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Shell Calcification and Growth of the Aquatic Snail Planorbella trivolvis Under Low Calcium Conditions Typical of Decalcified Northern Lakes

Shawn A. Witte
State University of New York College at Buffalo - Buffalo State College, shawnwitte72@gmail.com

Advisor
Alicia Pérez-Fuentetaja, Ph.D.
First Reader
Alicia Pérez-Fuentetaja, Ph.D.
Second Reader
Christopher Pennuto, Ph.D.
Third Reader
Randal Snyder, Ph.D.
Department Chair
Daniel L. Potts, Ph.D.

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An Abstract of a Thesis in Biology

Submitted in Partial Fulfillment

of the Requirements for the Degree of

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Abstract

The water calcium concentration of northern softwater lakes has declined due to numerous anthropogenic stressors. The current lake calcium concentrations have been deemed critically low for the survival of many aquatic organisms, and the availability of calcium is expected to decline further. To assess the impact that critically low water calcium has on aquatic snail shell calcification and growth, juvenile *Planorbella trivolvis* were raised for 60 days in one of four treatments, each containing a different amount of dissolved calcium (0.64, 1.3, 2.5, and 5.1 mg/L). Body mass, shell size, and shell calcium measurements were collected on a sample of snails halfway through and at the end of the experiment. There was no difference in tissue weight or shell size among treatments, suggesting low water calcium concentrations do not impact adult body mass or size. However, snails had reduced shell calcium when reared in water containing less than 2.5 mg/L calcium, with larger snails being more deficient. For snails in the highest calcium treatment (5.1 mg/L Ca), the amount of shell calcium relative to total dry weight increased during the experimental period; however, for snails in the two lowest calcium treatments (0.64 and 1.3 mg/L Ca), percent calcium decreased until day 30, after which it increased but to a lesser extent than snails in the two highest calcium treatments (2.5 and 5.1 mg/L Ca). Interestingly, smaller snails had shells with more calcium relative to total dry weight than larger snails and required less water calcium to biomineralize their shell. These results indicate that adult size and body mass are not affected by low water calcium, but shell calcification is negatively affected by declining water calcium concentration with the effects being greater for larger snails within a species. The implications of low water calcium in wild snail populations may be a balancing act between small, well-calcified snails with increased vulnerability to predation due to their small size and larger snails with decalcified shells but fewer potential predators.

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Approved by:

Alicia Pérez-Fuentetaja, Ph.D.
Professor of Biology
Chairperson of the Thesis Committee/Thesis Advisor

Daniel Potts, Ph.D.
Professor and Chair of Biology

Kevin J. Miller, Ed.D.
Dean of the Graduate School

THESIS COMMITTEE

Alicia Pérez-Fuentetaja, Ph.D.
Professor of Biology / Research Scientist
Thesis Advisor

Christopher Pennuto, Ph.D.
Professor of Biology / Research Scientist

Randal Snyder, Ph.D.
Professor of Biology

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Introduction

Background

Calcium (Ca) is an essential nutrient for life across both the animal and plant kingdoms. Its role in organisms ranges from structural reinforcement, including for the formation of exoskeletons and bony structures, to cellular signaling, including intracellular and enzymatic processes, muscle contractions, and neuronal activity (Ebashi & Endo, 1968; Greenaway, 1985; Jeziorski & Smol, 2017; Weyhenmeyer et al., 2019). For organisms to grow and survive, Ca must be acquired directly from the surrounding abiotic environment or through consumption of other Ca-containing organisms (Giardini et al., 2015; Weyhenmeyer et al., 2019). For freshwater fauna that obtain most of their required Ca from dissolved Ca in the water, Ca plays an additional role in driving community composition and structure. Many freshwater macroinvertebrates have critical Ca thresholds, and aqueous Ca concentrations below the threshold can result in a shift towards less Ca-dependent organisms or life history trade-offs to compensate for low ambient Ca (Korosi et al., 2012). Declining Ca concentrations in soft water lakes poses a novel threat to heavily calcified organisms, such as snails, that demand high levels of Ca for growth, reproduction, and survival.

Calcium Decline

The decline of Ca in soft water lakes is widespread and has emerged as a prominent environmental stressor due to past and present anthropogenic activities that influence Ca cycling. Industrialization in the 19th century led to large quantities of nitrogen and sulfur oxides being released, and these acidic gases accumulated in the atmosphere, soils, and waters (Samyn et al., 2012). The effects industrial emissions have on the acidity of precipitation and surface water

chemistry was recognized in the late 19th century (Smith, 1872), and legislation was enacted to mitigate lake water pH changes associated with acid deposition. Although there were widespread improvements in water quality from strict emission reduction programs, introduced H^+ ions displaced base cations (primarily Ca) and facilitated their export from the soil to surface waters, resulting in a depletion of Ca reservoirs in shallow catchments soils characteristic of soft water regions (Jeziorski & Smol, 2017). Soft water lakes on the Precambrian Shield in Canada and the United States, and throughout Scandinavia, overlay bedrock composed of granite or other felsic intrusive rocks. In these regions, Ca replenishment primarily comes from mineral weathering, but the catchment resists erosion and is therefore vulnerable to Ca deficiency (Giardini et al., 2015; Jeziorski & Smol, 2017). Although the consequences of Ca decline are site-specific and dependent on regional geology, modern day anthropogenic modifications to the catchment are causing Ca to decline further.

Timber harvesting and reductions in particulate deposition, such as dust from emissions, unpaved roads, and soil erosion, have further reduced the pool of Ca in watershed soils (Jeziorski & Smol, 2017). Timber harvesting has exacerbated the decline of Ca throughout eastern North America and parts of Europe because 60% of the Ca in trees is sequestered in the stemwood and bark, most of which gets exported during timber harvesting (Reid & Watmough, 2016). The removal of trees and subsequent forest regrowth depletes the little remaining soil Ca in soft water regions. Globally, 20% of lakes have Ca concentrations < 1.5 mg/L, a threshold considered critical for reproduction and survival of many aquatic organisms (Weyhenmeyer et al., 2019). Some of the lowest lake Ca concentrations occur in eastern Canada where active timber harvesting is predicted to result in a 10-40% decrease in water Ca from present-day values and low Ca concentrations (< 1.0 mg/L) are likely to become common throughout lakes in the

Canadian Shield (Jeziorski & Smol, 2017). Freshwater fauna with shells, especially molluscs, rely on large amounts of Ca from the water for successful calcification, and critically low concentrations will present novel ecological and physiological problems for shelled aquatic organisms.

Shell Calcification

The formation of a snail's shell is complex and poorly understood mechanistically; however, the importance of two ions, Ca^{2+} and HCO_3^-, are well studied. Ca in fresh waters is taken up from the environment against a concentration gradient (Greenaway, 1971; Ponder et al., 2019). The Ca concentration in the haemolymph of freshwater snails ranges from 3.2 to 8.55 mM, whereas the concentration in the water is between 0.1-1.0 mM. For this large concentration gradient to be passed, Ca must be actively transported from the water. This is contrary to marine molluscs where both the haemolymph and seawater have Ca concentrations around 10 mM, so there is no diffusion gradient against Ca uptake (Ponder et al., 2019). The hard part of the shell is a combination of calcium carbonate ($CaCO_3$) crystal layers and organic material. Ninety-five percent or greater of the shell consists of $CaCO_3$, while the remaining 0.01%-5% of the shell is organic glycoproteins, polysaccharides, and proteoglycans (Brown, 2001; Hasse et al., 2000; Marxen et al., 2003; White et al., 2007; Ponder et al., 2019). The primary $CaCO_3$ polymorphs comprising the shell are calcite and/or aragonite, and their percent composition varies by species and growing conditions. Molluscan shells are biomineralized under strict metabolic control by incrementally adding shell material to the aperture from mantle secretions. An organic matrix within the extrapallial space uses a variety of macromolecules to create a supersaturated solution of Ca^{2+} and HCO_3^- between the mantle edge and periostracum. Supersaturation forms crystals

which become superimposed and stacked within the sealed and environmentally isolated organic periostracum to form the shell. The periostracum prevents or slows shell erosion and is the framework that $CaCO_3$ is deposited on (Marin & Luquet, 2004; Ponder et al., 2019). Ca is the main cation in a snail's shell, and since the majority is obtained from the water, there must be sufficient Ca available for a snail to biomineralize its shell.

The ratio of water-derived *versus* food-derived Ca is likely to vary between species, but a greater amount is suspected to derive from the water under ideal conditions (Greenaway, 1971). *Lymnaea stagnalis* derives 80% of its shell Ca from the water column and 20% from the food it consumes (Van Der Borght & Van Puymbroeck, 1966). However, when *Lymnaea peregra* and *Planorbarius corneus* were reared in Ca-poor water, a greater percent of Ca was obtained from the diet (from 46% in Ca-rich water to 70% and 79% in Ca-poor water, respectively) (Young, 1975). Ca has also been studied in relation to morphological and behavioral inducible defenses in *L. stagnalis* in the presence of predators. These snails were found to grow larger with stronger shells when exposed to predatory cues, but only when sufficient Ca is available in the water (Rundle et al., 2004). Therefore, snails in low Ca environments may be unable to optimize protective adaptations and become more vulnerable to predation. The ability for snails to form long-term and intermediate-term memory is reduced in low Ca conditions, as are respiration and locomotion (Dalesman et al., 2010). Shell length and crush weights are also affected by low Ca concentrations. When reared in high Ca waters, *Pomacea paludosa* were found to grow longer with stronger shells when compared to low Ca conditions, with no genetic predisposition to growing faster in adverse parameters (Glass & Darby, 2009). These studies indicate that Ca is an essential element for numerous aspects of a snail's development and life history. However, the

impact of declining and critically low water Ca on the vital process of shell calcification has, to my knowledge, yet to be investigated.

Model Organism

Molluscs are one of the freshwater groups considered sensitive to acidification and calcium decline, and gastropods, the largest class in phylum Mollusca, are among the most sensitive. Entire lake populations of freshwater snails are at risk of extirpation because most northern populations are typically short-lived with semelparous reproductive strategies (Hunter, 1988, 1990). Of freshwater snails, Planorbidae is one of the most abundant and widespread families in the world, and *Planorbella* (formerly *Helisoma*) is a prevalent genus in this family (Martin et al., 2020). *Planorbella trivolvis* (Say 1817, Hygrophila: Planorbidae, also known as *Helisoma trivolvis*) is one of the most widely distributed planorbid snails with populations throughout North America, from Florida to arctic Canada, but it has been spread artificially around the world by aquarium hobbyists and water gardeners (Dillon, 2018; C. G. Norton & Wright, 2019). *P. trivolvis* is a simultaneous hermaphroditic species that thrives in eutrophic environments. Populations can be found in lakes, ponds, riverine backwaters, swamps, and wetlands with significant phenotypic plasticity in shell morphology between and within populations (Peterson, 2007; Dillon, 2018). The concentration of $CaCO_3$ in the shell of *P. trivolvis* is between 97-98% (White et al., 2007), well within the concentration of $\geq 95\%$ $CaCO_3$ known for molluscan shells (Marxen et al., 2003), making this snail an excellent model organism for a study on low water Ca.

P. trivolvis has been a model organism for studies in numerous disciplines, including population genetics and biogeography (Martin et al., 2020); growth and reproduction (C. G.

Norton et al., 2008, 2018; C. G. Norton & Bronson, 2006; C. G. Norton & Wright, 2019; C. Norton & Newman, 2015); ecotoxicology and parasitology (Mischke et al., 2021; Peterson, 2007); nutrition and dietary selection (Kimberly & Salice, 2012); and acidification of water with low calcium (Hunter, 1988, 1990). Hunter (1988) found a significant relationship between low pH and juvenile survival, shell Ca per unit dry weight, and shell Ca per unit shell diameter. Low pH and low Ca were lethal to *P. trivolvis* (Hunter, 1990), with eggs and juveniles having greater sensitivity and it was suggested that Ca as low as 2.0 mg/L (regardless of pH) would cause recruitment failure through juvenile mortality. With many lakes in soft water regions already below 2.0 mg/L, and a projection of continued decline, an investigation on the impact of critically low Ca on shell calcification of *P. trivolvis* is needed.

Research Aim and Objective

The goal of my research is to gain an understanding of how freshwater snails will respond to the continued decline of Ca in soft water lakes. While some studies have shown negative ecological impacts, including death, recruitment failure, and a reduction in protective defenses for snails raised in Ca-limited and/or low pH water, the impact that critically low Ca (less than 1.5 mg/L) has on snails is missing in the literature. In this study, I raised a common and widespread species of freshwater snail in four Ca concentrations and collected data on shell growth and calcification at three time-points. Furthermore, I sought to determine if there is a relationship between shell calcification and shell size within different Ca concentrations because of natural intraspecific plasticity. This investigation into shell growth and calcification of

Planorbella trivolvis after being reared in a range of Ca concentrations will add to the knowledge of organisms susceptible to Ca decline.

Hypotheses

1.) There will be an increase in body mass with increasing water Ca due to better developed muscles and tissues. Snails in the highest Ca concentration will have the largest ash-free dry weight, and snails in the lowest Ca concentration will have the smallest ash-free dry weight.

2.) *P. trivolvis* will grow a shorter and thinner shell with decreasing water Ca concentration. This effect will result in snails in the lowest Ca concentration having the smallest shell width, shell height, and aperture height, and snails in the highest Ca concentration having the largest shell width, shell height, and aperture height.

3.) Because the shell of a freshwater snail is ≥ 95% $CaCO_3$, and the majority of this is obtained from the water, *P. trivolvis* will have a less calcified shell with decreasing water Ca concentration. This effect will include a decrease in ash weight and percent Ca dry weight. Snails with a smaller shell due the species phenotypically plastic nature will be less calcified than larger snails.

Methods

Creation of Laboratory Monoculture

A colony of mixed ramshorn snails were obtained in early 2019 from an aquarium hobbyist in New Jersey and the snails reproduced freely in the author's home aquaria. In January 2020, two juvenile snails (approximately 3 mm shell width) were randomly selected from the

same aquarium and placed in a new aquarium with dechlorinated tap water and sponge filtration at ambient temperature. Due to the lack of literature on the age of first copulation in the suspected genus, the original pair may have been inseminated prior to isolation. Upon maturity, the original pair were allowed to reproduce and lay eggs freely for three weeks. Two neonate snails (F1) with shell widths < 1 mm were isolated within three days of hatching and reared in a new system. This process was repeated until the F3 generation was produced. The F3 generation was placed in a five-gallon glass aquarium with filtered tap water and allowed to reproduce freely to create a genetically similar monoculture. Several ages of the F4 generation were sent to Dr. D. Christopher Rogers at Kansas Biological Survey who identified the species to be *Helisoma trivolvis* (*personal communication*, March 13, 2021). *Helisoma* and *Planorbella* are equivalent genera, but *Planorbella* is consistent with current literature and the generic name I will use. Progeny of the F3 free-breeding *P. trivolvis* monoculture were used for this research.

Experimental Treatments

FLAMES, a synthetic soft water medium, replicates the conditions of Canadian Shield lakes and was used to create four media with different Ca concentrations (Celis-Salgado et al., 2008). Following this water formula, eleven stock solutions were prepared using deionized (DI) water. To create media with four Ca concentrations, the volume of calcium sulphate dihydrate ($CaSO_4 \cdot 2\ H_2O$) was manipulated. The FLAMES medium has a [Ca] of 2.54 mg/L, of which 2.531 mg/L comes from $CaSO_4 \cdot 2\ H_2O$, and the remainder (< 0.4%) is from calcium pantothenate in the vitamin solution that is part of the recipe (0.009 mg/L), which was not taken into consideration for the experiments as it is a constant amount. The FLAMES medium recipe

contains 20 mL of $CaSO_4 \cdot 2\ H_2O$ stock solution. By adjusting this volume, media containing 0.64, 1.3, 2.5, and 5.1 mg/L Ca were prepared and used for this experiment.

Experimental Setup

Twenty egg masses laid in the free-breeding monoculture were placed in a 500-mL glass beaker containing 400-mL of FLAMES ("rearing beaker") and incubated at 25 °C with an 8-hour photoperiod. Approximately 500 snails hatched over the next three days and were fed *ad libitum* on algae wafer (Hikari® Tropical Algae Wafers). Fifty percent of the media, feces, and uneaten food were removed from the rearing beaker every other day and replenished with fresh FLAMES. Twenty-four hours prior to the beginning of the experiment, the experimental beakers (25 per treatment) were labeled 1-100 and filled with 50 mL of fresh media. One milliliter of used media from the rearing beaker was added to spike the fresh media with a microbial population. One hour prior to experiment onset, pre-weighed food was added to each beaker.

