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Alix Melton
University of Puget Sound

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The effect of increasing ambient salinity on the heart rate and osmoregulatory ability of the invasive crayfish, *Orconectes sp.*

Alix Melton

Biology 334, University of Puget Sound

Professor Alexa Tullis

Abstract

Increased physiological tolerances to environmental factors such as salinity have been demonstrated to contribute to the success of invasive species. This study sought to investigate the salinity tolerance of *Orconectes sp.* (invasive to North America and Europe) as a method of characterizing a possible source of this genus' success. Heart rate and hemolymph osmolarity were measured in response to increasing environmental salinity. The results show that *Orconectes sp.* is an imperfect hyper-regulator of hemolymph osmolarity in up to 600 mOsm environments, at which point it transitions to be a slight hyporegulator. The heart rate of *Orconectes sp.* was affected only as the crayfish transitioned from 455 mOsm to 751 mOsm environmental osmolarity (50-75% sea water), at which point heart rate decreased. This, combined with elevated mortality rates in highly saline environments suggests that *Orconectes sp.* would not be able to extend its range to include brackish water or marine environments. However, its moderate tolerance may allow it to cross saline barriers to find novel freshwater environments or find habitats in areas with unstable salinity levels.

Introduction

The crayfish genus *Orconectes* includes several invasive species, including *Orconectes virilis* and *Orconectes rusticus*. *Orconectes virilis* is native to the Missouri, Mississippi, Ohio and Great Lakes drainages in the United States and has been introduced to other regions of North America as well as Europe (2010, *Orconectes virilis*). *Orconectes rusticus*, native to Ohio, Illinois, Indiana, and Kentucky watersheds, has spread to other parts of the northeast United States (2010, *Orconectes Rusticus*). The effect of the invasion of *Orconectes spp.* to freshwater lakes includes decrease in snail and macrophyte abundance and diversity (Rosenthal et al., 2006).

A California study on the characteristics of successful invasive fish species displayed that physiological tolerances to factors such as pH, temperature, and salinity were some of the most important attributes contributing to the invasiveness of species (Marchetti et al., 2004). These physiological tolerances allow invasive species to be habitat generalists and thus be more portable between environments where these factors may differ. Knowing the salinity tolerance of *Orconectes sp.* would allow us to determine its ability to invade habitats with unstable salinity levels and brackish waters. In addition, inland salinity levels in Australia have been shown to be increasing in some areas due to anthropogenic causes (Williams, 2001). If this phenomenon were

to spread to areas where *Orconectes sp.* inhabits (or the surrounding areas), an increased salinity tolerance would allow the genus to out-compete organisms with lower tolerance.

Physiological tolerance to changes in salinity is dependent on an organism's osmoregulatory ability. The challenges of osmoregulation vary depending on the relative osmolarities of the environmental and internal environments of an organism. Many crustaceans occupy marine environments and are osmoconformers—their hemolymph osmolarity is the same as environmental osmolarity. However, crustaceans such as *Orconectes sp.* that have adapted to freshwater environments face the challenges of hyperosmoregulation—they must minimize water gain and salt loss (Susanto and Charmantier, 2000).

The physiological mechanisms of hyperosmoregulation in various fresh water organisms have been described by several studies. These organisms rely in part on ion exchanges that allow for maintaining internal a high Na^+ and Cl^- gradient using a process that requires ATP production and suggests a role for mitochondria-rich cells found in the gills of many freshwater organisms (Kirschner, 2004). These organisms produce urine that is hypotonic to their blood to allow them to lose some of the water gained by flux, a process that also requires energy in the form of ATP (Potts, 1954). Changes in surface permeability have also been shown to be method of decreasing flux (Campbell and Jones, 1990). These physiological mechanisms of osmoregulation often require ATP, and thus an organism's tolerance of salinity changes may hinge on its ability to mediate the increased metabolic costs of osmoregulation.

