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Effects of Aquatic Acidification on Calcium Uptake in White River Shrimp Litopenaeus setiferus Gills

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Effects of Aquatic Acidification on Calcium Uptake in White River Shrimp *Litopenaeus setiferus* Gills

by

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ABSTRACT

Previous research regarding aquatic acidification has examined the protonation of the carbonate and does not consider calcium to be a limiting factor. This is the first study to suggest that pH may affect the uptake of calcium in crustacean gills. This project describes ion transport mechanisms present in the cell membranes of white river shrimp *Litopenaeus setiferus* gill epithelium, and the effects of pH on the uptake of calcium by these means. Partially purified membrane vesicles (PPMV) of shrimp gills were prepared through a homogenization process that has been used previously to define ion transport in crab and lobster gill tissues. In the current study, shrimp gill PPMV calcium uptake at 50 μM, and 250 μM was greatest at pH 7.0 (p=0.01, p=0.0001). A valinomycin/K^+ induced membrane potential (PD) at pH 7.0 significantly increased (p=0.003) calcium uptake from that observed in the absence of a PD. An induced PD at pH 8.0 significantly increased (p=0.003) calcium uptake from that observed in the absence of a PD, however, was not significantly greater than uptake at pH 7.0 in the presence of a PD (p=0.05). Amiloride (2mM) treatments, and amiloride (2mM) + verapamil (100μM) cocktail treatments showed significant decrease in calcium uptake from the control (p=0.03), however they were not different from each other. This indicates an electrogenic carrier with two driving forces: calcium concentration, and asymmetric exchange stoichiometry.

INTRODUCTION

At least one-third of anthropogenic carbon dioxide (CO_2) released by the burning of fossil fuels does not stay in the atmosphere, but dissolves into aquatic systems (Whitley, 2011). Since the beginning of the industrial era, aquatic systems have become a sink for anthropogenic CO_2, absorbing approximately 525 billion tons of this CO_2, with a current absorption rate of 22 million tons per day (Bennett, 2018). Twenty-two million tons of CO_2 emissions is equivalent to 53 billion miles driven by an average passenger vehicle, or the ability to charge a smartphone 2.8 billion times. It would take 364 million tree seedlings grown for 10 years to sequester the CO_2 absorbed into the ocean in one day (United States Environmental Protection Agency, 2018).

The last greatest increase of CO_2 into the atmosphere was about 56 million years ago during the Paleocene-Eocene Thermal Maximum. This was an abrupt warming event triggered by the release of massive amounts of CO_2 into the atmosphere resulting in a 200,000-year warm period with global temperatures increased by 8°C. During this event, the maximum CO_2 emissions were 0.6 gigatons per year. The current levels of CO_2 emissions are 10 gigatons per year (Meissner et al., 2014)

When CO_2 is absorbed by aquatic systems, carbonic acid is formed: $CO_2 + H_2O \leftrightarrow H_2CO_3$. Carbonic acid deprotonates to form bicarbonate, resulting in an increased concentration of hydrogen ions in aquatic systems: $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. Carbonate ions dissociate from calcium: $CaCO_3 \leftrightarrow Ca^{2+} + CO_3^{2-}$ and act as an antacid to neutralize the protons, forming more bicarbonate: $CO_3^{2-} + H^+ \leftrightarrow HCO_3^-$. This increase in protons increases aquatic acidity and causes a decrease in abundance of carbonate ions. A 30% increase in proton concentrations and a 16% decrease in carbonate ion concentrations have been observed in marine environments

because of the aquatic absorption of anthropogenic CO_2 emitted since the beginning of the industrial revolution (Whitely, 2011).

Since the beginning of the Carboniferous period, about 300 million years ago, oceanic pH has been relatively basic with an average pH of 8.2. The current ocean pH averages 8.1, corresponding to a drop of 0.1 pH units, or 30% increase in acidity since the 1800s (Orr et al., 2005). Freshwater systems vary in pH much more than oceanic systems, as acidification of a smaller volume takes place at a faster rate (Whitely, 2011). The complex biogeochemistry of freshwater systems makes it difficult to determine how much of the CO_2 increase in these systems is due to the increased CO_2 in the atmosphere. CO_2 levels in freshwater systems can vary widely based on a myriad of other factors such as the type of nearby ecosystem, amount of precipitation, buffering capacity of soil, land use such as agriculture or landfills, seasonal shifts as leaves drop into the water or as ice forms, the presence or absence of animal inhabitants through their life cycles, and the photosynthesis rates of algae and plants. A study by Weiss and colleagues (2018) monitored all the aforementioned factors over a 35-year period and found a significant increase in CO_2 and a correlating 0.3 pH unit decrease, or 100% increase in acidity, in German freshwater systems from 1981-2015.

Current ocean acidification projections for the year 2100 show a 100-150% increase in acidity, respective to a decrease in pH from current 8.1 to 7.8-7.9 (Orr et al., 2005). With these conditions, models constructed by Orr and colleagues (2005) predict that by 2050, surface waters will become undersaturated with calcium carbonate which is vital to shell building of aquatic calcifying species. This loss of calcium carbonate by increasing aquatic pH under projected 2100 conditions was experimentally observed with living pteropods by Orr and colleagues (2005) (Figure 1).

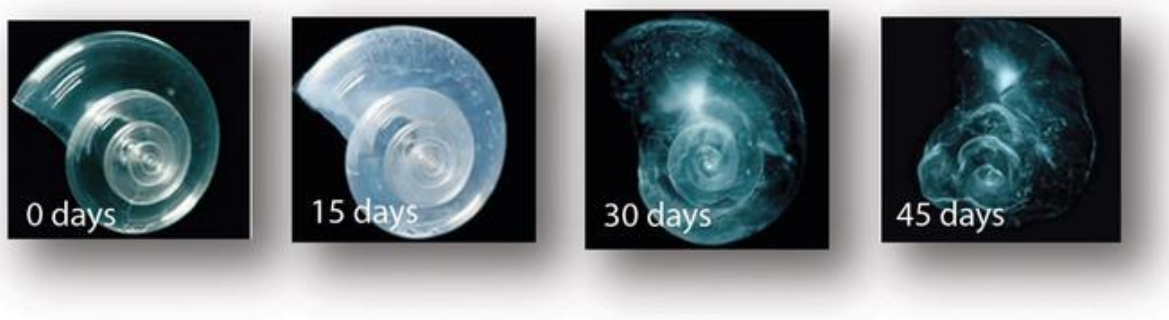

Figure 1 Effect of change in Southern Ocean seawater pH from 8.2 to 7.8 on pteropod *Limacina helicina* shell thickness over incubation of 45 days based on year 2100 IS92a business-as-usual CO_2 emissions projected by Orr and colleagues (2005). Photographed by David Liittschwager (2014) for collaboration with the NOAA PMEL Carbon Program Study.

Freshwater systems are projected to decrease in pH by the same magnitude as surface ocean through 2100 (Phillips et al., 2015). This poses a significant threat to important freshwater species that utilize calcium carbonate for shell building and growth, such as crustaceans like the juvenile white river shrimp *Litopenaeus setiferus* found in the northern region of the St. Johns River in Jacksonville, Florida.

Crustacean growth can only occur through molting, which requires external calcium. A lack of calcium may restrict crustacean growth (Hessen et al., 1991). Calcium is central to many steps in crustacean molting and calcification of the carapace. There are four organs that control the concentration of calcium in crustaceans: the hepatopancreas, the kidneys, the integumentary epidermis, and the gills (Ahearn et al., 1998). Gills are the primary site of calcium regulation in crustaceans, followed by the antennal glands and gut (Towle, 1993). Upon endocrine initiation of molting via ecdysones, calcium is reabsorbed from the carapace and transported into the hemolymph, and stored in gastroliths and the hepatopancreas, which allows the softening of the

carapace resulting in ecdysis. Post-molt, the calcium in the storage sites is transported back to the carapace to re-calcify it. Additional calcium from the environment is needed post-molt to account for carapace growth and calcification. Increase in proton concentration from aquatic absorption of anthropogenic CO_2 results in the dissociation of calcium from carbonate, and the protonation of carbonate into bicarbonate, which is not compatible in binding with calcium to form calcium carbonate shells that are essential to calcifying organisms such as *L. setiferus* (Henry and Wheatly, 1992).

The physiological characteristics of crustacean gill transport proteins on apical aspects of gill epithelia facing the aquatic environment have not been fully characterized. The basolateral aspect of gill epithelia (facing the hemolymph) has been shown to have a high affinity calcium ATPase and a low affinity calcium/sodium antiporter, both of which transfer Ca^{2+} from the cytoplasm of gill epithelium to the hemolymph after cellular uptake from the aquatic environment (Flik et al., 1994; Zhuang and Ahearn, 1998; Flik and Haond, 2000). Freire et al. (2008) proposed a Ca^{2+} transport model of the apical membrane of crustacean gill cells (Figure 2) based on previous work with the hepatopancreas of the lobster and crayfish (Wheatly et al., 2002; Ahearn et al., 2004), the antennal glands of the lobster (Ahearn et al., 1995), and the gut of the lobster (Wheatly et al., 2002 and Ahearn et al., 2004). This model suggests that there exist three possible kinds of calcium uptake carriers in crustaceans; an electrogenic Ca^{2+}/H^{+} antiporter, an electroneutral $Ca^{2+}/2H^{+}$ antiporter, or an open calcium channel. These transport systems may be present individually or in combination with each other. It is one of the objectives of this study to delineate which system (or systems) is the mechanism of calcium uptake in *L. setiferus* gills. It is possible that this carrier uses both ion (calcium and proton) gradients, as well as the membrane potential as driving forces for cation exchange.

