mg/L calcium environment so this treatment is not included in the maternal environment experiments.
entrance into stage 5 using time-lapse imaging (See Time-lapse imaging, page 61). This technique was also used to determine embryo survival.

Embryos isolated from their mothers and were rinsed 3x (app. 15mL total) in calcium-free COMBO medium and distributed individually into the wells of 24-well plates. Each well contained 2ml of COMBO medium (8 wells each of 0mg/L, 5mg/L or 25mg/L calcium adjusted COMBO medium). Once all wells had received an embryo, the time 0 visual stage interpretation was conducted. Subsequent visual analysis was conducted at 24 hour intervals until the embryos had reached stage 6.

*Time-lapse imaging*

Due to physical limitations regarding stage mobility and range, only 16 wells of a 24 well plate were usable during automated embryonic development monitoring. The 4 leftmost and 4 rightmost wells were excluded to accommodate. In a trial with 5mg/L and 25mg/L calcium adjusted treatments only, isolated and rinsed embryos were distributed into 1 of the 8 wells designated to each calcium concentration. In the case that there were 3 treatments in the trial (0mg/L, 5mg/L and 25 mg/L calcium, or 5mg/L, 10mg/L and 25mg/L) only 15 wells were used (5 wells per calcium treatment). The embryo containing well plate was then mounted and fixed to the microscope stage and sealed to prevent evaporation during the 72 hour experimental duration. The embryos were mapped using NIS-Elements Advanced Research Software. Images were taken every hour for 72 hours, or until the embryos had reached stage 5 (at which point the embryos become motile and move from their mapped position, making it impossible to capture images beyond this point). The images were analysed using gross-landmarks and events to ensure consistency (See Table 2.1). It is important to note that Stages 5 and 6 are not visible in the automated
technique as the embryos become motile at this time. Also embryos/juveniles kept in shallow containers, or in volumes that push the lower threshold space requirements for juveniles, will demonstrate a curved caudal spine phenotype. This makes the stage 6 caudal tail extension landmark conditional in that the embryos cannot be experiencing space related stress in order for this landmark to hold.

**Source of calcium required for embryogenesis and maternal calcium provisioning**

A series of radiotracer experiments using $^{45}\text{CaCl}$ were used in combination with flame AAS techniques to determine the source(s) of calcium used by embryos during embryogenesis (environmental/maternal) and to determine whether this transfer is influenced by changes in environmental calcium concentration (i.e. do quantities of calcium in mothers and embryos change?).

**Radiation experiments**

All radiation experiments used 10mL scintillation vials for processing and used Ca$^{45}$ (CaCl$_2$) as a calcium radioisotope. All collected samples were digested and combined with 5mL of prepared scintillation fluid (See Appendix, page 5), prior to vigorous mechanical agitation. Samples were given at least 8 hours to fully activate the scintillation fluid before analysis in a Beckman LS6500 scintillation counter. These experiments were conducted to capture the transfer of calcium into the embryos from the mothers and/or the ambient environmental medium.

1. **Calcium uptake kinetics**

This experiment was conducted to confirm that Ca$^{45}$ was being incorporated by *Daphnia magna*, and determine whether this uptake was active or passive. Sixty five
freshly molted adult females of the same cohort were placed in a common COMBO medium incubation solution spiked with 250kBq Ca$^{45}$ (CaCl$_2$) Five females were then collected at each 15 min interval for 3hrs (starting at timepoint 0 minutes). Each female was rinsed 4x in COMBO medium (total volume ~20mL) with a 2 min interval between washes to allow for swimming and ventilation. This was done to promote the removal of non-specific Ca$^{45}$. Females were then digested immediately and individually in 0.5 mL of 30% HNO$_3$ at 60°C for 6 hrs. A 0.5mL sample of the incubation fluid was collected at every 30 min interval to monitor the background radioactivity levels. A 0.5mL sample of the final wash solution per female was collected at every 30 min interval to ensure that there was no residual Ca$^{45}$ in solution when the females were considered ready for digestion.

2. *Embryo overnight exposure experiments*

These experiments were designed and conducted to determine whether embryos uptake calcium directly from the environment during embryogenesis and whether this uptake is influenced by environmental calcium concentration. Isolated embryos (stage 0 and 1) from the same cold calcium environment$^2$ were rinsed 3x (~15 mL volume total) in calcium-free COMBO before being distributed into either a 1MBq or 250kBq Ca$^{45}$ incubation treatment. Embryos were left overnight (16hrs) in the calcium treatment. Each replicate (of 20 embryos) was then rinsed 3x with COMBO (~15mL total volume) and digested in 0.5mL of 30% HNO$_3$ at 60°C for 6 hrs before being processed.

3. *Mother Embryo Transfer experiments*

These experiments were designed and conducted to determine whether embryos receive calcium from their mothers and whether or not this transfer of calcium from

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$^2$ Non-radioactive calcium environment.
mother to embryo is influenced by environmental calcium concentrations. Freshly molted females were collected and placed into either a 250kBq or 1MBq Ca$^{45}$ environment. Females were fed calcium-free algae rations (as per calcium adjusted culture maintenance) for the duration of the experiment. When the females became gravid, their broods were collected (as per embryo isolation), rinsed in COMBO medium thoroughly, and digested in 0.5mL of 30% HNO$_3$ at 60°C for 6 hrs. Mothers were also rinsed and digested.

Flame Atomic Absorption Spectrophotometry

Embryos were raised in either a 5mg/L, 10mg/L, or 25mg/L calcium medium. Once they were sexually mature mothers and their embryos (stage 0-2) were prepared for Flame AAS analysis. Mothers were processed individually. Embryos were pooled into samples of approximately 50 individuals. The samples were digested in a small volume of 80% HNO$_3$ for 2-3hrs (or until totally dissolved). The samples were diluted to 1% HNO$_3$ and were filtered using sterile syringes and 0.22µm syringe filters. Approximately 0.04g of CsCl was added to each of the filtered samples (~3.5 mL) to reduce the amount of noise that non-calcium divalent cations may cause in the read. Due to technical constraints stemming from the light weight of embryos and sensitivity of the available weighing scale (Fisher Scientific, accu-124D, Dual Range), dry weight was not a feasible or practical variable that could be used to normalize calcium concentration values collected from Flame AAS measurements. Protein content values were to be used instead of dry weight values. A modified BCA$^{TM}$ protein assay (Thermo Scientific, Micro BCA$^{TM}$ protein assay kit) protocol was developed for this specific function. Although the technique was successful in trials, it was unsuccessful in the final sample analysis. This is
likely due to improper sample storage. Therefore the data generated from the Flame AAS experiments is presented in mg/L calcium per specimen rather than percent calcium per dry weight, or per protein value.