Eighteen days after hatching began, all juvenile snails were removed from the rearing beaker with a turkey baster and placed in a 100-mm Petri dish. Visually smaller snails and snails with shell cracks or defects (approximately 200) were removed from the Petri dish and did not enter the experiment. Of the remaining ~300 snails, 15 were randomly selected for Day 0 measurements and were set aside in a separate Petri dish. From the population of remaining snails, one snail at a time was randomly selected and placed into a sterile 100-mL glass beaker containing one of the four media. Snails were transferred within a drop of media in a disposable plastic pipette, which preliminary trials showed was the least traumatic method of handling juvenile snails. After all 100 experimental beakers were inoculated with a snail, the beakers were placed into one of four forest green colored plastic trays grouped by Ca concentration (which had

shown to be the least disrupting coloration for the snails in previous trials). The trays were placed into one environmental incubator chamber (Percival Scientific, Model I-22VL) and maintained at 25 °C with an 8-hour photoperiod consisting of soft light by covering the upper 90% of each vertical lamp (which were at 10% light intensity) with aluminum foil.

In preliminary trials, the top portion of a frozen romaine lettuce leaf cut into equal sized squares and soaked in FLAMES for 24-48 hours was attempted as a food source unsuccessfully. Additional preliminary trials suggested that algae wafers are a better diet for this snail species in low Ca water. Therefore, each beaker contained an *ad libitum* aliquot of algae wafer that was broken into pieces and weighed to provide each snail an equal amount. On day 20, snails were dislodged from the side of the beaker using a rounded metal spatula to stimulate foot retraction and transferred in a small volume of used media to 236-mL borosilicate glass jars (8 oz. Mason® jars) containing 100 mL of fresh media and 1 mL of used media. On day 40, the volume of media was increased to 150 mL per snail. To limit evaporation from the beakers, a large tray of water was placed on the bottom of the incubator to raise the humidity.

Experiment Maintenance

Every two days, experimental water was changed and fresh food provided for the snails. In random order, all beakers of like media were removed from a tray. One beaker at a time, the old media was gently swirled for 3 sec to dislodge any uneaten food and waste from the bottom of the beaker, with care taken to leave the snail attached to the beaker. Beginning on day 40, a 3-mL disposable plastic pipette was used to pulse water within the beaker to dislodge uneaten food and waste from the bottom of the jar and wash away accumulated debris from the shell. All but approximately 1 mL of the remaining media was poured off prior to adding fresh media with

emphasis on lightly washing the snail and the side of the beaker. The fresh media at 25 °C was added to the beaker and the beaker was returned to the tray, and this process continued until all replicates received a water change. Pre-weighed algae wafer pieces were added to each beaker and stirred gently to submerge them. The trays were moved to a new location in the incubator in a clockwise pattern. All treatments were provided the same mass of algae wafer (see Appendix A) and were refreshed in random order on maintenance days.

Two snails died during the 60-day experiment due to handling damage: one on day 2 and one on day 22. Both were from the 0.64 mg/L Ca treatment (0.64 Ca).

Water Quality Assurance and Testing

Evaporation was limited to less than 2 mL of 50 mL of media per day by placing a large tray filled with water on the bottom of the incubator chamber to increase the humidity (see Appendix B). Water pH was tested periodically on random experimental beakers during the first 30 days and at each maintenance day thereafter (Mettler Toledo SevenExcellence™ with Mettler Toledo InLab® Expert Pro-ISM electrode). Three snails per treatment were subjectively less than half the size of a typical individual ("runts") and were not included in the population of randomly selected snails for pH measurements. Ammonia/ammonium levels were similar for all treatment media with snails (see Appendix C). Changes in media pH from algae wafer decomposition was assessed in a two-day experiment without snails present (see Appendix D).

Data collection

On day 0, data were collected on 12 of the 15 snails set aside during the setup of the experiment. Twelve silver capsules were individually pre-weighed, and the mass was recorded to

the nearest 0.0001 mg on a micro balance (Sartorius Cubis™ II). Each capsule was placed into a numbered porcelain tray well. Using a 3-mL disposable plastic pipette, one of the 15 snails was randomly selected and placed within a drop of water into a new 150-mm Petri dish. The snail was washed with a gentle stream of deionized water for 5 sec prior to moving the snail within a drop of water onto a Kimwipe tissue folded in half three times. After 5 sec, the snail was carefully rotated using a dissecting probe to its apertural surface on a dry portion of the tissue where it was allowed to dry for 30 seconds. After 30 seconds, the snail was picked up using the tip of a dissecting probe, placed into a silver capsule, and the combined wet weight and silver capsule weight was recorded to the nearest 0.01 mg after 10 sec of allowing the wet specimen to equilibrate within the micro balance weighing chamber. The silver capsule was returned to the porcelain tray. The porcelain tray was placed in a freezer overnight to euthanize the snails and was kept frozen until morphometrics were collected one week later.

After bringing the specimens to room temperature, shell morphometrics were collected using digital microscopy and integrated measuring functions (Olympus® DP21). At the time of measuring, the specimen was removed from the silver capsule and placed on a glass microscope slide on its apical surface. The greatest distance between the tip of the aperture and the outer whorl on the umbilical surface was measured three times (shell width; Figure 2a). The specimen was then rotated onto its outer whorl with the aperture facing upwards. Clay was used to aid in positioning the shell until a sliver of the outer whorl on both the apical and umbilical sides and a cross section of the aperture wall tips were all visible. The height of the shell was measured at the parietal aspect of the aperture at the connections to the outer whorl three times (shell height; Figure 2b). Without moving the shell, the aperture height was measured three times as the greatest length between the inside lip of the palatal wall beginning at the concave ridge on the

apical side and extending across to the umbilical side (Figure 2c). After morphometrics were collected, the specimen was returned to the silver capsule and porcelain tray, and the capsule was pinched closed using forceps, leaving 1-2 mm of the capsule open to allow off-gassing during the drying process. This process was repeated until morphometrics were collected for all 12 snails. One of the snails was damaged during the measuring process, therefore it was removed and not included in the day 0 population.

Similar to the protocol in Glass & Darby 2009 (see also Brodersen & Madsen, 2003; Hunter & Lull, 1977), the specimens were dried in a drying oven at 90 °C for at least 24 hr prior to cooling to 25 °C for one hour. The specimens were removed from the drying oven and placed in a glass desiccator for transport to the micro balance. The dry weight plus silver capsule of each specimen was found to the nearest 0.0001 mg. Finally, after ashing in a muffle furnace at 550 °C for 1.5 hr and cooling to room temperature in a drying oven and glass desiccator, the ash weight plus silver capsule of each specimen was found to the nearest 0.0001 mg.

On day 30, four snails per treatment (n = 16) were randomly selected and sacrificed for data collection. Runt snails were not included in the population of randomly selected snails. The water pH and ammonia concentration were determined for all two-day-old media prior to initial shell washing. All 16 beakers were individually swirled to dislodge waste and uneaten food prior to using a disposable plastic pipette to wash debris off the shell. Used media was poured out and each beaker was filled with 100 mL of fresh media. Within two hours, each snail was dislodged from the jar by probing the foot with a blunt metal spatula. Upon dislodgement, the snail was poured into a metal sieve and all surfaces of the shell were washed with a stream of deionized water for 30 seconds. The wet weight plus silver capsule mass, shell morphometrics, dry weight plus silver capsule mass, and ash weight plus silver capsule mass were found for all 16 snails as

described above. Data collected on day 60 for the 82 remaining snails in the experiment followed the same procedure.

Data Analysis

For the snails' shell morphometric evaluation, I took measurements of shell width, shell height, and aperture height for statistical analyses. The ash-free dry weight (AFDW) was calculated by subtracting the ash weight from the dry weight. Whole snail Ca was calculated by multiplying the ash weight by 0.40 because a snail shell consists of > 95% $CaCO_3$, of which 40% of the molecular weight is Ca (Glass & Darby, 2009; Hunter & Lull, 1977). Finally, percent Ca dry weight (% Ca dry weight) was calculated by dividing whole snail Ca by dry weight and multiplying by 100. For statistical analysis, % Ca dry weight values were transformed by taking the arcsine of their square root.

All statistical analyses were performed using R. Statistical analyses were based on differences in measurements (pH, shell morphometrics, wet weight, dry weight, ash weight, AFDW, and % Ca dry weight) between the four treatments on days 30 and 60 ("day 30" group and "day 60" group, respectively) and on two size groups separated by the median shell width (snails less than ["day 60 < MSW"] and snails greater than or equal to the median shell width ["day 60 ≥ MSW"]). All data were tested for normality with the Shapiro-Wilk test and homogeneity of variance with the Levene's test. When data met the assumptions of normality and homoscedasticity, one-way analysis of variance (ANOVA) was used. When data transformations did not improve normality, non-parametric statistics (Kruskal-Wallis test or Wilcoxon rank-sum test) were used with the exception of the pH measurement which was analyzed with ANOVA despite not meeting the assumption of normality because the data was

nearly a normal distribution and non-parametric statistics did not change any significance. If the null hypothesis of ANOVA was rejected ($p < 0.05$), Tukey's HSD test for multiple comparisons of means was used to determine which treatment groups differed. If the null hypothesis of the Kruskal-Wallis test was rejected ($p < 0.05$), Dunn's multiple comparison test was used with a Bonferroni adjustment to determine which treatment groups differed.

The measurements and groups that were analyzed with parametric statistics (one-way ANOVA) were: all pH treatment groupings; wet weight day 30 and day 60 ≥ MSW; shell width day 30; shell height day 30 and day 60 ≥ MSW; aperture height day 30 and day 60 ≥ MSW; dry weight day 30 and day 60 ≥ MSW; ash weight day 30; AFDW day 30 and day 60 ≥ MSW; and % Ca dry weight day 30. The measurements and groups that were analyzed with non-parametric statistics (Kruskal-Wallis test or Wilcoxon rank-sum test) were: wet weight day 60 and day 60 < MSW; shell width day 60, day 60 < MSW, and day 60 ≥ MSW; shell height day 60 and < MSW; dry weight day 60 and day 60 < MSW; ash weight day 60, day 60 < MSW, and day 60 ≥ MSW; AFDW day 60 and day 60 < MSW; and % Ca dry weight day 60, day 60 < MSW; and day 60 ≥ MSW. Linear regression was performed on pH. Linear regression was also used to get the slope of the % Ca dry weight for day 0 to day 30, and a separate linear regression for day 30 to day 60 for all treatments.

Snails #33 and #39 from the 2.5 Ca treatment and snail #89 from the 0.64 Ca treatment were removed from statistical analysis because they were damaged and bled during the transfer from the 100-mL glass beaker to the 236-mL borosilicate glass jar on day 20. Therefore, day 60 statistical analyses were performed on a total of 79 snails.

Data were collected on day 0 from 11 non-experimental snails reared for 18 days in the same conditions as the snails who entered the experiment. Experimental day 30 measurements (n

= 16) were from four snails randomly selected (excluding runts) and sacrificed from each treatment. Because of size discrepancies on the randomly selected snails at the beginning for the experiment, experimental day 60 measurements (n = 79) were analyzed in three ways: together as a population, and as two size groups, one group containing all snails less than the median-shell-width (MSW) (11.033 mm), and the other group containing all snails greater than or equal to MSW. Day 30, day 60, day 60 < MSW, and day 60 ≥ MSW snails were analyzed for significant differences between water Ca treatments (n = 4) for each measurement. Treatment conditions are abbreviated as follows:

0.64 Ca = 0.64 mg/L Ca

1.3 Ca = 1.3 mg/L Ca

2.5 Ca = 2.5 mg/L Ca

5.1 Ca = 5.1 mg/L Ca

Results

In this experiment, I tested the effects of low water Ca on the growth and shell calcification of *Planorbella trivolvis* over 60 days. Shell morphometrics (shell width, shell height, and aperture height) provided data on the size of the shell, while wet weight, dry weight, ash weight, and ash-free dry weight (AFDW) provided data on body growth and shell calcification. Ash weight measured shell calcification as mass of $CaCO_3$, and AFDW measured organic body mass. The calculation of % Ca dry weight determined the proportion of dry weight that is Ca from the shell. By comparing the size of the snail's shell to its organic body mass and total shell Ca, I could investigate which, if any, parameters were affected by the stress of low Ca water.

Wet Weight

Water Ca concentration did not have a statistically significant effect on the wet weight of snails on day 30 (one-way ANOVA, $F_{(3,12)} = 0.239$, $p = 0.868$, Figure 1) or day 60 (Kruskal-Wallis, $H(3) = 5.664$, $p = 0.129$, Figure 1; see Appendix E for descriptive statistics). When separated into two groups by the median shell width, there was no significant difference between treatments on day 60 < MSW group (Kruskal-Wallis, $H(3) = 0.722$, $p = 0.868$) or day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 2.583$, $p = 0.068$).

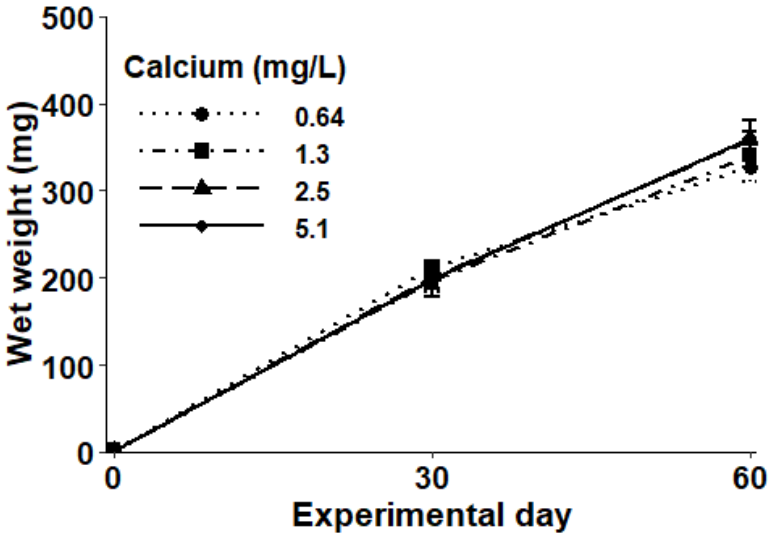

Figure 1: Growth curves of *P. trivolvis* mean wet weight in the four water Ca concentrations over 60 days. Error bars represent standard errors.

Shell Morphometrics

Shell morphometrics were determined at three shell locations: shell width, shell height, and aperture height (Figure 2; see Appendix E for descriptive statistics of each). Water Ca concentration did not have a significant effect on shell width on day 30 (one-way ANOVA, $F_{(3,12)} = 0.449$, $p = 0.723$, Figure 3a) or day 60 (Kruskal-Wallis, $H(3) = 3.315$, $p = 0.346$, Figure 3a).

When day 60 snails were separated into two size groups, there was not a significant difference between treatments on the day 60 < MSW group (Kruskal-Wallis, $H(3) = 0.703$, $p = 0.873$) or the day 60 ≥ MSW group (Kruskal-Wallis, $H(3) = 3.734$, $p = 0.292$).

Shell height was not significantly affected by water Ca concentration on day 30 (one-way ANOVA, $F_{(3,12)} = 0.587$, $p = 0.635$, Figure 3b). There was a slight difference in shell height between Ca treatments on day 60, but the difference was not significant (Kruskal-Wallis, $H(3) = 7.750$, $p = 0.051$, Figure 3b). There was not a significant difference in shell height between treatments in the day 60 < MSW group (Kruskal-Wallis, $H(3) = 3.592$, $p = 0.309$) or day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 1.908$, $p = 0.146$).

Water Ca concentration did not significantly affect aperture height on day 30 (one-way ANOVA, $F_{(3,12)} = 0.521$, $p = 0.676$, Figure 3c) or day 60 (Kruskal-Wallis, $H(3) = 1.129$, $p = 0.770$, Figure 3c). There was not a significant difference in aperture height between water Ca treatments in the day 60 < MSW group (Kruskal-Wallis, $H(3) = 1.050$, $p = 0.789$) or the day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 1.323$, $p = 0.282$).

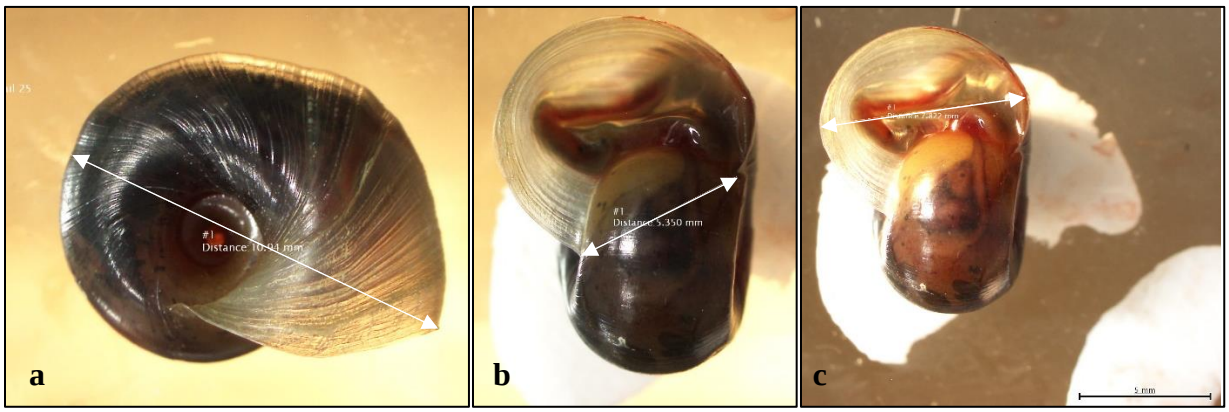

Figure 2: Photographs of snail #25 from the 5.1 Ca treatment showing locations of (a) shell width, (b) shell height, and (c) aperture height measurements.