While most crayfish species live in freshwater environments, some species have been shown to inhabit brackish waters. Additionally, several crayfish species have been shown to be capable of both hyper-regulation of hemolymph at low environmental salinities and hypo-regulation at high environmental salinities (Holdich et al., 1997). To our knowledge, no studies have investigated the particular capability of *Orconectes sp.* to osmoregulate their hemolymph in saline environments. In addition, no present studies appear to have investigated the response of the heart rate of this genus to changes in environmental osmolarity. This study will investigate the hemolymph osmolarity and heart rate responses of *Orconectes sp.* to increased environmental salinity after chronic acclimation. Measuring chronic acclimation, as opposed to acute response, provides a better description of what would occur if *Orconectes sp.* were to invade a more saline environment. Previous studies have shown that the heart rates of other crayfish genera increase acutely but then return to normal (Kozak et al., 2009). Thus we predict that chronically

acclimated *Orconectes sp.* will have stable heart rates across environmental osmolarities. We will also observe the hemolymph osmolarity in *Orconectes sp.* in response to varied environmental osmolarities. Previous studies have shown other crayfish genera to hyper-regulate their hemolymph up to a critical point at which they switch to hyporegulation (Holdich et al., 1997). This study will allow us to characterize how salinity tolerance may play a role in the invasiveness of *Orconectes spp.* and provide information about the ability of this crayfish to invade to saline environments. This study will also, by measuring heart rate, provide a model for predicting the metabolic cost of such an invasion.

Materials and Methods

Animal Acquisition and Care

The *Orconectes sp.* utilized in this study were obtained by and under the care of the University of Puget Sound biology department. The crayfish were farm raised and purchased from Kyle LeBlanc Crayfish Farms in Raceland, LA. The total sample included 10 females, 5 males, and 6 individuals that died before the conclusion of the study and we were unable to sex. Crayfish were stored 12"x18" bins in approximately 5 cm of 12° C de-ionized water. There were 4 crayfish assigned to each bin. Water was changed twice a week. The crayfish were fed shrimp/algae wafers (1 wafer per 4 crayfish) after each water change (Tullis, 2013).

General Experimental Procedure

A control group of four crayfish was kept in freshwater (0 mOsm) for the duration of the experiment. Water salinity in the experimental group's environment was incrementally increased by approximately 250 mOsm per week. This progression started at 0 mOsm and ended at approximately 750 mOsm. Hemolymph osmolarity, heart rate, and mass were determined for each crayfish at the end of each seven-day period. The salinity was changed each week immediately following data collection. Behavior of each crayfish was monitored twice a week prior to feeding. We aimed to have a sample size of 4 control and 12 experimental crayfish. When mortalities occurred, another crayfish was introduced to compensate, but not until after data was collected for that week. Thus, mortalities diminished sample sizes within testing periods.

Behavior Observations

Crayfish behavior was quantified according to the following scales: shelter, 1=inside tube, 2=under side of tube, 3= against the side of bin, 4= no shelter; movement (no outside

stimulation), 1=completely still, 2= twitching legs, 3= slow walking, 4= walking around a lot; movement (after waving hand 6" above tub), 1=no response, 2=backs off slightly, 3=moves quickly, 4=poised to attack; movement (after waving hand 1" above tub), 1=no response, 2=backs off slightly, 3=moves quickly, 4=poised to attack; movement (after prodding with pen near antennae), 1=no response, 2=backs off slightly, 3=moves quickly, 4=poised to attack.

Hemolymph Osmolarity Collection

Crayfish hemolymph osmolarity was determined according to the procedures described in Tullis (2013a). Hemolymph was drawn from the crayfish by inserting a 1cc syringe into the heart through the junction of the abdomen and cephalothorax and drawing up 50 μ L. This aliquot of hemolymph was injected into a microcentrifuge tube. Hemolymph osmolarity was then determined using a Wescor Vapor Pressure Osmometer. All syringes, microcentrifuge tubes, pipette tips, and samples were stored on ice to prevent clotting of the hemolymph.

Heart Rate Collection

Heart rate was collected according to the procedures laid out in Tullis (2013b). Crayfish were submerged in approximately 1" of water with the same osmolarity as they had been acclimated to and immobilized by rubber band restraints. Heart rate was measured by inserting impedance electrodes at the junction of the abdomen and cephalothorax on either side of the heart. These electrodes were connected to an impedance converter and data was collected using PowerLab 15T and LabChart 7. The heart rate was monitored for 1-2 minutes at each temperature.

Data Analysis

The mean heart rate (BPM) for each crayfish at each osmolarity was determined in LabChart 7. The mean was taken in areas of each trace where heart rate remained steady. Overall behavior scores for each day were determined by adding the scores from each subcategory. Statistical analysis was performed using R. A 2-way ANOVA was performed to analyze average behavior scores for the control group and experimental group for each day of behavior measurement. The change in heart rate and change in osmolarity were determined for each week. 2-way ANOVAs were used to determine if the treatment affected the crayfish over time. T-tests were used to compare the experimental and control groups each week. Absolute means for hemolymph osmolarity and heart rate at each environmental osmolarity were determined and analyzed using 1-way ANOVAs. Means are reported \pm SE.