**Embryonic carryover effects on growth and brood size**

*Calcium adjusted multi-generation experiments*

1. **Animal Husbandry and Experimental Set-up**

   Embryos were taken from females cultured in 10mg/L calcium COMBO medium and were raised in 24 well plates of either 5mg/L calcium COMBO medium or 25mg/L calcium COMBO medium. Once the embryos had become juveniles they were transferred to a larger pooled culture of the same calcium concentration where they were maintained as previously described. Juveniles were collected from the Generation 1 calcium adjusted cultures and were transferred to their respective calcium environments. They were maintained as previously described.

2. **Measurements**

   Every week growth and reproduction were measured. In order to accomplish this, females were removed from culture and placed on a clean shallow petri dish. Excess water in the dish was removed temporarily to immobilize the female. An image was taken and an on the spot evaluation of brood size and embryo stage was conducted. The process took no more than 60-90 seconds per female. Once females were analysed they were returned to culture. Size was measured from base of caudal tail to the top of the head above the compound eye using Image-J analysis software.

**Statistics**
The statistical program IBM SPSS Statistics 21, was used to analyse the data. ANOVA analyses were used to interpret whether treatment groups demonstrated significant differences from one another. Interactions and differences were considered significant when $p<0.05$. Where applicable, a Bonferroni post-hoc analysis was used to further test the significance among subdivisions of different experimental treatments. Error is presented as ± 1 standard error of the mean.

RESULTS

Influence of environmental calcium on embryogenesis and embryo survival

Developmental schedule was the parameter observed to determine the effects of environmental calcium concentrations on embryogenesis. Embryos were formed in either a low (5mg/L calcium), medium/normal (10mg/L calcium – concentration of the soft-COMBO medium that was used to maintain non-treatment cultures) or high (25mg/L calcium) calcium environment, then isolated from their mothers before being transferred into a no, low, or high calcium environment. The embryo development was then monitored using time-lapse photography. These experiments were designed to test whether embryos that were formed by mothers in a normal calcium environment experienced changes in developmental schedule as a result of developing in a low calcium environment; and also to test whether embryos formed by mothers in low or high calcium environments experienced a change in developmental schedule as a consequence of their formation environment. Embryos produced by mothers in a calcium reduced environment experienced a significant change in the duration of embryonic stages 1 and 2
(F=231.04, p<0.001, d.f.=1), whereby embryos reared in a low calcium environment\(^3\) progressed through these stages much more rapidly (Stage 1: 1 hour ± 0; Stage 2: 8 hours ± 0) than embryos reared in a high calcium environment (Stage 1: 6.71 hours ± 0.18; Stage 2: 13.14 hours ± 0.26; Figure 2.7). This acceleration averaged approximately 5 hours faster through both stages 1 and 2. This difference was not observed in embryos that were reared in a normal calcium environment (F=0.18, p=0.98, d.f.=2; Figure: 2.7), and were then transferred to a low calcium environment.

Onset of external/ambient environmental calcium acquisition appears to occur soon after the transition to stage 5 in embryos (post-hatching of the inner vitelline embryonic membrane, and in concurrence with the onset of motility). Embryos that were reared in normal calcium environments, and were subsequently isolated and transferred to a no calcium COMBO medium, demonstrated normal developmental progress and survival until the onset of stage 5 (Figure 2.8). Once the embryos had passed this point, the ‘no calcium’ treatment survival dropped off significantly when compared to the high and low calcium controls (% survival to 48 hours: no calcium: 90.28± 5.01, low calcium: 88.89 ±1.39, high calcium: 90.28±2.78; % survival to 72 hours: no calcium: 23.61±17.73, low calcium: 88.89±1.39, high calcium: 90.28±2.78; % survival to 96 hours: no calcium: 0±0, low and high treatments were maintained from 72 to 96 hours) suggesting that survival is influenced by time, as a consequence of the zero-calcium environment (F=13.71, p<0.001, d.f.=8).

**Source of calcium required for embryogenesis and maternal calcium provisioning**

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\(^3\) Embryo rearing environment refers to the calcium environment to which the embryos’ mothers had been acclimatized during their formation and subsequent deposit into the brood chamber.
$^{45}$CaCl radiotracer experiments were conducted to capture the transfer of calcium from mothers to their embryos, and to determine whether the embryos uptake calcium from the external environment. In order to confirm that the females were actively up-taking the $^{45}$CaCl from the ambient environment, it was important to conduct a calcium uptake kinetics experiment. The uptake curve generated from this experiment is presented (Figure 2.1). Also, since active uptake trends are predominantly logarithmic, the information from this experiment was necessary to ensure that null results weren’t generated as a consequence of natural uptake saturation of $^{45}$CaCl by the females.

The results from these experiments suggest that embryonic calcium is largely (if not entirely) maternally provisioned between stages 0 and 4. Embryos that were reared in the same non-radioactive calcium environment, and then transferred to a $^{45}$CaCl environment overnight (~12-16 hours), showed no significant difference in recorded CPM (scintillation counts per minute) when compared to controls (CPM means 250 kBq $^{45}$CaCl treatment: embryo sample: 16.33±1.45, wash fluid control: 17.8±0.37, COMBO control: 16±1.69; F=0.05, p=0.95, d.f.=2; Figure 2.2). This result was consistent even when the radioactivity of the calcium environment was increased 4x (CPM means 1 MBq $^{45}$CaCl treatment: embryo sample: 14.38±1.70, wash fluid control: 13.57±1.19, COMBO control: 16±1.69; F=0.65, p=0.53, d.f.=2; Figure 2.2). In contrast, embryos that were reared in a radioactive calcium environment showed a significant difference in $^{45}$Ca when compared to controls (CPM means 250 kBq treatment: embryo sample: 72.88±12.18; wash fluid control: 18.39±0.92; COMBO control: 13.50±0.62; F=13.64, p<0.001, d.f.=2; Figure 2.3), and showed an increase in $^{45}$Ca concentration when environmental $^{45}$Ca concentration was increased from 250kBq to 1MBq (CPM means 1 MBq treatment:
embryo sample: 138.22±20.68, wash fluid control: 54.94±4.14; COMBO control: 11.80±1.77; F=41.64, p<0.001, d.f.=1; Figure 2.4). This is further supported by Flame AAS measurements of embryos formed in different maternal environments as they too demonstrate a significant difference in calcium concentrations (F=15.12, p=0.008, d.f.=2; Figure 2.5), where the lowest calcium environment produced embryos with a significantly lowered calcium content (mean embryo calcium content mg/L: low: 0.002±1x10^{-4}, med: 0.00245±7x10^{-5}, high: 0.0034 ± 4x10^{-4}). Interestingly, although there appeared to be a similar phenomenon occurring in the mothers (Figure 2.6) this difference was not statistically significant in the mothers (mean female calcium content mg/L: low: 1.38±0.14, high: 2.00±0.40; F=2.17, p=0.18, d.f.=1). This demonstrates that a significant calcium shift can occur at the embryonic level that is statistically undetectable at the maternal level.

