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The Effects of Calcium Concentration and Food Levels on Daphnia

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The Effects of Calcium Concentration and Food Levels on the Growth and Reproduction of *Daphnia*

By

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ABSTRACT OF THESIS

Many physiological processes of *Daphnia* are negatively affected at low calcium concentrations. The concentration of calcium within lake water influences how *Daphnia* populations will survive and reproduce in natural environments. An experiment was designed to test the effects of calcium concentration and food level on the growth and reproduction of a *Daphnia* hybrid. *Daphnia pulex x Daphnia pulicaria* were reared in a soft water medium at three calcium concentrations (2.5mg/L, 1.0mg/L, 0.50mg/L) and high and low food levels (5.6 x 10^5 and 5.6 x 10^4 cells of the algae *Ankistrodesmus sp.*) in a 2 x 3 factorial design. The experiment results show that at low calcium concentrations (< 1.0 mg/L), survival, reproduction, intrinsic rate of natural population increase "r", time until first reproduction, molting and length of adult individuals and neonates were all negatively affected. Some of the physiological mechanisms exhibited interacting effects between calcium concentration and food level. In the case of the lipids accumulated and ovary condition of an individual, food was more important than calcium concentration. This experiment offers insight into how daphniids in natural settings are responding to the multiple stressors that they are already facing in many freshwater lakes.

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To my sisters, my Grandmother my Uncle Dan Myers and my longtime friends, who throughout my entire college career did whatever they could to be helpful and supportive, thank you. Whether it was to be understanding, help out with things that needed to get done in regular life, to buying text books, you all have been wonderful.

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Chapter 1 Introduction

Background of the Issue

Aquatic acidification alters the mineral composition and the biological nutrient availability of soft-water boreal lakes. Of particular interest is an essential mineral for many crustacean organisms, calcium, whose rate of leaching from soils and surface waters is accelerated by acid stress. As acidification continues over time, the leaching rate of calcium from soils surrounding the lakes exceeds its replenishment rate, the soil-base saturation becomes depleted and calcium becomes deficient in lake water (Jeziorski et al., 2008). Calcium deficiency can be even more pronounced in geographical regions where forest harvesting from watersheds also removes the nutrients contained within the trees. Aquatic crustaceans need calcium for their life processes and some of them have a high specific need for this nutrient and are highly susceptible to environmental calcium depletion. Besides the direct effects of lack of calcium for their anatomical and physiological needs, there is an indirect effect when their food sources become altered as well, either in quantity or quality due to the overall changes in nutrient availability resulting from the above stressors.

The cladoceran *Daphnia* is one such crustacean invertebrate with a high need for calcium. Daphniids are a group of widespread zooplankton species that use calcium to build their carapace, which is molted regularly for growth. Compared to other crustacean zooplankton, *Daphnia* have body concentrations of calcium that are orders of magnitude higher than in other species (Jeziorski and Yan, 2006). In soft water lakes, these zooplankton species are at risk from the combined effects of water acidity, low calcium concentrations and changes in the composition of their food sources. These environmental stressors may result in an overall reduction or species substitution of the *Daphnia* species in a lake. A decrease in numbers or extirpation of acid-sensitive daphniids from a lake will result in long term effects that will cascade throughout the food web (Jeziorski et al. 2008).

The Calcium Content of *Daphnia*

Daphniids are keystone herbivores in pelagic food webs and are a food source for predatory zooplankton species, fish larvae and planktivorous fish. Most calcium in *Daphnia* is found in the carapace or exoskeleton and is acquired primarily from the surrounding water and minimally through the diet. Because *Daphnia* molt their carapace regularly, the uptake of calcium from the surrounding medium is critical. For some species of *Daphnia*, the calcium content within the body is higher than in other species and they have an increased need for this mineral (Tan and Wang, 2010). Myussen (2009) found that when calcium levels in the water decreased, total body calcium in daphniids decreased as well. *Daphnia* use calcium for a variety of physiological processes including support, protection, growth, egg production, formation of soft tissues, cell signaling and muscle contractions. However, about 80-90% of the calcium they accumulate is deposited in the exoskeleton for support (Porcella et al., 1969) while 8% to 26% is found in soft tissues. With decreasing calcium concentrations, the carapace strength is compromised and *Daphnia* become vulnerable to mechanical damage and to predation. Therefore, decreasing calcium concentrations in lakes poses a great threat to vital processes of *Daphnia*'s growth and development and increases their risk of damage and predation.

Growth and Molting

Body size in daphniids varies due to many different factors. The body size of individual *Daphnia* may vary because of environmental factors such as temperature, food quantity or quality, the presence of invertebrate or vertebrate predators and constraints from environmental stressors. As juvenile daphniids grow with each molt in a stepwise fashion, body size increases with age until a certain age or size is met, at which time resources are then allocated to reproduction rather than growth (Ebert, 1994; Enserik et al., 1995; McCauley et al., 1990; Perrin, 1989)

Calcification of the exoskeleton begins prior to the shedding of the old one and is associated with molting and growth (Porcella, 1969). Total accumulation of calcium is directly related to the calcium content within the water (Porcella 1969, Tan and Wang, 2010). An experiment by Porcella et al. (1969) illustrates the fate of calcium within *Daphnia*'s bodies. These researchers studied the uptake of calcium throughout the molting period in *Daphnia magna*. Isotopes of strontium- 85 (which is chemically similar to calcium and is cycled in a similar manner) and calcium- 45 were added to a food-free medium, so that uptake of calcium through time and the places in the body where it was deposited could be analyzed. They found that 95% of the strontium isotope was deposited in the carapace of *D. magna*. Thus, the amount of calcium in the water is critical to build a strong carapace regularly. In addition, after about 17 hours, calcium uptake reaches a plateau in *Daphnia* and not much more is accumulated (Porcella, 1969).

Calcium uptake is most important during the post-molt time (Hessen et al. 2000). *D. magna* reared at concentrations below the calcium saturation level (5.3 mg/L Ca) had considerably less calcified exoskeletons than individuals reared at the optimal calcium concentrations (10 mg/L Ca). When calcium levels were reduced from 10 to 5.3 mg/L, dry weights of the animals were reduced from 4.2 % to 1% (Alstad et al. 1999). In addition, individuals reared at lower calcium concentrations could not compensate by prolonging the period of post-molt calcium uptake (Alstad et al. 1999). Specific thresholds for calcium concentration needs in crustaceans have been studied in *D. magna*, *Gammarus lacustris* and *Astacus astacus* (Hessen et al. 2000, Rukke 2002). Below a threshold of 0.1 – 0.5 mg/L Ca, growth rates, specific dry weights and length were all reduced. Hessen et al. (2000) found that when lake water has low calcium concentrations, crustaceans need to compensate for the loss of calcium available for the molting process. Compensation for low calcium in the water is made by three mechanisms: delayed reproduction, change in the makeup of the exoskeleton to one that is less calcified, or reduction in body size.

Survival in *Daphnia*

Due to high variation in the calcium requirements among different *Daphnia* species, survival under low calcium conditions will vary. Species with lower body calcium requirements will most likely survive better in low-calcium lakes than those with higher calcium requirements. In a laboratory experiment, *D. pulex* required between 0.1 and 0.5 mg/L Ca to survive 15 days (Ashforth and Yan, 2008). Other factors that affected survival in this experiment included food quantity and temperature. Temperatures between 24-32 °C and decreasing food quantity each negatively affected the survival of *D. pulex*. Similarly, in an experiment by Alstad et al. (1999) the effects of calcium concentration on the calcification process was investigated by rearing *D. magna* under five calcium concentrations ranging from 0.5 to 2.04 mg/L. Results showed that as calcium decreased, the specific calcium content of the animal decreased as well. Incomplete calcification under very poor conditions could thus affect survival of *Daphnia*.

Although survival has been shown experimentally to be affected by calcium levels in the water, laboratory studies have been found inadequate to estimate survival in natural settings (Walthall and Stark, 1997; Raimondo et al., 2007). To assess how daphniid populations will respond under natural conditions to calcium decline, conducting field studies would be the best approach; however, these types of studies are subject to confounding factors and specific thresholds and stressors are hard to pinpoint. Jeziorski and Yan (2006) studied lake sediment cores to better understand how daphniid populations have been responding to calcium decline in lakes. They found a positive correlation between lake water calcium and zooplankton calcium concentrations. Therefore, the species present in a lake may be indicators of ambient calcium concentrations and *vice versa*. Since ambient concentrations of calcium correlate with zooplankton species composition, as lake water calcium concentrations approach laboratory-derived low thresholds, survival of the species with high calcium demands will decrease, and ultimately they will be extirpated from these lakes.

Species that show reproductive elasticity, for example delaying reproduction to increase individual survival, will suffer from greater impacts to their populations than those species who maintain high reproductive rates. Even if survival is minimally affected, delays in maturity, decreased clutch size and decreased number of clutches will reduce the intrinsic rate of population increase and lead to a decrease of the affected

Daphnia populations even when death rates are stable (Walthall and Stark, 1997; Raimondo et al., 2007).

***Daphnia*'s Reproduction**

Daphnia reproduce via parthenogenesis; when conditions are optimal *Daphnia* can mature in about 3-7 days, and can produce broods of 10-20 neonates or more every 2-5 days (Taylor and Gabriel, 1992). In addition to the calcification process, calcium content of the medium can affect the intrinsic rate of population growth and possibly the maturation of daphniids (Tan and Wang, 2010). Additionally, low calcium levels combined with low food quantity or quality can also affect reproductive rates. When *D. pulex* were experimentally exposed to different calcium concentrations, food levels and rising temperatures, it was found that calcium levels between 0.1 and 0.5 mg/L were needed for survival. However, the animals exposed to 0.5 mg/L of calcium and the lowest temperature of 20 °C were the only individuals at the lower calcium concentrations able to reproduce, indicating a reproductive disadvantage at decreasing calcium concentrations and at higher temperatures (Ashforth and Yan, 2008). Below 1.5 mg/L Ca, the onset of reproduction was delayed and the magnitude of the negative effects of low calcium became greater as the concentration decreased further, in addition, when food levels were reduced from high to low levels, the basic reproductive rate of the individuals decreased by 90% (Ashforth and Yan, 2008). It seems there may be a size threshold for *Daphnia* at which sustained reproduction can begin. In stressful conditions, such as low food and/ or low calcium, this size threshold may decrease so reproduction is not delayed, maximizing population fitness in terms of rate of population increase. Alternatively, individuals may prolong juvenile instars in order to reach this size threshold (Ebert, 1992; Enserik et al., 1995 Gliwicz and Lampert, 1990; McCauley et al. 1990).