Results

Behavior did not significantly correlate with change in environmental salinity. The interaction term between treatment and week was insignificant (2-way ANOVA, $F_{5,74}=1.8406$, $p=0.11532$; Figure 1). Additionally, t-tests comparing the control and experimental groups each week confirmed that the experimental group did not differ from the control at $\alpha=0.05$. Lethargic behaviors (low activity scores) were often noted in the testing period prior to the death of crayfish. Heart rate and osmolarity measurements did not appear to change significantly prior to death.

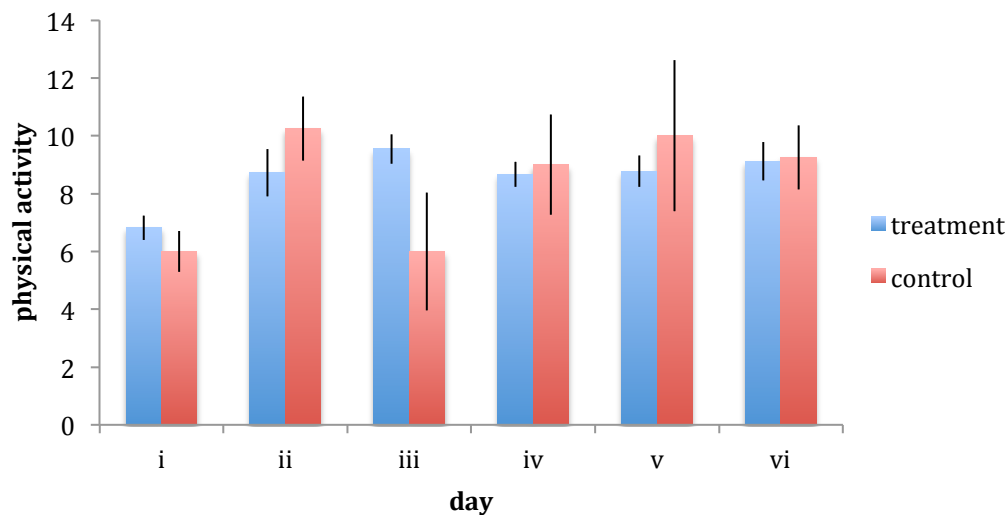


Figure 1. Physical activity level of *Orconectes sp.* when subjected to increasing environmental salinity. Behavior measurements were taken three days and seven days after introduction to each salinity. Environmental osmolarities: i- 231.5 mOsm, ii-231.5 mOsm, iii-455 mOsm, iv-455 mOsm, v-751 mOsm, vi-751 mOsm. Behavior scores are represented as mean \pm SE. Higher scores indicate more active behavior. Behavior of the experimental group did not differ significantly from that of the control group.

Over the course of the experiment, we had nine mortalities. Two of these mortalities were in the control group during week 2. Of the seven mortalities in experimental groups, one occurred in the 232 mOsm environment, two at 455 mOsm, and three at 751 mOsm. Two of the control group individuals and six of the experimental group individuals survived through the duration of the experiment.

One heart rate data point was dropped from the experimental group at 231.5 mOsm (Grubbs Test, $p<0.05$). Average heart rate of the crayfish changed as salinity increased but this

change was not directional and was only marginally significant (1-way ANOVA, $F=2.804$, $df=3$, 43 , $p=0.051$; Figure 2). The average heart rate was highest at 231.5 mOsm (117.4 ± 6.5 BPM) and lowest at 750.5 mOsm (104.2 ± 15.5 BPM; $p=0.070$). The interaction term for average change in heart rate of each treatment with week was significant (2-way ANOVA, $F=6.3007$, $df=2$, 32 , $p=0.005$; Figure 3). However, t-tests show that the heart rate in the experimental group only differed significantly from that of the control when in the 751 mOsm environment. Change in heart rate of the experimental group initially was positive (as environmental osmolarity increased from 0-250 mOsm) but became increasingly negative as osmolarity increased to ≈ 500 then ≈ 750 mOsm.

