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Effect of Hyposaline Stress on Metabolic Rate of the Invasive Mussel Mytilus galloprovincialis

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ABSTRACT

Climate change is predicted to decrease ocean salinity as the polar ice caps melt and the occurrence of precipitation events increases. Decreased ocean salinity (hyposalinity) may negatively impact marine invertebrates, especially marine mussels, as they are osmoconformers and their internal osmolarity depends on the solute concentration of their environment. Hyposalinity stress may influence cell function and alter mussel physiology. To compensate for the change in the environment, mussels may expend more energy to restore internal osmolarity, which can be assessed by quantifying metabolic rate. Thus, the purpose of our research was to examine the physiological response of *Mytilus galloprovincialis*, an invasive mussel species in Southern California, to varying salinity conditions. Mussels (N=39 total) were collected from Marina Del Rey, CA and acclimated in tanks to control conditions (34 ppt, 18°C seawater). Following acclimation, mussels were placed in individual closed-system respirometers to measure their oxygen consumption under different salinity conditions (35, control; 28 and 25 ppt, hyposaline) and subsequently used to calculate metabolic rate (MO_2 as mg O_2/min/g wet weight). Mussels exposed to hyposalinity (28 and 25 ppt) showed a trend towards increasing metabolic rate relative to the controls, however these data were not statistically significant (1-Way ANOVA, P=0.0975). Our data suggests that exposure to hyposalinity may cause a physiological response in mussels, where energy may be used to restore internal osmolarity. Coastal marine organisms will experience hyposaline stress as climate change progresses and our data begins to suggest that this may require a greater energy output as seawater conditions change.

INTRODUCTION

Climate change influences on the marine environment

Climate change will cause increases in land and ocean temperatures, increased extreme precipitation events, and an increased probability of drought in some areas of the world (IPCC, 2019). In marine environments, organisms will face challenges such as ocean acidification, increasing temperature, sea level rise and decreasing ocean salinity as climate change progresses (IPCC, 2019; Cole, 2016). In order to survive, an organism's physiology must change to cope with these conditions, both in the short and in the long term (Cole, 2016). For example, an organism may increase gene expression of heat shock proteins when exposed to high temperatures in order to maintain cellular function. In the short term, organisms with greater physiological plasticity will be able to withstand changes in the environment while acclimatization will be necessary if the changes are sustained over a long period (Somero, 2011). One of the most notable responses to climate change is a shift in species range to an area with more tolerable conditions (Tomanek, 2012; Doney *et al.*, 2012). The physiology and survival of marine life will be greatly affected by changing environmental conditions, necessitating immediate physiologic changes and eventual adaptation.

With increasing global temperature, organisms will likely experience greater metabolic rates (Somero, 2012). Increases in metabolic rate elevate heart rate to adequately supply tissues with oxygen, which can be sustained up to a certain critical point depending on the species (Somero, 2012). For each species, there is a critical temperature at which heart rate is maximized (T_{max}), above which the heart rate will decrease rapidly (Somero, 2012). Investigating this maximum temperature can help establish upper temperature limits for a species and can be used to predict the range of habitats in which they can live (Somero, 2012). Additionally, gene transcription and protein expression experience a variety of changes as a result of stress conditions (Somero, 2012). The effects that a particular species will experience due to climate change are unique to their tolerance of environmental conditions, therefore study is needed to determine the exact range of conditions in which a species can live to predict its future range as conditions in its current range change.

Blue mussels of the genus Mytilus and differential stress tolerance

Blue mussels are marine invertebrates that reside in intertidal environments and bays where conditions are frequently changing (Braby and Somero, 2016). On the west coast of the United States, the native mussel species *Mytilus trossulus* lives from Alaska to Central California, coexisting at the southern end of its range with the invasive species *Mytilus galloprovincialis*, which ranges from Central California to Mexico (Braby and Somero, 2016). Additionally, the invasive *M. galloprovincialis* forms a hybrid zone with *M. trossulus* in Japan, and lives on the East Coast of the United States, forming a hybrid zone with the native *M. edulis* (Braby and Somero, 2016). The ranges of each species that are currently observed are a direct result of the species' environmental tolerance. *Mytilus trossulus* is native to the North Pacific Ocean and is adapted to live in an environment with cold water temperatures and relatively low salinity, whereas *Mytilus galloprovincialis* emerged in the relatively warm and salty Mediterranean (Braby and Somero, 2016). The wide range of *M. galloprovincialis* spanning multiple continents indicates its tolerance for a wider range of environmental conditions than each of the native species with which it coexists, due to the environmental conditions of the area in which it developed (Braby and Somero, 2016).

To investigate the differences in tolerance of environmental conditions between these two species, a study by Tomanek et al. (2012) investigated the protein changes behind the responses of *M. galloprovincialis* and *M. trossulus* to salinity stress. After being exposed to one of three salinity treatments (24.5, 29.8 or 35 ppt) for four hours, some mussels were dissected while others were allowed to recover in 35 ppt salinity (control) for 24 hours (Tomanek *et al.*, 2012). The results of this study indicate that recovery from hyposaline stress (24.5 and 29.5 ppt treatments with a 24-hour recovery period) in *M. trossulus* involves a greater decrease of NADH production and oxidation than it does in *M. galloprovincialis*, which experienced downregulation in these pathways only at the 24.5 ppt salinity treatment with 24-hour recovery (Tomanek *et al.*, 2012). These responses indicate that the response to hyposaline stress in both species involves altering the mussel's metabolism, as NADH is an important molecule in metabolic processes. The same mussels used in this study were used to investigate the transcriptomics of salinity stress between these two congeners, finding that only 12 genes differed between the species in response to hypoosmotic stress (Lockwood and Somero, 2011). These studies indicate that *M.*

trossulus and *M. galloprovincialis* vary slightly in their cellular responses to stress, but the effects of these differences on their habitat tolerance are pronounced.