Reproductive thresholds can be compensated for in a number of ways, although the response to low food and/or low calcium can be species-specific and can even have inter-clonal differences. For example, larger *Daphnia* can produce larger clutches and some *Daphnia* may allocate resources to growth and maintenance and prolong juvenile instars in order to attain a larger size and more reproduction (Ebert, 1992 and 1994, Gliwicz and Lampert, 1990). However, some *Daphnia* species will produce fewer but larger neonates per clutch, which will give them the advantage of higher resistance to starvation (Enserik et al., 1995; McCauley et al. 1995; Tessier and Consolatti, 1989; Ebert, 1993).

Food Quality and Quantity

Periods of decreased grazable phytoplankton or blooms of blue-green algae can cause daphniids to face periods of starvation or low food quality in their environment. Acidification and the resulting decrease in nutrients can also affect phytoplankton quantity. The question arises about how a decrease or change in phytoplankton species composition or biomass alters grazer composition, particularly *Daphnia* species. Decrease in nutritional quality or overall shifts in phytoplankton community structure happen synchronously with acidification and calcium decline (Stokes, 1986), so daphniids are exposed to both types of limiting factors at the same time in affected lakes. Food quantity alone can affect growth and reproduction rates in *Daphnia*. For instance, Vanni and Lampert (1992) experimentally raised *D. galeata* on *Scenedesmus acutus* at varying food densities. Growth and reproduction rates were delayed at the lowest food concentrations. As the densities of *Scenedesmus* fed to *Daphnia* increased, their intrinsic rate of population growth increased as well. Changes in metabolic processes, such as energy allocation and changes in life histories may also result when *Daphnia* are raised at low food levels (Glazier and Calow, 1992). Typically, at low food levels, energy will be allocated to body maintenance and carapace formation over growth and reproduction; however, this can change depending on the species, the clone, age of the individual, population density and whether exposure was chronic or for a short period of time (Burns, 1994, Glazier and Calow 1992).

Ecological Impacts

There is a correlation between the pH and the calcium content of soft-water lakes. Soft water lakes that receive acidic deposition from surrounding industry experience a drop in pH, and their surrounding soils also become acidified and, as a result, calcium is leached (Jeziorski et al., 2008). In a study by Locke and Sprules (1994), community biomass, species richness, predator-prey interactions and competitive links decreased when pH dropped in lakes. Data from 46 Precambrian Shield lakes in the Sudbury area of Ontario (Canada) were collected in the 1970's when pH was 3.8, and in the 1990's when pH had increased to 7.0. The greatest decreases and disturbances in the overall interactions of the food web were seen at pH of 5, which corresponds to a calcium content of 1.2 mg/L (Locke and Sprules 1994, Jeziorski 2008). In the 1990's, when pH had increased about two units from the 1970's, species richness, predator-prey interactions, competitive linkages and density of zooplankton all increased. The decrease in pH and consequent depletion of calcium levels impaired important ecological interactions (Locke and Sprules, 1994). For instance, when a predator such as the larval midge *Chaborous* is present in a lake, their kairomones will induce an increase in body size in new generations of *Daphnia*, so they are too big to be consumed, and also they may develop antipredator defenses (Dodson, 1989). However, at calcium levels below 1.5 mg/L, *Daphnia* have been shown to be unable to produce predator-mediated responses (Riessen et al., 2012). Larger body size, the production of neck spines and the hardening of the carapace have all been found to be negatively affected at 1.5 mg/L Ca and these defense mechanisms could not be induced at 0.50 mg/L Ca. This effect particularly influences juvenile *Daphnia*, which are usually very susceptible to predation because of their small body size; under decreased calcium concentrations their predation risk increases by their slow growth and by a lack of defenses (Riessen et al., 2012).

The study of *Daphnia's* life history changes under altered food and calcium scenarios may be used to elucidate and predict what will happen to aquatic communities in lakes exposed to acidification and/or deforestation. In this study, I focused on the effects on survival, reproduction and allocation of resources in *Daphnia* exposed to varying calcium levels and food quantities. To assess the effects of decreasing calcium and food quantity on *Daphnia*, I focused on three main questions:

1) What is the effect on survival of decreasing calcium levels below the optimum for *Daphnia's* physiological processes when food availability is also limiting?

I expected to see the most significant effects on the survival of *Daphnia* at very low calcium levels. In addition, I expected food limitation to add to the stress in *Daphnia* and further decrease their survival rates. However, survival may not be affected greatly if individuals delay reproduction or do not prioritize allocation of resources to growth.

2) How is *Daphnia*'s reproduction affected by decreasing calcium levels under food limitation? I expected that as calcium decreased, *Daphnia*'s reproductive output (in terms of number of neonates per clutch), would decrease in a stepwise fashion. Also, I expected the intrinsic rate of population growth to decrease at each lower level of calcium and be compounded by low food levels. Thus, I expected that *Daphnia* exposed to low levels of calcium and low levels of food would experience less reproductive output and delayed reproduction or no reproduction at all.

3) How do calcium levels and food quantity affect *Daphnia*'s condition? I expected individuals exposed to high food levels to be in better conditions than those under low food levels. The condition of the animals, in this case, would be reflected by the amount of lipid reserves and by their ovary condition. Similarly, in regards to calcium levels, I expected that results would parallel those from the food levels and that low calcium would stress *Daphnia* further, decreasing their ability to store lipids and impairing the production of ovarian tissue.

Chapter 2. Methods and Materials

Laboratory Cultures:

A hybrid, *Daphnia pulex x Daphnia pulicaria* naturally occurring in McFarlane Lake, Ontario in the Canadian Shield was obtained from Dr. Norman Yan (FLAMES Laboratory at Dorset, Ontario, Canada). I cultured these *Daphnia* in FLAMES medium that resembles the natural chemical composition of two non-acidic undeveloped Canadian Shield Lakes (Blue Chalk Lake and Red Chalk Lake); this medium has a calcium content of 2.5 mg/L (Celis-Salgado et al. 2008). The *Daphnia* culture was maintained in an incubator at 20° C. *Daphnia* were inspected for presence of embryos in the brood chamber. Those with mature eggs were isolated and observed for eight hours; neonates born within that time period were isolated. Neonates from this cohort were randomly assigned to 20 mL glass vials, containing one neonate per vial with different calcium concentrations. These *Daphnia* were monitored daily until they produced the first clutch of eggs, at which point neonates that were born into a given treatment medium were used to start the experiment. Six treatments (3 calcium concentrations and 2 food levels) were used with 10 individuals per treatment separated into individual vials, for a total of 60 experimental units.

For the algal culture, a fish tank containing one goldfish was inoculated with *Ankistrodesmus sp.* The culture was allowed to grow until the tank was a dark green color, after which water was siphoned from the tank and filtered. Algae were harvested once a week inspected for species composition and stored in a refrigerator. The algae were allowed to settle in a centrifuge tube; the aquarium water was then poured out of the tube and it was replaced with experimental treatment media.

Experimental Treatments:

The experiment was a 2 x 3 factorial design to explore how the combined effects of calcium and food affect the growth, reproduction and survival of *Daphnia.* I considered the natural calcium content of the reference soft-water lakes (FLAMES medium, 2.5 mg/L Ca) as the most optimum calcium treatment for *Daphnia* to which other treatments should be compared. Each calcium treatment was combined with two food levels (high and low) as follows:

- 2.5 mg/L calcium medium, with high food.
- 2.5 mg/L calcium medium, with low food.
- 1.0 mg/L calcium medium, with high food.
- 1.0 mg/L calcium medium, with low food.
- 0.5 mg/L calcium medium, with high food.
- 0.5 mg/L calcium medium, with low food.

The 1.0 mg/L and 0.5 mg/L calcium concentrations for the experimental treatments were modified from the original FLAMES medium recipe (Celis-Salgado et al. 2008) by adjusting the concentrations of $CaSO_4$ to reflect the experimental concentrations. Densities of algae for the high and low food level treatments were determined counting the cells with a hemocytometer. The treatment with low food levels had 5.6×10^4 cells/mL of *Ankistrodesmus sp.* and the treatment with high food concentration had 5.6×10^5 cells/mL. The concentration of algal cells for the high food treatment was chosen after conducting a preliminary study to determine the density at which *Daphnia* were growing quickly, with no evidence of stress, and were producing large clutches of eggs.

Alternatively, for the low food concentration treatment I chose the threshold of food at which adults survived and were able to reproduce but at a reduced rate. In this pilot study, results from the statistical analysis (two tailed t-test) of the number of neonates produced in each clutch for the 2.5 mg/L Ca high food treatment and 2.5 mg/L Ca low food treatment showed that the high food treatment produced significantly more neonates than the low food treatment ($p = 0.0015$). The 2.5 mg/L Ca. high food treatment produced on average 20 neonates after the second clutch and the low food treatment produced on average 7.33 neonates per clutch. The average from the high food treatment shows that food is sufficient for optimal reproduction (Taylor and Gabriel, 1992). Thus, the algal concentrations outlined above were deemed appropriate to induce a food-level effect in the experimental *Daphnia*.

In the experimental setup, each experimental unit (a glass vial with one neonate in medium containing the calcium and food treatments) was randomly placed in a test-tube rack and placed in a 20° C incubator with a photoperiod of L:D = 15:9 h. Test-tube racks containing the experimental vials were placed in the same incubator shelf and rotated daily. The experimental *Daphnia* were placed daily in a clean vial and given fresh food and media. Data were collected for survival, molting, presence of eggs, number of neonates produced (neonates born were preserved for further analysis), lipid and ovary indices, and length of preserved neonates and of adults surviving to the end of the experiment. Length of neonates and adults was recorded as the measurement from the top of the head to the end of the tail spine. The experiment ran for 19 days and data were collected every day with the exception of adult length measurements and lipid and ovary indices, which were collected once at the end of the experiment.

Lipid and ovary indexes were assigned to each individual by visual inspection. These indexes are each ranked from 0 to 3, with 0 being the lowest score and 3 being the highest. For this assessment, I followed the pictorial descriptions of Tessier and Goulden (1982). A score of 0 for the lipid index indicates that individuals are starved for resources and are in poor health, a score of 3 indicates a very healthy individual with adequate nutrition and resources, which shows as multiple lipid droplets within the animal. For the ovary index, a score of 0 indicates that the individual is under stress from low resources and cannot allocate lipid reserves to ovary provisioning; a score of 3 indicates adequate resources and an individual with this score would show large opaque ovaries when visually inspected.