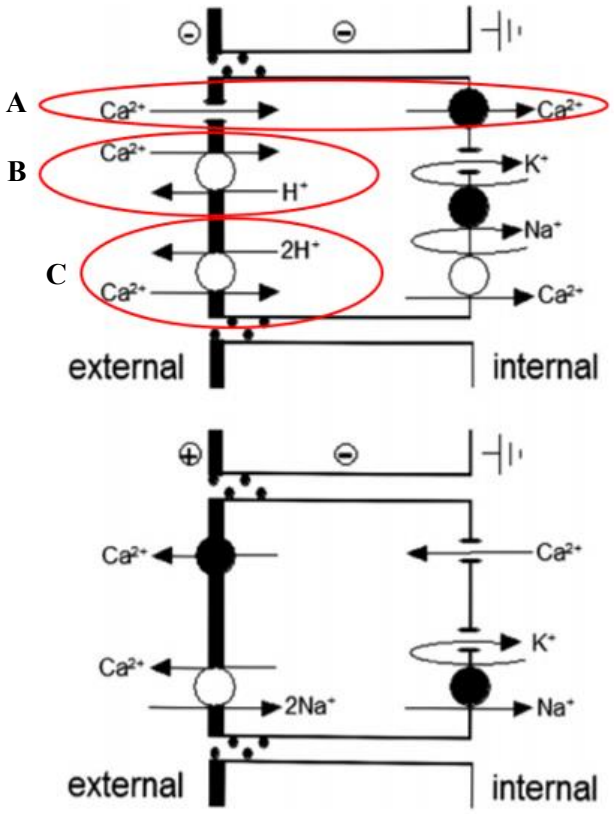

Figure 2 Proposed Ca^{2+}/ H^{+} crustacean gill calcium transport model for assumed calcium transport protein assembly in outwardly facing crustacean gills on earlier work with lobster and crayfish hepatopancreas and kidney epithelia. Apical absorption of Ca^{2+} is proposed to proceed via **A**) open Ca^{2+} channel, **B**) electrogenic Ca^{2+}/H^{+} exchange, and/or **C**) electroneutral $Ca^{2+}/2H^{+}$ exchange (Freire et al., 2008).

Valinomycin was used to distinguish the presence of an electrogenic or electroneutral system. Valinomycin is a calcium ionophore, shown in Figure 3, that carries K^{+} across membranes with a high selectivity and has been extensively used to impose high K^{+} permeability on cellular membranes. Valinomycin has a hydrophilic pocket that forms a binding site with K^{+}, and a hydrophobic exterior that allows it to cross the hydrophobic membrane. If calcium uptake

through plasma membranes with a membrane potential created by valinomycin is faster than calcium uptake through plasma membrane without a membrane potential, then the uptake of calcium is electrogenic because under these experimental conditions, the only driving force for calcium uptake is the membrane potential. This would support the electrogenic Freire et al., (2008) model and will rule out system C in Figure 2.

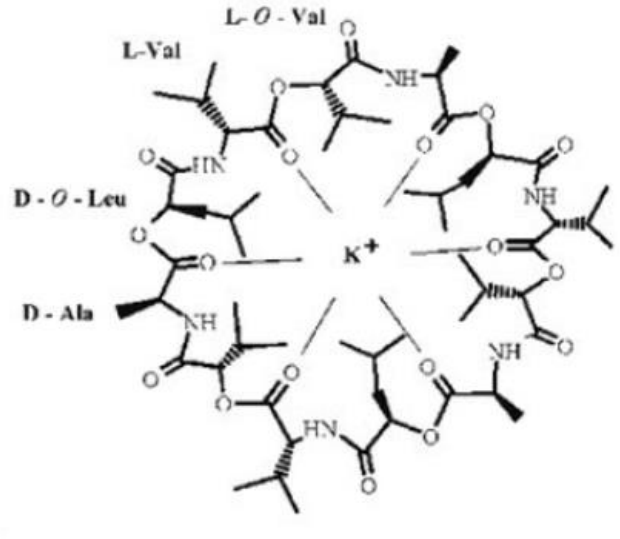

Figure 3 Molecular structure of potassium ionophore valinomycin with outwardly facing hydrophobic exterior with a K^+ ion bound in the central hydrophilic pocket (Saris et al., 2009).

Amiloride is a calcium channel blocker commonly used to treat high blood pressure. It has been shown to inhibit ^{45}Ca uptake via an electrogenic antiporter in lobster hepatopancreas by Ahearn and Franco, (1993) and Ahearn and Zhuang (1996). Amiloride allows for identification of specific antiporters as it has no effect on electroneutral antiporters. If sensitivity to amiloride is observed, there should be a decrease in calcium uptake in the presence of amiloride. Sensitivity to amiloride would suggest the presence of the electrogenic system of calcium uptake

(Figure 2: System B) and would rule out the electroneutral system of calcium uptake (Figure 2: System C).

Verapamil is another type of calcium channel blocker commonly used as an antihypertensive drug. It has been shown by Ahearn and Franco, (1993) and Ahearn and Zhuang (1996) to be an inhibitor of calcium channels in organs where calcium uptake occurs in crustaceans. If open calcium channels exist, then there should be a decrease in calcium uptake in the presence of verapamil. If insensitivity to verapamil is observed, then the verapamil sensitive open calcium channel (Figure 2: System A) can be ruled out.

The goal of this study was to delineate the effects of acidification on the uptake of calcium through the gill epithelia of the freshwater crustacean, *Litopenaeus setiferus*, to examine the effects of acidification on calcium uptake. Current research regarding the effects of aquatic acidification does not examine how the aquatic acidification process affects calcium balance in animals who are dependent upon it, such as crustaceans. This is the first study to examine whether pH affects the uptake of calcium in crustacean gills.

To address this goal, the specific objectives of this project were to: 1) determine how pH influences the uptake of calcium through the gill epithelia of white river shrimp *L. setiferus*, which will delineate the optimal pH for calcium uptake and describe how a decrease in pH, similar to what has been projected as a result of aquatic acidification, affects the ability of calcium uptake from water into the gill cytoplasm 2) determine the mechanism of calcium uptake (electrogenic or electroneutral); and 3) identify the calcium uptake mechanism in *L. setiferus* gill epithelia (an electrogenic Ca^{2+}/H^{+} antiporter, an electroneutral $Ca^{2+}/2H^{+}$ antiporter, or an open calcium channel).

Increasing emissions of CO_2 are changing the chemistry of aquatic systems. Oceans are projected to drop 0.2-0.3 pH units by the year 2100 (Orr et al., 2005) with freshwater systems expected to respond in the same magnitude. It is therefore important to understand how this change is affecting animals in these ecosystems. The identification of the mechanism of calcium uptake will allow for better understanding of how aquatic acidification influences how crustaceans are able to absorb calcium from their environment with respect to changing climate and aquatic conditions.

METHODS

Specimen collection

Live white river shrimp (*Litopenaeus setiferus*) (Figure 4) were obtained from northern Mayport St. Johns River water with a salinity of 5‰, and a pH ranging from 6.5-7.0 at Browns Creek Fish Camp in Jacksonville, Florida. Local measurements of freshwater calcium concentration by the St. Johns River Water Management District (1994-2019) show a median concentration of 1.165 mM, a maximum concentration of 2.925 mM, and a minimum concentration of 0.7025 mM.

Figure 4 A live white river shrimp *Litopenaeus setiferus* caught from the St. Johns River in Jacksonville, Florida.

Litopenaeus setiferus was chosen as the specimen of study because it is sustainably managed (NOAA, 2016), easily obtained, and serves as a basis of comparison to a marine relative, *Homarus americanus*, by work from the same laboratory (Nagle et al., 2018). *Litopenaeus setiferus* has a juvenile stage in upper reaches of the St. Johns River estuarine nursery where salinity is low, and an adult stage in the ocean. This allows for future work comparing effects of aquatic acidification in freshwater and marine environments with the same species. The northern region of the St. Johns River where specimens were collected measured an average pH of 7.89 ± 0.30, with a minimum and maximum pH of 5.37 and 9.11 respectively. The pH of the St. Johns River has been measured to decrease at a rate of 5.0×10^{-4} pH units per year (Robbins and Lisle, 2017).