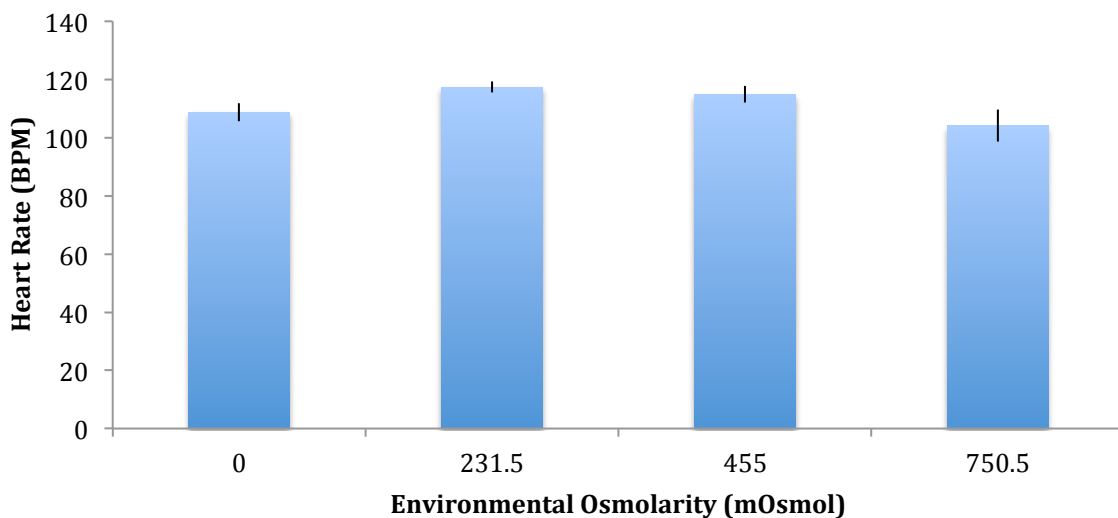


Figure 2. Cardiac response of *Orconectes sp.* to increasing environmental osmolarity. Heart rate is represented as mean \pm SE. Average heart rate showed only marginally significant change with environmental osmolarity.

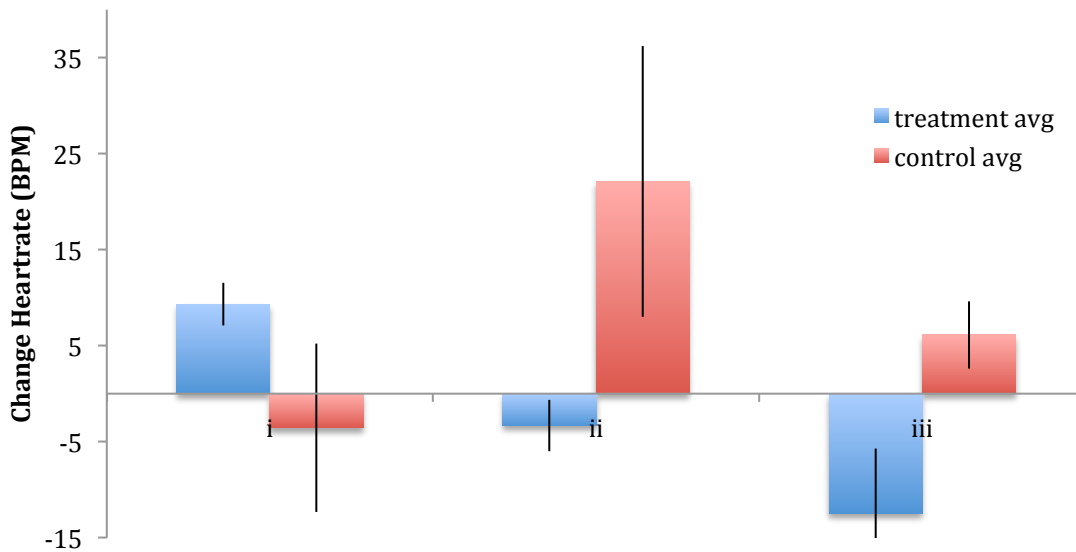


Figure 3. Average change in heart rate of *Orconectes sp.* per 250 mOsm incremental increase of environmental osmolarity. Changes in environmental osmolarity (x-axis): i= 0-231.5 mOsm, ii=231.5-455 mOsm, iii=455-751 mOsm. Change in the heart rate of the experimental group was significantly different from the control group during interval iii.

Average hemolymph osmolarity increased significantly with each ≈ 250 mOsm elevation in the environmental salinity (1-way ANOVA, $F=142.7$, $df= 3,44$, $p<0.001$; Figure 4). The average change in hemolymph osmolarity increased significantly in the experimental group each week (2-way ANOVA, $F= 16.262$, $df= 2,32$, $p<0.001$; Figure 5). T-tests for each interval of environmental osmolarity increase show that the change in hemolymph osmolarity of the experimental group differed from that of the control group at all points. The average hemolymph osmolarity of *Orconectes sp.* was hyperosmotic up to environmental osmolarity of 455 mOsm and slightly hyposmotic in the 751 mOsm environment (Figure 4). The transition from hyper- to hyporegulation was calculated to occur at 600 mOsm.