Statistical Analyses:

Initially, I used exploratory data analysis: descriptive statistics, distributional analysis and evaluation of any relationships. 2 x 3 factorial ANOVAs were used to evaluate significant differences between treatments. Intrinsic rate of population increase, *r*, was calculated using a computer program developed by Dr. Javier Peñalosa that uses jackknifing as a measure of accuracy to sample the distribution of *r*. The equation used was:

$$\sum e^{-rx} l_x m_x = 1$$

Where l_x is the proportion of individuals surviving at time x, and m_x is the mean number of offspring produced per surviving individual at time x. Positive values for the intrinsic rate of population increase, *r*, would indicate growth in a population and a negative value would indicate that there are more individuals dying than being born in that population.

All data was tested for normality (Kolmogorov- Smirnov test) and for homogeneity of variance (Levene's test). When data violated these assumptions it was transformed. If transformations did not improve normality, non-parametric statistics were used (Kruskal-Wallis test and Mann-Whitney test). The variables that were not transformed and analyzed using ANOVA's were: intrinsic rate of population increase, *r*, number of neonates in each clutch, total number of clutches, total number of molts, body length of experimental *Daphnia*, and neonate length. The variables transformed and analyzed using non-parametric tests included: total number of neonates produced per treatment, length of life in days for experimental *Daphnia*, time until first reproduction, number of molts until first reproduction and the lipid and ovary indices. For these data sets, post-hoc tests were used for pairwise comparisons using the Bonferroni correction at $p<0.0167$ (for three comparisons) for significance.

Chapter 3 Results

Intrinsic Rate of Population Increase

The intrinsic rate of population increase, *r,* was calculated using data on survivorship and number of neonates produced by the experimental *Daphnia.* Two treatment factors, calcium concentration and food level, were analyzed for their effects on *r* as well as their interaction effects. Calcium concentration had significant effects on the intrinsic rate of population increase for *Daphnia* (two-way ANOVA, Table 1, Figure 1). Food level also had significant effects on *r* (two-way ANOVA, Table 1, Figure 1). Interaction between calcium concentration and food level was marginally significant (two-way ANOVA, Table 1, Figure 1). *Daphnia* in the higher food level treatments had higher *r* values than those in the lower food level treatments (post-hoc, Tukey HSD $p<0.05$, Figure 1) except in the highest calcium treatment (2.5 mg/l high food and low food) where no significant differences were observed.

Table 1. Two-way ANOVA for the intrinsic rate of population increase *r* for *Daphnia*. Factors: Calcium concentration (2.5, 1.0, 0.50 mg/L), food level (high and low).

Effect	**df**	**MS**	**F**	**p-value**
Ca concentration	2	0.317	52.78	<0.0001
Food level	1	0.334	55.618	<0.0001
Ca conc. * Food level	2	0.016	2.731	0.074
Error	54	0.006		
Total	60			

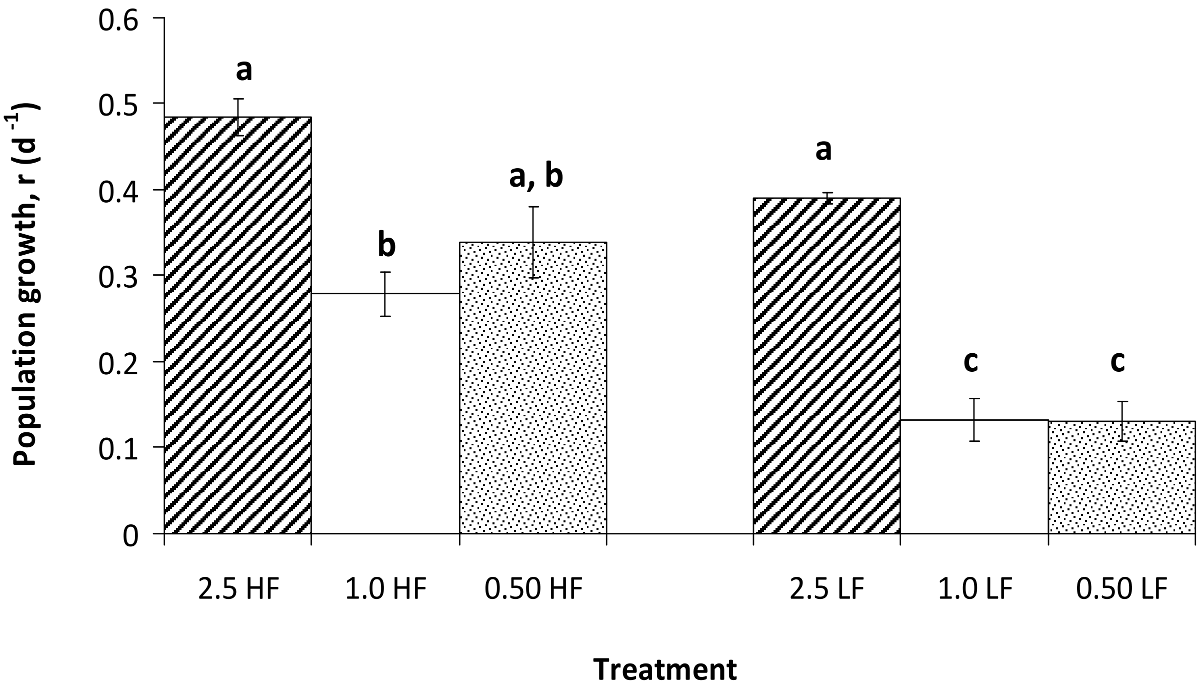

Figure 1.

The intrinsic rate of population increase, *r*, for *Daphnia* (mean± SE) at different calcium and food levels. Different letters indicate significant differences (post-hoc Tukey HSD, $p < 0.05$). Treatments are: 2.5 HF= 2.5 mg/L calcium and high food, 1.0 HF= 1.0 mg/L calcium and high food, 0.50 HF= 0.50 mg/L calcium and high food, 2.5 LF = 2.5 mg/L calcium and low food, 1.0 LF= 1.0 mg/L calcium and low food, 0.50 LF= 0.50 mg/L calcium and low food.

Number of Neonates Produced per Treatment

The total number of neonates produced per treatment was significantly affected by calcium concentration (two-way ANOVA, Table 2, Figure 2) and by food level (two-way ANOVA, Table 2, Figure 2). No significant interaction effects between food and calcium levels were observed. *Daphnia* in media with higher levels of calcium produced more neonates. However, lower calcium concentrations limited the number of neonates produced in the lifetime of the adult and this calcium deficiency was not overcome by the availability of high food (post-hoc Tukey HSD, $p < 0.05$, Figure 2).

Table 2. Two-way ANOVA for the number of neonates produced per treatment by the experimental *Daphnia*. Factors: Calcium concentration (2.5, 1.0, 0.50, mg/L) and food level (high and low).

Effect	**df**	**MS**	**F**	**p- value**
Ca concentration	2	35.026	23.15	< 0.0001
Food level	1	10.785	7.128	0.01
Ca conc.* Food level	2	0.033	0.022	0.979
Error	54	1.513		
Total	60			

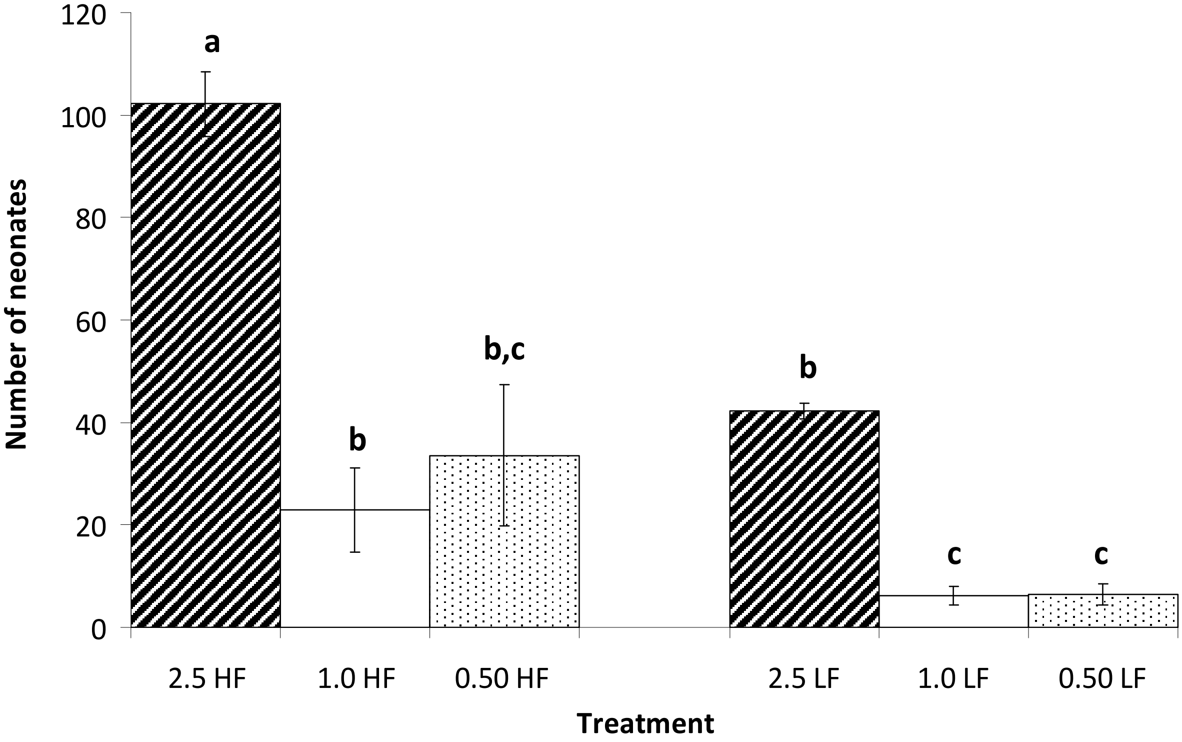

Figure 2.

Number of neonates produced per individual by the experimental *Daphnia* (mean ± SE). Letters indicate significant differences between treatments (post-hoc Tukey HSD, $p < 0.05$). For treatment notations see Figure 1.