Preparation of membrane vesicles

Partially purified membrane vesicles (PPMV) of shrimp gills were prepared using a centrifugation and homogenization process, described in Lucu (2008). Flik and Haond (2000) found that this process produced vesicles of plasma membrane from both basolateral and apical aspects of cells from lobster *Homarus americanus* gills. Basolateral calcium transport occurs via calcium-ATPase and Ca^{2+}/Na^{+} antiporters. Because this project focused on calcium uptake from the apical epithelium, membrane vesicles were prepared using buffer solutions containing HEPES/Tris, and mannitol containing no sodium or ATP. This minimized Ca^{2+} transport from basolateral calcium-ATPase and Ca^{2+}/Na^{+} antiporters allowing for distinction of apical transport properties and minimizing basolateral contributions.

Animals were euthanized via denervation and removal of the heart. The carapace was removed followed by excision of the gills (Figure 5), which were placed in cold hypotonic mannitol salt buffer at pH 7.0 [Buffer 1: 300 mM mannitol, 125 μM PMSF

(phenylmethylsulfonylfluoride), and 5 mM EGTA (ethyleneglycol-bis[β-amino-ethylether]-N,N'-tetraacetic acid), 125 mL MiliQ filtered water] and homogenized via blender for three minutes.

Figure 5 Exposed gill chamber of dissected shrimp *Litopenaeus setiferus* with carapace and abdomen removed.

The gill slurry was then centrifuged for 20 minutes at 3,000 RPM in a 4°C Beckman Avanti centrifuge with a JA25.5 rotor. Supernatant from this spin was decanted into clean centrifuge tubes and the pellet containing gross intracellular components was discarded. Supernatant was centrifuged again for 30 minutes at 30,000 RPM using a JA30.0 rotor at 4°C. The supernatant was discarded, and the resulting pellet was resuspended in 35 mL of pH 7.0 Buffer 2 [300 mM mannitol, 12 mM HEPES/Tris (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid/ Tris), 500 mL MiliQ filtered water]. Pellet suspension was homogenized with 10 strokes using a Potter Elvehjem tissue homogenizer attached to a hand-held power drill. This homogenized pellet solution was centrifuged at 30,000 rpm for 30 minutes at 4°C using the same rotor. The resulting supernatant was discarded, and the pellet was resuspended in 600 µl of

Buffer 2 via homogenization with 20 passes through a 22-gauge syringe in an Eppendorf tube. Vesicles were placed on ice to equilibrate for 15 minutes.

Protein concentration of the PPMV solution was determined after 15-minute equilibration using a Bradford protein assay for use in normalizing uptake results. PPMV incubation buffers varied in composition with respect to the objective being tested.

Calcium uptake experiments

Following incubation (buffer components, incubation time, and pH varied with respect to the objective being tested), uptake of radioisotope ^{45}Ca into *L. setiferus* PPMV was measured via liquid scintillation counting of Millipore filters in a Beckman LS6500 liquid scintillation counter, following the Millipore filtration technique developed by Hopfer and colleagues (1973). Incubation of PPMV with ^{45}Ca using liquid scintillation counting methods were previously described (Zhuang and Ahearn, 1996).

Data calculations

The data from the Bradford Protein Assay was converted into milligrams per 20 µl. Picomoles of ^{45}Ca per 20 µl were calculated for each concentration of calcium tested. Averages of the background (BKD) and baseline radiation (IO) were calculated for reach replicate to obtain counts per minute (cpm) per 20 µl. Specific activity is the amount of radiolabeled mass in a sample. The specific activity was calculated for each replication using the average baseline radiation (IOs) for each replicate, and the concentration of ^{45}Ca as follows:

$$Specific\ Activity\ in\ \ cpm/picomoles = \ \ (Average\ of\ IOs\ )/([45Ca]per\ 20\mu l)$$

The cpm for each time point was divided by the specific activity to obtain the picomoles of radiation uptake. Using the specific activity of the isotope and protein concentration from the Bio-Rad Bradford protein assay, average uptake values of ^{45}Ca were expressed as pmol/mg protein. Left and right gills from 12 shrimp were used for each experiment, and 3-4 replicates of each experiment were performed. Time course graphs of these averages were created using Sigma Plot 10.0 (Systat Software, Inc., San Jose, CA), showing the time to calcium equilibrium in vesicles incubated in different proton concentrations.

Statistical analysis

To determine the existence of significant differences in calcium uptake between pH treatments (objective 1), a repeated measures ANOVA was conducted for each concentration of calcium because there were multiple measures of the same variable (calcium uptake) taken from the same batch of gill vesicles over a time-course. Because there exists no biological rationale for change existing in only one direction for this experiment, a targeted two-tailed t-test between pH 7 and 8, and between pH 6 and 7 was the most conservative approach to determine where the significant differences exist within the data.

To determine significant differences in valinomycin treatments (objective 2), a two-factor ANOVA with replication was conducted because there were multiple measures of calcium uptake taken from the same batch of gill vesicles at single time points.

A repeated measures ANOVA was used to determine significant differences between calcium uptake in amiloride and verapamil treatments in comparison with controls over a time-course (objective 3). A targeted two-tailed t-test between the control and amiloride only treatments were used as a post-hoc test to determine where the significant differences exist within the data. Significant differences between treatments were established at $p<0.05$.

Chemicals

Reagent grade chemicals were purchased from Fisher Scientific, Waltham, MA and Sigma Chemical, St. Louis, MO.

RESULTS

The questions addressed by this project were 1) What pH is optimal for the uptake of calcium through the gill epithelia of white river shrimp *Litopenaeus setiferus*? 2) What is the mechanism of calcium uptake in this animal? 3) How are the calcium channels and/or carriers functioning in the presence of increasing proton concentrations?

Question 1 was addressed by loading shrimp gill PPMV with Buffer 2 solution containing 300 mM mannitol, 12 mM HEPES/Tris at pH 7.0 and incubating 600 µl of the PPMV in the same buffer medium with added 2 µl ^{45}Ca and 50 µM $CaCl_2$ or 250 µM $CaCl_2$ at pH 6.0, 7.0, and 8.0 for time intervals of 1 minute, 5 minutes, 20 minutes, and 30 minutes. Uptake of radioisotope ^{45}Ca, at both 50 µM and 250 µM, by purified gill vesicles subject to varying pH levels delineated that pH 7.0 is optimal for calcium uptake by gills of juvenile *L. setiferus* (Figures 6 and 8). Time course graphs were fit to begin at zero and there was no significant effect of time on calcium uptake at either 50 µM or 250 µM $CaCl_2$, so one-minute influxes of calcium were determined via the slope of uptake versus time at one minute to show the initial uptake at each pH tested. One-minute influx at both 50 µM and 250 µM ^{45}Ca is in keeping with greatest calcium uptake occurring at pH 7.0 (Figures 7 and 9).

Because calcium concentrations vary in freshwater, low and high (relative to freshwater) calcium concentrations were tested to verify that the pH where maximum calcium uptake occurred did not change with calcium concentration. Shrimp gill PPMV calcium uptake at 50 µM (p =0.01), and 250 µM (p =0.001) was highest at pH 7.0.

A repeated measures ANOVA was conducted to compare the effects of pH 6, pH 7, and pH 8 on calcium uptake at 50 µM and at 250 µM by shrimp gill PPMV over a time course (Figure 6 and 8). There was a significant effect of pH on calcium uptake at the $p<0.05$ level for the three conditions at 50 µM [$F_{2,6}=10.65$, $p=0.01$] and at 250 µM [$F_{2,6}=56.65$, $p=0.0001$], however no significant effect of time on calcium uptake was observed. A two-tailed t-test showed a significantly greater uptake in calcium at one minute by pH 7 than 8 at 50 µM ($p=0.04$) and 250 µM ($p=0.03$). This shows that deviating from optimal pH can decrease calcium uptake. Further experiments proceeded with pH 7 conditions as a control.