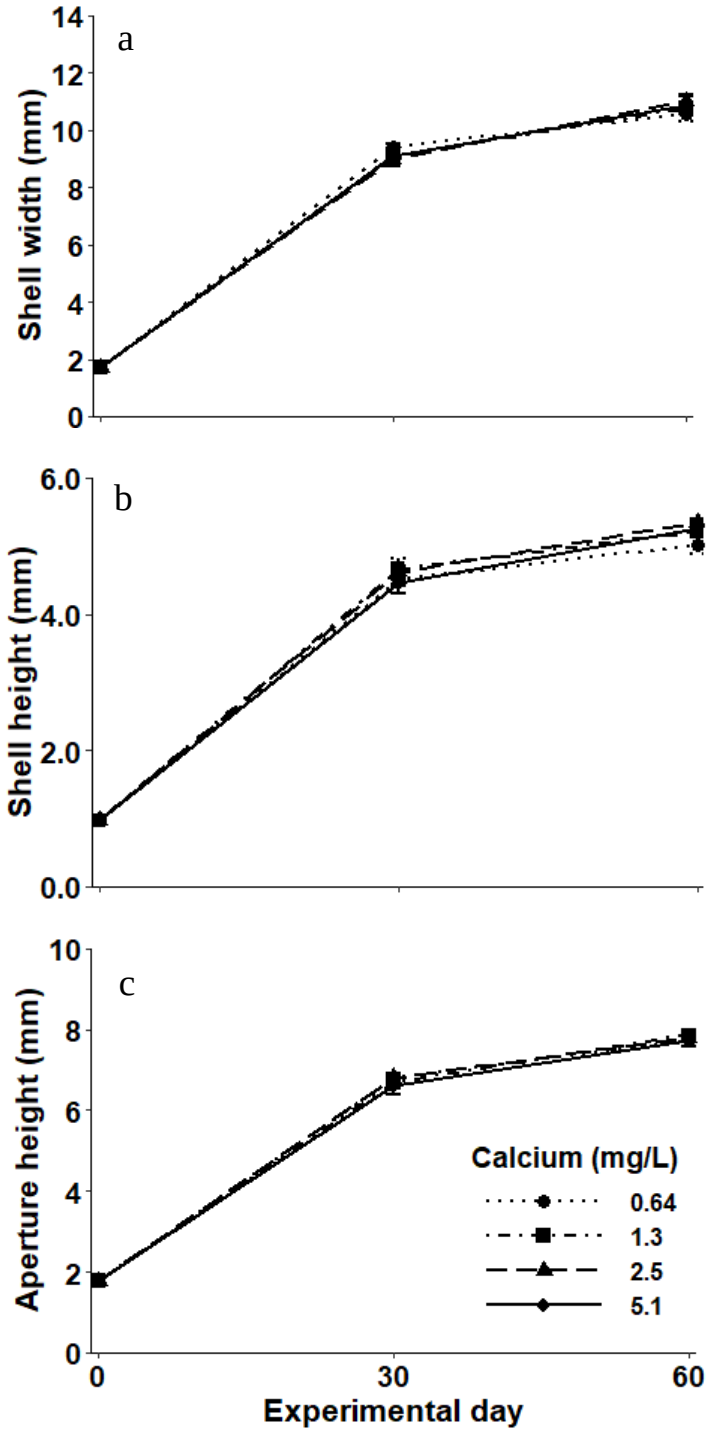

Figure 3: Growth curves of *P. trivolvis* mean (a) shell width, (b) shell height, and (c) aperture height in the four water Ca concentrations over 60 days. Error bars represent standard errors.

Dry Weight

Water Ca concentration did not have a significant effect on the dry weights of snails on day 30 (one-way ANOVA, $F_{(3,12)} = 1.323$, $p = 0.282$, Figure 4, Tables 1, 2a). However, by day 60 there was a significant difference in the dry weight of snails in the different treatments (Kruskal-Wallis, $H(3) = 20.256$, $p < 0.001$, Figure 4, Tables 1, 2b). Pairwise comparisons using Dunn's test with a Bonferroni adjustment indicated that on day 60, snails in 0.64 Ca had significantly lower dry weights than snails in 2.5 Ca ($p = 0.016$, Figure 5) and 5.1 Ca ($p < 0.001$, Figure 5). There was nearly a significant difference on day 60 between 1.3 Ca and 5.1 Ca snails (Dunn's test, $p = 0.058$, Figure 5).

There was no difference in the dry weight of snails between treatments in the day 60 < MSW group (Kruskal-Wallis, $H(3) = 4.6525$, $p = 0.199$, Figure 6a, Tables 1, 2b); however, there was a significant difference in snail dry weight between Ca treatments in the day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 9.775$, $p < 0.001$, Tables 1, 2a). Tukey's HSD test for multiple comparisons found that, in the day 60 ≥ MSW group, the mean dry weight of snails in 5.1 Ca was significantly greater than the mean dry weight of snails in 0.64 Ca ($p < 0.001$, Figure 6b, Table 1), 1.3 Ca ($p = 0.003$, Figure 6b, Table 1), and 2.5 Ca ($p = 0.006$, Figure 6b, Table 1).

Table 1: Descriptive statistics for the dry weight of all snail groups throughout the experiment.

MSW = Median Shell Width

Dry weight (mg)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	0.285	0.298	0.198	0.351	0.047	0.014
Day 30:	Ca 0.64	4	33.953	34.027	32.424	35.334	1.218	0.609
	Ca 1.3	4	34.293	35.829	27.421	38.092	5.010	2.505
	Ca 2.5	4	37.915	39.491	31.028	41.651	4.706	2.353
	Ca 5.1	4	37.068	38.858	27.844	42.713	6.793	3.396
	(all)	16	35.807	35.723	27.421	42.713	4.702	1.176
Day 60:	Ca 0.64	18	65.829	67.437	36.911	78.584	9.874	2.327
	Ca 1.3	21	73.379	73.073	48.747	102.467	12.079	2.635
	Ca 2.5	19	77.276	78.367	48.954	88.725	9.644	2.212
	Ca 5.1	21	82.361	86.674	10.238	102.668	21.350	4.659
	(all)	79	74.983	74.819	10.238	102.668	15.263	1.717
Day 60 < MSW:	Ca 0.64	12	61.509	63.534	36.911	71.301	9.251	2.671
	Ca 1.3	11	66.403	67.603	48.747	77.147	7.9989	2.412
	Ca 2.5	7	68.331	72.275	48.954	76.739	9.523	3.599
	Ca 5.1	9	67.059	81.075	10.238	85.750	24.085	8.028
	(all)	39	65.395	66.325	10.238	85.750	13.614	2.180
Day 60 ≥ MSW:	Ca 0.64	6	74.469	74.102	71.065	78.584	2.968	1.212
	Ca 1.3	10	81.052	81.502	58.268	102.468	11.324	3.581
	Ca 2.5	12	82.494	83.465	74.726	88.724	4.702	1.357
	Ca 5.1	12	93.837	95.780	73.613	102.668	8.463	2.443
	(all)	40	84.333	83.465	58.268	102.668	10.184	1.610

Table 2: One-way ANOVA for (a) dry weight of all snails on day 30 and on day 60 ≥ MSW, and (b) mean dry weight ranks on day 60 and on day 60 < MSW group.

a.) Dry weight ANOVA

	Effect	df	MS	F	p-value
Day 30	Ca concentration	3	15.686	0.9914	0.592
	Residuals	12	0.6804		
Day 60 ≥ MSW	Ca concentration	3	605.290	9.775	< 0.001
	Residuals	36	61.920		

b.) Dry weight ranks

Day 60		Day 60 < MSW	
Treatment	Rank mean	Treatment	Rank mean
Ca 0.64	22.500	Ca 0.64	14.417
Ca 1.3	36.000	Ca 1.3	20.636
Ca 2.5	45.158	Ca 2.5	23.429
Ca 5.1	54.333	Ca 5.1	24.000

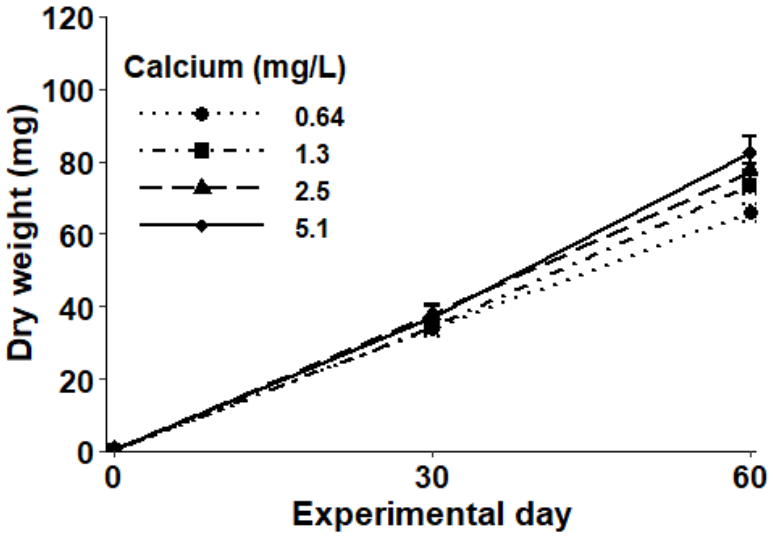

Figure 4: Growth curves of *P. trivolvis* mean dry weight in the four water Ca concentrations over 60 days. Error bars represent standard errors.

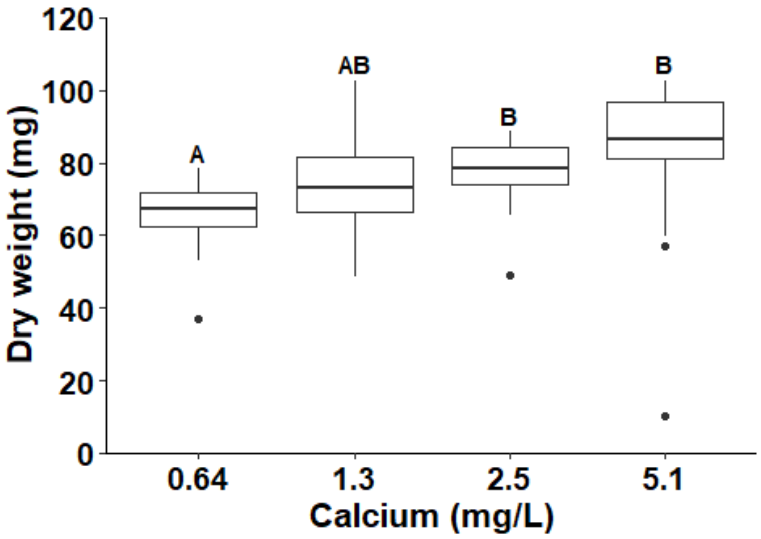

Figure 5: *Planorbella trivolvis* dry weight in the four water Ca concentrations on day 60. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

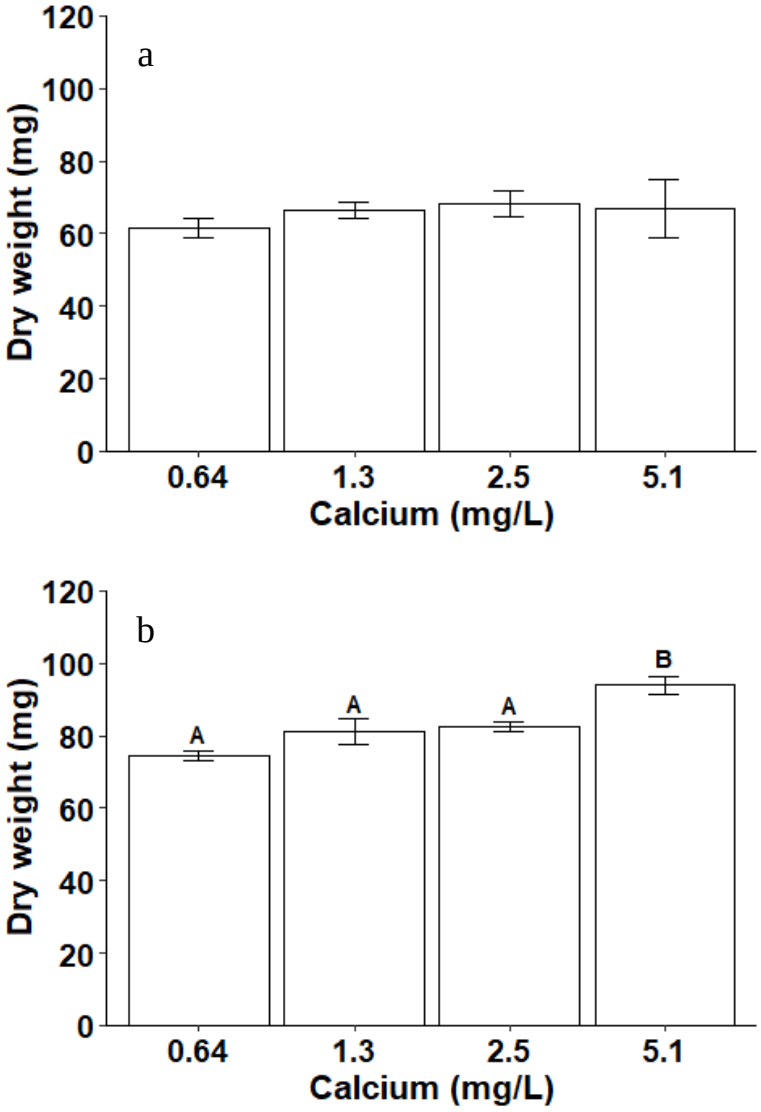

Figure 6: *Planorbella trivolvis* dry weight in (a) day 60 < MSW group and (b) day 60 ≥ MSW group. Different letters indicate significant differences among treatments. Error bars represent standard errors.

Ash Weight and Whole Snail Calcium

Water Ca concentration had a significant effect on the ash weight of snails on day 30 (one-way ANOVA, $F_{(3,12)} = 19.616$, $p < 0.001$, Figure 7a, Tables 3, 4). Snails in 0.64 Ca had significantly less ash than snails in both 2.5 Ca and 5.1 Ca (*post hoc*, Tukey HSD, $p < 0.001$, Figure 8a). Snails in 1.3 Ca had a significantly lower amount of ash than snails in 2.5 Ca (*post hoc*, Tukey HSD, $p = 0.011$, Figure 8a) and 5.1 Ca (*post hoc*, Tukey HSD, $p = 0.001$, Figure 8a). The was no statistically significant difference in the ash weight of snails on day 30 when comparing the 0.64 Ca treatment to the 1.3 Ca treatments or the 2.5 Ca treatment to the 5.1 Ca (*post hoc*, Tukey HSD, $p > 0.05$, Figure 8a).

On day 60, there was a statistically significant difference in ash weight between Ca treatments (Kruskal-Wallis, $H(3) = 60.791$, $p < 0.001$, Figure 7a, Tables 3, 5). Pairwise comparison of ranks indicated that snails in 0.64 Ca had significantly lower ash weights than snails in 1.3 Ca (Dunn's test, $p = 0.037$, Figure 9a), 2.5 Ca (Dunn's test, $p < 0.001$, Figure 9a), and 5.1 Ca (Dunn's test, $p < 0.001$, Figure 9a). Snails in 1.3 Ca had significantly lower ash weights than snails in 2.5 Ca (Dunn's test, $p = 0.025$, Figure 9a) and 5.1 Ca (Dunn's test, $p < 0.001$, Figure 9a). The ash weight of snails in 2.5 Ca did not differ significantly from snails in 5.1 Ca (Dunn's test, $p = 0.540$, Figure 9a).

In the day 60 < MSW group, there was a statistically significant difference in the ash weight of snails between water Ca treatments (Kruskal-Wallis, $H(3) = 25.893$, $p < 0.001$, Tables 3, 5). Pairwise comparisons indicated that snails in 0.64 Ca had significantly lower ash weights than snails in both 2.5 Ca and 5.1 Ca (Dunn's test, $p < 0.001$, Figure 10a).