Neonate Length

The length of the neonates was significantly affected by calcium concentration (two-way ANOVA, Table 3), food levels (two-way ANOVA, Table 3) and there was a significant interaction between calcium and food levels (two-way ANOVA, Table 3). At the calcium level of 2.5 mg/L there were no significant differences in neonate length between high and low food levels, however, in lower calcium treatments high food levels produced neonates with larger bodies than those neonates in low food level treatments (including all clutches, mean length: 1.0 HF = 0.9148 mm vs. 1.0 LF= 0.8539 mm, 0.50 HF= 0.9692 mm vs. 0.50 LF= 0.7843 mm) (Figure 3). Clutch order also affected the length of the neonates (one-way ANOVA, $F_{5, 184} = 4.541$, $p < 0.001$, Table 4). Overall, the neonates born in the first clutch for all treatments were significantly smaller than neonates born in the 3rd, 4th and 5th clutches (post-hoc Tukey HSD, $p<0.05$, Figure 3).

Table 3: Two-way ANOVA for average length of neonates produced by experimental *Daphnia.* Factors: Calcium concentration (2.5, 1.0, 0.50 mg/L), and food levels (high and low).

Effect	**df**	**MS**	**F**	**p-value**
Ca concentration	2	0.240	37.930	<0.0001
Food level	1	0.140	22.098	<0.0001
Ca conc. * Food level	2	0.210	33.170	<0.0001
Error	184			
Total	190			

Table 4: One-way ANOVA for average length of neonates by clutch produced by experimental *Daphnia.* Factor: Clutch order.

Effect	**df**	**MS**	**F**	**p- value**
Clutch Order	5	0.045	4.541	<0.001
Error	184	0.01		
Total	190			

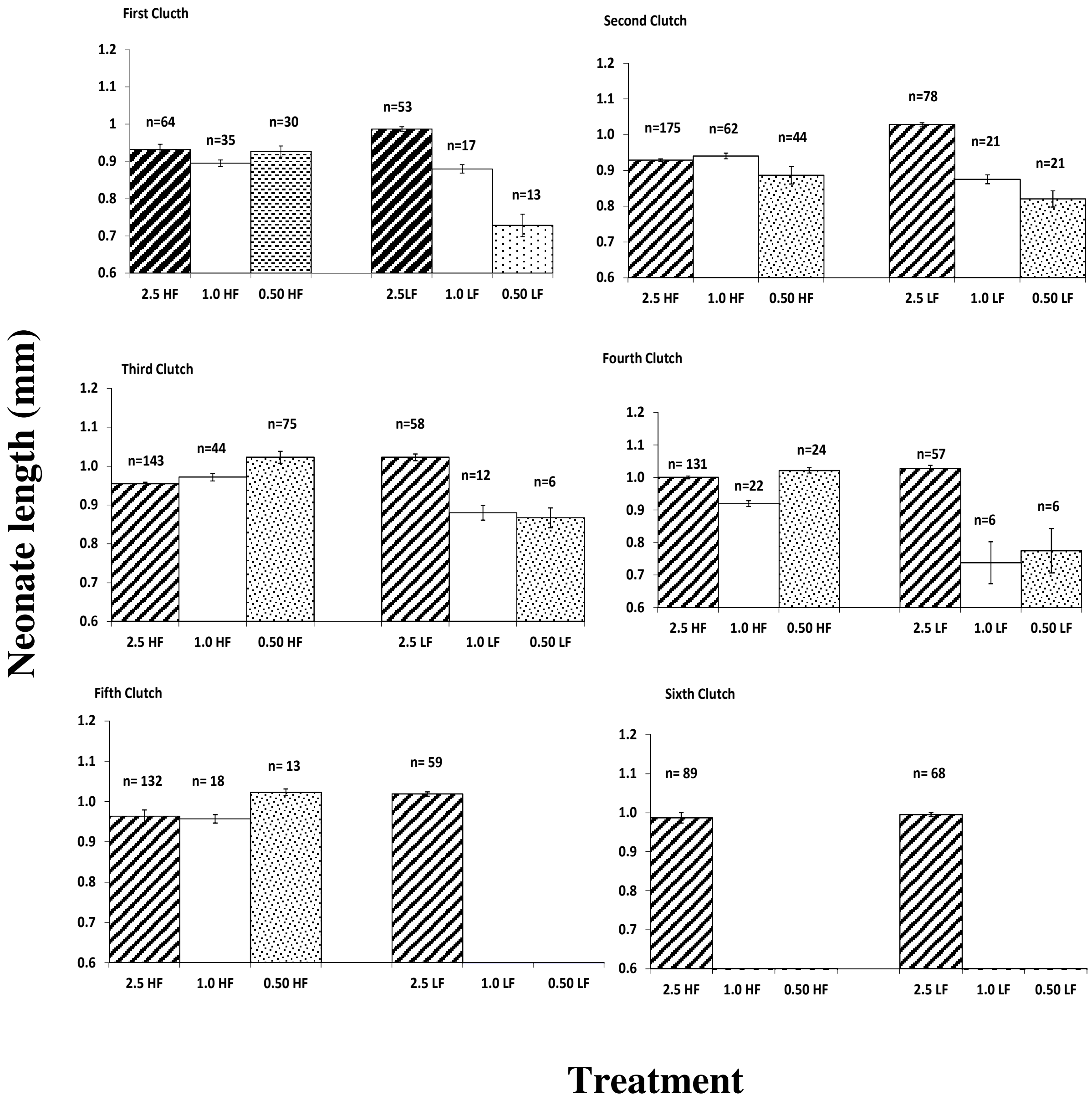

Figure 3: Length of neonates (mean± SE) by clutch order. “n” denotes number of animals measured per clutch. For treatments’ notation see Figure 1.

Survivorship in Experimental *Daphnia*:

The length of the life (number of days alive) of the experimental *Daphnia* was measured as the number of days they were alive during the study period. How long they lived was significantly affected by calcium concentration (Kruskal-Wallis, p=0.02, Figure 4) and food level (Mann-Whitney, p=0.002, Figure 4). *Daphnia* reared in higher calcium treatments (2.5 mg/L Ca) lived significantly longer than those reared in lower calcium treatments (). Interestingly, animals in low food treatments overall had significantly higher survival rates (total mortality of seven individuals) than those in high food treatments (total mortality of 18 individuals). These results were largely driven by a higher survivorship in the 0.5 mg/L Ca low food (total mortality of one individual) over the high food treatment (total mortality of eight individuals) (post-hoc Mann-Whitney, p=0.001).

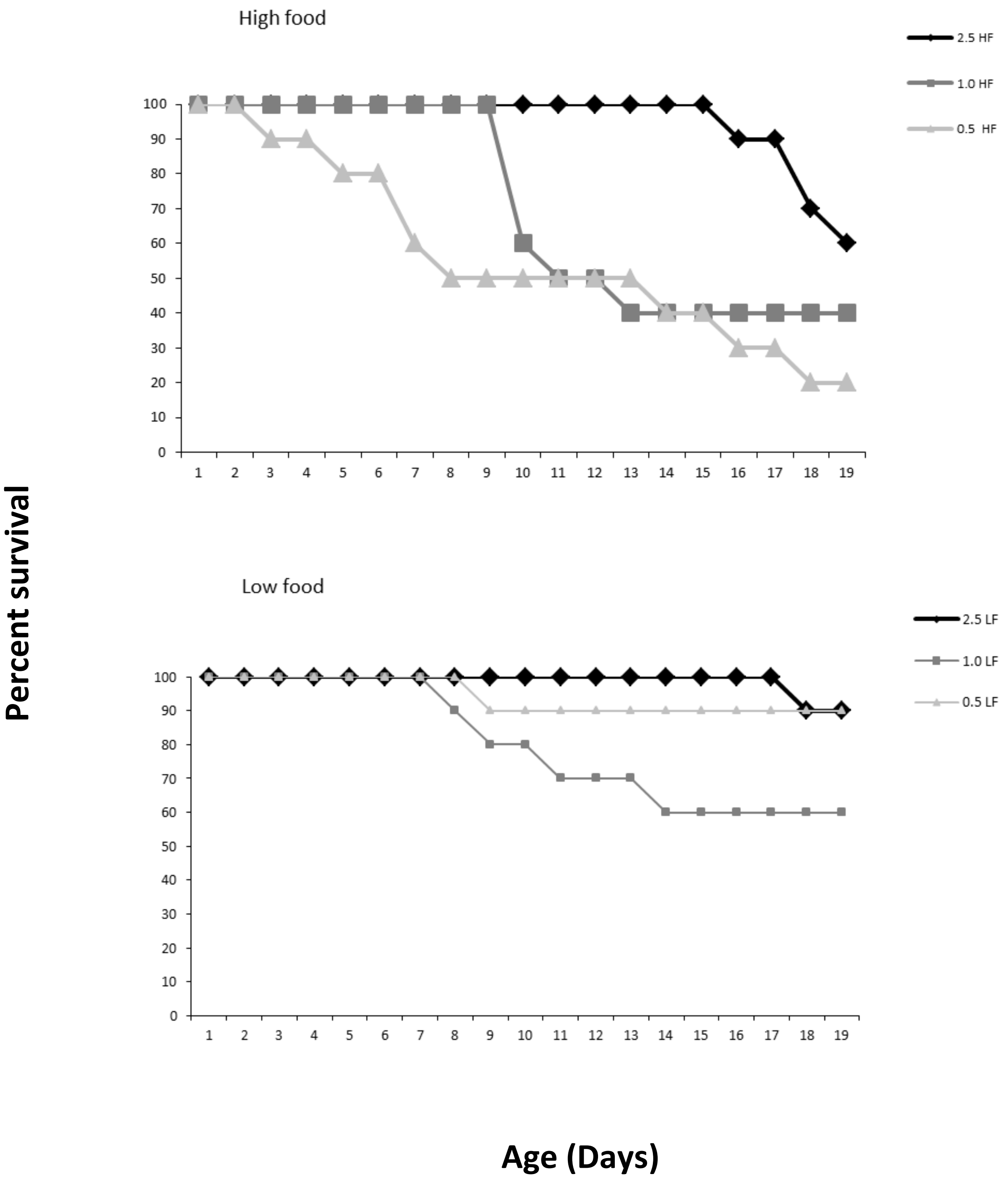

Figure 4: Percent survivorship of experimental *Daphnia* in high food and low food treatments at different calcium concentrations. For treatment notations see Figure 1.