**Embryonic carryover effects on growth and brood size**

In the multigenerational experiments size was significantly affected by treatment (F=17.16, p<0.001, d.f. =1), and size was also significantly different across the two generations (mean difference in size between high and low treatments: generation 1: 0.13 mm ± 0.08, generation 2: 0.07 mm ± 0.03; F=6.41, p=0.01, d.f. = 1). However no interacting effect between treatment and generation was found. With respect to brood size, there was a significant difference in brood sizes from week to week (e.g. generation 1: week 3: 1.70 ± 0.21, week 4: 8.44 ± 0.74; F=15.62, p<0.001, d.f.=5), and when comparing similar weeks across generations (e.g. week 4: generation 1: 8.44 ± 0.74, generation 2: 3.28 ± 0.61; F= 4.23, p<0.001, d.f.=5); however, this suggests nothing regarding the effects of calcium environment and brood size, as these results are
independent of calcium treatment. A Pearson’s correlation analysis demonstrated only a small positive correlation between brood size and body size ($\rho=0.089$).
FIGURES AND TABLES

Table 2.1: A visual and written summary of the staging scheme developed for *Daphnia magna* embryogenesis.

<table>
<thead>
<tr>
<th>Stage and Time</th>
<th>Image</th>
<th>Features</th>
</tr>
</thead>
</table>
| Stage 0 0-15hrs | ![Image](image1.png) | - Visible yolk granules and fat droplets  
- Droplets will be visually preserved until roughly stage 4  
- Scale ~85 µm |
| Stage 1 15-25hrs | ![Image](image2.png) | - Egg membranes become discernable from the internal cell mass  
- Early cephalic invaginations mark the locations of the antennae, mandibular region, and carapace buds  
- Posterior invagination visible (future anus and post abdomen)  
- Thoracic segmentation visible  
- Scale ~85 µm |
| Stage 2 25-35hrs | ![Image](image3.png) | - Hatching of the chorion occurs, and carapace bulges are prominent  
- Thoracic appendages are developing  
- Posterior invagination gives rise to a more prominent posterior furrow  
- Scale ~85 µm |
| Stage 3 35-40hrs | ![Image](image4.png) | - Body shape is bulbous and gently fusiform; the head and rostrum become distinguishable  
- Cephalic organization of internal structures (early brain, eyes, etc)  
- Secondary antennae become bilobed, and thoracic appendages become more sophisticated  
- Earliest organization of the post abdomen (posterior bulge)  
- Carapace begins rapid extension  
- Scale ~85 µm |
| Stage 4 40-50hrs | ![Image](image5.png) | - Rapid carapace extension occurs and the embryo is fully enclosed in carapace  
- Embryo begins moving  
- Ostracum nearly fully developed and begins to beat  
- Compound eye spots become more sophisticated and the small ocellus begins to develop  
- Primary antennae visible, thoracic appendages are segmented and setae are visible at the distal tips of antennae and among appendages  
- Scale ~100 µm |
| Stage 5 45-55hrs | ![Image](image6.png) | - The vitelline membrane hatches and the secondary antennae are free  
- Embryo can swim in short bursts  
- Eye spots, ocellus, and thoracic appendages are mature  
- Body has lengthened and straightened  
- Brain and oesum are nearly mature (nervature begins to invest in the compound eye)  
- Scale ~150 µm |
| Stage 6 55+hrs | ![Image](image7.png) | - Caudal spine extends  
- Final maturation of organs  
- Setae are extensive and antennae are held fully extended in a normal posture  
- Scale ~200 µm |
Figure 2.1: This graph demonstrates the calcium uptake kinetics of *Daphnia magna*. The data are presented as scintillation counts per minute over a 3 hour uptake period. Adult females were sampled from a 1Mbq $^{45}$CaCl treatment every 15 minutes for 3 hours. The females were processed individually. A quadratic regression was applied to the data and gave an $r^2=0.812$. 
Figure 2.2: This graph demonstrates the scintillation counts of embryos that were formed in non-radioactive environments and then transferred to either a high or low radioactive environment for 16 hours before being processed for counting. This was conducted to determine whether embryos can uptake $^{45}$Ca directly from the environment. Embryos were collected from mothers reared in non-radioactive 10mg/L calcium environments and placed in either a high (1Mbq) or low (250kbq) $^{45}$CaCl overnight exposure treatment. The low treatment had 9 replicates (20 embryos per replicate) and the high treatment had 16 replicates. Embryos were washed 3 times in COMBO medium and were processed for scintillation measurements. The wash value is a sample of the last wash of the embryos and serves as a control to ensure all unspecific $^{45}$CaCl is removed from the sample and solution. The COMBO value also serves as a background scintillation control.
Figure 2.3: This graph demonstrates maternal provisioning of calcium to her embryos. The different experimental (coloured) and control treatments (hatch-marked/stippling) are presented on the x-axis. The values given are scintillation counts per minute (log transformed). Embryos were collected from pre-gravid mothers who were acclimated for ~3 days in 250kbq $^{45}$CaCl$_2$ cultures. Embryos were collected at stage 0 which means they could not have been exposed to ambient radioactive calcium for much longer (if longer) than the embryos used in the overnight exposure experiments. Last wash and COMBO values are presented as background controls (as in Figure 2.2). A univariate analysis of variance was conducted on the data, along with a post-hoc Bonferroni correction to investigate the specific relationships among the different treatments. In these treatments embryo CPM values were statistically significant from the COMBO control (p<0.001) and the last wash control (p<0.001). This (in combination with results presented in Figure 2.2) suggests that early stage embryos acquire calcium from their mothers, rather than the external environment.
Figure 2.4: Log transformed scintillation CPM for embryos and mothers that were formed in high and low $^{45}$CaCl environments. Embryos were collected from pre-gravid mothers that were acclimated in either a high (1Mbq) or low (250kbq) $^{45}$CaCl environment for ~3days. The embryos were then processed for scintillation measurement as described in Figure 2.2. Mothers were rinsed 3x (~5-7ml per rinse) in COMBO medium and were processed individually. A univariate analysis investigating the relationship between CPM and $^{45}$CaCl environment demonstrated that $^{45}$CaCl environment has a significant effect (p<0.001) on the CPM values. This suggests that mothers that are exposed to higher concentrations of $^{45}$CaCl produce embryos with higher concentrations of $^{45}$CaCl.
Figure 2.5: Embryo calcium content (mg/L) as it relates to different calcium environments. Embryos were collected from mothers reared in one of three different calcium environments: low (5mg/L); medium (10mg/L); high (25mg/L). Approximately 50 embryos were processed per replicate, and 2-4 replicates were collected for each of the calcium treatments. Samples were processed as described for Flame AAS. The data are presented as calcium (mg/L) per embryo. The data suggest that calcium environment of the mother has a significant (p<0.01) effect on embryo calcium content.
Figure 2.6: Female calcium content (mg/l) as it relates to environmental calcium concentrations. Females were reared in low (5mg/L) and high (25mg/L) calcium environments. Once their embryos were collected for Flame AAS analysis (Figure 2.5), mothers were processed individually in the same manner as the embryos and measurements were made using a Flame AAS. A univariate analysis of variance yielded no significant (p>0.05) difference in calcium content between mothers raised in low and high calcium environments.
**Figure 2.7:** The data from two different experiments is presented above. (Env) experiments: Embryos formed by mothers in a common ~10mg/L calcium environment were isolated from their mothers and placed in a 0mg/L, 5mg/L or 25mg/L calcium environment. (Mat) Experiments: Embryos were collected from mothers that were kept in either a 5mg/L or 25mg/L calcium environment*, and were individually redistributed into a calcium environment that matched the environment in which they were formed. Due to an interruption of one experimental run, the sample size at stage 4 in the environmental treatment was less than that of the previous 3 stages. The apparent decrease in cumulative time experience by the 0mg/L and 5mg/L calcium treatments is most likely related to this interruption (and reduced sample size) and also to the fact that the embryos of the complete/uninterrupted runs were individuals nearer to the lower SE threshold the stage 3 mean.