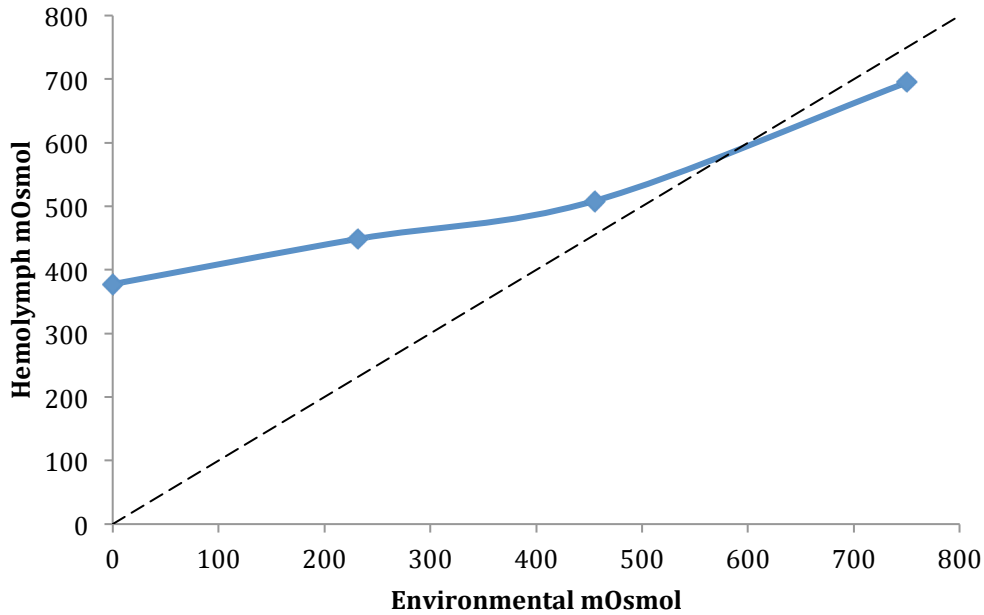


Figure 4. Osmoregulatory abilities of *Orconectes sp.* in increasing environmental osmolarity. Hemolymph osmolarities are represented as mean \pm SE. The dashed line represents the isosmotic line. The experimental group's trendline intercepts the isosmotic line at 600 mOsm. Hemolymph osmolarity increased significantly with each elevation in environmental osmolarity.