Final Body Length of Experimental *Daphnia*

The experimental *Daphnia* body lengths (data collected on individuals that survived the duration of the experiment only) were significantly affected by calcium concentrations (two-way ANOVA, Table 5, Figure 5) and food levels (two-way ANOVA, Table 5, Figure 5). *Daphnia* reared in higher calcium media had on average larger bodies than those reared in lower calcium treatments (pairwise comparisons $p<0.05$, Figure 5). *Daphnia* reared in high food treatments had significantly larger bodies than those reared in low food treatments (pairwise comparisons $p<0.05$). At low food levels, however, calcium did not significantly increase adult body length (Figure 5).

Table 5: Two-way ANOVA for experimental *Daphnia* body length. Factors: calcium concentration (2.5, 1.0, 0.5 mg/L) and food levels (high and low).

Effect	**df**	**MS**	**F**	**p- value**
Ca concentration	2	0.091	12.17	<0.0001
Food level	1	0.032	4.326	0.043
Ca conc. * Food level	2	0.003	0.398	0.674
Error	48	0.007		
Total	54			

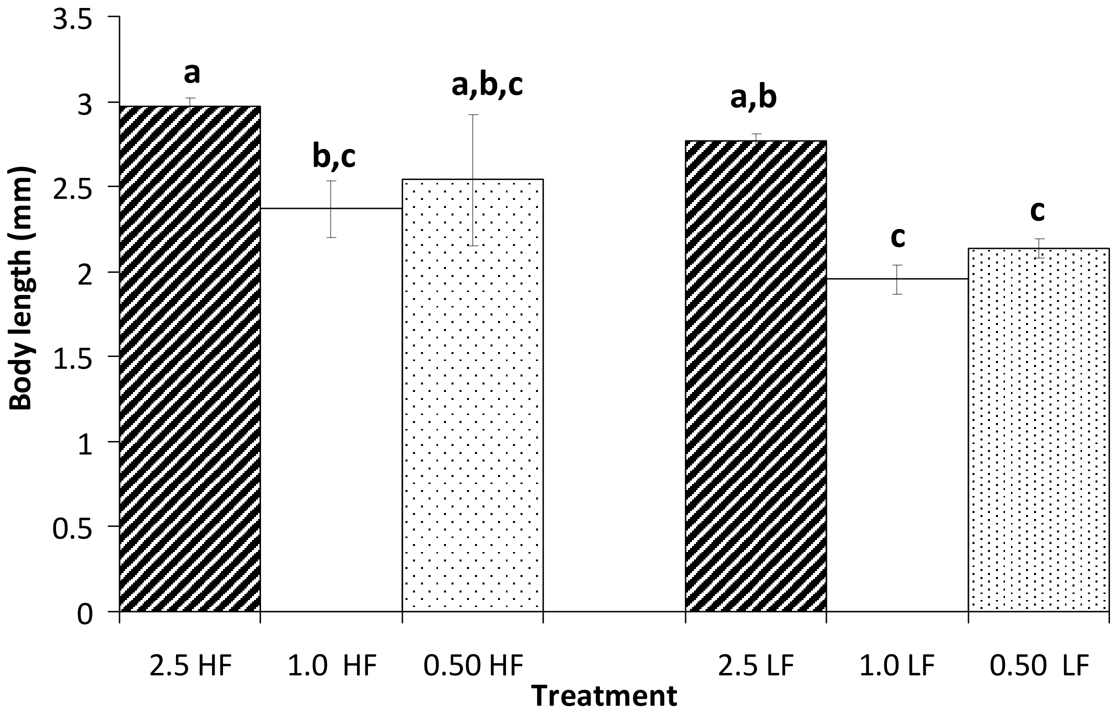

Figure 5: Body length of experimental *Daphnia* (mean ± SE). Different letters indicate significant differences between treatments (post-hoc Tukey HSD, $p<0.05$). For treatment notations see Figure 1.

Total Number of Instars in Experimental *Daphnia*

The physiological process of molting and thus number of instars was significantly affected by calcium concentration (two-way ANOVA, Table 6) and food level (two-way ANOVA, Table 6) and an interaction effect was present between calcium concentrations and food level (two-way ANOVA, Table 6). *Daphnia* exposed to 2.5 mg/L calcium underwent significantly fewer instars than *Daphnia* in the other two calcium treatments (post-hoc Tukey HSD, $p<0.05$, Figure 6). *Daphnia* in treatments with high food levels also underwent significantly fewer instars than those in treatments with low food levels (pairwise comparison LSD, $p=0.001$, Figure 6). The number of instars that the experimental animals underwent before their first reproduction was not significantly affected by calcium concentrations (2.5 HF=3.5, 2.5 LF=3.8, 1.0 HF= 4.5, 1.0 LF= 6.8, 0.5 HF= 4.1, 0.5 LF= 7.1) (Kruskal-Wallis, $p>0.05$) or food levels (Mann-Whitney, $p>0.05$). Therefore, before reproduction, *Daphnia* underwent a similar number of instars in all treatments.

Table 6: Two-way ANOVA for total number of molts produced by the experimental *Daphnia.* Factors: calcium concentration (2.5, 1.0, 0.50 mg/L) and food levels (high and low).

Effect	**df**	**MS**	**F**	**p- value**
Ca concentration	2	17.117	4.275	0.019
Food level	1	45.067	11.256	0.001
Ca conc. * Food level	2	12.717	3.176	0.05
Error	54	4.004		
Total	60			

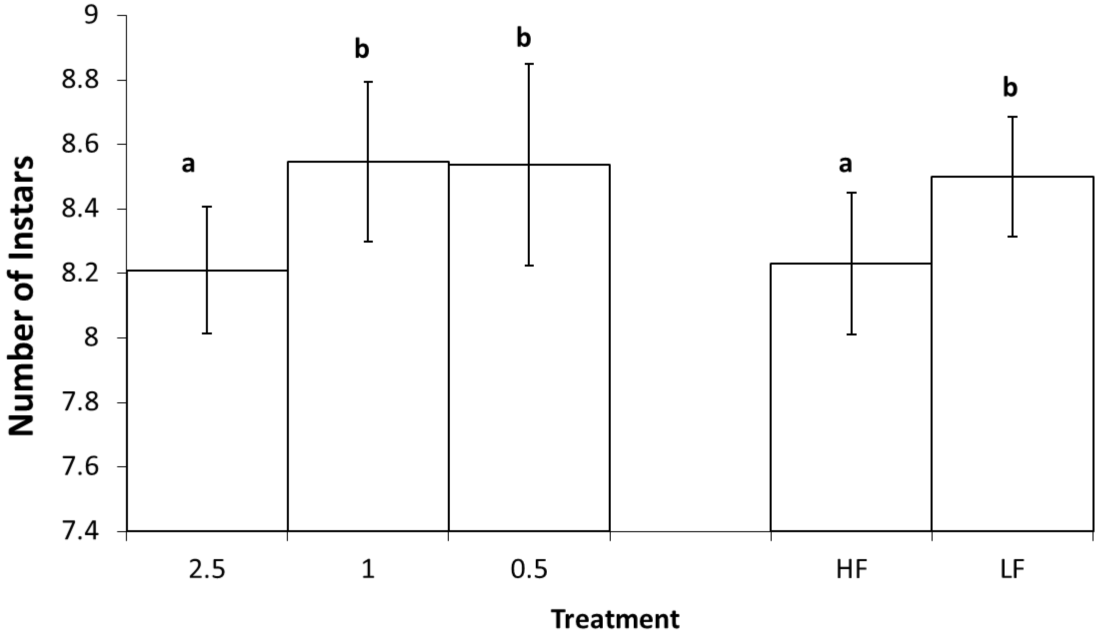

Figure 6: Number of instars by *Daphnia* (mean ± SE). Different letters indicate significant differences between treatments.

Time Until First Reproduction in Experimental *Daphnia*

The time elapsed until first reproduction in *Daphnia* was significantly affected by calcium concentration (Kruskal-Wallis, $p=0.056$, $\alpha=0.10$, Figure 7) but not by food levels (Mann-Whitney $p>0.1$). Those *Daphnia* reared in lower calcium concentrations of 1.0 and 0.50 mg/L Ca experienced delays in maturity and thus the onset of first reproduction occurred later than in those *Daphnia* reared in the 2.5 mg/L Ca treatments (post-hoc Mann-Whitney, $p<0.0167$). However, the time until first reproduction at the lowest calcium concentration of 0.50 mg/L Ca was not significantly different from the time it took at the two higher calcium concentrations (Figure 7). This finding is a reflection of the short time that the *Daphnia* in the 0.5 mg/L Ca high food treatment took until first reproduction (mean=7.7±0.4 days) compared to the 2.5 mg/L Ca treatments (mean=7±0 days). The treatment of 2.5 mg/L Ca had a total of 20 individuals reproducing at day 7, while the treatment of 0.50 mg/L Ca had only 4 individuals who reproduced at day 7 out of 16 that reproduced at all.

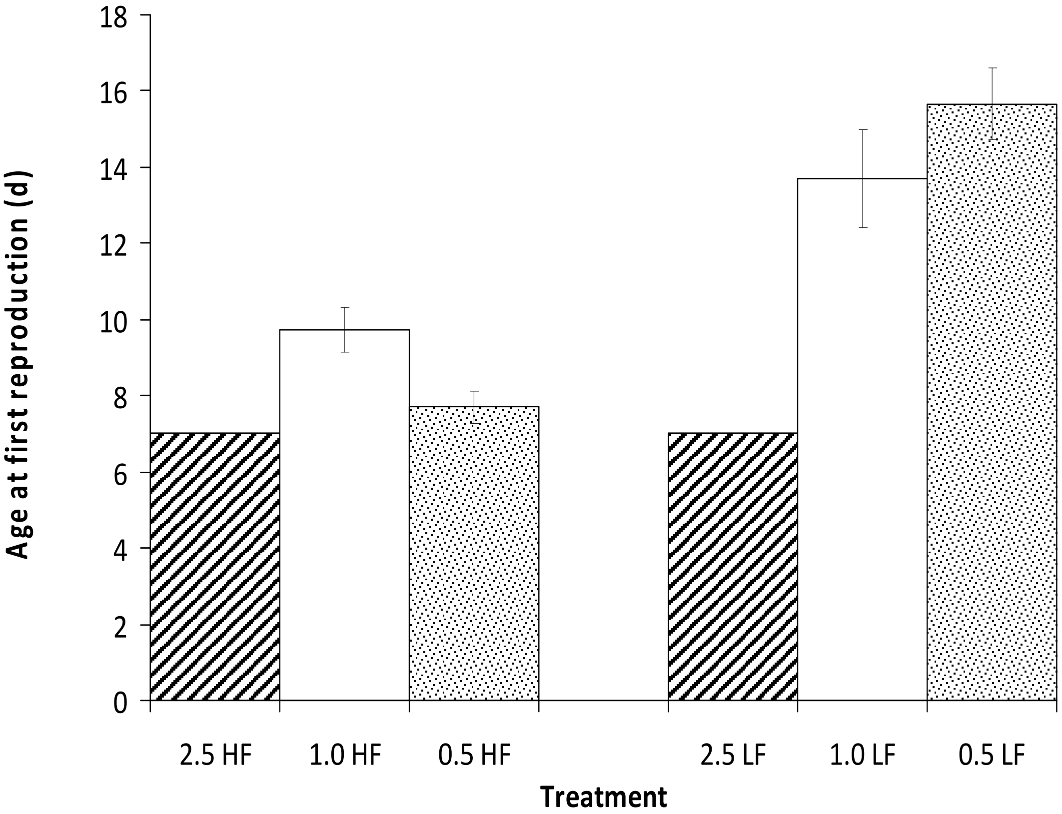

Figure 7: Number of days until first reproduction (mean ± S.E) Error bars, are not present in both of the 2.5 mg/L Ca treatments because all individuals reproduced at an age of 7 days.