*Mothers and juveniles cannot survive and reproduce in a 0mg/L calcium environment so this treatment is not included in the maternal environment experiments.

All embryos were monitored using time-lapse photography (1 photo/hr for 72 hours or until fully developed) and their developmental progress was monitored using a developmental staging scheme as a template (Table 2.1). These data can only be collected to the completion of Stage 4 as the embryos become motile at Stage 5 and move from the field of view. Data are presented as both cumulative time (sum of progress in hours) and relative time (absolute time in hours spent in a given stage).

Embryos formed in the same calcium environment developed with temporal consistency across all calcium treatments. Embryos formed by mothers in calcium deprived environments demonstrated an accelerated rate of development (cumulative time) which appears to be a consequence of acceleration within stages 1 and 2 (relative time).
Figure 2.8: In order to track developmental progress past Stage 4 manual developmental schedule experiments were conducted (sampled at 24hr time intervals). The data up to the stage 4 level were consistent with previous automated findings. However, when the ambient environmental calcium data (former) is coupled with survival trends (latter) it becomes apparent that as embryos transition from stage 5 to stage 6 they may begin interacting with the external calcium environment. Until this stage survival (and development) are unaffected by the ambient calcium concentrations explored in this study.
Figure 2.9: The line graphs demonstrate *Daphnia magna* size/growth as it relates to calcium treatment and generation (low=5mg/L, high=25mg/L). The bar graphs represent brood size as it relates to treatment and generation. Size is significantly affected by treatment in generation 1 (p<0.005), and size is significantly affected by generation. However, there is no significant effect of generation and treatment on size. There are significant differences in the brood sizes from week to week, and when comparing similar weeks across generations (p<0.005). However, there is no significant effect of treatment detected.
DISCUSSION

Influence of environmental calcium on embryogenesis and embryo survival

The developmental time-lapse experiments conducted for this study provide evidence that embryos formed in different calcium environments can experience temporal changes in developmental schedule. Embryos that came from mothers in low calcium environments experienced an accelerated rate of development through stage 1 and 2 of embryogenesis, completing each stage approximately 5 hours faster than embryos formed in 25mg/L calcium environments. Although this temporal shift occurred, there were no visual, physical abnormalities observed in the developing embryos. Studies in mice have shown that increased levels of calcium can accelerate early blastocyst morphogenesis processes (Stachecki et al. 1994) demonstrating that temporal shifts in development are possible as a consequence of changes in calcium levels. My data show specifically that decreased environmental calcium concentration might be affecting processes in early development (i.e. pre-organogenesis) and altering overall developmental schedule. Perhaps by reducing the amount of calcium available for certain developmental processes, the embryos of Daphnia magna are forced to complete calcium dependent developmental processes more rapidly due to limited calcium availability. Because a basal amount of calcium is likely required for proper cell adhesion in Daphnia embryos, calcium limitation may reduce the amount of free, signaling calcium in the embryos. It would be interesting to investigate the calcium signalling signatures of early embryo morphogenesis via live-fluorescent or aequorin imaging techniques, to determine whether there are changes in embryonic calcium signalling wave frequency as a result of decreased environmental calcium.
Embryo survival was only affected by the 0mg/L external environmental calcium treatment (Figure 2.8) and embryo mortality is first significant at $t_3 = 72$ hours. Embryos that were formed in the same, 10mg/L calcium environment were placed in different ambient calcium conditions (0mg/L, 5mg/L and 25mg/L). Survival was consistent across treatments until the embryos approached stage 5. At this time the 0mg/L calcium treatment experienced a rapid drop in survival ($t_3 = 72$ hours: $23.61\% \pm 17.73\%$), and all animals in this treatment had expired by 96 hours ($t_4$). This was not seen in the low and high calcium treatments where survival (89 and 90% respectively) was maintained from 72-96 hours. The high mortality in the 0mg/L calcium treatment could be related to the onset of direct environmental calcium interaction with newly formed osmoregulatory organs after hatching the vitelline membrane (Charmantier and Charmantier-Daures, 2001). It is possible that the embryos of *D. magna*, upon formation, might contain a finite amount of calcium that is exhausted by stage 5, and must be supplemented with environmental calcium. This would account for the dramatic drop in survival that occurs once embryos pass stage 5. Oviparous, squamate reptiles are an example of organisms that also possess a finite amount of calcium upon their formation. The yolk accounts for 19-86% of embryonic calcium content, while the remaining calcium is extracted from the eggshell (Stewart and Ecay, 2000). More studies will have to be conducted to clarify the underpinnings of this finding regarding calcium’s relationship to embryo mortality.