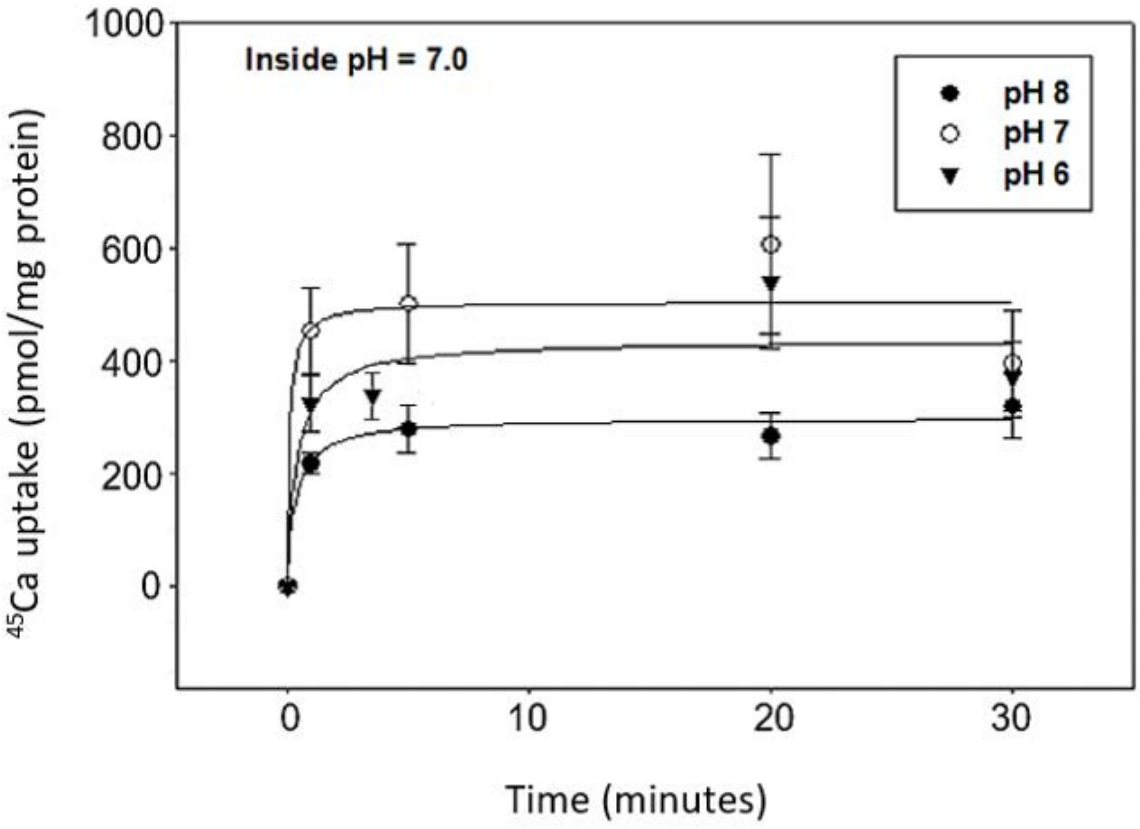

Figure 6 Effect of external pH on uptake of 50µM ^{45}Ca by shrimp gill partially purified membrane vesicles (PPMV). PPMV were loaded with Buffer 2 solution containing 300mM mannitol, 12mM HEPES/Tris at pH 7.0 and incubated in the same medium with added 50µM calcium at pH 6.0, 7.0, and 8.0 for time intervals of 1 minute, 5 minutes, 20 minutes, and 30 minutes. Graphs were fit using hyperbola plus a linear function using Sigma Plot 10.0. Values are displayed as means ± 1SEM and *n*=3 trials per treatment.

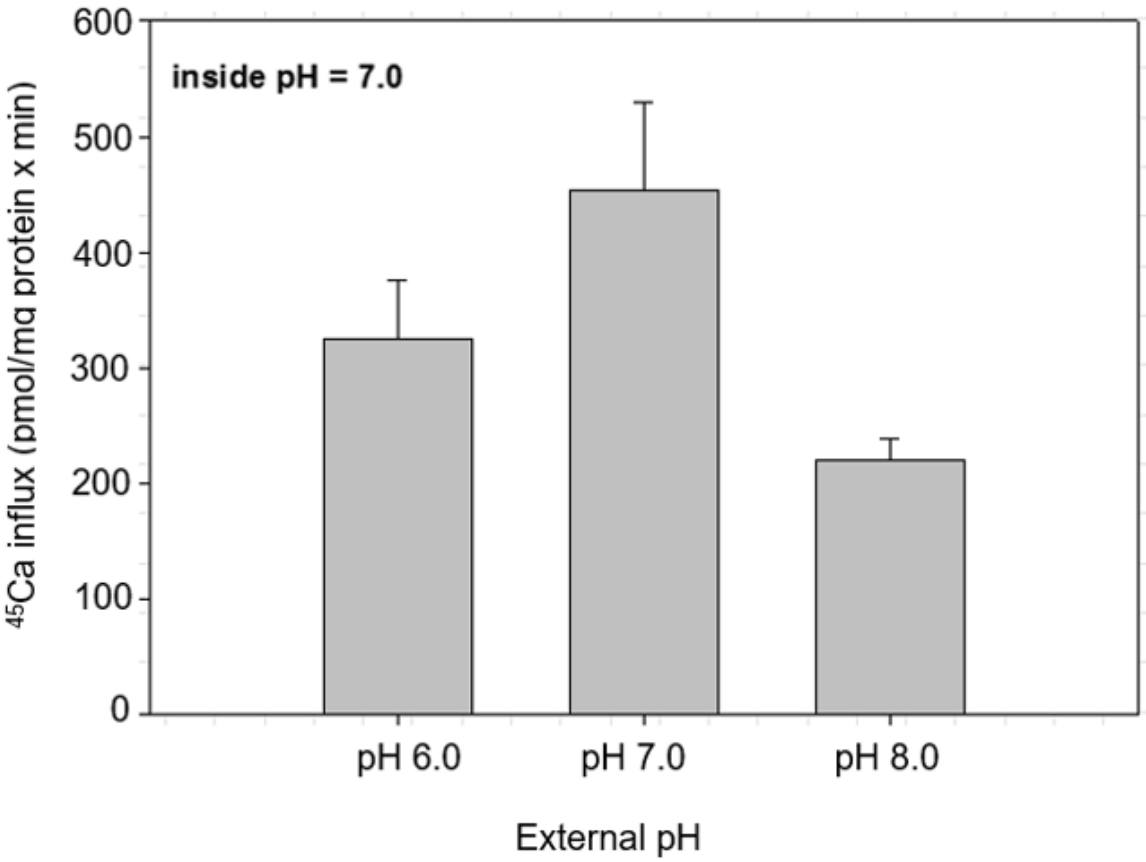

Figure 7 Effect of external pH on one-minute influx of 50 µM ^{45}Ca into shrimp gill vesicles. PPMV were loaded and incubated in the same media as in Figure 6, for one minute. Values are averages of one-minute ^{45}Ca influx for triplicate experiments ± 1SEM.

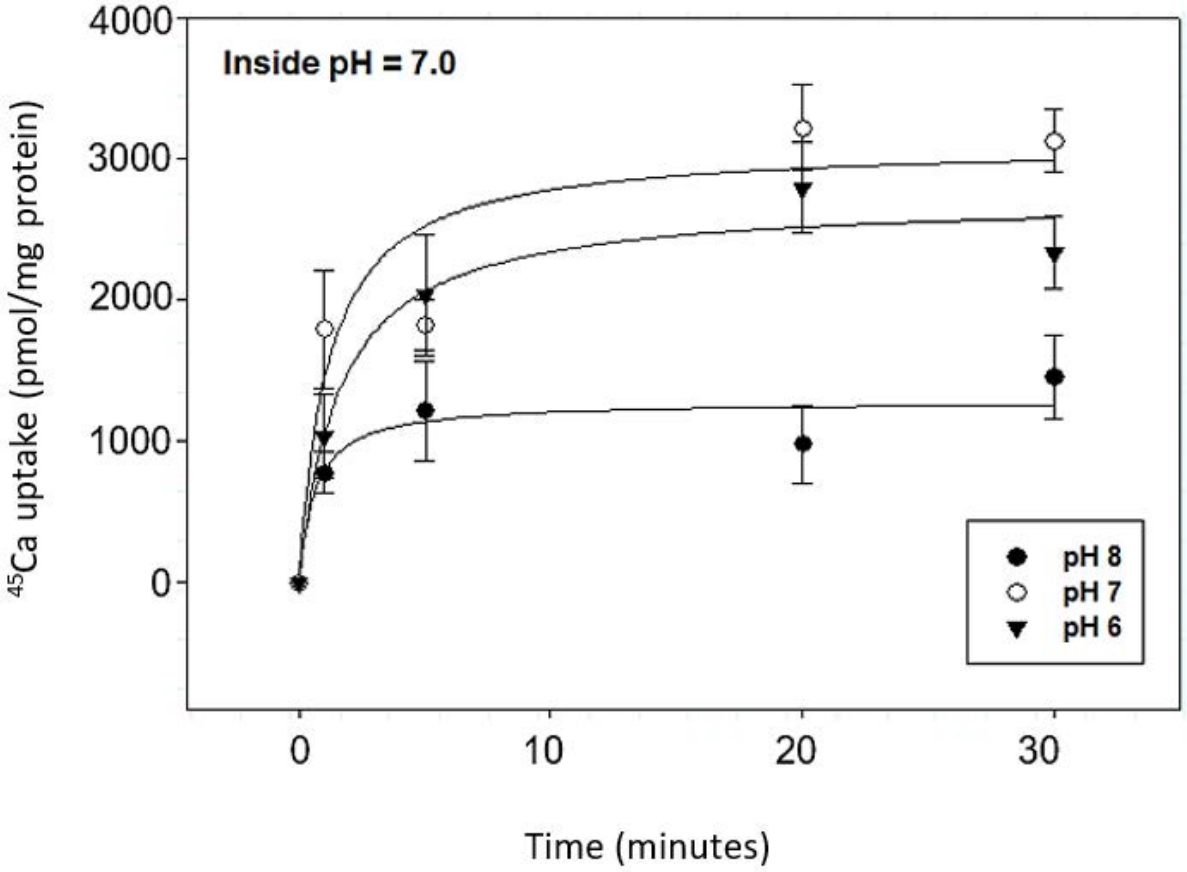

Figure 8 Effect of external pH on uptake of 250 μM ^{45}Ca uptake by shrimp gill PPMV. PPMV were loaded with Buffer 2 solution containing 300 mM mannitol, 12 mM HEPES/Tris at pH 7.0 and incubated in the same medium with added 250 μM calcium at pH 6.0, 7.0, and 8.0 for time intervals of 1 minute, 5 minutes, 20 minutes, and 30 minutes. Graphs were fit using hyperbola plus a linear function using Sigma Plot 10.0. Values are displayed as means ± 1SEM and n=3 trials per treatment.