Lipid and Ovary Index for Adult *Daphnia*

The lipid and ovary indexes were recorded only for those *Daphnia* that survived to the end of the experiment; therefore, the analyses were performed only on surviving animals. The lipid and ovary indexes for *Daphnia* were significantly affected by food levels (Mann-Whitney, lipid p =0.001, ovary p <0.0001, Figure 8). However, no significant effects from calcium concentration were present (Mann-Whitney, p>0.05). Even at low levels of calcium, if sufficient food was present, the *Daphnia* were able to accumulate lipid droplets and allocate resources to ovaries (Figure 8). The two most extreme treatments in this experiment (2.5 Ca HF and 0.5 Ca LF) show opposing strategies in *Daphnia*. On the one hand, at high calcium and food, *Daphnia* allocated a lot of their energy to reproduction. However, in the opposite scenario, at low calcium and food, *Daphnia* put a lot of their energy into accumulating lipids and many more individuals survived to the end of the experiment than in other intermediate treatments.

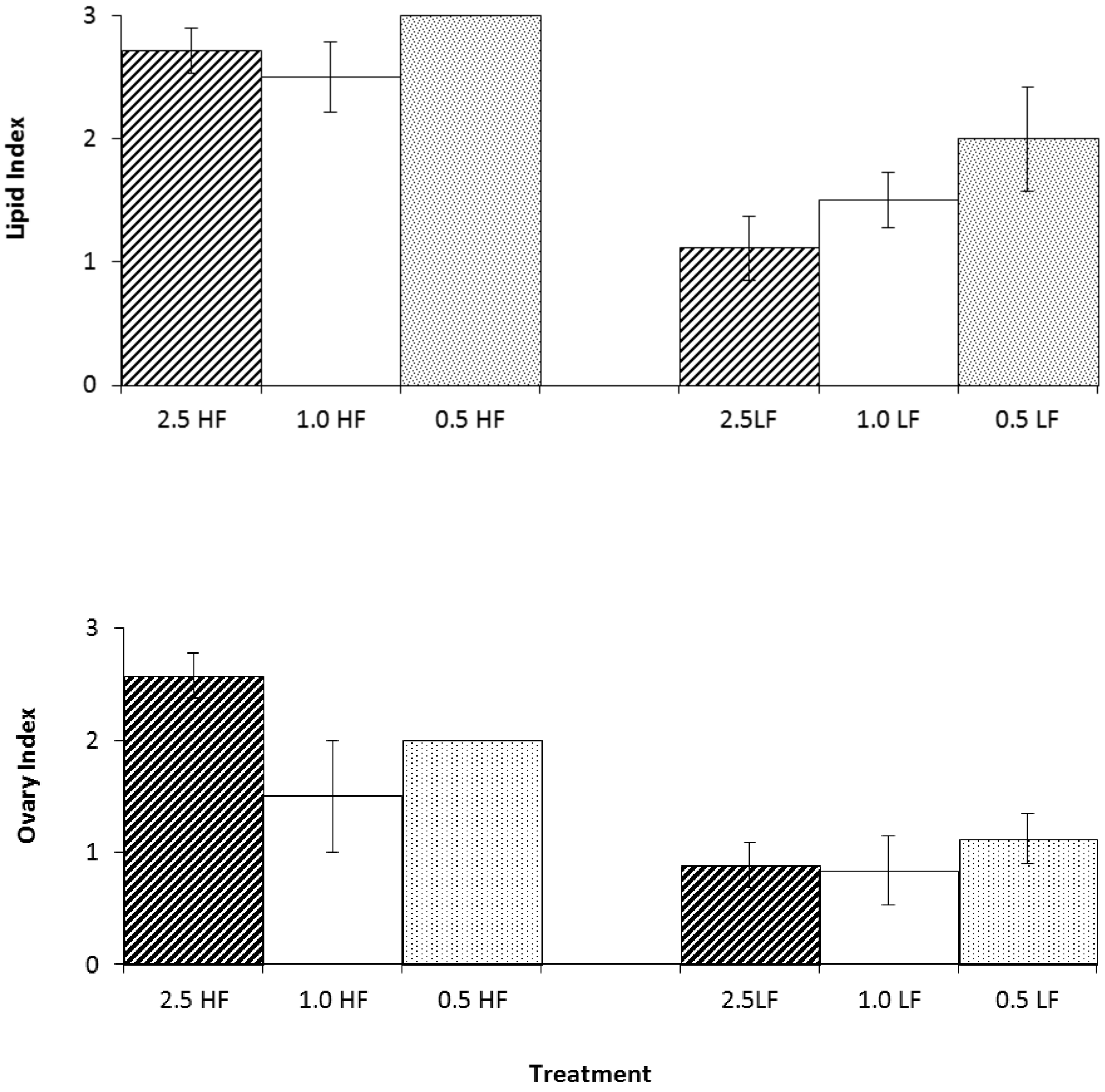

Figure 8: Lipid and ovary rank by individual *Daphnia* for the different treatments. A score of 0 indicates very poor resources; scores from 1-3 indicate increasing resources and ovary condition. Only individuals that survived to the end of the experiment are depicted in these graphs. Error bars are not represented in the 0.5 HF treatment because data was taken from only one individual.

Total Number of Clutches Produced by Experimental *Daphnia*

The total number of clutches produced by *Daphnia* per treatment was significantly affected by calcium concentration (Table 7) but not by food levels. *Daphnia* reared in calcium concentrations of 2.5 mg/L produced significantly more clutches (mean = 6 clutches) than the other two calcium concentrations (1.0 mg/L Ca mean= 4.5 clutches; 0.50 mg/L Ca mean= 4.5 clutches) (post-hoc Tukey HSD, p< 0.0001).

Table 7: Two-way ANOVA for number of clutches produced by *Daphnia*. Factors: calcium concentration (2.5, 1.0, 0.50 mg/L) and food levels (high and low).

Effect	**df**	**MS**	**F**	**p- value**
Ca concentration	2	101.6	43.716	<0.0001
Food level	1	2.017	0.868	0.356
Ca conc.* Food level	2	0.267	0.115	0.892
Error	54	2.324		
Total	60			

Number of Neonates Produced by Clutch Order

The number of neonates produced in the first three clutches was significantly affected by calcium and food levels; however, after the third clutch the number of neonates produced was affected mostly by food level only. In the first clutch, the number of neonates was significantly affected by calcium concentration (two-way ANOVA, $F_{2, 44}$ =7.115, p= 0.002, Figure 9) and food level (two-way ANOVA, $F_{41, 47}$ =5.635, p= 0.022, Figure 9), but there were no interaction effects of calcium and food levels. The number of neonates in the second clutch was significantly affected by calcium concentrations (Kruskal- Wallis, p=0.003, Figure 9) and food level (Mann-Whitney, p< 0.0001, Figure 9), but there were no interaction effects. In the third clutch, the number of neonates produced was also significantly affected by calcium concentration (Kruskal- Wallis, p=0.030, Figure 9) and food level (Kruskal- Wallis, p<0.0001, Figure, 9). After the third clutch, high food treatments produced significantly more neonates than low food treatments: fourth clutch (two-way ANOVA, $F_{5,23}$ =72.697, p< 0.001, Figure 9), fifth clutch (two-way ANOVA, $F_{1,22}$ =36.847, p< 0.0001, Figure 9) and sixth clutch (Mann-Whitney, p=0.007, Figure 9).