Under the assumption that gas exchange is still occurring across both embryonic membranes in *Daphnia magna* (consistent with other crustacean embryos; Charmantier and Charmantier-Daures, 2001) it is likely that small ions and gases can easily penetrate these embryonic barriers. However, it might be the case that larger, complex molecules
are unable to penetrate the embryonic barriers. In the chick embryo it has been observed that calcium is transported from the eggshell by the chorionallantoic membrane through a complex calcium transport mechanism that makes use of calcium-dependent ATPase, carbonic anhydrase and calcium-binding protein (Tuan and Ono, 1986). It is likely that calcium transport is regulated by similar complex transport processes in *D. magna*, given that calcium is an important signalling molecule whose fundamental transport mechanisms (i.e. calcium pumps and transmembrane channels) are likely stringently conserved over evolutionary time.

Recent studies in ecotoxicology have investigated the efficacy of *D. magna* embryos in bioassays used to indicate water quality and/or endocrine disrupter contamination (e.g. Wang, Lu and Chang 2011, Kast-Hutcheson *et al.* 2001, personal previous unpublished data). If the embryonic membranes of *Daphnia magna* are involved in early embryonic osmoregulation (Charmantier and Charmantier-Daures, 2001), and the transport of larger minerals such as calcium across these membranes is regulated by complex transport mechanisms as seen in the chick embryo (Tuan and Ono, 1986), bioassays that employ simple pharmacological exposures may not demonstrate the true effects of the disruptive compounds on embryos if the compounds are unable to cross these barriers. Properly incorporating compounds into the embryos might require acclimating mothers to the compound, and having them transfer it naturally to the embryos as seen with calcium. Alternatively, it might require microinjection of the compound into the embryo to mimic maternal provisioning. In any case, based on the information gathered from this study, embryonic membrane permeability, and maternal provisioning pathways should be taken into consideration when designing
pharmacological experiments and bioassays aimed at investigating the effects of endocrine disrupters and biological contaminants on embryos of *Daphnia magna*.

**Source of calcium required for embryogenesis and maternal calcium provisioning**

$^{45}$CaCl radiotracer experiments demonstrated that calcium is transferred to the embryo from the mother (Figure 2.3 and 2.4), and that little if any calcium is acquired directly from the environment (Figure 2.2) until stage 5 (Figure 2.8). Flame atomic absorption spectrophotometry was used to quantify calcium provisioned to embryos by mothers, under different environmental calcium conditions. Consistent with my hypothesis, embryos formed in low (5mg/L) calcium environments had 1.7x less calcium than embryos formed in high calcium environments (25 mg/L; Figure 2.5). The reproductive mechanism that allows for maternal reduction of calcium loss to asexual embryos is unknown. However it is possible (and theoretically most parsimonious) that asexual embryo calcium reduction occurs via the same resting egg calcium reclamation mechanism serendipitously demonstrated in a study by Jeziorski and Yan (2008). It would be interesting to pursue this possibility in future research by trying to localize and interact with (i.e. activate and knock-out) the genes that encode for calcium mobilization and binding proteins. The 1.7 fold difference in calcium content found between embryos formed in low and high calcium environments suggests that *D. magna* are capable of modifying calcium efflux via modifications to reproductive calcium provisioning. This finding is consistent with Tan and Wang (2009) who also demonstrated that *D. magna* can compensate for low calcium availability by reducing calcium efflux. Finally, there is potentially something interesting happening around stage 5 regarding the significance of the vitelline membrane and calcium transport. Future studies could investigate whether
embryos encapsulated in the vitelline membrane contain more or less calcium than embryos that have hatched from the vitelline membrane in different calcium environments.

Another interesting result is the lack of significant difference between calcium content of mothers reared in the low (5mg/L) and high (25mg/L) calcium treatments (~1.4 mg/L and 2 mg/L respectively; Figure 2.6). There is still a notable difference between the two treatments, but the lack of statistical significance may suggest that the mothers are able to modify reproductive calcium loss in a way that supports their other calcium demands (i.e. calcium required for muscular contraction and carapace reinforcement). This might be a means of promoting female survival over reproduction, as a consequence of a calcium dependent life history trade-off that exists between female survival (longevity) and reproduction. It is not uncommon that organisms opt for survival over propagation and it has been demonstrated (in both primitive and derived organisms) that survival can be compromised by reproduction (Stony Coral: Oren et al. 2001; Asian timber elephants: Robinson et al. 2012). This trade-off in a parthenogenic organism is particularly interesting as it potentially compromises the success of tens of genetic maternal clones (Baker and Hebert, 1985). From a genetic survival perspective, it would seem most advantageous to invest energy into embryogenesis to promote propagation and mass replication of the genome. However, since juveniles are more vulnerable to calcium deficiency than adults (Porcella et al. 1969; Hessen et al. 2000), perhaps this strategy of maternal preservation is optimal at the calcium concentrations explored in this study (5mg/L, 10mg/L and 25mg/L). It promotes the survival of a reproductive female, rather than embryos and juveniles that may have reduced fitness as a consequence of reduced
environmental calcium concentration. Regardless of the fundamental driving causes of this strategy, it is not surprising that females can adjust embryonic calcium provisioning given the frequency with which epigenetic modifications and plasticity are observed in *Daphnia magna* (Colbourne et al 2011; Harris, Bartlett and Lloyd 2012).