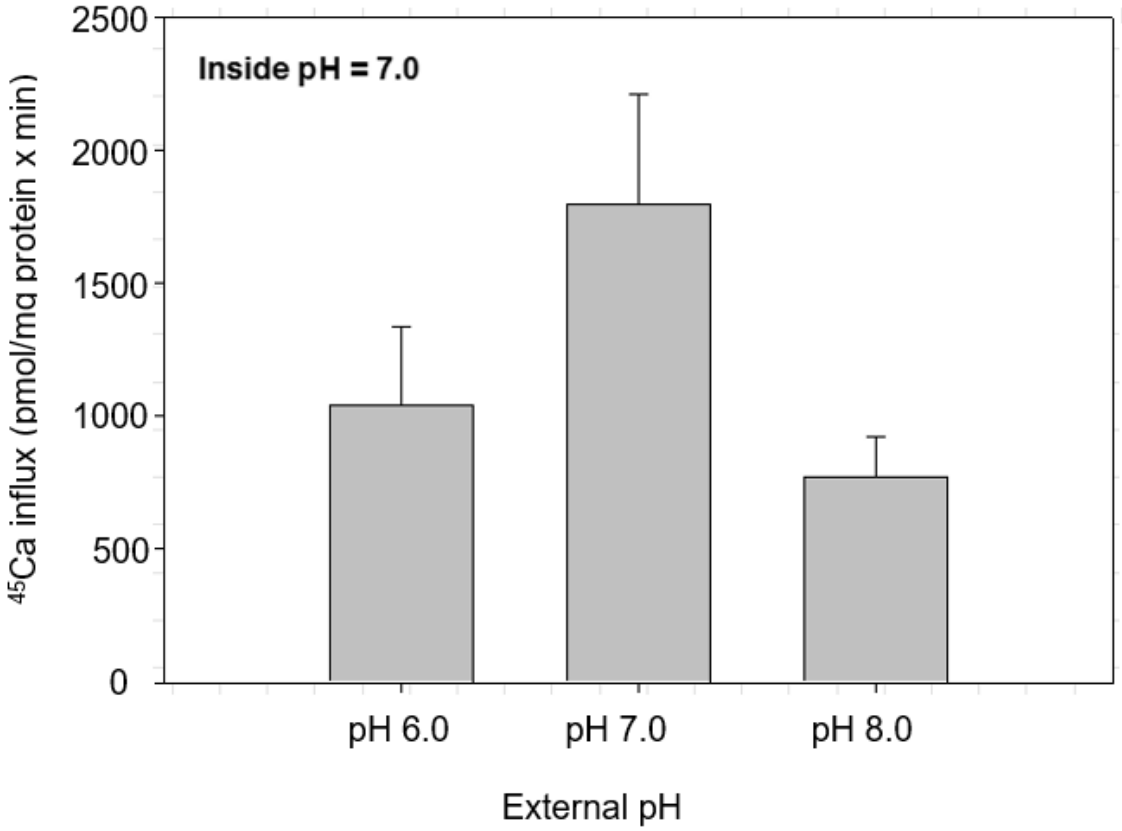

Figure 9 Effect of external pH on one minute influx of 250 μM calcium into shrimp gill PPMV. PPMV were loaded and incubated in the same media as Figure 8, for one minute. Values are averages of one-minute ^{45}Ca influx for triplicate experiments ± 1SEM.

Question 2 sought to determine the presence of electrogenic uptake of calcium via electrogenic carrier mediated uptake, or if the calcium uptake system is electroneutral in nature. This was addressed by testing the effects of a valinomycin (a potassium ionophore) induced membrane potential (PD) on the uptake of 250 μM calcium by shrimp gill PPMV at either pH 7.0 or 8.0 All vesicles were loaded with buffer adjusted to either pH 7.0 or 8.0 containing 300 mM mannitol, 12 mM HEPES/Tris, 50 mM k-gluconate, and 50 μM valinomycin.

Vesicles were then divided into four groups, each subject to a different incubation media. A membrane potential difference (PD) was generated by incubating preloaded vesicles for 10 minutes in media adjusted to pH 7.0 or 8.0 containing 400 mM mannitol, 12 mM HEPES/Tris, and 250 µM calcium. The two control incubation conditions (non-PD inducing media) at pH 7.0 or 8.0 contained 300mM mannitol, 12mM HEPES/Tris, 250µM calcium, and 50 mM k-gluconate. This experimental layout is shown in Figure 10.

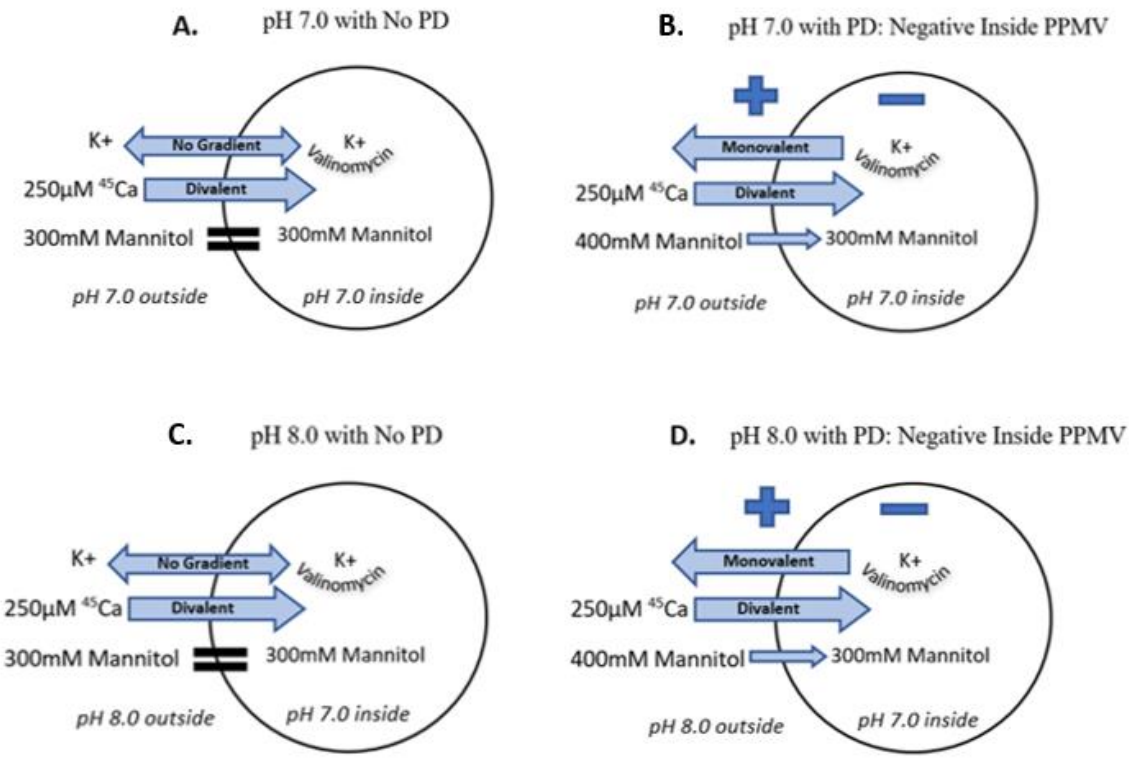

Figure 10 Vesicle diagram of experimental conditions testing the effects of an induced membrane PD on 10-minute uptake of 250 µM ^{45}Ca at incubation pH of 7.0 and 8.0. **A**: Mannitol and potassium (in the form of k-gluconate) concentrations were equal on both sides of the membrane. No gradient was present, thus no PD induced. This condition served as the control for pH 7.0. **B**: Both a mannitol and potassium gradient were present, inducing a PD at pH 7.0. **C**: No

gradient was present as in **A**, thus no PD induced. This condition served as the control for pH 8.0. **D**: Both a mannitol and potassium gradient were present, inducing a PD at pH 8.0.

A two-factor ANOVA with replication was conducted that examined the effects of an induced membrane potential on calcium uptake at pH 7 and pH 8 (Figure 11). This analysis showed that vesicles with an induced membrane potential had significantly greater uptake of calcium than vesicles without an induced membrane potential (p=0.003). Vesicles with pH 7 externally and internally showed greater calcium uptake than vesicles with pH 8 externally and pH 7 internally (p=0.01). However, there is no interaction between PD and pH on calcium uptake [$F_{1, 12}$ =4.38, p=0.05].