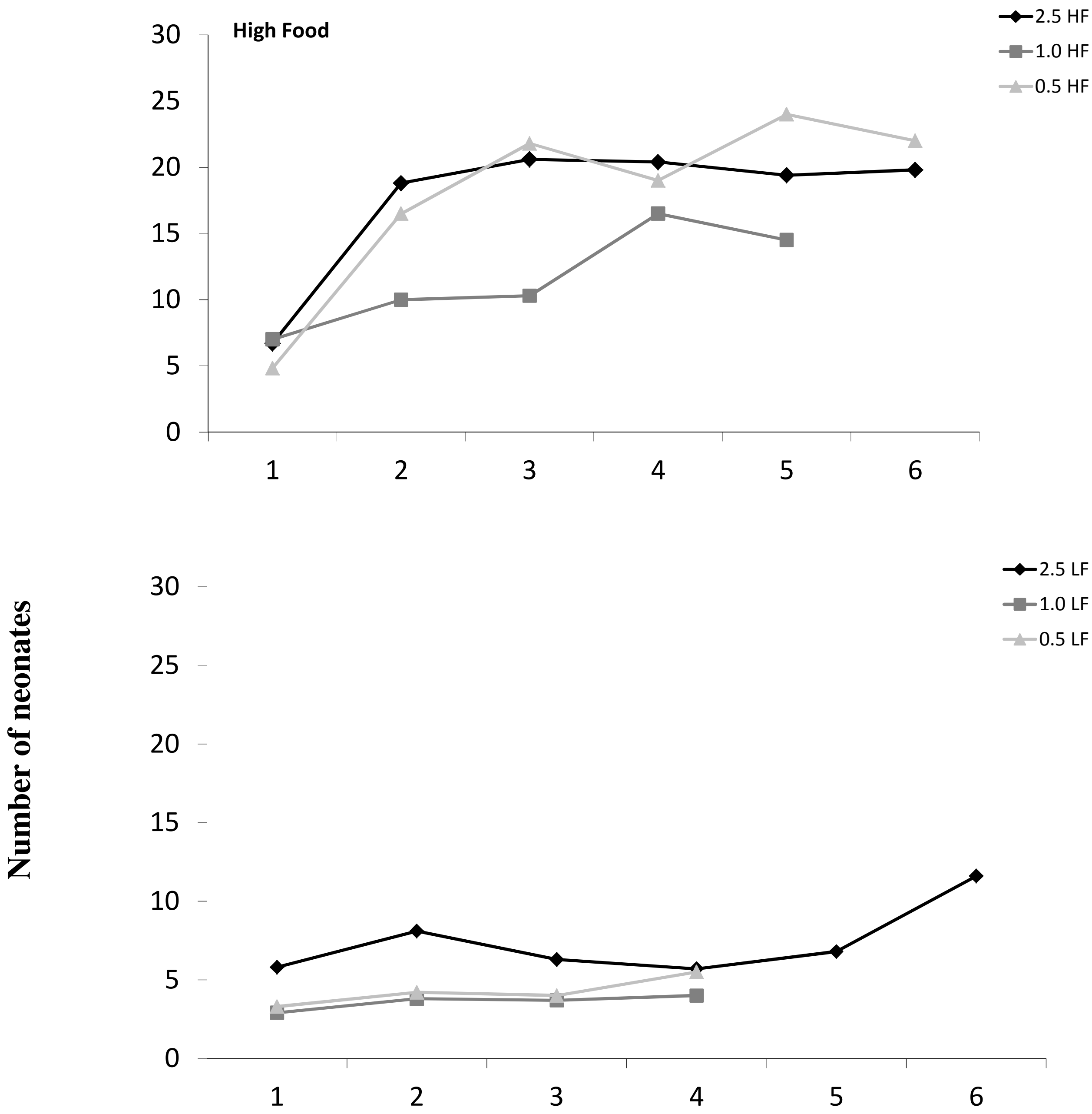

Figure 9: Number of neonate xperimental *Daphnia* per treatment (mean ± SE).

Chapter 4

Discussion

Physiological processes in *Daphnia* like molting, reproduction and allocation of resources are dependent on a variety of factors including lake water calcium concentration, food quantity and quality, presence of toxins such as heavy metals and many more. My experiment was designed to study the effects of decreasing calcium and food levels on the growth and reproduction of *Daphnia,* and to determine if these two selected factors had any interacting effects. Results indicate that as calcium declines, *Daphnia*'s growth and reproduction are altered and physiological stress is carried on to future generations as well. However, my findings support the idea that for some physiological processes, the effects of low calcium can be compensated for if adequate food is present.

The Intrinsic Rate of Population Growth

Reproduction can be measured as how much each individual *Daphnia* is contributing to the growth of the overall population. My results show that calcium and food significantly affected the r value for each treatment and that food and calcium also marginally interacted, affecting the rate of population growth. As calcium is reduced from 2.5 to 1.0 to 0.50 mg/L in the high food treatments, r differs from 0.48 to 0.28 and 0.34. In spite of the interaction between calcium and food within the high food treatments, in the low food treatments the trend of decreasing r values from 2.5 to 1.0 mg/L Ca and then leveling off between 1.0 and 0.5 mg/L Ca is similar; the respective values of r for the calcium levels of 2.5, 1.0, 0.50 mg/L are 0.39, 0.13 and 0.13. The interaction effect is marginal due to the fact that individuals in the 0.5 mg/L Ca HF treatment had a high r value, however notsignificantly higher than 1.0 mg/L Ca HF.As calcium was reduced in the treatments, if high food levels were present the effect on reproduction would not be as large as when the food was low. Similarly, Ashforth and Yan (2008) found that in *D. pulex,* food had the strongest effects on *r*, but as calcium levels decreased below 1.5 mg/L calcium the effects on reproduction were greater.

In terms of reproduction endpoints, my results indicate that as calcium concentration decreases, overall reproduction is negatively affected. If, however, high food levels are present, it can mask these effects or compensate for low calcium levels. Enserik et al. (1990) found that under the same low food conditions, *D. magna* of different clones had opposite responses. Some individuals reproduced early and at a small body size, while other individuals reabsorbed ovaries and gave priority to maintenance and growth, indicating that there may not be a size threshold for reproduction but a resource threshold. Also if the theory of a resource threshold is true, then clones under various environmental stressors could show differences in life history and reproductive strategies. Under stressful conditions, a variety of phenotypes may be expressed in order to maximize fitness. Tessier and Consolatti (1990) also found that different daphniid species responded differently to environmental stressors. Food levels in *D. pulex* and *D. pulicaria* caused very different responses between the two; under low food levels *D. pulex* produced neonates with smaller mass and *D. pulicaria* produced neonates with a greater mass. These differences were also observed by Glazier (1992).

In my experiments, the low food treatments seemed to have a more predictable response in reproduction, whereas those *Daphnia* exposed to high food had a more varied response. For instance, those individuals reared at high food and the lowest calcium treatment of 0.5 mg/L had a higher *r* value than those reared at 1.0 mg/L Ca and high food. In most cases, the treatment of 0.5 mg/L Ca with high food contained *Daphnia* that produced more neonates and had a higher *r* value than the *Daphnia* reared at 1.0 mg/L Ca and high food (although not statistically different, Fig. 1), which was unexpected. Enserink et al. (1995) proposed that there may be a trade-off at low food between growth and reproduction. Although an individual may meet a minimum size to allocate energy to ovary provisioning, the energy needed to develop eggs or release a mature clutch may be too great and the individual will be better off delaying reproduction.

Reproduction

Daphnia suffer a reproductive disadvantage at low levels of calcium and low levels of food, causing them to produce fewer neonates (Ashforth and Yan, 2008;Gliwicz and Guisande, 1992). I found in my experiments that when calcium was reduced to 1.0 mg/L and below, *Daphnia* had a reduced reproductive output. Results similar to these have been found in other studies of daphniids, where starvation and food shortage caused *Daphnia* to produce fewer and smaller neonates (Glazier, 1992; Gliwicz and Guisande, 1992; Tessier and Consolatti, 1991). Mechanisms for dealing with the stress of food scarcity can be to delay maturation (thus delaying the onset of reproduction), to produce fewer neonates or to produce a lower number of clutches (Ashforth and Yan, 2008).

Previous studies support the hypothesis that there is a size threshold for maturity among *Daphnia* species (Ebert, 1992; Gliwicz and Lampert, 1990). Calcium is an important factor for growth and thus, length of and individual. If an individual does not achieve a certain minimum length, the energy needed to produce a clutch may be too great and reproduction cannot begin (Ebert, 1992). For the first three clutches produced in my experiments, the larger *Daphnia* (those exposed to high calcium and high food levels) produced more neonates per clutch because more resources were available to grow larger and to initiate reproduction. After the *Daphnia* produced the third clutch, the individuals in the lower calcium treatments most likely had enough time to reach a minimum body length to support reproduction of optimal clutch sizes (about 20 neonates), but at this time food became the factor that would significantly affect the number of neonates produced per clutch. *Daphnia* reared at high food levels can produce clutches that have more neonates, while those individuals reared at low food levels will produce on average fewer neonates per clutch.

The lipid index ranks show how much resources an individual has stored within the body, while the ovary index shows how resources have been allocated to reproduction The lipid and ovary indices were significantly affected by food level; even individuals that were reared at low levels of calcium could attain healthy lipid and ovary indexes if high food levels were present. Individuals reared at 0.50 mg/L Ca with high food levels were able to sustain a high lipid index; the ovary index was intermediate, although these data only came from one surviving individual in the treatment. Individuals that were exposed to high food levels were able to produce larger neonates, more neonates and more clutches on average because they had more resources stored from abundant food, which allowed them to allocate more resources to reproduction.

My experimental observation that fewer neonates are produced by individuals exposed to low food levels was also found by Glazier (1992) in an experiment where food shortage caused *D. magna* and *D. pulicaria* to produce fewer and smaller eggs per clutch than those produced by *Daphnia* not exposed to food shortages (Glazier, 1992). In natural settings, *Daphnia* are often exposed to periods of very low food concentrations, which are possibly lower than those of laboratory settings, because in the lakes there is a mix of algal species and not all species are equally edible. Because the maximum number of eggs produced per individual is highly correlated with food quality and quantity (Lampert, 1978), individuals reared at low food levels should produce fewer neonates than those exposed to high food levels. This response to low food becomes more pronounced as calcium decreases as well. Generally, I found that under low calcium conditions, the experimental *Daphnia* produced fewer neonates than under higher calcium, and when reared in low food treatments as well, they produced even fewer neonates.

Lipid and ovary indices also show that resources at low food levels are not sufficient to sustain high levels of reproduction even when calcium levels are high. Individuals exposed to high food have sufficient resources to form healthy ovaries, even when calcium levels are low. The size of an animal may also affect the lipid and ovary indices, since larger animals have a lower mass-specific metabolic rate and they may have more energy available for storage and, therefore, have an advantage in food shortage situations. My results of very high lipid indices under 0.50 mg/L Ca and low food may be because these individuals on average had very small body sizes, did not allocate resources to reproduction and therefore had excess nutrients for storage. Some studies show that smaller individuals may have increased assimilation efficiency and therefore these individuals can assimilate food very efficiently causing them to be able to store excess lipids; however, they never allocate this energy to ovary provisioning (Urabe and

Watanabe, 1991). Under low calcium concentrations, building functional carapaces for large individuals or offspring is not supported, so survival would be the only advantage to maintaining a small body size mechanism (Urabe and Watanabe, 1991).

Additionally, my results show that calcium affects the total number of clutches a *Daphnia* can produce during the experimental period of 19 days. As the stress of low calcium grows with each molt, it becomes harder for a *Daphnia* to produce the next clutch. For instance, *Daphnia* reared at 2.5 mg/L of Ca produced six clutches in both high and low food media; *Daphnia* reared at 1.0 mg/L and 0.50 mg/L Ca produced five clutches when exposed to high food and four clutches when exposed to low food. Thus, when sufficient calcium was present, *Daphnia* grew larger and allocated sufficient resources to reproduction. However, those *Daphnia* reared at lower calcium concentrations generally produced the first brood later than those individuals exposed to higher levels of calcium and overall produced fewer clutches because of a reproductive delay. Similar results were observed by Ashforth and Yan (2008), where *D. pulex* had delayed reproduction at calcium levels below 1.5 mg/L as a result of slower growth rates at decreasing calcium levels. Other studies have also found delayed reproduction in *Daphnia* because they did not reach the size threshold to reproduce (Ebert, 1992; Urabe and Watanabe, 1991).