In the day 60 ≥ MSW group, there was a statistically significant difference in the ash weight of snails in different water Ca treatments (Kruskal-Wallis, $H(3) = 35.328$, $p < 0.001$,

Tables 3, 5). Pairwise comparison of ranks indicated that snails in the 0.64 Ca treatment had significantly less ash than snails in both the 2.5 Ca treatment (Dunn's test, $p = 0.005$, Figure 11a) and 5.1 Ca treatment (Dunn's test, $p < 0.001$, Figure 11a), and snails in the 1.3 Ca treatment had lower amounts of ash than snails in 5.1 Ca (Dunn's test, $p < 0.001$, Figure 11a).

The statistical analyses for whole snail Ca are not provided because they are equivalent to dry weight (Figures 7b, 8b, 9b, 10b, 11b; see Appendix E for descriptive statistics).

Table 3: Descriptive statistics for the ash weight of all snail groups throughout the experiment.

Ash weight (mg)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	0.082	0.086	0.060	0.100	0.012	0.004
Day 30:	Ca 0.64	4	7.475	7.419	7.384	7.678	0.138	0.069
	Ca 1.3	4	8.410	8.831	6.889	9.090	1.024	0.512
	Ca 2.5	4	10.659	10.924	9.623	11.166	0.704	0.352
	Ca 5.1	4	11.339	11.604	9.953	12.197	1.076	0.538
	(all)	16	9.471	9.356	6.889	12.195	1.793	0.448
Day 60:	Ca 0.64	18	23.257	23.701	18.571	24.072	1.263	0.298
	Ca 1.3	21	27.087	27.536	24.035	28.254	1.161	0.253
	Ca 2.5	19	32.080	32.294	26.703	34.672	1.814	0.416
	Ca 5.1	21	35.029	36.536	7.610	40.247	7.022	1.532
	(all)	79	29.526	28.143	7.610	40.246	5.874	0.661
Day 60 < MSW:	Ca 0.64	12	22.961	23.264	18.571	23.984	1.470	0.424
	Ca 1.3	11	26.541	26.999	24.035	27.845	1.355	0.409
	Ca 2.5	7	30.749	30.872	26.703	33.683	2.128	0.804
	Ca 5.1	9	31.114	35.441	7.610	37.040	9.304	3.101
	(all)	39	27.250	26.999	7.610	37.040	5.639	0.903
Day 60 ≥ MSW:	Ca 0.64	6	23.849	23.909	23.486	24.073	0.207	0.085
	Ca 1.3	10	27.687	27.775	26.790	28.254	0.437	0.138
	Ca 2.5	12	32.856	32.917	31.208	34.672	1.064	0.307
	Ca 5.1	12	37.964	38.781	33.346	40.247	2.184	0.630
	(all)	40	31.745	32.563	23.486	40.246	5.273	0.834

Table 4: One-way ANOVA for the ash weight of all snails on day 30.

Ash weight ANOVA					
	Effect	df	MS	F	p-value
Day 30	Ca concentration	3	13.3476	19.616	< 0.001
	Residuals	12	0.6804		

Table 5: Mean ash weight ranks for all four treatments on day 60 and in the day 60 < MSW and ≥ MSW groups.

Ash weight ranks		
	Treatment	Mean rank
Day 60	Ca 0.64	10.556
	Ca 1.3	30.762
	Ca 2.5	51.632
	Ca 5.1	63.952
Day 60 < MSW	Ca 0.64	7.500
	Ca 1.3	19.636
	Ca 2.5	28.857
	Ca 5.1	30.222
Day 60 ≥ MSW	Ca 0.64	3.500
	Ca 1.3	11.500
	Ca 2.5	22.917
	Ca 5.1	34.083

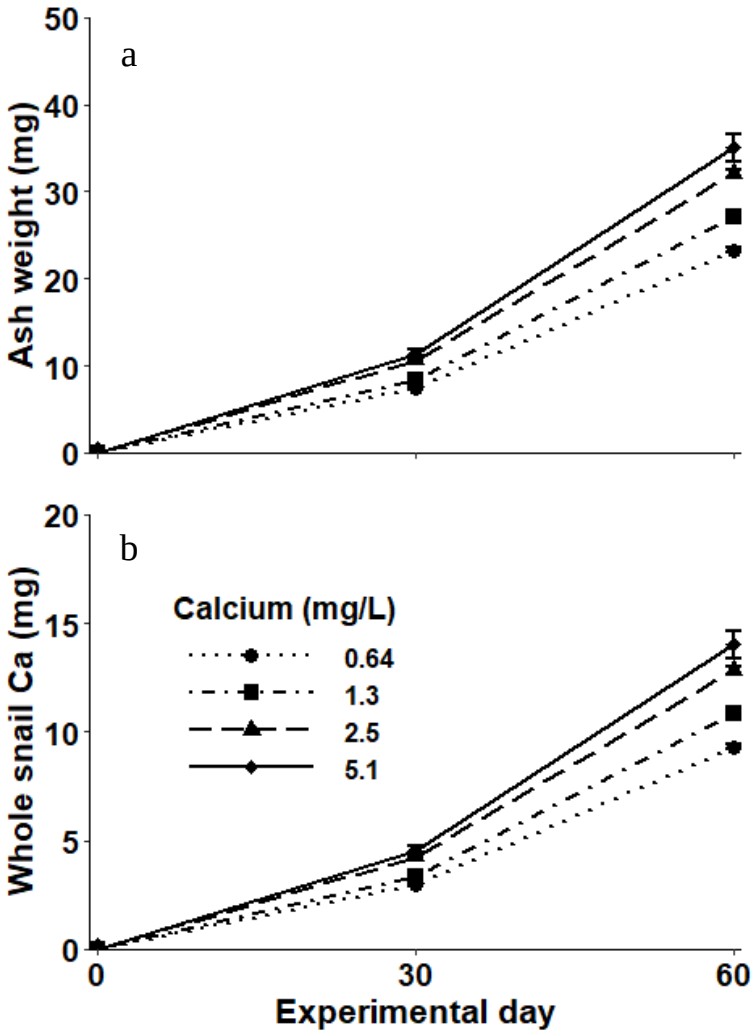

Figure 7: Growth curves of (a) *P. trivolvis* mean ash weight and (b) whole snail calcium in the four water Ca concentrations over 60 days. Error bars represent standard errors.

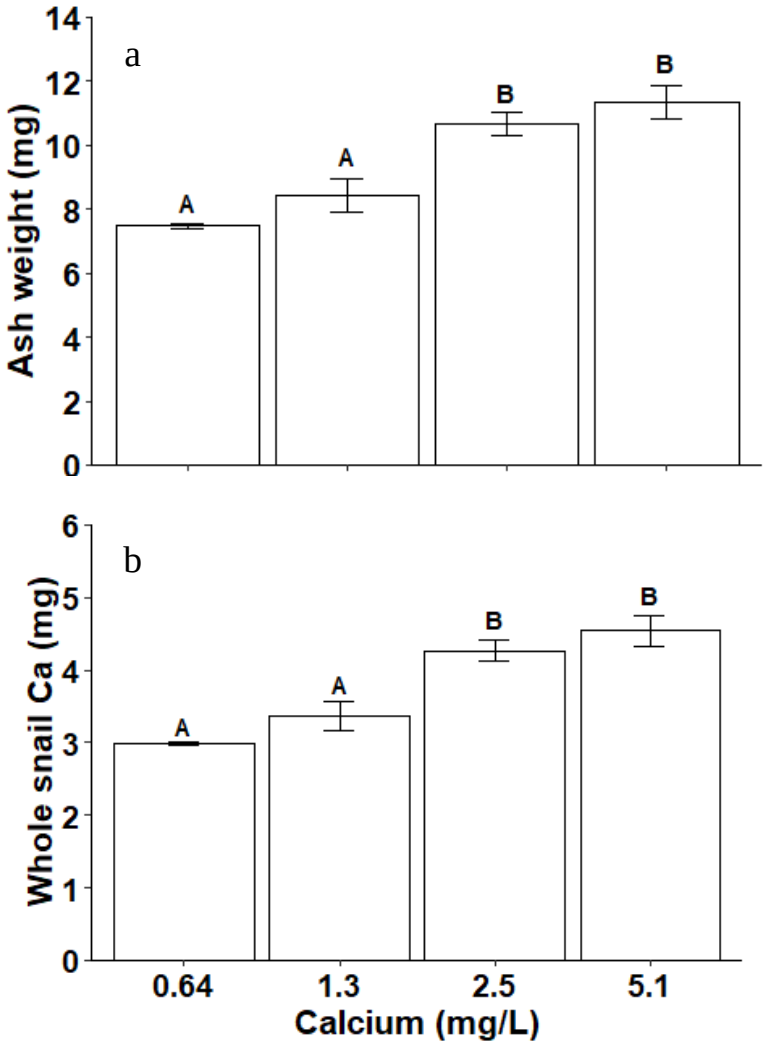

Figure 8: *Planorbella trivolvis* (a) ash weight and (b) whole snail calcium in the four water Ca concentrations on day 30. Different letters indicate significant differences between treatments. Error bars represent standard errors.

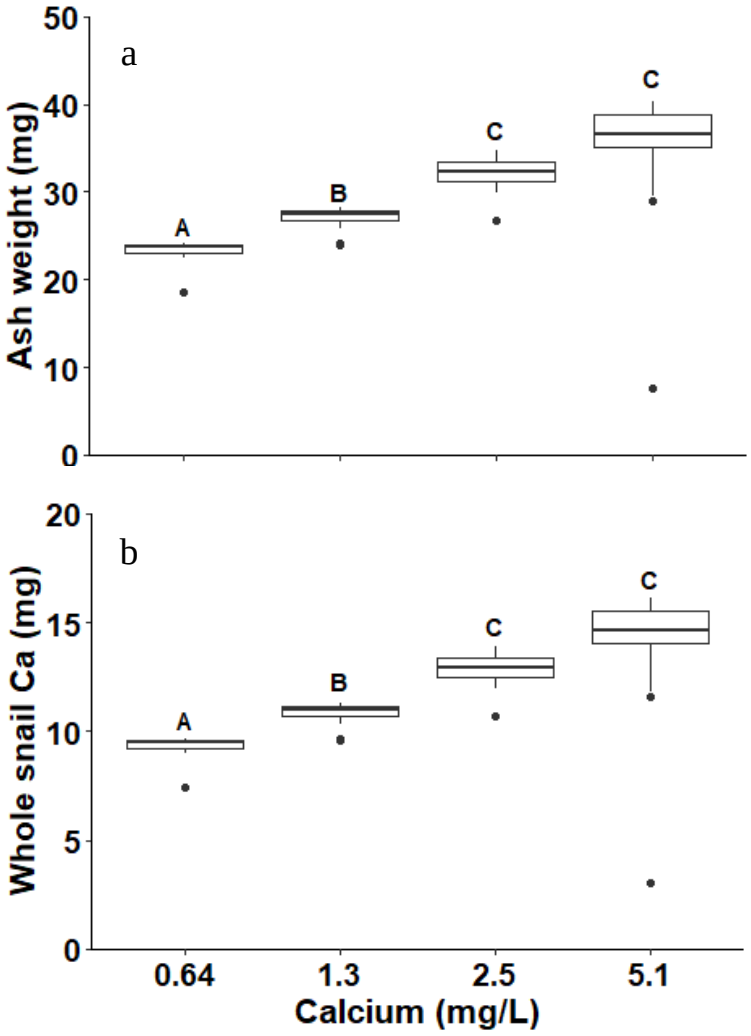

Figure 9: *Planorbella trivolvis* (a) ash weight and (b) whole snail calcium in the four water Ca concentrations on day 60. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

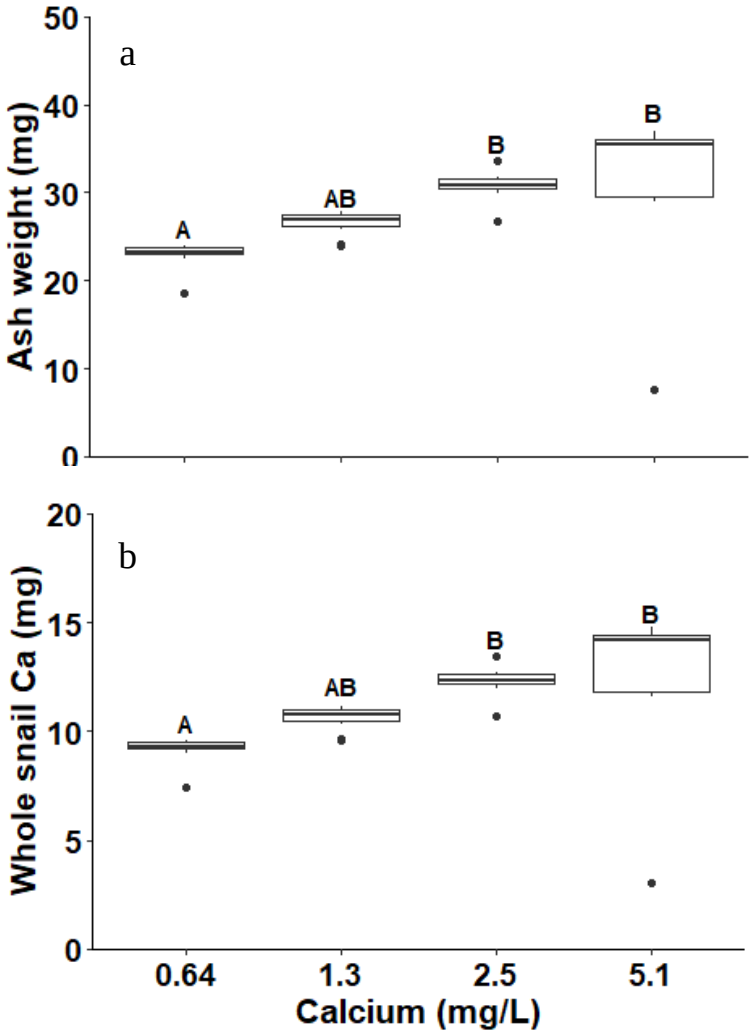

Figure 10: *Planorbella trivolvis* (a) ash weight and (b) whole snail Ca in the four water Ca concentrations in the day 60 < MSW group. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

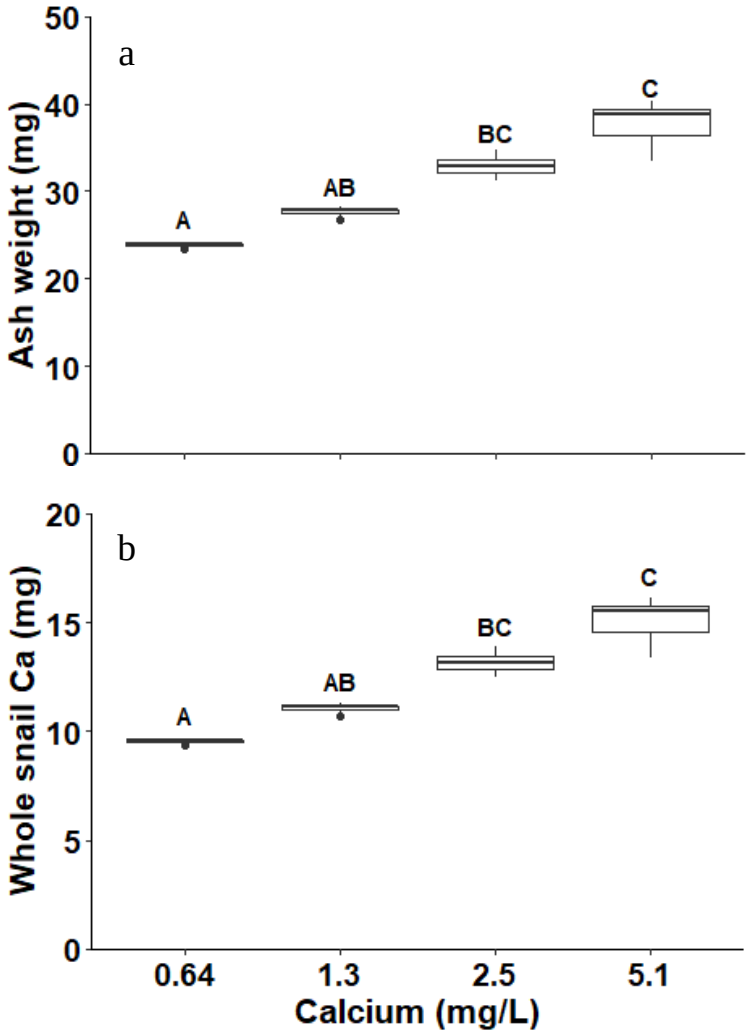

Figure 11: *Planorbella trivolvis* (a) ash weight and (b) whole snail Ca in the four water Ca concentrations in the day 60 ≥ MSW group. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

Ash-free dry weight

Water Ca concentration did not have a statistically significant effect on AFDW (see Appendix E for descriptive statistics). There was not a significant difference in mean AFDW between Ca treatments on day 30 (one-way ANOVA, $F_{(3,12)} = 0.1157$, $p = 0.942$, Figure 12). Also, there was no significant difference in AFDW ranks between treatments on day 60 (Kruskal-Wallis, $H(3) = 4.465$, $p = 0.215$, Figures 12), or in the day 60 < MSW group (Kruskal-Wallis, $H(3) = 0.542$, $p = 0.910$), or in the day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 1.803$, $p = 0.164$).