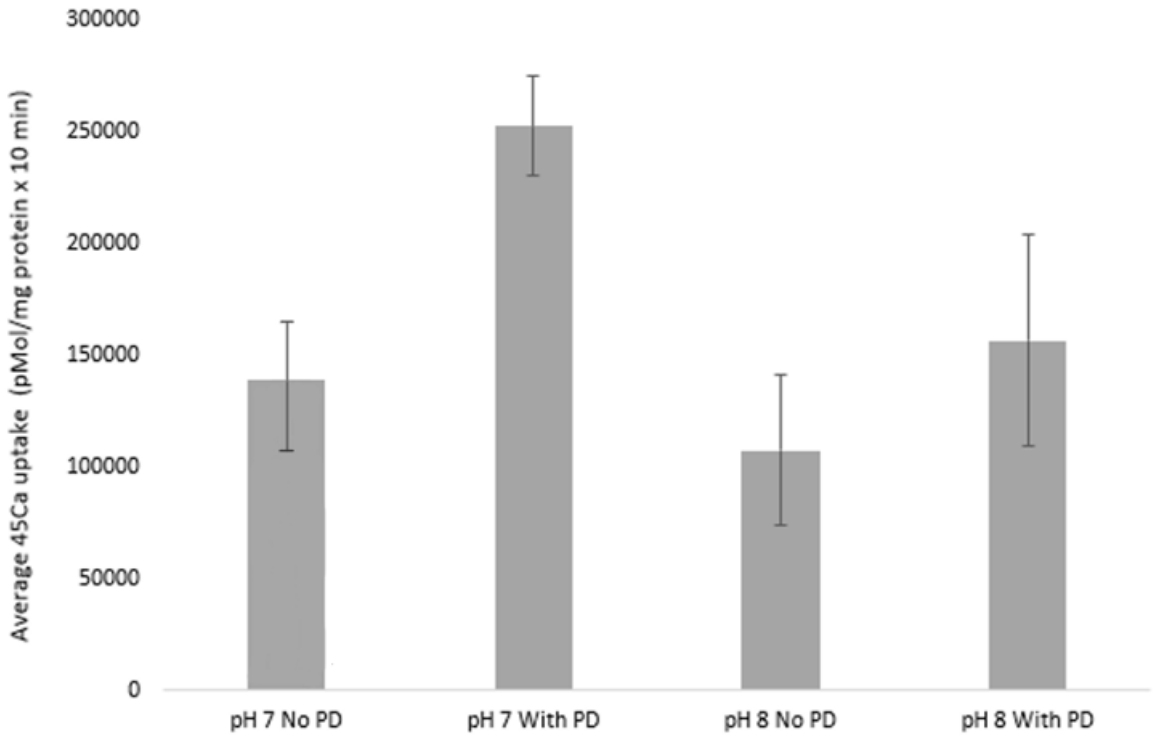

Figure 11 Effects of induced membrane potential difference (PD) on 10-minute uptake of 250μM calcium at pH 7.0 and 8.0. Vesicles were loaded with pH 7.0 buffer containing 300mM mannitol, 12 mM HEPES/Tris, 20 mM k-gluconate, and 50 μM valinomycin. Vesicles were divided into four groups as shown in Figure 10, each group subject to a different incubation media. Two of the groups were incubated in non-PD inducing media at either pH 7.0 or 8.0 that contained 300 mM mannitol, 12 mM HEPES/Tris, 250 μM ^{45}Ca, and 50 mM k-gluconate. The remaining two groups were incubated in PD-inducing media at either pH 7.0 or 8.0 that contained 400 mM mannitol, 250 μM ^{45}Ca, and 12 mM HEPES/Tris. Values are average ± 1SEM, n=4 replicates per treatment.

Question 3 was addressed by treating shrimp gill PPMV to a mannitol control, 2 mM amiloride, or a 2 mM amiloride and 100 μM verapamil cocktail (Figure 12). A repeated measures ANOVA was conducted to compare the effects of no inhibitors, 2 mM amiloride only, and 2 mM amiloride with 100 μM verapamil cocktail on calcium uptake by shrimp gill PPMV. There was a significant effect by inhibitors on calcium uptake at the $p<0.05$ level for the three conditions [$F_{2,8} = 6.81$, $p=0.01$]. A two-tailed t-test showed a significant decrease in calcium uptake by 2 mM amiloride only than the no-inhibitor control at 250 μM ($p=0.03$). Because there was no difference in calcium uptake inhibition between amiloride alone, and the amiloride and verapamil cocktail, the presence of a verapamil-sensitive calcium channel (Figure 2: system A) was ruled out.

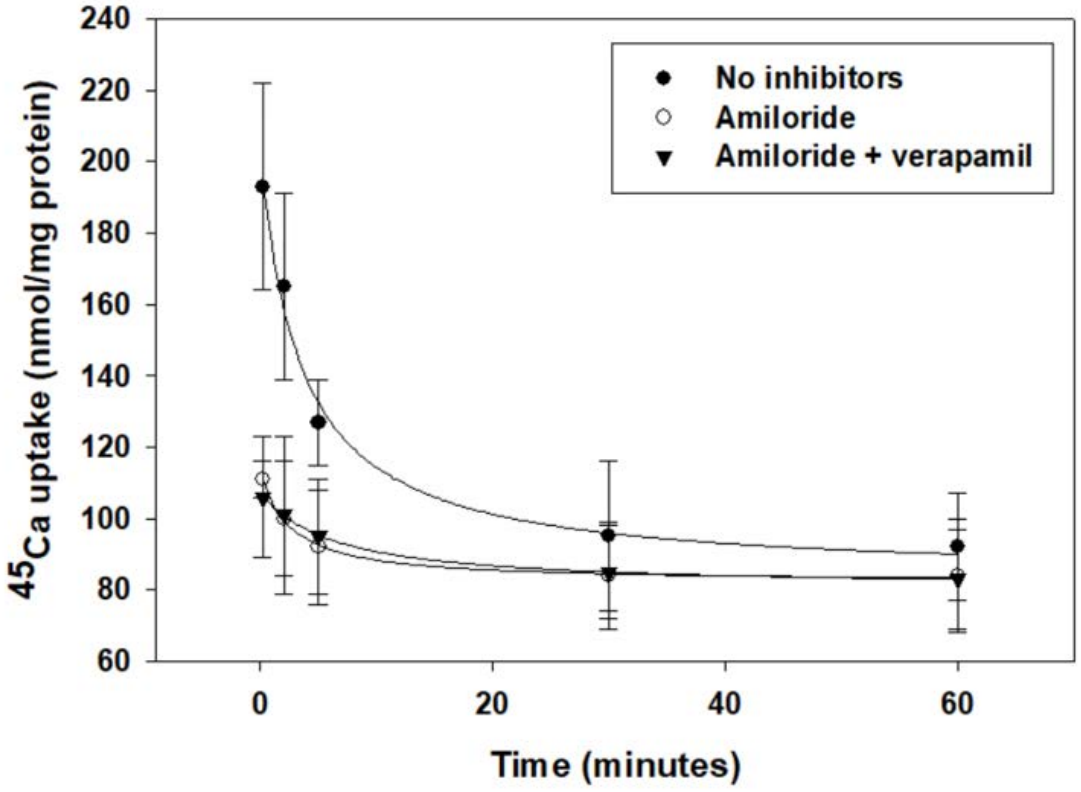

Figure 12 Effects of 2 mM amiloride and 100 µM verapamil on the time course of 250 µM ^{45}Ca uptake. Vesicles were loaded with Buffer 2 containing 300 mM mannitol, 12 mM HEPES/Tris, and 250 µM calcium at pH 7.0. Vesicles were divided into three incubation groups. The first was a control buffer medium (300 mM mannitol, 12 mM HEPES/Tris, at pH 7.0) with no inhibitors. The second buffer medium had the control components plus 2 mM amiloride. The third buffer medium had the control components plus 2 mM amiloride and 100 µM verapamil. Vesicles were incubated in their respective medium for time intervals of 0.25 minutes, 1 minute, 2 minutes, 5 minutes, 30 minutes, and 60 minutes. Values are averages ± 1SEM, n=3 trials per treatment.

DISCUSSION

The Freire et al., (2008) proposed model of crustacean absorption and secretion of calcium suggested that the outward membranes of gill epithelia in crustaceans may possess amiloride-sensitive, electrogenic, Ca^{2+}/H^{+} antiporter, and amiloride-insensitive, electroneutral $Ca^{2+}/2H^{+}$ antiporters, or verapamil-sensitive calcium channel. The results of this study suggest that *L. setiferus* gill epithelia are sites of calcium uptake from freshwater using an electrogenic, amiloride-sensitive, Ca^{2+}/H^{+} antiporter (Figure 2: System B), for which there exist two driving forces: the calcium concentration gradient directed toward the cytoplasm, and asymmetric exchange stoichiometry (electrogenic transport driven by membrane potential). Unlike what has been previously reported for the hepatopancreas and the antennal glands of lobster (Ahearn and Franco, 1993; Zhuang and Ahearn, 1996), there is no evidence for a verapamil-sensitive calcium channel or an electroneutral, amiloride-insensitive, $Ca^{2+}/2H^{+}$ antiporter in the gills of this animal.

Figures 6-9 show that pH 7.0 resulted in the greatest calcium uptake in *L. setiferus* gills. It is important to note that these animals were obtained from waters with a pH ranging from 6.5-7.0 with expected pH decrease of 100-150% by 2100. Thus juvenile *L. setiferus* presently exist in an environment that reaches levels that are too acidic for optimal calcium uptake through gill epithelia. This poses a significant threat to proper development and survival of these animals whom of which are reliant on the uptake of calcium in order to molt, allowing for appropriate growth and protection from predators.