Delaying the onset of first reproduction is a compensatory mechanism for those individuals reared at low calcium and low food. Their growth rates are slow and, although they may have started to produce eggs, do not have sufficient energy for the full development of the eggs or to release them. Therefore, the eggs can be reabsorbed to conserve resources and energy (Ebert, 1992, McCauley et al., 1990). If ovary development has not started yet, *Daphnia* under stressful conditions can increase their number of juvenile instars in order to attain a larger body size that can support proper reproduction (Ebert, 1992; Gliwicz and Lampert, 1990; McCauley et al., 1990; Tessier et al., 1983).

Number of instars, Body Length and Survival

As an individual *Daphnia* ages and grows, molting is an essential process. Calcium is needed to build a strong and larger carapace. My results show that as calcium decreased, the experimental *Daphnia* increased the frequency of molting; similarly, as food level decreased, the *Daphnia* molted more often. Indicating that these Daphnia underwent more instars, undergoing more juvenile instars takes less energy than undergoing adult instars, allowing these individuals to conserve energy and live longer, however reproduction is delayed. However, at low levels of calcium with high food levels, the *Daphnia* molted less frequently than their counterparts exposed to low calcium and low food, the extra energy resources they receive from high food levels allows these individuals more ATP to actively uptake the calcium ions in the water and thus they molt less often. *Daphnia* exposed to low levels of calcium cannot actively uptake the needed calcium to build a proper carapace and to overcome this stress the individual will molt more frequently, possibly because wear of the carapace may be more extensive in the less calcified molts, or perhaps because they are trying to reach a certain body size. The result of more frequent molting in my experiment contrasts to the results found by Hessen and Rukke (2000) and Lynch (1989), whose experiments on crustacean zooplankton showed that the frequency of molting was independent of calcium concentration and food level.

However, one can argue that at low concentrations of calcium and low food levels, growth is slow. In order to gain a minimum body size to support reproduction, *Daphnia* may allocate more energy to maintenance and growth and accumulate reserves of energy to support future reproduction, thus molt more to attain larger body size and replace damaged carapaces. Normally, juvenile *Daphnia* molt daily at 20 ° C and as they mature the molting frequency can level off to about every other day (Hessen and Rukke, 2000). Those *Daphnia* exposed to low calcium and low food, whose growth rates were slowed down, maintained a molting frequency similar to juveniles and hence molted more often than those who had sufficient calcium and food resources.

Body length of adult *Daphnia* was affected by calcium and food. As calcium decreased, the average body length was smaller; in addition, *Daphnia* reared in low food treatments had on average smaller bodies as well. Calcium is required to rebuild the carapace after molting and each subsequent molt allows for growth in body size. Thus, as calcium decreases, less calcium is available to build a larger body, but when food levels are high; food can compensate for lost calcium and provide additional resources to actively uptake calcium and build a larger body. Although calcium is obtained from the surrounding medium, additional food resources provide additional energy and ATP to actively transport more calcium ions into the body than at low food levels (Neufeld and Cameron, 1993). The cost of molting increases with increasing body size, and with each molt there is a constant drain of needed resources (Hessen and Rukke 2000; Lynch et al 1989). The larger an individual *Daphnia* becomes, the more energy is needed to complete a molt and to regain essential minerals. *Daphnia* reared at calcium levels below 1.0 mg/L had significantly smaller bodies than those reared at 2.5 mg/L Ca, at both high and low food levels. As calcium decreases, sufficient resources to build a functional carapace are lacking and the energy needed to build a larger body size becomes too great for the already stressed animal. Therefore, individuals exposed to low levels of calcium can only sustain a smaller body size. My results are consistent with Glazier and Calow (1992) where *D. pulex* reared at low food levels allocated more energy to maintenance than growth or reproduction. Larger individuals consume significantly more food, therefore, gaining more resources as well, to be allocated to growth (Urabe and Watanabe, 1991). Overall, under the constraints of low calcium, *Daphnia* do not have sufficient calcium to build large carapaces, and when individuals are also reared under low food conditions excess resources are not present to increase body length.

Many studies have focused on the resources that *Daphnia* allocate to future generations under stressful conditions, since it is important to know how future generations will be affected and how overall populations will respond. My results show that as calcium concentrations decrease, *Daphnia* produce neonates that have significantly smaller bodies. However, when calcium levels are low but high food is present, the neonates born under these conditions will have significantly longer bodies than those neonates whose mothers were exposed to low food levels. But results vary when looking at the size of neonates whose mothers were exposed to different stressors. For example, Glazier (1992) found that food shortages caused *D. magna* and *D. pulicaria* to produce smaller neonates, while *D. pulex* produced larger neonates. Variation in the size of the neonates born illustrates that each species and maybe even individuals, respond differently depending on the tradeoffs made. Adult *Daphnia* are capable of assessing resource availability and can, therefore, allocate resources to neonates differently to maximize their survival (Gliwicz and Guisande, 1992). At low food levels, *Daphnia* may produce fewer but larger neonates that will be more resistant to starvation because of the abundant lipids they received from maternal reserves. In contrast, my results show that *Daphnia* reared under low food levels produced fewer neonates and these neonates were on average smaller than those born to *Daphnia* under high food conditions. Most likely the *Daphnia* in media with low food concentrations allocated fewer resources to their neonates, resulting in smaller body sizes. Interestingly, some of the clutches produced in the 0.50 mg/L Ca treatment had somewhat larger neonates or neonates the same size as those produced in the 1.0 mg/L treatment, for both high food and low food treatments, indicating that *Daphnia* reared under 0.50 mg/L Ca and high food may be allocating more resources to their neonates to maximize survival (Tessier and Goulden, 1982; Gliwicz and Guisande, 1992). On one hand, *Daphnia* may allocate resources to grow large neonates that will be more resistant to starvation, in which case they may suffer more from decreased calcium concentrations. Or, alternatively, they may not have enough resources to grow larger and, thus, the neonates they produce have small body sizes as well. However, these smaller neonates may benefit from not having to obtain as much calcium for their smaller carapaces. This strategy could be seen as a positive response for a population that has to deal with low calcium levels.

Clutch order also affected the length of the neonates. Those neonates who were born in the first clutch had significantly smaller bodies than those born in later clutches; neonate length was also more variable in the first clutch, which is consistent with McCauley et al. (1990). *Daphnia* need calcium not only to build their bodies but to build the bodies of the neonates they produce; however, *Daphnia* may still be using resources to grow a larger body themselves and may not have enough stored resources to produce large neonates. As more and more clutches are produced, the variation in size of neonates decreases (Ebert, 1993). However, as this happens, the adult *Daphnia* will accumulate resources to continually produce larger neonates, and as these reproducing *Daphnia* continue to grow, the energy strain will become greater, resulting in a more constant neonate size. In the case of *Daphnia* raised at lower levels of calcium, the neonates they produce are even smaller because resources have been restricted throughout their lifetime and they cannot be stored for future generations.

Daphnia reared in higher calcium treatments lived longer on average than those *Daphnia* reared in the lower calcium treatments. After 19 days, *Daphnia* exposed to high food levels and 2.5 mg/L Ca had a 60% survival rate, while those reared at 1.0 mg/L calcium had a survival rate of 40% and those reared at 0.50 mg/L Ca had a survival rate of 20%. Also, the low food level treatments had on average a higher survival rate. The higher survival rates in the 0.50 mg/L Ca compared to 1.0 mg/L Ca in low food treatments may be because these individuals allocated more resources to maintenance than growth and reproduction. As the age at maturity increases, the risk of death before reproduction decreases because more resources are allocated to maintenance (Ebert, 1992); although this strategy decreases overall reproduction, it also increases overall survival, which is seen in my experiment. With fewer resources being allocated to growing larger and producing neonates, these individuals had more resources for survival and maintenance. Lipid reserves are the lowest after egg production because the triglycerides that form lipid droplets move through the body during egg development and combine with proteins to form the opaque ovaries, thus, if resources are not allocated to ovary provisioning more resources are available for maintenance survival (Tessier and Goulden, 1982). Ashforth and Yan (2008) showed somewhat similar results in their experiment with *D. pulex*. They found that this species of daphniid requires a minimum calcium concentration of 0.1-0.50 mg/L to survive a 15-day experiment. They also found that the metabolic demands of acquiring calcium become too great at low food levels and the individuals exposed to these conditions cannot build a fully functional carapace (Ashforth and Yan, 2008). The tendency for the individuals in the low food treatments to be significantly smaller than their high food counterparts proved to be a positive response in terms of overall survival.

Conclusions

As calcium is currently approaching threshold levels in many soft-water lakes, the growth and reproduction of daphniid species that require calcium to build their carapace will be negatively affected. In addition, the naturally occurring periods of low or undesirable food quality will contribute further to these negative effects. Although a tradeoff between growth and reproduction exists under the stressful conditions of low calcium and varying food quantity, their effects on future *Daphnia* generations is not thoroughly understood. While the adult *Daphnia* in my experiments reproduced in all treatments, I did not test the development of the neonates to determine whether their survival or reproduction was affected by these stressors. In the low calcium and low food treatments, the very low reproductive output of these individuals would most likely result in reduced increases population size. If *Daphnia* are too stressed to allocate essential resources to their neonates, future generations may not have the necessary reserves to survive and reproduce, possibly resulting in the extirpation of these species from the affected lakes.

Daphnia in nature are met with multiple stressors at one time, such as food availability, calcium concentrations, pH-related stresses and competition and predation, among others. Predation is perhaps one of the most imminent threats to survival in nature, and the strategies necessary to lessen predation, like increasing body size or building neck spines, can be assumed to suffer under low calcium conditions (Riessen et al., 2012). Body size was greatly affected in my experiments at low calcium concentrations, where growth rates slowed down and the animals were unable to molt; these effects will surely affect the development of predator defenses as well. Because *Daphnia* species make up the lower ranks of the trophic chain of aquatic food webs, a calcium decrease in lake water also lowers their bodily calcium content and this effect can cascade to their predators, which include vertebrates such as larval fish. In addition, the shed carapace of *Daphnia* also contribute to the phosphorous, carbon and calcium recycling in the lake, and the poor quality of the carapaces may have an impact in nutrient cycling in soft-water lakes.

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