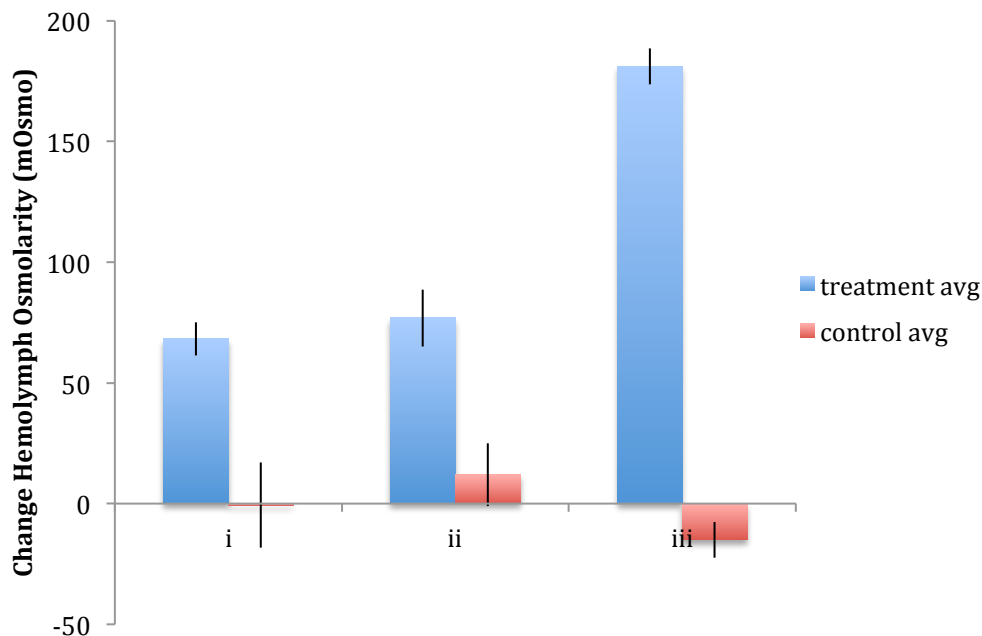


Figure 5. Average change in hemolymph osmolarity in *Orconectes sp.* per 250 mOsm increase in environmental osmolarity. Changes in environmental osmolarity (x-axis): i= 0-231.5 mOsm, ii=231.5-455 mOsm, iii=455-751 mOsm. Change in hemolymph osmolarity is represented as mean \pm SE. *Orconectes sp.* hemolymph osmolarity increased with each elevation of environmental osmolarity, with the largest increase occurring during interval iii.

Discussion

This study sought to characterize the osmoregulatory ability of an invasive species of crayfish, *Orconectes sp.* to see if physiological tolerance to salinity may be a contributing factor to its invasiveness. The results of this study demonstrate that *Orconectes sp.* is able to hyper-regulate its hemolymph osmolarity up to a critical point at 600 mOsm, when it transitions to slight hyporegulation. Although *Orconectes sp.* demonstrated the ability to hyper-regulate below this critical point, it was not a perfect hyper-regulator. With each ~250 mOsm increase in environmental osmolarity, we saw a significant increase in hemolymph osmolarity. This increase was similar during the first two increases in environmental osmolarity (to ~250 and ~500 mOsm) but more than doubled when environmental osmolarity was increased from ~500-750 mOsm (Figure 5). This pattern is similar to what has been reported for other crayfish genera. However, the 600 mOsm threshold of *Orconectes sp.* is higher than that reported for several crayfish genera, including other invasive crayfish (Holdich et al., 1997). *Orconectes sp.* increased threshold for this transition might make it a more successful invader by allowing it to function somewhat normally in more saline environments.

The heart rate of *Orconectes sp.* was not universally affected by environmental osmolarity. The only point at which the experimental group heart rate changed significantly compared to the change in the control group was when environmental osmolarity was increased from 455 to 751 mOsm. This also is the interval at which the crayfish switched from hyper- to hyporegulation of hemolymph osmolarity. If hyporegulation were a more energetically costly activity than hyper-regulation, we would expect to see the opposite effect. This is expected because blood would circulate faster to provide oxygen for the tissue's metabolic activities. Thus, the decrease in heart rate could mean that the osmoregulation at higher salinities is less energetically costly. However, the lack of correlation between heart rate and changes in environmental osmolarity at lower salinities suggests that it is more likely the crayfish were stressed to the point where their bodies began to fail (causing their heart rates to decrease).

While we did not have enough mortalities to perform statistical analysis of mortality rates, it appears that mortality rates rose as environmental salinity rose. In the experimental group, we saw zero mortalities in freshwater, one mortality at 232 mOsm, two mortalities at 455 mOsm, and three mortalities at 751 mOsm. A separate study on the survival of *Orconectes virilis* when subjected salinated environments found that mortality increased significantly as

environmental osmolarity approached that of ocean water (35 ppt NaCl, 1000 mOsm)(Kendall and Schwartz, 1964). This information, combined with the decrease in heart rate observed in our testing at 750 mOsm, suggests that *Orconectes sp.* may not be suited for the colonization of highly salinated environments. However, even if *Orconectes sp.* were unable to tolerate high salinities for long enough time periods to colonize brackish or marine environments, its ability to survive for days at a time in these environments might allow it to expand its range by being able to cross salinity barriers to new freshwater habitats. For example, if a marine bay contains the mouths of two rivers, one of which is inhabited by *Orconectes sp.* and one which is not, the crayfish's salinity tolerance may allow it to cross the bay and gain access to another freshwater environment (Kendall and Schwartz, 1964).

To further characterize the osmoregulatory abilities of *Orconectes sp.*, it would be useful to extend the time period of acclimation to each experimental condition as well as test osmolarities up to 1000 mOsm. This would provide more information about the long-term ability of *Orconectes sp.* to inhabit these highly saline environments. Additionally, a study on another crayfish, *Cherax quadricarinatus* examined the effect of salinity on at different points in the life cycle and found that growth rates in this species are slowed by chronic exposure to saline environments (Meade et al., 2002). Although our results suggest that adult *Orconectes sp.* could likely inhabit partially saline environments without severe physiological disruption, we were unable to examine the effect of salinity across the crayfish lifecycle. This information would be crucial in determining the extent to which *Orconectes sp.* could expand its range to include salinated waters.

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