**Embryonic carryover effects on growth and brood size**

In order to understand the real-world ecological consequences of environmental constituents on different organisms it is critical to examine their emergent effects in combination with each other. But by isolating single stressors, the individual mechanisms influenced by a specific environmental factor can be revealed and explored. Many studies have investigated the impact of multiple stressors on *Daphnia magna*. Some of this work has examined the synergistic consequences of a combination of different environmental factors such as: heavy metal toxicity, UV radiation exposure, food shortage, calcium shortage and temperature (Bodar *et al.* 1989; Altshuler *et al.* 2011; He and Wang, 2009; Ashforth and Yan, 2008). To my knowledge no studies have explored the isolated consequences of calcium deficiency over multiple generations. Based on the experimental design and parameters chosen in this study, no conclusions can be drawn regarding the significance of calcium environment (between the range of 5mg/L-25mg/L) on growth and brood size (Figure 2.8) over multiple generations. Although some significant relationships were found, they reveal little if anything about the potential carryover effects of embryonic calcium deficiency. It is possible that within the chosen range of calcium concentrations (5mg/L-25mg/L) effects that would manifest as growth and brood related consequences are not disparate enough to detect. Calcium deficiency tolerance is dramatically reduced when present in synergy with other stressors (Altshuler
et al. 2011; Ashforth and Yan, 2008). Perhaps in our experiment, in a controlled laboratory where no other stressors exist, the chosen calcium range does not have statistically significant, detectable effects on the chosen life history traits. A study by Lewis and Maki (1981) explored the effects of water hardness on growth and reproduction in *Daphnia magna* and used a low calcium threshold of 20mg/L and an upper threshold of 140mg/L. Across this range they obtained evidence suggesting a positive relationship between calcium concentration and reproductive output. I wanted to explore the consequences of calcium decline when approaching the suggested reproductive threshold (Muysen et al. 2008; Hessen et al. 2000) and within an environmentally relevant range for softwater lakes. Across Canada calcium content in lakes ranges from 1-336mg/L and averages 21.8mg/L (Health Canada, 1987). Perhaps future studies exploring the multigenerational effects of embryonic calcium deficiency should conduct the experiment over a wider range of calcium concentrations to satisfy the possibility that the resolution of calcium concentrations used in this experiment was too fine to detect long term consequences on growth and reproduction. Environmental stressors seldom operate in isolation in the natural environment so it is important not to make the assumption that these values are necessarily transferable to Daphnia calcium tolerance in their home environments. It might also be possible that the *Daphnia magna* used in this particular study have been acclimatized to calcium concentrations which might make them more resistant to the onset of consequences related to calcium stress. A study investigating the low calcium tolerance of two different populations of *D. galeata* (one from a lake of approximately 10mg/L Ca\(^{2+}\), and one from a lake of approximately 2-3mg/L Ca\(^{2+}\)) found that Daphnia acclimatized to higher calcium concentrations had a
higher tolerance to calcium stress when compared to populations acclimatized to low calcium environments (Rukke, 2002b). Where many protocols require that *Daphnia magna* are kept in media with calcium concentrations above 25mg/L (e.g. Reconstituted water, see Appendix, page 7), the soft-COMBO medium used in our lab results in a final calcium concentration of 8-10mg/L. However, even calcium concentrations of 10mg/L can buffer the effects of low environmental calcium concentration on *Daphnia* (Rukke, 2002b). This could be further examined by running a series of tests monitoring growth and reproduction on our laboratory *Daphnia magna* strain, and on a new imported strain of females from a different source, over a wider range of calcium concentrations than those explored in the present study.
CHAPTER 4 - GENERAL DISCUSSION

Calcium decline in soft-water lakes is a major issue in the Canadian Shield and in Scandinavia. Although extensive research has focused on the consequences of calcium decline on adult and juvenile *Daphnia*, and similar crustacean zooplankton species, few studies have investigated the effects of calcium decline on embryos and embryogenesis. The objective of the present study was to investigate the consequences of calcium decline on *Daphnia magna* embryogenesis. More specifically this study aimed to determine how low ambient calcium concentrations relate to embryogenesis, embryo survival, maternal calcium provisioning to embryos, and later life consequences on growth and brood size (as a result of embryonic carryover effects), while investigating the main source of calcium required by *Daphnia magna* for embryogenesis. In order to interpret embryogenesis in a repeatable, measurable manner, development was examined thoroughly using time-lapse imagery and manual monitoring techniques and a discursive account of phenotypic progress through embryogenesis was provided. These features were used to generate an embryonic staging scheme that reduces embryological progress to a numerical framework. This simplification allows researchers to begin teasing apart the inter-related complex processes that underlie each stage by binning similar events and major transitions into discrete stages. This work may prove valuable as novel approaches to ecotoxicology have used embryonic assays to explore the effects of endocrine disrupters and wastewater effluents on development of *Daphnia* (Wang *et al.* 2011; Kast-Hutcheson *et al.* 2001). Future work in ecotoxicology may benefit from the use of this staging scheme to explore sub-lethal temporal shifts in developmental schedule that result from exposure to different environmental contaminants.
Localizing calcium in adult and embryo *Daphnia magna* and developmental staging

Alizarin Red-S was used to localize calcium within *D. magna* embryos and adults. The results from this work provide a first glimpse at the tissues and cells that contain calcium in *D. magna*. In embryos calcium was localized in active, animal pole cells. Calcium was consistently found in the cephalic region and structures, thoracic appendages, ventral neuroectoderm, carapace and gut tube. This suggests that calcium may be involved in signalling processes of these organisms, or in the structural elements of the staining regions. To my knowledge calcium has never before been localized in *D. magna* embryos. Calcium staining in the described regions, and the developmental staging scheme described in Chapter 2, lay the foundation for further investigation of calcium’s functions in *Daphnia magna* embryogenesis (as this relates to decreased environmental calcium availability). Alizarin Red-S staining in adults suggests that calcium is present in a network of potential muscle support cells (stellate cells and thoracic cells) that may assume either structural or nutritional support functions. Calcium was also localized at the base of the thoracic appendages in the region of the epipodites. Based on location this calcium may be involved in osmoregulation and/or respiration processes as this area contains the gill epithelia (Kikuchi, 1983). Calcium was also localized in the gut epithelium with less prominence/signal strength than the stellate or thoracic cells. Crustaceans have been recorded in many instances to store calcium in the cardiac stomach as gastroliths during premoult preparation processes (Zanotto and Wheatly, 2003). This calcium is then mobilized and channelled to the carapace again postmoult. It is possible that gut staining is a reflection of this process occurring to a lesser extent. Calcification and moulting in *Daphnia* occur so frequently that the process...
is much more continuous in *Daphnia* when compared to other crustaceans (Halcrow, 1976). Perhaps calcium in the gut epithelium is being bound to calcium-sequestering proteins as a means of temporary storage due to the quick turn-around of calcium during calcification. Future studies may want to investigate whether daphniids possess gene homologues that encode calcium sequestering proteins and whether gene expression in the gut epithelium is up-regulated during premoult and down-regulated during postmolt.