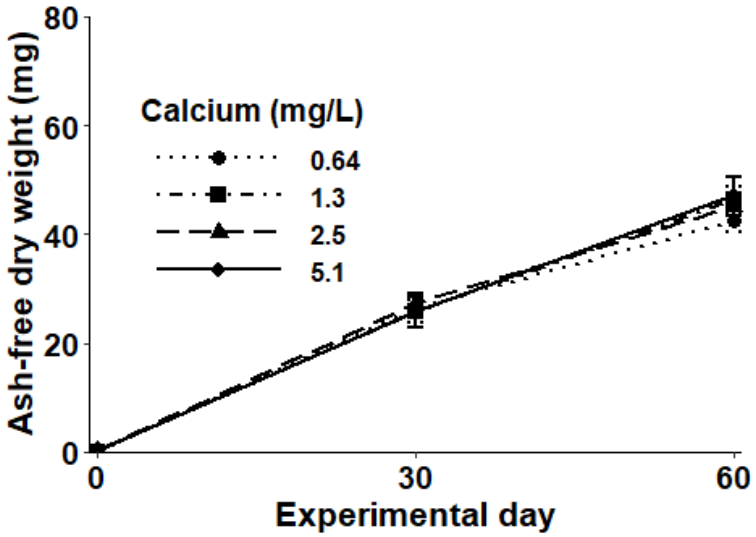

Figure 12: Growth curves of *P. trivolvis* mean AFDW in the four water Ca concentrations over 60 days. Error bars represent standard errors.

Percent Calcium Dry Weight

The proportion of Ca relative to dry weight differed significantly between treatments on day 30 (one-way ANOVA, $F_{(3,12)} = 18.220$, $p < 0.001$, Figure 13, Tables 6, 7). Snails in 0.64 Ca had significantly lower % Ca dry weight than snails in Ca 2.5 (*post hoc*, Tukey HSD, $p = 0.002$, Figure 14) and Ca 5.1 (*post hoc*, Tukey HSD, $p < 0.001$, Figure 14). Snails in Ca 1.3 had significantly lower % Ca dry weight than snails in Ca 5.1 (*post hoc*, Tukey HSD, $p = 0.002$, Figure 14). Snails in Ca 2.5 had higher % Ca dry weight than snails in Ca 1.3, but the difference was almost significant (*post hoc*, Tukey HSD, $p = 0.067$, Figure 14). Snails in the two lowest Ca waters (Ca 0.64 and Ca 1.3) did not differ significantly in their % Ca dry weight (*post hoc*, Tukey HSD, $p = 0.216$, Figure 14). Likewise, snails in the two highest Ca waters (Ca 2.5 and Ca 5.1) did not differ significantly in their % Ca dry weight (*post hoc*, Tukey HSD, $p = 0.253$, Figure 14).

On day 60, % Ca dry weight was significantly different between treatments (Kruskal-Wallis, $H(3) = 30.856$, $p < 0.001$, Tables 6, 8). Pairwise comparison of ranks indicated that snails in the Ca 0.64 treatment had significantly lower % Ca dry weight than snails in both Ca 2.5 and Ca 5.1 (Dunn's test, $p < 0.001$, Figure 15), and snails in the Ca 1.3 treatment had significantly lower % Ca dry weight than snails in both Ca 2.5 (Dunn's test, $p = 0.030$, Figure 15) and Ca 5.1 (Dunn's test, $p = 0.001$, Figure 15). As on day 30, snails in the two lowest Ca waters did not have a significant difference in % Ca dry weight, and neither did snails in the two highest Ca waters (Dunn's test, $p = 1.000$, Figure 15).

Snails in the day 60 < MSW group had significant differences in % Ca dry weight between treatments paralleling snails on day 30 (Kruskal-Wallis, $H(3) = 21.901$, $p < 0.001$, Tables 6, 8). Pairwise comparison of ranks indicated that snails in the 0.64 Ca treatment had

significantly lower % Ca dry weight than snails in the 2.5 Ca treatment (Dunn's test, $p = 0.004$, Figure 16a) and 5.1 Ca treatment (Dunn's test, $p < 0.001$, Figure 16a). Snails in the 1.3 Ca treatment had significantly lower % Ca dry weight than snails in the 5.1 Ca treatment (Dunn's test, $p = 0.025$, Figure 16a).

Snails in the day 60 ≥ MSW group had significant differences in % Ca dry weight between treatments paralleling snails on day 60 (Kruskal-Wallis, $H(3) = 23.084$, $p < 0.001$, Tables 6, 8). Pairwise comparison of ranks indicated that snails in the 2.5 Ca treatment had significantly higher % Ca dry weight than snails in the 0.64 Ca treatment (Dunn's test, $p = 0.003$, Figure 16b) and 1.3 Ca treatment (Dunn's test, $p = 0.040$, Figure 16b). Snails in the 5.1 Ca treatment had significantly higher % Ca dry weight than snails in the 0.64 Ca treatment (Dunn's test, $p < 0.001$, Figure 16b) and 1.3 Ca treatment (Dunn's test, $p = 0.006$, Figure 16b).

Among all treatments, snails in the day 60 < MSW group had two-percent higher % Ca dry weight than snails in the day 60 ≥ MSW group, and this difference was significant (Wilcoxon, $W = 1116$, $p < 0.001$, Table 6, Figure 16). Additionally, there was a significant difference in % Ca dry weight between the day 60 < MSW and day 60 ≥ MSW groups within each treatment (Figure 16). In the 0.64 Ca treatment, smaller snails (day 60 < MSW group) had significantly higher % Ca dry weight than larger snails (day 60 ≥ MSW group) (Wilcoxon, $W = 70$, $p < 0.001$). This pattern existed for all treatments with smaller snails having significantly more Ca per mg of dry weight than larger snails in 1.3 Ca (Wilcoxon, $W = 98$, $p = 0.002$), 2.5 Ca (Wilcoxon, $W = 82$, $p < 0.001$), and 5.1 Ca (Wilcoxon, $W = 103$, $p < 0.001$).

In addition to the differences in % Ca dry weight between treatments on days 30 and 60, the change in % Ca dry weight over time differed between treatments (Figure 13, see Appendix F). In general, all but the snails in the 5.1 Ca treatment lost Ca during days 0 to 30 but gained Ca

for the second part of the experiment from days 30 to 60. Simple linear regression showed that for snails in 0.64 Ca between days 0 and 30, the regression was significant ($F_{(1,13)} = 40.730$, $p < 0.001$) with an R^2 of 0.758 and snails lost 0.092% Ca dry weight (negative slope) for each day between days 0 and 30 but gained 0.186% Ca dry weight per day between days 30 and 60 as indicated by a significant regression equation ($F_{(1,20)} = 33.520$, $p < 0.001$) with an R^2 of 0.626 and a positive slope (Table 9). For snails in 1.3 Ca between days 0 and 30, the regression equation ($F_{(1,13)} = 14.930$, $p = 0.002$) had an R^2 of 0.535 and the snails lost 0.057% Ca dry weight for each day between days 0 and 30 but gained 0.174% Ca dry weight per day between days 30 and 60 as indicated by a significant regression equation ($F_{(1,23)} = 25.950$, $p < 0.001$) with an R^2 of 0.530 and a positive slope (Table 9). Snails in 2.5 Ca lost 0.008% Ca dry weight for each day between days 0 and 30, but the regression equation was not significant ($F_{(1,13)} = 0.263$, $p = 0.617$) with an R^2 of 0.020. The snails in 2.5 Ca gained 0.182% Ca dry weight per day between days 30 and 60 as indicated by a significant regression equation ($F_{(1,21)} = 46.330$, $p < 0.001$) with an R^2 of 0.688 and a positive slope (Table 9). Snails in 5.1 Ca had an increase of 0.028% Ca dry weight per day between days 0 and 30, but the regression equation was not significant ($F_{(1,13)} = 2.315$, $p = 0.152$) with an R^2 of 0.151. Between days 30 and 60, snails in 5.1 Ca had an increase of 0.179% Ca dry weight per day indicated by a significant regression equation ($F_{(1,23)} = 10.900$, $p = 0.003$) with an R^2 of 0.322 and positive slope (Table 9).

Table 6: Descriptive statistics for the percent Ca dry weight of all snail groups throughout the experiment.

Ca dry weight (%)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	11.558	11.597	9.835	12.864	0.832	0.251
Day 30:	Ca 0.64	4	8.811	8.760	8.617	9.109	0.217	0.108
	Ca 1.3	4	9.842	9.797	9.407	10.367	0.445	0.223
	Ca 2.5	4	11.314	11.064	10.723	12.405	0.746	0.373
	Ca 5.1	4	12.411	11.965	11.414	14.299	1.300	0.650
	(all)	16	10.595	10.545	8.617	14.299	1.586	0.396
Day 60:	Ca 0.64	18	14.398	13.948	12.193	20.125	1.891	0.446
	Ca 1.3	21	15.075	15.073	10.858	19.810	2.011	0.439
	Ca 2.5	19	16.782	16.483	15.260	21.819	1.548	0.355
	Ca 5.1	21	17.769	16.820	15.449	29.733	3.150	0.687
	(all)	79	16.047	15.967	10.858	29.733	2.593	0.292
Day 60 < MSW:	Ca 0.64	12	15.184	14.711	13.136	20.125	1.844	0.532
	Ca 1.3	11	16.137	15.996	14.438	19.810	1.427	0.430
	Ca 2.5	7	18.201	17.623	16.505	21.819	1.745	0.660
	Ca 5.1	9	19.812	17.767	17.278	29.733	3.985	1.328
	(all)	39	17.063	16.505	13.136	29.733	2.956	0.473
Day 60 ≥ MSW:	Ca 0.64	6	12.826	12.780	12.193	13.487	0.478	0.194
	Ca 1.3	10	13.906	13.750	10.858	18.391	1.958	0.619
	Ca 2.5	12	15.955	15.896	15.260	16.752	0.479	0.138
	Ca 5.1	12	16.237	16.140	15.449	18.120	0.715	0.206
	(all)	40	15.058	15.650	10.858	18.391	1.704	0.269

Table 7: One-way ANOVA for the percent Ca dry weight of all snails on day 30.

% Ca dry weight ANOVA					
	Effect	df	MS	F	p-value
Day 30	Ca concentration	3	0.003	18.220	< 0.001
	Residuals	12	< 0.001		

Table 8: Mean % Ca dry weight ranks for all four treatments on day 60 and in the day 60 < MSW and ≥ MSW groups.

% Ca dry weight ranks		
	Treatment	Mean rank
Day 60	Ca 0.64	21.000
	Ca 1.3	30.286
	Ca 2.5	50.684
	Ca 5.1	56.333
Day 60 < MSW	Ca 0.64	10.417
	Ca 1.3	16.091
	Ca 2.5	28.714
	Ca 5.1	30.778
Day 60 ≥ MSW	Ca 0.64	5.833
	Ca 1.3	12.5
	Ca 2.5	26.083
	Ca 5.1	28.917

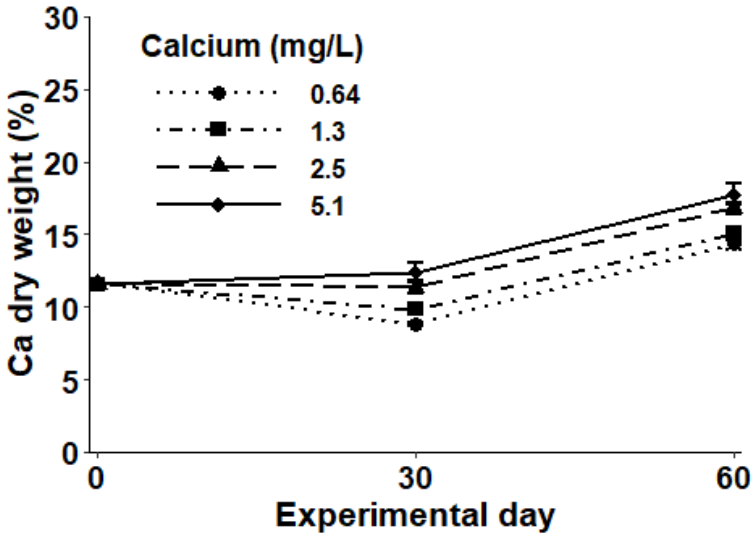

Figure 13: Growth curves of *P. trivolvis* mean % Ca dry weight in the four water Ca concentrations over 60 days. Error bars represent standard errors.

Table 9: Slopes of the linear regression lines between days 0 and 30 and 30 and 60 in figure 19. See Appendix F for linear regression equations.

% Ca dry weight slopes				
	Treatment			
	0.64 Ca	1.3 Ca	2.5 Ca	5.1 Ca
Days 0 – 30:	-0.092	-0.057	-0.008	0.028
Days 30 – 60:	0.186	0.174	0.182	0.179

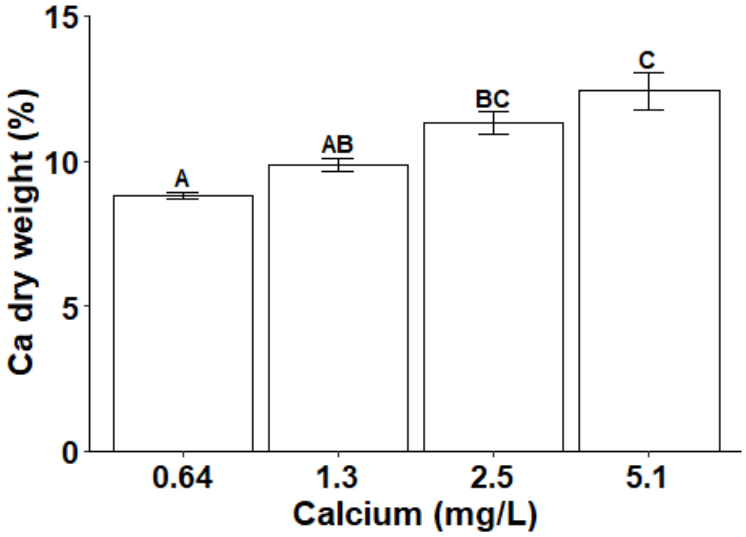

Figure 14: *Planorbella trivolvis* % Ca dry weight on day 30 in the four water Ca concentrations. Error bars represent standard errors.

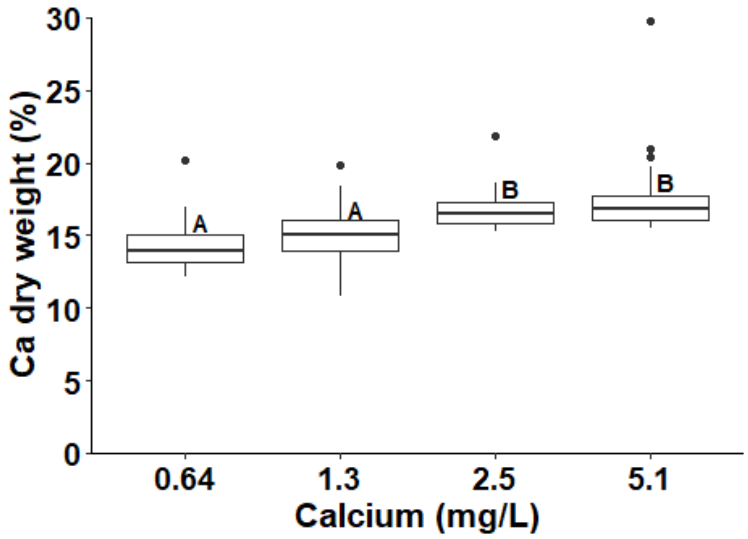

Figure 15: *Planorbella trivolvis* % Ca dry weight on day 60 in the four water Ca concentrations. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

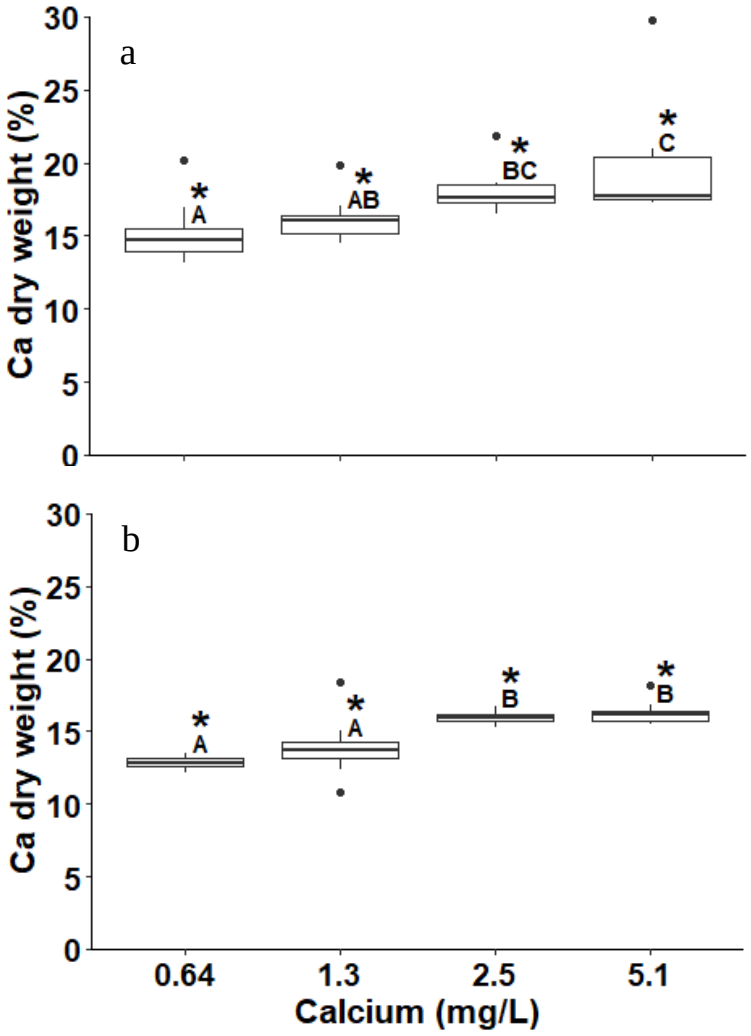

Figure 16: *Planorbella trivolvis* % Ca dry weight in (a) the day 60 < MSW group and (b) day 60 ≥ MSW group in the four water Ca concentrations. Asterisks indicate significance when comparing the day 60 < MSW to day 60 ≥ MSW groups within each treatment. Different letters indicate significant differences between treatments. In each boxplot, the horizontal thick black line is the median, the horizontal thin black lines are the upper and lower quartiles, the tips of the vertical thin black lines are the minimum and maximum values that are not outliers, and the black circles are outliers.