Diffusion through a calcium channel should respond to an imposed transmembrane electrical potential difference established by K^+ diffusion potential using Valinomycin. The results from Figure 11 show that when an outwardly directed (protons driven out of the vesicle creating a negative charge inside of the vesicle) membrane potential (PD) was imposed across the membrane of shrimp gill PPMV with pH 7.0 on both sides, a significant increase in uptake of calcium was shown compared to the control without PD (p=0.003). When the external pH was 8.0, an induced PD also significantly increased calcium uptake compared to the control with an absence of PD (p=0.01), however, uptake at pH 8.0 with or without an induced PD was not as great as calcium uptake at pH 7.0 for both conditions. The greatest calcium uptake still occurred at pH 7.0, in keeping with the results from Figures 7-10.

Uptake of calcium at pH 7.0 with an imposed membrane potential (K^+ inside and mannitol outside) was faster than similar vesicles made at the same time without a membrane potential (Figure 11). Under these experimental conditions, the only driving force for calcium uptake is the membrane potential, indicating that the uptake of calcium is electrogenic transport at pH 7.0, supporting the electrogenic Freire et al., (2008) model (Figure 2: System B).

Previous research by Ahearn and Franco, (1993) and Ahearn and Zhuang (1996) performed on lobster hepatopancreas and antennal glands have shown that 2 mM amiloride inhibits calcium uptake via an electrogenic antiporter and has no effect on electroneutral antiporters, allowing for identification of specific antiporters. Verapamil (100 μM) is an inhibitor of calcium channels in organs where calcium uptake occurs in crustaceans. The rapid uptake followed by a fall to equilibrium in Figure 12 is indicative of a driving force for calcium. There exists strong inhibition by amiloride, and no effect of verapamil. Thus, there is no open channel and no verapamil sensitivity. This indicates an electrogenic amiloride-sensitive carrier with two

driving forces: concentration of calcium, and asymmetric exchange stoichiometry (Figure 2: System B). The mechanism of calcium uptake into *L. setiferus* gill PPMV therefore is through amiloride-sensitive Ca^{2+}/H^+ electrogenic exchange, thus supporting the hypothesis of electrogenic carrier mediated uptake (Figure 13).

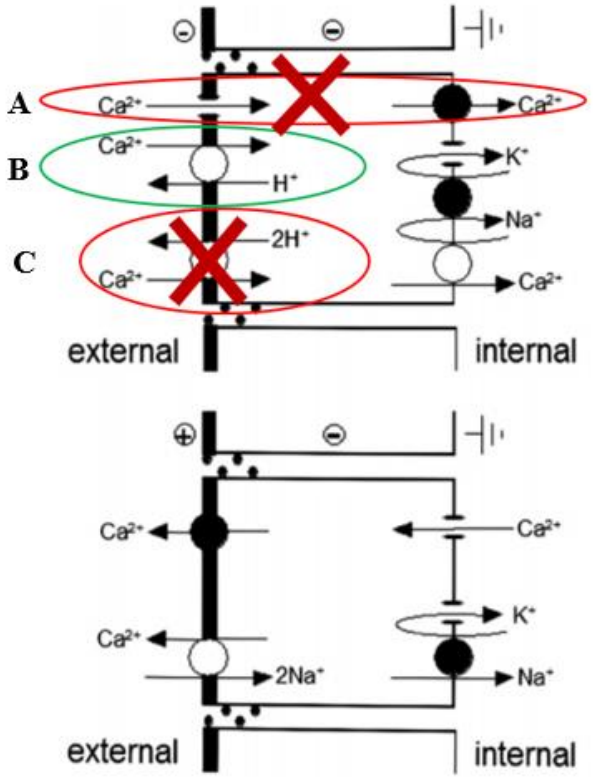

Figure 13 Uptake of calcium into *L. setiferus* gill PPMV is through amiloride-sensitive Ca^{2+}/H^+ electrogenic exchange (**B**). There was an increase in calcium uptake when a membrane potential was induced by valinomycin, suggesting that membrane potential is a driving force for calcium uptake as the proton electrochemical gradient drives calcium into the vesicles. These findings, along with the findings that calcium channel blocker amiloride inhibited calcium uptake into vesicles, system **C** of the Freire model (calcium uptake by amiloride insensitive $Ca^{2+}/2H^+$ electroneutral exchange) was ruled out. Calcium uptake must be electrogenic. To determine the

presence of an open calcium channel, verapamil was tested with amiloride to observe if there was any greater inhibition of calcium uptake from verapamil. Because verapamil had no effect on calcium uptake, there was no evidence for a verapamil-sensitive calcium channel, and system **A** of the Freire model was ruled out (Freire et al., 2008).

Previous work has been conducted by Nagle and colleagues (2018) studying calcium uptake through branchiostegite epithelia of lobster *Homarus americanus*, a marine relative of the freshwater subject of this study *L. setiferus*. Branchiostegites are the extended pleural aspect of the carapace which forms the wall of the gill chamber. The results of this report suggest that the calcium uptake mechanism of marine lobster branchiostegite epithelia is accomplished through the combination of an open calcium channel, as there existed no verapamil sensitivity, and an electroneutral $Ca^{2+}/2H^{+}$ antiporter, as there existed no amiloride sensitivity. There was no evidence for the presence of an electrogenic, amiloride-sensitive, Ca^{2+}/H^{+} antiporter in the lobster tissue as has been reported in this study for the gill epithelia of *L. setiferus*, and which has been reported for hepatopancreas and antennal glands in the lobster (Ahearn and Franco 1993; Zhuang and Ahearn 1996).

The animal used in this experiment, *L. setiferus,* has a juvenile stage in the upper reaches of St. Johns River estuarine nursery where salinity is low, and an adult stage in the ocean. This allows for future work comparing effects of aquatic acidification in freshwater that were addressed in the current study, to effects of aquatic acidification in marine environments with the same animal. Further work is needed to determine if a change from the electrogenic calcium uptake mechanism occurs as *L. setiferus* enters the adult marine stage, and if that change in calcium uptake mechanism reflects the open calcium channel and electroneutral carrier mediated uptake found by Nagle and colleagues (2018) in marine lobster *H. americanus*.

Many previous studies regarding aquatic acidification examine protonation of carbonate and do not consider calcium to be a limiting factor. In studying shrimp larvae (*Pandalus borealis*) under aquatic acidification conditions predicted for the year 2100 by Orr et al., (2005), Bechmann and colleagues (2011) observed decreased calcification of shells and delay in development in low-pH water, addressing undersaturation of calcium carbonate and not considering a potential decrease in the uptake of calcium as the limiting factor. In a review of physiological responses of crustaceans to aquatic acidification, Whiteley (2011) described that crustacean calcification processes are dependent on the altered aquatic carbonate chemistry, and little is known about how calcium uptake is affected under conditions of increasing aquatic acidification. The research focus regarding effects of aquatic acidification on crustacean calcification are mechanisms of acid-base regulation, requiring electroneutral ion exchange across gill epithelia where H^+ exchanges for Na^+, and HCO_3^- exchanges for Cl^- (Cameron et al., 1983; Mantel and Farmer, 1983; Wheatly and Henry, 1992), and not calcium uptake.

Pooled data from 1984-2009 by Whiteley (2011) show that the growth of several other shrimp species, in addition to other crustaceans, exhibit decreased growth rates and decreased egg production (which also require calcium uptake to make calcium carbonate shells) in acidic environments. Specifically, regarding shrimp species *Penaeus occidentalis* and *Penaeus monodon*, exposure to acidic pH ranging from 6.4 to 7.9 (compared to environmental conditions of 8.1) for 56 and 36 days respectively resulted in decreased growth rates (Whiteley, 2011; Wickens, 1984) (Figure 14).