**Limiting environmental calcium and its effects on *Daphnia magna* embryonic developmental schedule, maternal calcium provisioning, and embryonic calcium content**

*Developmental Schedule*

Time-lapse and manual embryo monitoring were the techniques employed to investigate changes in embryonic developmental schedule in response to environmental calcium concentrations. It was determined that embryos formed in low ambient calcium environments experienced accelerated development through stages 1 and 2 of embryogenesis (~5 hours faster than high calcium treatments in each stage). The embryos resumed control speeds after the onset of processes related to organogenesis in stage 3. It has been demonstrated in birds and mammals that accelerated embryogenesis can ultimately reduce an organism’s lifespan (Ricklefs, 2006). Future studies related to this finding could investigate how acceleration through early embryonic stages influences *D. magna* total lifespan. Studies could also use calcium imaging or calcium chelating methods to determine what processes are being affected by calcium availability in early embryonic development, and whether they are responsible for this acceleration. Some visualization techniques include aequorin imaging, or incubation in fluorescently tagged calcium-binding proteins (e.g. cameleon imaging) that allow for live, real-time...
visualization of embryonic calcium mobilization. These techniques would help to link particular calcium signalling patterns with their functions during embryogenesis based on variables such as calcium quantity, wave frequency and spatial distribution (i.e. localized signalling or broadcast, general signalling). Studies could also attempt to microinject a calcium chelator into embryos to determine what structures and mechanisms are dependent on calcium, and whether these processes can be recovered upon microinjection of calcium. These techniques could provide valuable insight as to the causes of the observed acceleration through developmental stages 1 and 2 in embryos formed in calcium deficient environments.

Embryo survival was significantly impacted by environmental calcium concentration with the onset of stage 5. This suggests that embryos begin to interact with their external calcium environment at this time, and up to this point, the embryos are visually unaffected by their ambient calcium environment. This result reiterates that ecotoxicological and pharmacological experiments exploring embryonic effects of environmental endocrine disruptors should consider the permeability of the embryo to the chemicals, and the pathways the chemicals must take in order to reach the developing *Daphnia*, as it seems that transport of molecules across the embryonic membranes is well regulated.

*Radiation experiments*

Radiation experiments were used to investigate the transfer of calcium from mothers to embryos and the uptake of environmental calcium by embryos. Before hypothesis specific experiments using $^{45}$CaCl could be conducted, a series of experiments studying the calcium uptake kinetics of $^{45}$CaCl were completed to ensure that the females
were able to uptake and hold the radiotracer. The results gathered from this study suggest that *D. magna* are able to uptake the calcium radioisotope $^{45}\text{CaCl}$ and that calcium uptake is an active process (rather than a passive diffusive process). Embryos formed in a non-radioactive calcium environment (~10mg/L calcium) were subject to overnight radiation exposures to determine whether these embryos were uptaking calcium directly from their environment. It was determined that these embryos had CPM reads that weren’t significantly different from the controls. Given that the embryos are not using ambient environmental calcium it is only logical to assume that the embryos are receiving the calcium necessary for development from their mothers. In order to confirm this, embryos were collected from mothers who had been acclimatized to a radioactive calcium environment, and were processed in the same manner as the embryos from the overnight exposure experiments. These embryos had significantly higher CPM reads when compared to the controls, suggesting that embryos are receiving the calcium they require for development from their mothers.

*Flame AAS*

Flame atomic absorption spectrophotometry was used to determine how much calcium is provisioned to embryos by mothers in different calcium environments. The results demonstrate that mothers are able to provision significantly less calcium to their embryos in calcium limiting environments (5mg/L) when compared to embryos formed in higher calcium environments (25mg/L). Future studies might consider investigating the ratio of embryonic calcium to maternal calcium to determine whether this ratio is maintained, or whether it proportionally decreases with decreased environmental calcium and increases with increased environmental calcium.
Embryonic carryover effects on growth and brood size

Multigenerational studies investigating the effects of environmental calcium concentration (5mg/L and 25mg/L) on brood size and growth yielded no conclusive evidence that there are carryover effects of limiting calcium at the tested concentrations on subsequent generations, nor any consequences of chronic exposure to reduced calcium for that matter. It is advisable that future studies explore a wider range of calcium concentrations, fitness parameters, and possibly an additional generation to better understand what effect environmental calcium concentration has on *Daphnia magna* fitness.

Concluding Remarks

In summary this thesis was intended to investigate the consequences of a real environmental threat, calcium decline, on the embryogenesis of a keystone, freshwater invertebrate: *Daphnia magna*. It has led to the creation of a developmental staging scheme that is founded on unbiased, visually interpretable, descriptive features that make it ideal for multiple applications. This study is also the first to localize calcium in adult female and embryo *Daphnia magna* using cryosectioning techniques and the calcium stain Alizarin Red-S. The localization of calcium is a valuable first step toward pinpointing the specific mechanisms, processes and tissues that could be affected by decreased environmental calcium. Calcium was specifically and interestingly localized in the gut epithelium of *D. magna*. As the gut epithelium is a known region of calcium storage in crustaceans (Zanotto and Wheatly, 2003) it is possible that this staining could be a related to some form of calcium storage in the gut epithelia of *Daphnia magna*: a crustacean that previously had no evidence supporting the existence of a calcium storage
mechanism. It was also determined through a series of calcium radiotracer experiments that the calcium required for development comes predominantly (if not exclusively) from the mother, and not the external environment. Flame AAS measured samples of embryos formed in low calcium environments contained significantly less calcium than embryos formed in high calcium environments, while mothers acclimatized to the same high and low calcium environments did not have statistically significant differences in calcium content. Also embryos formed in low calcium environments experienced accelerated development through stages 1 and 2 of embryonic development. This suggests that the pre-organogenesis processes (i.e. involved in blastocyst and/or morula formation) are experiencing a disproportionately influential effect of limiting calcium, and the specific mechanisms affected should be explored in future studies. Finally, a multigenerational study was conducted to investigate the embryonic carryover effects of limiting environmental calcium on growth and brood size. Although the results from this experiment provide no support for carryover effects, it is advised that similar future studies make amendments to the experimental design (and possibly expand the investigated parameters) to more thoroughly investigate the possible relationship between early life calcium experiences, and later life stages in *Daphnia magna*. 
REFERENCES


McCormick MI and Gagliano M. 2009. Carry-over effects – the importance of a good start. 11th International Coral Reef Symposium. 305-310.