Water pH Change

The pH of the used media by randomly selected snails was measured at random intervals before day 30, and at each water change (every 48 hours) beginning on day 30. On day 30, there was a statistically significant difference in the pH of used water between treatments (one-way ANOVA, $F_{(3,12)} = 30.990$, $p < 0.001$, Figure 17, Table 10, see Appendix E). The 5.1 Ca water acidified significantly more than the water in 0.64 Ca, 1.3 Ca, and 2.5 Ca (*post hoc*, Tukey HSD, $p \leq 0.004$, Figure 18a). The 2.5 Ca water also acidified significantly more than 0.64 Ca and 1.3 Ca waters (*post hoc*, Tukey HSD, $p \leq 0.032$, Figure 18a).

On day 60, there was a statistically significant difference in 48-hour used water between all Ca treatments (one-way ANOVA, $F_{(3,75)} = 97.033$, $p < 0.001$, Figure 17, Table 10, see Appendix E). The 5.1 Ca water acidified more than the water in 0.64 Ca, 1.3 Ca, and 2.5 Ca (*post hoc*, Tukey HSD, $p \leq 0.002$, Figure 18b, Table 17). The other treatments were also significantly different in acidification levels (*post hoc*, Tukey HSD, $p < 0.001$, Figure 18b).

In the two size subgroups, these differences in water pH were maintained. There was a statistically significant difference in 48-hour used water between Ca treatments in the day 60 < MSW group (one-way ANOVA, $F_{(3,35)} = 22.695$, $p < 0.001$, Figure 18c, Table 10, see Appendix E) as well as in the day 60 ≥ MSW group (one-way ANOVA, $F_{(3,36)} = 289.230$, $p < 0.001$, Figure 18d, Table 10, see Appendix E)

The change in pH over time differed between treatments (Figure 17, see Appendix F). A simple linear regression was created for each Ca treatment to predict pH based on the experimental days on which the used water pH was measured. For the 0.64 Ca water, the regression equation was significant ($F_{(1,100)} = 20.190$, $p < 0.001$) with an R^2 of 0.168. Snails in the 0.64 Ca water caused a -0.003 pH-unit change per day during the experiment (Table 11). For

the 1.3 Ca water, the regression equation was also significant ($F_{(1,103)} = 12.120$, $p < 0.001$) with an R^2 of 0.105. Snails in the 1.3 Ca water caused a -0.003 pH-unit change per day during the experiment (Table 11). For the 2.5 Ca water, the regression equation was also significant ($F_{(1,101)} = 10.560$, $p = 0.002$) with an R^2 of 0.095. Snails in the 2.5 Ca water caused a -0.003 pH-unit change per day during the experiment (Table 11). For the 5.1 Ca water, the regression equation was also significant ($F_{(1,102)} = 4.245$, $p = 0.042$) with an R^2 of 0.040. Snails in the 5.1 Ca water caused an increase of 0.002 pH-units per day during the experiment (Table 11).

Table 10: One-way ANOVA for the used-media pH of all snails on day 30 and day 60 and in the day 60 < MSW and day 60 ≥ MSW groups.

pH ANOVA					
	Effect	df	MS	F	p-value
Day 30	Ca concentration	3	0.764	30.990	< 0.001
	Residuals	12	0.025		
Day 60	Ca concentration	3	2.320	97.033	< 0.001
	Residuals	75	0.024		
Day 60 < MSW	Ca concentration	3	0.899	22.695	< 0.001
	Residuals	35	0.040		
Day 60 ≥ MSW	Ca concentration	3	1.418	289.230	< 0.001
	Residuals	36	0.005		

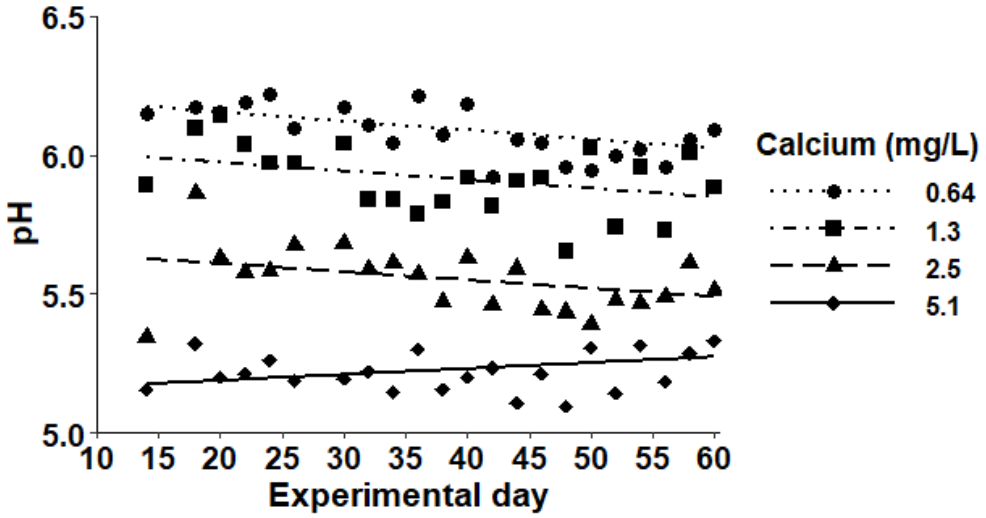

Figure 17: pH of 48-hour used experimental water for each treatment. Linear regression lines are fit individually for each treatment. The pH of fresh media averaged 6.5.

Table 11: Slopes of the linear regression lines for all four treatments in Figure 17. See Appendix F for linear regression equations.

pH linear regression slopes				
	Treatment			
	0.64 Ca	1.3 Ca	2.5 Ca	5.1 Ca
Slope:	-0.003	-0.003	-0.003	0.002

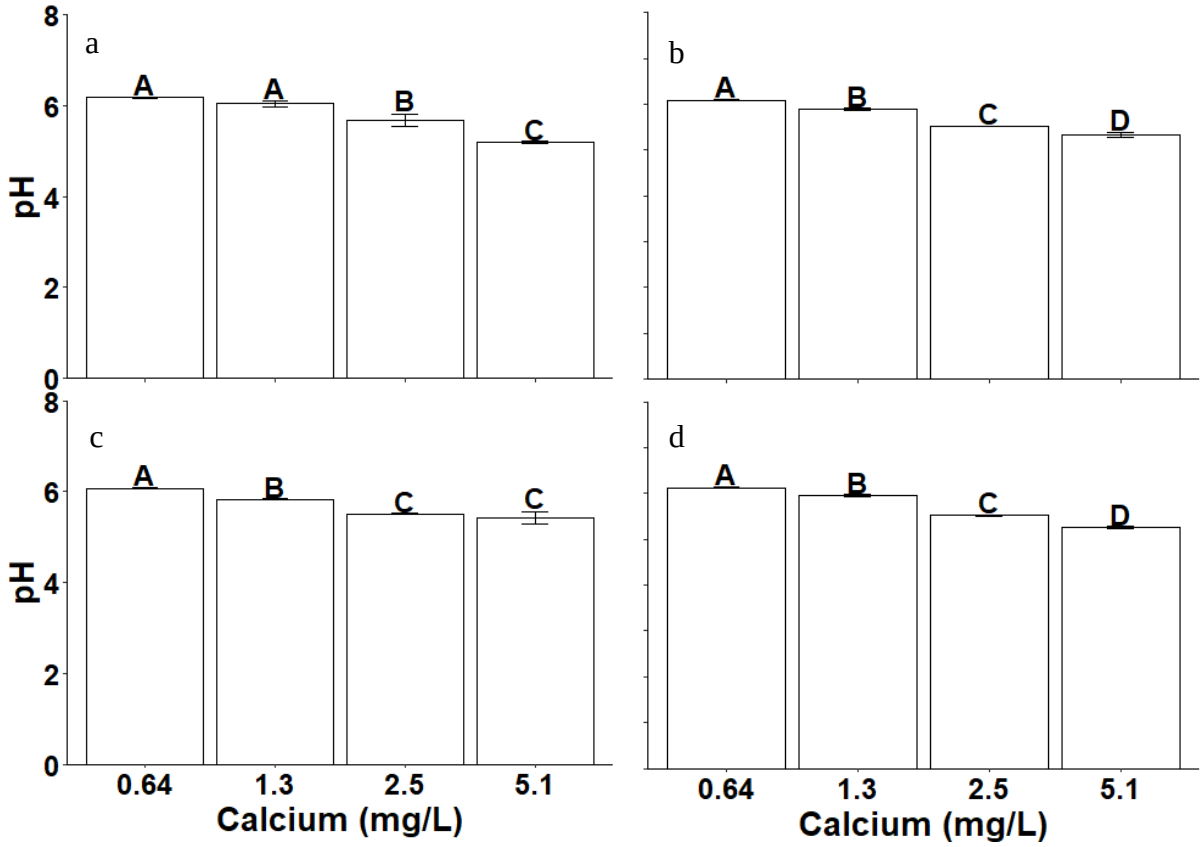

Figure 18: pH of 48-hour old water for each treatment on (a) day 30 and (b) day 60 and in (c) the day 60 < MSW group and (d) day 60 ≥ MSW group. Error bars represent standard error.

Discussion

Shell biomineralization of freshwater snails is dependent on a variety of environmental factors but, because Ca is the most abundant cation in the shell and is actively taken up from the external medium, the availability of sufficient Ca in the water is a crucial factor for shell calcification and growth (Van Der Borght & Van Puymbroeck, 1966; Greenaway, 1971; Young, 1975; Marxen et al., 2003; Marin & Luquet, 2004; White et al., 2007; Ponder et al., 2019). My experiment was designed to study the effects of decreasing water Ca concentration on the shell calcification and growth of *Planorbella trivolvis*. My results indicate that declining water Ca negatively affects *P. trivolvis* shell calcification and the impact will vary depending on snail size.

Shell Size and Organic Body Mass

Although a snail's shell requires a high input of Ca for its formation, surprisingly shell size (independently of its thickness and Ca content) was not affected by water Ca concentration in my experiment, contrary to my hypothesis. Shell width, shell height, and aperture height were not different among treatments with different Ca concentrations at any time during the experiment. This suggests that when grown in waters characteristic of softwater lakes, *P. trivolvis* strives to biomineralize a shell at a given rate regardless of water Ca availability. These results conflict with the results of Hunter (1990), when the shell of adult *P. trivolvis* grew less per day in low Ca water (~2 mg/L Ca) than in high Ca water (~60 mg/L Ca). However, Hunter (1990) placed adults from a high Ca environment into low Ca water and measured growth over time, rather than rearing *P. trivolvis* from juvenile to adult stage in treatment Ca concentrations as in my experiment. It is possible that adult snails' growth rates are more affected by low Ca water, however, snails in decalcified northern lakes will reside in the same water Ca

concentration for their entire lifespan, and relationships between snail Ca content and the environment are more valid under active growing periods (Mackie & Flippance, 1983). Therefore, my experiment better predicts the effects of low water Ca on snail shell growth rate because snails were reared in low Ca water from the juvenile to adult stage. However, Brodersen & Madsen (2003) found that *Biomphalaria sudanica*, also a pulmonate snail with a planispiral shell, was significantly smaller as an adult when raised in low water Ca concentrations than high water Ca concentrations (seven treatments ranging from 3.6 mg/L Ca to 360 mg/L Ca). In my experiment, three treatments contained water with less Ca than the lowest in Brodersen & Madsen (2003); therefore, it is possible if my experiment included more than one treatment with greater than 3.6 mg/L Ca, a difference in snail shell growth between treatments may have been observed. However, softwater lakes rarely have Ca concentrations higher than 3.6 mg/L, and freshwaters with Ca concentrations ≤ 4.0 mg/L predominate in the world's lakes, with over 20% of lakes having Ca concentrations ≤ 1.5 mg/L and continuing to decline (Jeziorski et al., 2008; Jeziorski & Smol, 2017; Weyhenmeyer et al., 2019). My results suggest that *P. trivolvis* can reach its maximum size, within the range of Ca concentrations characteristic of soft water lakes, irrespective of water Ca availability. Fragile snail shells of adult animals (unknown species) have been observed in Gull Lake (Ontario, Canada), where Ca has been steadily declining (A. Pérez-Fuentetaja, *personal communication*). Therefore, declines in water Ca below *P. trivolvis*' critical threshold will not impact adult shell size, although the thickness of the shell would likely be reduced.

Wet weight consists of the entire snail, including the calcified shell, organic tissues, and moisture. Hunter (1990) found that when *P. trivolvis* was reared in high Ca water (~60 mg/L Ca), live weight (equal to wet weight in my experiment) increased more per day than when

reared in low Ca water (~2 mg/L Ca), only when water pH was circumneutral to slightly acidic. In my experiment, the difference between the highest and lowest Ca treatments is four-fold, rather than 30-fold in Hunter (1990), which could explain why water Ca did not impact snail wet weight in my treatments. My results suggest that in the Ca concentrations characteristic of soft water lakes, *P. trivolvis* can reach its maximum potential wet body mass.

The measurement of dry weight includes the inorganic and organic body mass with moisture removed. Snail dry weight was negatively affected by water Ca concentration with an increasing effect over time. Water Ca concentration did not affect dry weight on day 30 of my experiment, but on day 60 snails in the lowest Ca water (0.64 Ca) had lower dry weights than snails in the two highest Ca waters (2.5 Ca and 5.1 Ca). My results are similar to the results of Brodersen and Madsen (2003) when the dry weight of *B. sudanica* increased with increasing Ca concentration. However, Brodersen and Madsen (2003) found a linear relationship between dry weight and Ca concentrations when snails were reared in seven different treatments with Ca ranging from 3.6 to 360 mg/L. My experiment included three Ca concentrations lower than theirs, so it is possible that the linear relationship between water Ca concentration and dry weight becomes disrupted below a certain Ca threshold. As expected, larger snails within my experiments were more affected by low Ca than smaller snails. On day 60, snails with a shell width greater than the median shell width of the population required a higher concentration of water Ca because snails reared in 5.1 mg/L Ca had significantly higher dry weights than snails in 0.64, 1.3, and 2.5 mg/L Ca. There was no difference in dry weight between Ca treatments in the smaller snail population containing all snails with shell widths less than the median shell width. My results suggest that the dry weight of larger snails was more affected by low water Ca than that of smaller snails.