Species	pCO_2 (kPa)	pH	Time	Effect	Source
Adults					
Acartia tsuensis	0.20	7.4	27 d	No effect on survival, body size, development rate, or egg production	Kurihara & Ishimatsu (2008)
Calanus finmarchicus	0.8	6.85	72 h	No effect on adult growth, decrease in egg production	Mayor et al. (2007)
Acartia steueri	0.20–1.0	7.4–6.8	8 d	Decreased egg production at <pH 6.8	Kurihara et al. (2004a,b)
Acartia erythraea	0.51–1.0	7.0–6.8	8 d	Decreased egg production at <pH 6.8	Kurihara et al. (2004a,b)
Amphibalanus amphitrite	–	7.4	8–11 wk	No effect on growth or egg production	McDonald et al. (2009)
Semibalanus balanoides	0.09	7.7	104 d	Decreased survival	Findlay et al. (2009)
Penaeus occidentalis	–	7.6 & 7.3	56 d	Decreased growth rates	Wickins (1984)
Penaeus monodon	–	7.9–6.4	36 d	Decreased growth rates	Wickins (1984)
Palaemon pacificus	1.0	7.9	30 wk	No effect on growth	Kurihara et al. (2008)
	0.20	7.6	15 wk	Decreased growth and egg production	Kurihara et al. (2008)
Eggs/larvae					
Acartia erythraea	0.20–1.0	7.4–6.8	2d	Increase in nauplius mortality rates and hatching rate	Kurihara et al. (2004a,b)
Acartia tsuensis	0.20	7.4	27 d	No effect on development rate or hatching success	Kurihara & Ishimatsu (2008)
Calanus finmarchicus	0.81	6.95	72 h	Decreased hatching success	Mayor et al. (2007)
Euphausia superba	1.0–2.0	7.7/7.4	26 d	Decreased hatching success	Kurihara et al. (2008)
Amphibalanus amphitrite	–	7.4	8–11 wk	No effect on larval condition, cyprid size and attachment, or metamorphosis	McDonald et al. (2009)
Semibalanus balanoides	0.09	7.7	104 d	Decreased rates of embryonic development, hatching and post-larval growth	Findlay et al. (2009, 2010b)
Echinogammarus marinus	0.20	7.5	18–20 d	No effect on rates of embryonic development or hatchling number	Egilsdottir et al. (2009)
Gammarus locusta	0.10	7.6	–	No effect on growth rates to maturity	Hauton et al. (2009)
Palaemon pacificus	0.20	7.6	–	Decreased body size in settling juveniles	Kurihara et al. (2008)
Homarus gammarus	0.12	–	–	No effect on hatchling number or rate of development	Arnold et al. (2009)

Figure 14 Effects of increased aquatic CO_2 and corresponding pH decrease (compared to current oceanic pH of 8.1) over time on growth, reproduction, and development in commercially important crustacean species (Whiteley, 2011).

It is currently unknown what proportion of calcium uptake is performed by each of the four organs (hepatopancreas, kidneys, integumentary epidermis, and gill epithelia) that regulate calcium in crustaceans, and whether one organ can compensate for calcium uptake deficit by another organ. Further studies are needed to determine this relationship between calcium regulating organs, and if calcium regulating function between organs changes with aquatic acidification.

Aquatic acidification is intertwined in the physiology of crustacean calcium dependency, and it is imperative to further research these important issues so that awareness of the severity of these changes in aquatic conditions can lead to changes in current policies and practices.

WORK CITED

Ahearn, A. G., Duerr, M. J., Zhuang, Z., Brown, J. R., Aslamkhan, A., Killebrew, A. D., 1998. Ion transport processes of crustacean epithelial cells. *Invited Perspectives in Physiological and Biochemical Zoology*, 72, 1-18.

Ahearn, A. G., Franco, P., 1993. Ca2+ transport pathways in brush-border membrane vesicles of crustacean glands. *The American Journal of Physiology*, 64, 206-213.

Ahearn, G.A., Mandal, P.K., Mandal, A., 2004. Calcium regulation in crustaceans during the molt cycle: a review and update. *Comparative Biochemistry and Physiology A* ,137, 247–257.

Ahearn, A. G., Zhuang, Z., 1995. Cellular Mechanisms of Calcium Transport in Crustaceans. *Society for Integrative and Comparative Biology,* 69, 383-402.

Bechmann, R., Taban, I., Westerlund, S., Godal, B., Arnberg, M., Vingen, S., Ingvarsdottir, A., Baussant, T., 2011. Effects of ocean acidification on early life stages of shrimp (*Pandalus borealis*) and mussel (*Mytilus edulis*). Journal of Toxicology and Environmental Health, 74, 424-438.

Bennett, Jennifer. “Ocean Acidification.” Ocean Portal | Smithsonian, Smithsonian's National Museum of Natural History, 18 Dec. 2018, ocean.si.edu/ocean-life/invertebrates/ocean-acidification.

Cameron, N., Mangum, P., Bliss, E., 1983. Environmental adaptations of the respiratory system: ventilation, circulation and oxygen transport. In: Vernberg J, Vernberg WB (eds) The Biology of Crustacea: Environmental adaptations. Academic Press, New York, pp 43–63.

Flik, G., Haond, C., 2000. Na+ and Ca2+ pumps in the gills, epipodites, and branchiostegites of the European lobster *Homarus gammarus*: effects of dilute seawater. *Journal of Experimental Biology*, 203, 213-220.

Flik, G., Verbost, P., Atsma, W., 1994. Calcium transport in gill plasma membranes of the crab *Carcinus maenas*: evidence for carriers driven by ATP and NA+ gradient. *Journal of Experimental Biology*, 195, 109-122.

Freire, A. C., Onken, H., McNamara, C.J., 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comparative Biochemistry and Physiology A*, 151, 272-304.

Henry, P. R., Wheatly, G. M., 1992. Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist*, 32, 407-416.

Hessen, D., Kristiansen, G., Lid, I., 1991. Calcium uptake from food and water in the crayfish *Astacus astacus* measured by radioactive ^{45}Ca. *Crustaceana*, 60, 76-83.

Hopfer, U., Nelson, K., Perrotto, J., Isselbacher, K., 1973. Glucose transport in isolated brush border membrane from rat intestine. *Journal of Biological Chemistry*, 248:25032.

Liittschwager, D., 2014. Pteropod Experiment. NOAA PMEL Carbon Group. https://www.pmel.noaa.gov/co2/file/Pteropod+shell+experiment

Lucu, Č., 2008. Changes in Na^+/K^+-ATPase activity, unsaturated fatty acids and metallothioneins in gills of the shore crab *Carcinus aestuarii* after dilute seawater acclimation. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 149(4), 363-372.

Mantel, H., Farmer, L., 1983. Osmotic and ionic regulation. In: Mantel LH (ed) The biology of Crustacea. Internal anatomy and physiological regulation. Academic Press, New York., pp 53–16.

Meissner, K., Bralower, T., Alexander, K., Dunkley Jones, T., Sijp, W., Ward, M., 2014. The Paleocene-Eocene Thermal Maximum: How much carbon is enough? *Paleoceanography*, 29(10), 893-1001.

Nagle, L., Brown, S., Krinos, A., Ahearn, G., 2018. Ocean acidification: effects of pH on ^{45}Ca uptake by lobster branchiostegites. *Journal of Comparative Physiology B*, DOI:10.1007/s00360-018-1173-2

NOAA Fisheries. “White Shrimp.” Species Directory| NOAA Fisheries, 2016, https://www.fisheries.noaa.gov/species/white-shrimp

Orr, James., Fabry, V., Aumont, O., Bopp, L., Doney, S., Feely, R., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R., Lindsay, K., Maier-Reimer, E., Matear, R., Monfrey, P., Mouchet, A., Najjar, R., Plattner, G., Rodgers, K., Sabine, C., Sarmiento, J., Schlitzer, R., Slater, R., Totterdel, I., Weirig, M., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, 437, 681-686.

Phillips, J., McKinley, G., Bennington, V., Bootsma, H., Pilcher, D., Sterner, R., Urban, N., 2015. The potential for CO_2-induced acidification in freshwater: A Great Lakes case study. *Oceanography*, 28, 136-145.

Robins, L., Lisle., 2017. Regional acidification trends in Florida shellfish estuaries: a 20+ year look at pH, oxygen, temperature, and salinity. *Estuaries and Coasts*, 41:5, 1268-1281.

Saris, N., Andersson, M., Mikkola, R., Andersson, L., Teplova, V., Grigoriev, P., Salkinoja-Salonen, M., 2009. Microbial toxin's effect on mitochondrial survival by increasing K^+ uptake. *Toxicology and Industrial Health*, 25(7):441-446.

St. Johns River Water Management District. 1999. Ground-water quality of the surficial aquifer system and the upper Floridian aquifer, Ocala National Forest and Lake County, Florida, 1990-99.

Towle, D., 1993. Ion transport systems in membrane vesicles isolated from crustacean tissues. *The Journal of Experimental Zoology*, 265: 387-396.

United States Environmental Protection Agency. "Greenhouse Gas Equivalencies Calculator." Energy and the Environment| EPA, 2018, https://www.epa.gov/energy/greenhouse-gas-equivalencies-calculator

Weiss, L., Potter, L., Steiger, A., Kruppert, S., Frost, U., Tollrain, R., 2018. Rising pCO_2 in freshwater ecosystems has the potential to negatively affect predator-induced defenses in *Daphnia*. *Current Biology*, 28, 327-332.

Wheatly MG, Henry RP (1992) Extracellular and intracellular acid-base regulation in crustaceans. *Journal of Experimental Zoology*, 263,127–142.

Wheatly, M.G., Zanotto, F.P., Hubbard, M.G., 2002. Calcium homeostasis in crustaceans: subcellular Calcium dynamics. *Comparative Biochemistry and Physiology B*, 132, 163–178.

Whitley, N. M., 2011. Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series*, 430, 257-271.

Wickins, F., 1984. The effect of hypercapnic seawater on growth and mineralization in penaeid prawns. *Aquaculture*, 41, 59-75.

Zhuang, Z., Ahearn, G., 1998. Energized Ca^{2+} transport by hepatopancreatic basolateral plasma membranes of *Homarus americanus*. *Journal of Experimental Biology*, 201, 211-220.

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