Figure A-1: *Daphnia* life history schematic. Asexual cycle: An asexual embryo develops into a cloned female who parthenogenetically reproduces a clutch (or brood) of clones. Sexual cycle: Environmentally induced epigenetic modifications result in male and female juveniles. The product of sexual reproduction is the dark, chitinous ephippium that contains two feminized embryos. Ephippia overwinter (or cope with environmental stress). Environmental triggers (such as light, temperature, etc) stimulate embryogenesis in the two ephippial embryos. Females are born and if conditions are favourable the asexual cycle commences (personally developed image).
Figure A-2: Schematic of immobilization technique. A pipette mostly filled with air, uses negative pressure to hold the female still. The point of attachment is posterior to the secondary antenna, on the carapace. A second pipette filled with water gently jets water into the brood chamber once the post-abdomen and abdomen are lifted. The gentle current carries the embryos out of the brood chamber without mechanical damage (personally developed image).
2. Protocols

1. Gelatin Coating Slides

Materials: slides, 70% EtOH, Kimwipes®, Type B gelatin, Chromium (III) Potassium Sulfate Dodecahydrate, distilled or nanopure water, stir bar, stir/hotplate, coplin jar or dipping jar/beaker, clean jar for solution storage.

Procedure: For 200ml of solution

Heat water to 60°C (the stir plate heat can be set higher at first but make sure to avoid overheating the solution). Add 1 g of gelatin (0.5% Gelatin) and add 0.1g of Chromium (III) Potassium Sulfate Dodecahydrate (0.05%) and allow to stir and mix completely (may take ~20 minutes). Once the mixture is clear and homogeneous, pour it into a dipping container (Coplin jar or something tall).

WEAR GLOVES when handling slides as dirt and oily residues can reduce the adhesion of slides to sections and they may flake off in preparation. You can clean slides in a hot soapy water bath and rinse with hot water for a long time (~hour or two of running water) and then dip slides or you can use a Kimwipe© and minimum 70% EtOH. Once slides are cleaned they should be placed label side down (Figure A-3: opposite to this orientation demonstrated in this photograph. These were slides before they were cleaned or treated with gelatin medium). While holding the label of the slide, dip the entire mounting surface of the slide in the gelatin medium. Move it around or dip several times. Then remove from the medium, and allow the excess to drip off (or touch the corner of the slide on the edge of the coplin jar to speed this up). Then carefully, without touching the face of the slide (ie/ handle only the edges) place the slides on the drying rack in the proper orientation. It's probably a good idea not to fill both sides of the slide rack with slides to prepare. Only have one side filled at a time so that slides can be transferred to the other side once they have gone through a certain step. Place in a drying oven at 60°C overnight. Do this quickly to avoid letting dust settle on the gelatinous slides. The slides will be ready for use the next day. Do not over heat. If/when the slides begin flaking, they will not hold specimens/sections reliably anymore and should be discarded.
2. **DABCO Mounting Medium**

Final Concentration: 90% Glycerol, 0.1M Tris-HCl, 10g/L Dapco

1. Measure out 0.1g of DABCO (1,4-Diazabicyclo[2.2.2]octane) and transfer to a 15ml Falcon tube
2. Add 1ml of 1M Tris-HCl (@pH 8.5)
3. Mix well until DABCO is dissolved
4. In a **glass** container heat 100% glycerol in a microwave for 10 seconds (glycerol will be quite fluid)
5. Add the glycerol to the falcon tube to the 10ml mark.
6. **Gently** mix by inversion, avoid creating bubbles
7. Cool at room temperature, store at 4°C
3. Scintillation fluid

Materials:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Order Code</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>105 L-12984</td>
<td>Fisher Chemical</td>
</tr>
<tr>
<td>Triton x100 proteomics grade</td>
<td>M143-4L</td>
<td>AMRESCO</td>
</tr>
<tr>
<td>2, 5-Diphenyloxazole (PPO) (PPO)</td>
<td>D4630-100g</td>
<td>Sigma</td>
</tr>
<tr>
<td>1, 4-Bis (5-phenyl-2-oxazolyl) benzoate (POPOP)</td>
<td>P3754-25G</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Side notes
Tx100 comes in 'wine box' like cardboard/plastic bag containers. This is a very clean and efficient way to order the chemical specifically for this purpose

Non-chemical Materials:

1. Stir plate (in fume hood)
2. Medium stir bar
3. 4L Erlenmeyer flask
4. 2-3L graduated cylinder
5. funnel
6. weigh boats for chemicals

Procedure:

1. In a well-ventilated area (under a hood) transfer 2L of Toluene to a light protected 4L Erlenmeyer Flask containing a large/medium stir bar (via graduated cylinder and funnel)

2. Add powder chemicals to toluene

12g PPO
0.6g POPOP

3. Stir the chemicals at medium speed in the toluene while completing the next step
4. (this step doesn't have to be completed under a hood, but the flask containing the toluene should be on a stir plate in the fume hood and should remain under the fume hood)
Collect 1L of Tx100 in a graduated cylinder. This may take up to 10 minutes.
5. Using the funnel carefully and slowly transfer the viscous Tx100 to the toluene PPO and POPOP solution
6. Allow the mixture to stir overnight to ensure full mixing of the chemicals and triton x

4. **Calcein-AM staining**

Six live female *Daphnia magna* were placed in each well of a 6 well plate (~8mL volume per well). Calcein-AM was dissolved in either 5mg/L, 10mg/L or 25mg/L calcium adjusted COMBO medium to yield Calcein-AM concentrations of either 50mg/L or 100mg/L. This produced the 6 different treatments used in this experiment. This well design was replicated 3x so that females could be collected at 3 different timepoints (1 hour, 3 hours and 6 hours post incubation). The samples were fixed and processed for cryosectioning and were mounted in a light reduced environment using a glycerol DABCO Tris-HCl buffered mounting medium. Slides were then visualized using NIS-Elements AR software and a Nikon-Ti inverted microscope equipped with argon fluorescent light and FITC range fluorescent imaging filters.
5. *Reconstituted Water*

Quantities of Reagent-grade chemicals required for preparation

Taken from ASTM designation: E 729 – 96
“Standard guide for conducting acute toxicity tests with macroinvertebrates”
Tim Boudreau, 2001

<table>
<thead>
<tr>
<th>Hard Water Salts required</th>
<th>Quantity (mg)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>In 1L</td>
<td>In 20L</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>192</td>
<td>3840</td>
</tr>
<tr>
<td>CaSO₄·2H₂O</td>
<td>120</td>
<td>2400</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>120</td>
<td>2400</td>
</tr>
<tr>
<td>KCl</td>
<td>8</td>
<td>160</td>
</tr>
</tbody>
</table>

After a required 24 hour aeration period, the water has a pH range of 7.8-8.0 and a Hardness of 160-190 and Alkalinity of 110 -130.