Organic dry weight (ash-free dry weight) consists of all dry organic components, including body muscles, tissues, and a small amount of shell proteins, and is the most accurate predictor of biologically active tissue mass for snails (Zhao et al., 2009; Eklöf et al., 2017). AFDW was not affected by water Ca concentration, contrary to my hypothesis (Figure 19). Since there was no difference in AFDW between treatments at any time during my experiment, my results suggest that muscle and tissue development are not controlled by the availability of water Ca within waters characteristic of softwater lakes. This contradicts the results of Brodersen and Madsen (2003) which found a positive linear relationship between AFDW and water Ca concentration in *B. sudanica*, which was suspected to be from abundant Ca not limiting other physiological processes. Explanations for the disrupted relationship in my experiment include interspecific differences and the much lower water Ca concentrations used in my experiment. Moreover, there is inter- and intraspecific variation in shell calcification of freshwater molluscs across the lakes in the Canadian Shield that is strongly, and most often positively, correlated to the Ca availability in the water (Nduku & Harrison, 1976; Mackie & Flippance, 1983). My results suggest that, within the Ca levels characteristic of soft water lakes, organic growth is constant over time regardless of how little Ca is available in the water. Therefore, since there was no treatment difference in the organic dry weight of the snails on day 60, the differences in total dry weights among treatments can be explained by a difference in inorganic material ($CaCO_3$) in the shell.

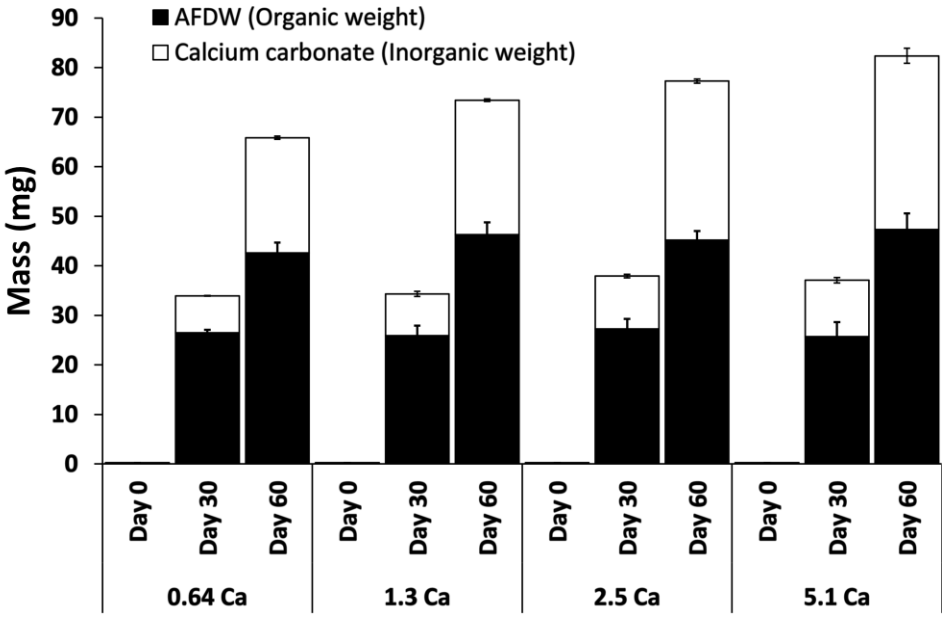

Figure 19: Comparison of the organic weight (AFDW) and inorganic weight (ash) of snails from the four Ca treatments over the 60-day experiment. Error bars represent standard errors.

Inorganic Body Mass and Shell Calcification

In freshwater snails, ash (inorganic dry weight) is 95% or more $CaCO_3$, of which the majority is in the shell, and since 40% of the ash is Ca, ash weight can assess shell calcification (Hunter & Lull, 1977; Glass & Darby, 2009). In my experiment, ash weight is analogous to shell $CaCO_3$, and whole snail Ca is analogous to shell Ca. My results indicate that whole snail Ca is affected by water Ca concentration with an increasing effect over time (Figure 7b). On day 30, snails in the two highest Ca concentrations had more shell Ca than snails in the two lowest Ca media. The negative effect of low Ca increased by day 60 with snails reared in 0.64 mg/L Ca water having less shell calcium than snails in all other treatments. Furthermore, snails in 1.3 mg/L Ca water had less shell Ca than snails in the two highest Ca treatments, which had similar amounts of shell Ca. My results suggest that shell calcification of *P. trivolvis* is negatively

affected by water containing less than 2.5 mg/L Ca with the effect becoming greater with declining Ca and age. Glass and Darby (2009) found that *Pomacea paludosa*, the Florida apple snail, can sequester sufficient Ca from water containing 7 mg/L Ca, but there is no significant benefit to shell Ca content when water Ca is increased to 70 mg/L, although shell growth was faster. In a separate experiment, there was lower shell Ca in *P. paludosa* grown in 3.6 mg/L Ca water (compared to 33.9 mg/L Ca), but the independent effects caused by the lower pH of this softer water were not investigated. Glass and Darby (2009) propose that reduced shell Ca, despite continued shell growth, may have been caused by low water pH (accompanying low water Ca) resulting in shell erosion. In my experiment, pH differed with water Ca concentration because of changes caused by the presence of live snails, and the pH decreased more with the more Ca that was available in the water. Adult snails are tolerant of short-term low pH (Hunter, 1990); therefore, differences in shell calcification among treatments in my experiment were caused by differences in water Ca availability. Furthermore, my experiment stressed snails by testing Ca levels known to be critically low to aquatic organisms which could explain why *P. trivolvis* benefitted from increases in water Ca. Brodersen and Madsen (2003) found comparable results to mine with *B. sudanica*, in that inorganic dry weight increased linearly with Ca concentration. In my experiment, mean ash weight increased with increasing water Ca, but there was not a significant difference between the two highest Ca treatments. A possible explanation for this is that the Ca threshold for *P. trivolvis* is between 2.5 and 5.1 mg/L Ca, and a slight deviation from the threshold does not result in significantly better shell calcification.

Shell calcification differed within the experimental population with larger snails having a higher demand for water Ca than smaller snails. When comparing the same Ca concentration treatments between the two size groups (Day 60 < MSW and Day 60 ≥ MSW), snails calcified

similarly regardless of shell size, but the Ca threshold for shell calcification differed between small and large snails. The smaller snails in the population on day 60 did not grow a less calcified shell unless reared in water containing less than 1.3 mg/L Ca (Figure 10b). The larger snails grew a less calcified shell in all water below 2.5 mg/L Ca (Figure 11b). My results suggest that, within the Ca levels characteristic of softwater lakes, snails in water with Ca concentrations less than 2.5 mg/L will have less calcified shells, and the demand for Ca for shell calcification increases with increasing shell size. Since smaller snails require less Ca to grow a shell with an amount of Ca comparable to larger snails, soft water lakes with declining water Ca may experience a population shift towards smaller snails.

Percent Ca dry weight is the amount of Ca per mg of dry body weight, and this percentage differed among treatments and over time. Snails in the two lowest Ca treatments had a decrease in % Ca dry weight between days 0 and 30. This can be explained by a reduction in water Ca availability from the original beaker, where they grew for 18 days prior to the beginning of the experiment and had a concentration of 2.5 mg/L Ca. The snails entering the 0.64 Ca and 1.3 Ca treatments were stressed due to the sudden lower water Ca availability, as their metabolism was already adapted for growth in 2.5 mg/L Ca since hatching. In contrast, snails grown in the 2.5 Ca treatment had a slight decrease in % Ca dry weight, but this loss was not significant, suggesting that the water Ca threshold for *P. trivolvis* is slightly above 2.5 mg/L Ca. Hunter (1990) proposed that low Ca levels (1-2 mg/L) are lethal to *P. trivolvis*, especially juveniles. My experiment refutes this finding because no snails in 0.64 Ca or 1.3 Ca died due to the experimental conditions. Nduku and Harrison (1976) found that *Biomphalaria pfeifferi* reached its physiological limit for extraction of Ca from the water at 2.0 mg/L Ca, but lower water Ca concentrations were partially masked by an increase in media Ca concentration due to

Ca leaching from the lettuce that was provided as food. It is possible that snails in my 0.64 Ca and 1.3 Ca treatments benefitted from additional water Ca that leached from the algae wafers they were fed. However, my treatments were effective at stressing the snails as shown by snails in both 0.64 Ca and 1.3 Ca having less shell Ca than the snails in the two higher Ca treatments (Figure 9b). Although water Ca levels may have been slightly elevated from the expected values due to Ca leaching from the algae wafer, both 0.64 Ca and 1.3 Ca water would have been elevated by the same amount, yet there was still a difference in calcification among treatments.

Hunter (1990) identified the critical Ca threshold for *P. trivolvis* to be less than 2.0 mg/L which is comparable to my findings. On day 30, snails in all the treatments had similar % Ca dry weight. However, the amount of Ca relative to dry weight increased more with increasing water Ca concentration up to 2.5 mg/L, suggesting that the Ca threshold for *P. trivolvis* is around 2.5 mg/L Ca.

Against my expectations, smaller snails in the experimental population had, on average, greater than two percent more Ca per mg of dry weight than larger snails. This trend was consistent among all Ca treatments, with the mean % Ca dry weight difference between size groups greatest in the highest water Ca concentration (Table 6, Figure 16). This suggests that small snails biomineralize a shell with more Ca per mg of dry weight than larger snails do, regardless of water Ca concentration, and can do so with less Ca available in the water. Larger snails required a two-fold increase in water Ca concentration to have a mean % Ca dry weight similar to that of smaller snails. Smaller snails required only 0.64 mg/L Ca to have a % Ca dry weight > 15%, whereas larger snails required 2.5 mg/L Ca for the same level of calcification. Likewise, larger snails required 5.1 mg/L Ca to have a % Ca dry weight > 15%, but smaller snails obtained this proportion of shell Ca relative to dry weight in 1.3 mg/L Ca water. Brodersen

and Madsen (2003) found that the crush weight of *B. sudanica* increased with both inorganic weight and shell diameter (equal to shell width in my experiment). Likewise, the crush weight of *P. paludosa* was positively correlated to the amount of Ca in the shell and shell size (Glass & Darby, 2009). A higher percentage of Ca (as in the smaller snails) should represent a stronger shell in *P. trivolvis*, but the larger snails may be more difficult to consume despite being less calcified. Larger snails experience a higher encounter rate from predators than smaller snails, but larger snails have some protection from predation due to predator gape limitations (Brodersen & Madsen, 2003). Therefore, while smaller *P. trivolvis* may be better protected against damage and possibly predation in low Ca waters from having a more calcified shell, larger snails may persist due to protection associated with their greater shell size.

During the experiment, the pH of the water was measured at different times. Among all treatments and throughout the duration of the experiment, snails in higher Ca waters caused the water to become more acidic over 48 hours. Adult snails are relatively tolerant to short-term exposures to low pH, and sublethal effects, such as shell erosion, do not occur until longer term exposure (Hunter, 1990), so the short-term acidification in my experiment should not have altered my results. The difference in pH between the lowest and highest Ca treatments decreased as the experiment progressed. The most likely explanation for these pH decreases with increasing Ca in the water is a difference in the snails' metabolic activity. Snails with sufficient Ca were more metabolically active biomineralizing their shell compared to snails with limited water Ca. This idea is supported by snails having higher % Ca dry weight and ash weight in higher Ca water. As snails take up Ca to biomineralize their shell, ammonia (NH_3) and hydrogen (H^+) ions are released which are acidic and lower the water pH (Thomas et al., 1976). In low Ca water, low bicarbonate (HCO_3^+) concentration results in a pH drop as snails respire and give off carbon

dioxide (Nduku & Harrison, 1976). In addition, NH_3 production increases with increasing snail body mass, as it is the main excretory product of aquatic snails and does not result in negative growth effects at low concentrations (< 1 ppm) (Thomas et al., 1976). However, there was no difference in biologically active tissue (AFDW) among treatments, or large amounts of ammonia produced by snails in the higher Ca treatments (see Appendix C), so it is unlikely for the pH change to be caused by larger snails producing more ammonia.

Conclusion

My research demonstrates that *Planorbella trivolvis* is sensitive to further declines in water Ca concentration in softwater lakes. Although *P. trivolvis* can reach maximum shell size and organic body mass regardless of water Ca availability, the shell is less calcified and contains less Ca per mg of dry weight with decreasing ambient Ca availability. Interestingly, smaller snails had more shell Ca relative to dry weight than larger snails did in the same water Ca concentration, which suggests that with continued declines in water Ca concentration, there may be a trade-off between shell size and shell calcification with smaller snails in the population requiring less water Ca to biomineralize a shell with a comparable amount of Ca to larger snails. Therefore, the snail community in softwater lakes may shift towards smaller snails that have less demand for water Ca, but larger snails may persist because their size provides some protection against predation, although larger snails have higher encounter rates with predators (Brodersen & Madsen, 2003). Applying these results broadly to other snail species suggests that, with further declines in water Ca, the snail community may become composed of one or two size classes: smaller snails that have tougher shells or, larger snails able to avoid predation due to predator gape limitations.

Interestingly, fish that ingest snails often do so without crushing the shell. In the case of consuming small snails, the inorganic and indigestible shell material would use stomach space that would prevent the ingestion of better quality food (Brodersen & Madsen, 2003). Additionally, the swim bladder will have to increase in size to mitigate the negative buoyance resulting from the higher density of shell material compared to organic material, leaving even less stomach space available (Brodersen & Madsen, 2003). Because of this, snails from low Ca lakes may have an ideal small size for fish predators, but there may be nutritional consequences resulting from their consumption. Also, if fish predation were heavier on small snails in low Ca lakes, this selection pressure could result in a size shift to larger snails.

Snails play an important role in nutrient cycling in freshwater habitats because they are generalist feeders and involved in the breakdown of organic matter through the consumption of algae and detritus (Covich et al., 1999; Lombardo & Cooke, 2002; Osborne et al., 2020). In my experiment, organic tissue development was not affected by continued declines in water Ca availability; therefore, regardless of water Ca concentration, snails will develop their normal body mass.

Overall, my research has demonstrated that species-level effects in snails will occur due to declines in Ca concentrations in softwater lakes. How these effects will cascade through the food web will depend on predator-prey interactions and the strength of the connections in the aquatic community.

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Appendix A

Each snail was fed an equal portion (+/- 0.3 mg) of crushed algae wafer after each water change. The quantity of food was increased for all snails when at least one snail had less than approximately 10% of the previous feedings' food remaining.

Daily algae wafer aliquot			
Day	Mass (mg)	Day	Mass (mg)
0	3.5	30	18
2	3.5	32	20
4	3.5	34	20
6	4	36	20
8	4.5	38	20
10	6	40	20
12	7	42	22
14	9	44	22
16	10	46	22
18	11	48	24
20	12	50	24
22	14	52	24
24	15	54	24
26	16	56	24
28	17	58	24

Appendix B

To estimate evaporation from the experimental beakers, three 100-mL glass beakers containing 50 mL of deionized water, or three 236-mL borosilicate glass jars containing 100 mL or 150 mL of deionized water, were placed in random locations in the experimental chamber on random days. The beaker or jar and the volume of deionized water matched the experimental design on that day of the experiment. Deionized water was added to the beaker or jar which was then weighed on a standard laboratory balance. Since deionized water has a density of 1.0 g/mL, 1.0 g loss is equivalent to 1.0 mL loss from the beaker. The mass of each beaker and the day it was weighed is listed in the table below, along with the average volume lost in 48 hours. The volume lost in 48 hours was divided by two to estimate the volume lost in 24 hours. The volume lost from the 100 mL and 150 mL was divided by two and three, respectively, to estimate the volume lost per 50 mL of deionized water.

Evaporative loss from incubator (50 mL)

Replicate	Day 14 mass (g)	Day 16 mass (g)	Day 18 mass (g)	Mean loss per 48 hr (g)
1	98.364	95.826	92.482	2.941
2	105.408	100.536	97.521	3.944
3	106.746	104.385	97.593	4.576
			Mean 48-hour loss (per 50 mL):	3.820 mL
			Mean 24-hour loss (per 50 mL):	1.910 mL

Evaporative loss from incubator (100 mL)

Replicate	Day 28 mass (g)	Day 30 mass (g)	Day 32 mass (g)	Day 34 mass (g)	Day 36 mass (g)	Day 38 mass (g)	Day 40 mass (g)	Mean loss per 48 hr (g)
1	276	268	259	255 (276)	273	269	261	6.4
2	278	273	261	254 (278)	275	271	256	8.4
3	277	271	257	253 (277)	273	270	265	6.6
						Mean 48-hour loss (per 100 mL):		7.13 mL
						Mean 24-hour loss (per 100 mL):		3.57 mL
						Mean 24-hour loss (per 50 mL):		1.78 mL

Evaporative loss from incubator (150 mL)							
Replicate	Day 40 mass (g)	Day 42 mass (g)	Day 44 mass (g)	Day 52 mass (g)	Day 54 mass (g)	Day 56 mass (g)	Mean loss per 48 hr (g)
1	330	324	311	326	312	307	9.50
2	330	324	314	326	314	309	8.25
3	330	312	295	326	312	306	13.75
						Mean 48-hour loss (per 150 mL):	10.5 mL
						Mean 24-hour loss (per 150 mL):	5.25 mL
						Mean 24-hour loss (per 50 mL):	1.75 mL

Appendix C

Concentration (ppm) of ammonia/ammonium in the water was measured from snails sacrificed on experimental day 30. The API® Ammonia Test Kit uses two reagents that yield colors ranging from yellow to dark green to indicate 0 ppm to 8 ppm ammonia/ammonium. The color of the test tube is compared to an indicator card to estimate the concentration of ammonia/ammonium present. Due to the values not being very high, it was deemed that ammonia was not a significant contributor to any results of the experiment and it was not considered further.

Ammonia/ammonium (ppm)							
Treatment	N	Mean	Median	Min	Max	SD	SE
Ca 0.64	4	1.250	1.000	1.000	2.000	0.500	0.250
Ca 1.3	4	1.500	1.500	1.000	2.000	0.577	0.289
Ca 2.5	4	1.375	1.500	0.500	2.000	0.750	0.375
Ca 5.1	4	1.000	1.000	1.000	1.000	0.000	0.000
(all)	16	1.281	1.000	0.500	2.000	0.515	0.129

Appendix D

To determine if the algae wafers had any impact on media pH, a two-day experiment was setup with three replicates per medium. Each replicate contained 50 mL of media and 10-mg crushed algae wafer. The pH was measured initially and after 24 and 48 hours, and the measurements are listed in the table below. On average, the media pH increased by 0.39 after 48 hours. How much of this change is from the algae wafer and evaporation was not investigated; however, since evaporation in 48 hours remained < 4 mL per 50 mL (< 10%), the increase in pH is most likely to be primarily from the algae wafer.

Media pH change from algae wafer

Treatment	pH – hour 0	pH – hour 24	pH – hour 48	24-hour pH change	48-hour pH change
0.64 Ca	6.52	6.41	6.93	-0.11	+0.41
1.3 Ca	6.63	6.61	6.95	-0.02	+0.32
2.5 Ca	6.53	6.49	6.96	-0.04	+0.43
5.1 Ca	6.49	6.49	6.89	-0.00	+0.40
			Mean:	-0.04	+0.39

Appendix E

Descriptive statistics for the wet weight of all snail groups throughout the experiment.

MSW = Median Shell Width

Wet weight (mg)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	1.649	1.670	1.110	2.250	0.336	0.101
Day 30:	Ca 0.64	4	212.085	211.060	203.940	222.280	7.714	3.857
	Ca 1.3	4	196.443	198.085	166.660	222.940	26.967	13.484
	Ca 2.5	4	200.915	209.690	163.870	220.410	25.213	12.606
	Ca 5.1	4	198.933	204.930	149.340	236.530	42.136	21.068
	(all)	16	202.09	209.795	149.340	236.530	26.033	6.508
Day 60:	Ca 0.64	18	325.951	344.000	134.740	411.390	69.000	16.264
	Ca 1.3	21	340.419	335.680	178.930	428.90	62.090	13.549
	Ca 2.5	19	357.935	369.800	235.390	422.930	45.0592	10.337
	Ca 5.1	21	360.971	375.700	71.830	450.830	89.021	19.426
	(all)	79	346.799	361.280	71.830	450.830	68.719	7.731
Day 60 < MSW:	Ca 0.64	12	298.109	324.420	134.740	361.280	68.320	19.722
	Ca 1.3	11	299.412	316.790	178.930	363.960	50.881	15.341
	Ca 2.5	7	313.237	326.780	235.390	342.170	36.563	13.819
	Ca 5.1	9	292.933	337.850	71.830	370.040	96.993	32.331
	(all)	39	299.997	321.820	71.830	370.040	65.340	10.463
Day 60 ≥ MSW:	Ca 0.64	6	381.635	380.550	364.090	411.390	18.3746	7.5013
	Ca 1.3	10	385.527	389.155	307.710	428.900	37.074	11.724
	Ca 2.5	12	384.008	384.425	337.110	422.930	24.086	6.953
	Ca 5.1	12	412.000	408.020	346.290	450.830	30.639	8.845
	(all)	40	392.430	389.890	307.710	450.830	30.963	4.896

Descriptive statistics for the shell width of all snail groups throughout the experiment.

MSW = Median Shell Width

Shell width (mm)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	1.705	1.654	1.529	1.918	0.12	0.039
Day 30:	Ca 0.64	4	9.409	9.405	9.121	9.705	0.249	0.124
	Ca 1.3	4	9.112	9.087	8.636	9.637	0.524	0.262
	Ca 2.5	4	9.000	9.178	8.356	9.290	0.434	0.217
	Ca 5.1	4	9.115	9.112	8.244	9.990	0.753	0.377
	(all)	16	9.159	9.256	8.244	9.990	0.493	0.123
Day 60:	Ca 0.64	18	10.544	10.885	7.275	11.667	1.118	0.264
	Ca 1.3	21	10.765	11.017	7.771	11.883	0.973	0.212
	Ca 2.5	19	11.026	11.097	9.646	12.387	0.700	0.154
	Ca 5.1	21	10.890	11.173	6.027	12.403	1.458	0.318
	(all)	79	10.811	11.033	6.027	12.403	1.093	0.123
Day 60 < MSW:	Ca 0.64	12	10.098	10.497	7.275	10.993	1.124	0.325
	Ca 1.3	11	10.082	10.253	7.771	11.017	0.872	0.263
	Ca 2.5	7	10.303	10.350	9.646	10.583	0.323	0.122
	Ca 5.1	9	9.808	10.647	6.027	10.987	1.663	0.554
	(all)	39	10.063	10.410	6.027	11.017	1.091	0.175
Day 60 ≥ MSW:	Ca 0.64	6	11.438	11.465	11.120	11.667	0.181	0.0740
	Ca 1.3	10	11.515	11.548	11.147	11.883	0.257	0.081
	Ca 2.5	12	11.447	11.409	11.033	12.387	0.388	0.112
	Ca 5.1	12	11.702	11.579	11.150	12.403	0.423	0.122
	(all)	40	11.539	11.472	11.033	12.403	0.354	0.056

Descriptive statistics for the shell height of all snail groups throughout the experiment.

MSW = Median Shell Width

Shell height (mm)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	0.970	0.988	0.836	1.088	0.073	0.022
Day 30:	Ca 0.64	4	4.493	4.451	4.339	4.733	0.170	0.085
	Ca 1.3	4	4.658	4.772	4.18	4.908	0.325	0.163
	Ca 2.5	4	4.619	4.584	4.496	4.813	0.153	0.077
	Ca 5.1	4	4.454	4.495	4.042	4.784	0.321	0.161
	(all)	16	4.556	4.555	4.042	4.908	0.245	0.061
Day 60:	Ca 0.64	18	5.016	5.102	2.763	5.633	0.597	0.141
	Ca 1.3	21	5.218	5.320	4.15	5.901	0.459	0.100
	Ca 2.5	19	5.339	5.314	4.830	5.807	0.272	0.062
	Ca 5.1	21	5.258	5.421	3.484	5.853	0.561	0.123
	(all)	79	5.212	5.296	2.763	5.901	0.493	0.055
Day 60 < MSW:	Ca 0.64	12	4.909	5.047	2.763	5.633	0.710	0.205
	Ca 1.3	11	5.059	5.286	4.150	5.608	0.483	0.146
	Ca 2.5	7	5.371	5.521	4.830	5.729	0.364	0.138
	Ca 5.1	9	4.937	5.132	3.484	5.853	0.744	0.248
	(all)	39	5.041	5.132	2.763	5.853	0.611	0.098
Day 60 ≥ MSW:	Ca 0.64	6	5.230	5.225	5.065	5.458	0.141	0.058
	Ca 1.3	10	5.392	5.503	4.887	5.901	0.380	0.120
	Ca 2.5	12	5.320	5.268	5.076	5.807	0.218	0.063
	Ca 5.1	12	5.498	5.470	5.269	5.787	0.152	0.044
	(all)	40	5.378	5.363	4.887	5.901	0.254	0.040

Descriptive statistics for the aperture height of all snail groups throughout the experiment. MSW = Median Shell Width

Aperture height (mm)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	1.783	1.809	1.459	1.995	0.166	0.050
Day 30:	Ca 0.64	4	6.809	6.781	6.671	7.003	0.140	0.070
	Ca 1.3	4	6.687	6.692	6.468	6.896	0.211	0.105
	Ca 2.5	4	6.803	6.823	6.561	7.005	0.183	0.092
	Ca 5.1	4	6.604	6.460	6.258	7.237	0.450	0.225
	(all)	16	6.726	6.781	6.258	7.237	0.260	0.065
Day 60:	Ca 0.64	18	7.744	7.932	5.482	8.350	0.663	0.156
	Ca 1.3	21	7.862	8.063	6.227	8.788	0.616	0.134
	Ca 2.5	19	7.798	7.690	7.007	8.510	0.442	0.101
	Ca 5.1	21	7.735	7.864	4.870	8.680	0.756	0.165
	(all)	79	7.786	7.887	4.870	8.788	0.622	0.070
Day 60 < MSW:	Ca 0.64	12	7.607	7.685	5.482	8.350	0.768	0.222
	Ca 1.3	11	7.665	7.982	6.227	8.463	0.709	0.214
	Ca 2.5	7	7.826	7.819	7.007	8.404	0.482	0.182
	Ca 5.1	9	7.359	7.725	4.870	8.111	1.001	0.334
	(all)	39	7.605	7.725	4.870	8.463	0.758	0.121
Day 60 ≥ MSW:	Ca 0.64	6	8.018	8.092	7.571	8.248	0.254	0.104
	Ca 1.3	10	8.079	8.131	7.273	8.788	0.428	0.135
	Ca 2.5	12	7.782	7.681	7.058	8.510	0.439	0.127
	Ca 5.1	12	8.016	7.971	7.528	8.680	0.328	0.095
	(all)	40	7.962	7.987	7.058	8.788	0.388	0.061

Descriptive statistics for the whole snail calcium of all snail groups throughout the experiment. MSW = Median Shell Width

Whole snail Ca (mg)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	0.033	0.034	0.024	0.040	0.005	0.001
Day 30:	Ca 0.64	4	2.990	2.968	2.954	3.071	0.055	0.028
	Ca 1.3	4	3.364	3.532	2.756	3.636	0.410	0.205
	Ca 2.5	4	4.264	4.369	3.849	4.466	0.282	0.141
	Ca 5.1	4	4.536	4.642	3.981	4.878	0.431	0.685
	(all)	16	3.788	3.743	2.756	4.878	0.717	0.179
Day 60:	Ca 0.64	18	9.303	9.480	7.428	9.629	0.505	0.119
	Ca 1.3	21	10.835	11.014	9.614	11.302	0.464	0.101
	Ca 2.5	19	12.832	12.918	10.681	13.869	0.726	0.166
	Ca 5.1	21	14.011	14.614	3.044	16.099	2.809	0.613
	(all)	79	11.810	11.257	3.044	16.099	2.350	0.264
Day 60 < MSW:	Ca 0.64	12	9.184	9.306	7.428	9.593	0.588	0.170
	Ca 1.3	11	10.616	10.800	9.614	11.138	0.542	0.163
	Ca 2.5	7	12.300	12.349	10.681	13.473	0.851	0.322
	Ca 5.1	9	12.446	14.177	3.044	14.816	3.721	1.240
	(all)	39	10.900	10.800	3.044	14.816	2.255	0.361
Day 60 ≥ MSW:	Ca 0.64	6	9.540	9.564	9.394	9.629	0.083	0.034
	Ca 1.3	10	11.075	11.110	10.716	11.302	0.175	0.055
	Ca 2.5	12	13.142	13.167	12.483	13.869	0.426	0.123
	Ca 5.1	12	15.186	15.512	13.338	16.099	0.874	0.252
	(all)	40	12.698	13.025	9.394	16.099	2.109	0.334

Descriptive statistics for the ash-free dry weight (AFDW) of all snail groups throughout the experiment. MSW = Median Shell Width

AFDW (mg)								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Day 0:	Ca 2.5	11	0.203	0.212	0.137	0.265	0.037	0.011
Day 30:	Ca 0.64	4	26.478	26.608	25.040	27.657	1.112	0.556
	Ca 1.3	4	25.883	26.991	20.532	29.018	4.042	2.021
	Ca 2.5	4	27.256	28.567	21.405	30.485	4.005	2.003
	Ca 5.1	4	25.729	27.251	17.891	30.525	5.726	2.863
	(all)	16	26.337	27.298	17.891	30.525	3.697	0.924
Day 60:	Ca 0.64	18	42.572	43.971	18.340	54.631	8.785	2.071
	Ca 1.3	21	46.292	45.537	24.605	74.652	11.224	2.449
	Ca 2.5	19	45.196	46.074	22.252	54.877	7.960	1.826
	Ca 5.1	21	47.332	51.617	2.628	62.726	14.672	3.201
	(all)	79	45.457	46.074	2.628	74.652	11.046	1.243
Day 60 < MSW:	Ca 0.64	12	38.549	40.166	18.340	47.885	7.905	2.282
	Ca 1.3	11	39.862	40.568	24.605	49.301	6.777	2.043
	Ca 2.5	7	37.582	40.432	22.252	43.947	7.578	2.864
	Ca 5.1	9	35.944	45.154	2.628	48.711	15.048	5.016
	(all)	39	38.144	40.568	2.628	49.301	9.435	1.511
Day 60 ≥ MSW:	Ca 0.64	6	50.620	50.427	47.104	54.631	2.892	1.181
	Ca 1.3	10	53.365	53.487	31.478	74.652	11.081	3.504
	Ca 2.5	12	49.637	50.424	43.431	54.877	3.733	1.078
	Ca 5.1	12	55.873	56.773	40.267	62.726	6.408	1.850
	(all)	40	52.587	52.067	31.478	74.652	7.188	1.137

Descriptive statistics for the pH of fresh and used media of all snail groups. MSW = Median Shell Width

Media pH								
Group	Treatment	N	Mean	Median	Min	Max	SD	SE
Fresh:	Ca 0.64	40	6.518	6.540	6.290	6.640	0.084	0.013
	Ca 1.3	40	6.480	6.490	6.230	6.720	0.105	0.017
	Ca 2.5	40	6.506	6.500	6.430	6.650	0.051	0.008
	Ca 5.1	43	6.502	6.490	6.440	6.570	0.040	0.006
	(all)	163	6.502	6.500	6.230	6.720	0.075	0.006
Day 30:	Ca 0.64	4	6.173	6.165	6.140	6.220	0.036	0.018
	Ca 1.3	4	6.040	6.065	5.86	6.170	0.147	0.074
	Ca 2.5	4	5.683	5.645	5.410	6.030	0.264	0.132
	Ca 5.1	4	5.195	5.180	5.120	5.300	0.077	0.039
	(all)	16	5.772	5.920	5.120	6.220	0.415	0.104
Day 60:	Ca 0.64	18	6.090	6.075	5.990	6.240	0.076	0.018
	Ca 1.3	21	5.883	5.860	5.770	6.080	0.098	0.021
	Ca 2.5	19	5.514	5.520	5.440	5.590	0.047	0.011
	Ca 5.1	21	5.331	5.280	5.150	6.470	0.271	0.059
	(all)	79	5.695	5.770	5.150	6.470	0.335	0.038
Day 60 < MSW:	Ca 0.64	12	6.073	6.040	5.990	6.240	0.083	0.024
	Ca 1.3	11	5.824	5.820	5.770	5.900	0.051	0.015
	Ca 2.5	7	5.503	5.490	5.470	5.550	0.034	0.013
	Ca 5.1	9	5.424	5.300	5.150	6.470	0.340	0.133
	(all)	39	5.751	5.790	5.150	6.470	0.328	0.052
Day 60 ≥ MSW:	Ca 0.64	6	6.123	6.120	6.060	6.180	0.050	0.020
	Ca 1.3	10	5.949	5.945	5.830	6.080	0.096	0.030
	Ca 2.5	12	5.520	5.525	5.440	5.590	0.054	0.015
	Ca 5.1	12	5.261	5.260	5.160	5.380	0.067	0.019
	(all)	40	5.640	5.560	5.160	6.180	0.337	0.053

Appendix F

Equations of the linear regression lines in Figures 13 and 17 are listed in the tables below.

% Ca dry weight linear regression equations (from Figure 13)				
Days	Treatment			
	0.64 Ca	1.3 Ca	2.5 Ca	5.1 Ca
0-30:	y = -0.092x + 11.558	y = -0.057x + 11.558	y = -0.008x + 11.558	y = 0.028x + 11.558
30-60:	y = 0.186x + 3.224	y = 0.174x + 4.610	y = 0.182x + 5.846	y = 0.179x + 7.053

pH linear regression equations (from Figure 17)			
Treatment			
0.64 Ca	1.3 Ca	2.5 Ca	5.1 Ca
y = -0.003x + 6.220	y = -0.003x + 6.035	y = -0.003x + 5.667	y = 0.002x + 5.145