M. trossulus has been shown to have a greater tolerance for lower salinity conditions than the native *M. galloprovincialis*, which could prove essential in predicting changes in each species' range as climate change affects global ocean salinity levels (Tomanek *et al.*, 2012). As conditions change, the potential for the ranges of each of these two species to shift arises. Range shift has already occurred in *M. edulis* on the East Coast of the United States, attributed to a rise in temperature that the mussels are unable to survive (Somero, 2012). It is important to study the responses of each of these two species to salinity stress as previous research indicates that *M. galloprovincialis* is less tolerant to lower salinities, which could lead to changes in its species range (Tomanek *et al.*, 2012). Increases in extreme precipitation events due to climate change will decrease the salinity of the waters they inhabit, perhaps to the point where the mussels can no longer survive in the areas in which they currently live.

Mussels are osmoconformers, which means their internal osmotic pressure matches that of their external environment, so they are particularly sensitive to changes in ambient salinity (Freitas, 2017; Hamer et al., 2008). As ambient salinity decreases as a result of climate change, mussels alter their physiology to cope with hypoosmotic stress. When the external osmolarity is greater than the internal osmolarity, water passively diffuses out of cells while salts are passively transported into cells by Na^+ and Cl^- channels. Mussels use taurine as an osmolyte to regulate their internal cellular volume such that when the external osmotic pressure increases, the concentration of taurine is increased to maintain internal cellular volume without affecting other intracellular contents, preventing the passive loss of intracellular water to the environment (Hosoi et al., 2005). In previous studies of *M. galloprovincialis*, decreases in salinity conditions have been shown to produce physiological responses in mussels (Freitas *et al.*, 2017). A study by Freitas *et al.* (2017) exposed *M. galloprovincialis* mussels to two different pH conditions (7.8 and 7.3, both at control 28 ppt salinity) and three different salinity conditions (14, 28 and 35 ppt, all at control 7.8 pH) by gradually acclimating them over 2-3 days by decreasing the pH and increasing or decreasing the salinity away from the control value. The electron transport chain activity and energy reserves of the mussels were measured after the mussels were exposed to their respective conditions for 28 days, finding that mussels exposed to hyposaline stress (14 ppt) at control pH 7.8 experienced increased electron transport chain activity, indicating increased

metabolic rate under hyposaline conditions (Freitas *et al.*, 2017). This suggests that maintaining intracellular osmotic pressure under hyposaline conditions requires energy.

Assessing physiological impacts of environmental stress through respirometry

Physiologic changes in energy consumption of marine organisms can be assessed by calculating metabolic rate. Metabolic rate is a measure of the rate at which metabolism occurs in an organism under a set of conditions, which can be calculated after performing respirometry (Svendsen, 2016). Closed-system respirometry is a controlled experimental method used to detect an aquatic animal's consumption of oxygen (Svendsen, 2016). The animal of interest is placed in a chamber located in a closed loop with a recirculation pump and an oxygen sensor (Svendsen, 2016). The same water is recirculated through the chamber, therefore as the animal consumes oxygen, the sensor detects the change in the amount of dissolved oxygen within the water (Svendsen, 2016). The data collected from a respirometry experiment are then used to calculate metabolic rate (MO_2 as mg O_2/min*g).

The purpose of our study is to measure the metabolic rate of *M. galloprovincialis* mussels under decreasing salinity conditions. We expect that mussels exposed to hyposaline stress will have greater metabolic rates than mussels under control conditions. It is predicted that energy will be used to create a physiologic response compensating for the experienced hyposaline stress as mussels acclimate to varying salinity conditions. This study can be used to make predictions regarding the response of *M. galloprovincialis* to decreasing salinity as climate change progresses and project potential shifts in their range due to changes in ambient salinity.

MATERIALS AND METHODS

Animal Care and Maintenance

Mytilus galloprovincialis mussels (*N*=80) were collected from Marina del Rey, CA following guidelines set by the California Department of Fish and Wildlife (Permit GM-182770006-18277-001). Mussels were selected if their shell length was between 4.5 and 6.5 cm. The mussels were acclimated to aquarium tanks in control conditions (34 ppt, 18ºC) at Loyola Marymount University for at least 2 weeks without food prior to experimentation.

Respirometry Setup

Closed-system respirometry (Loligo Systems, Viborg, Denmark) was performed using AutoResp (v. 2.0, Loligo Systems) software to measure mussel oxygen consumption. The respirometry took place in two 50 gallon tanks filled with 30 gallons of seawater adjusted to the experimental salinity. Seawater was created using Instant Ocean sea salt mixed with DI water and salinities verified using a refractometer. DI water was added to decrease salinity and Instant Ocean was added to increase the salinity as appropriate. A total of 8 respirometry chambers (500 mL each) were used, each containing a mussel sealed inside. Each mussel was placed in a mesh sleeve before being placed in the chamber to prevent excessive movement that would influence metabolic rate.

Each chamber was attached to a recirculation pump and a flush pump using plastic tubing (Fig. 1). These pumps were connected to a Bluetooth enabled power strip that was controlled by the AutoResp software throughout experimentation. An oxygen sensor was connected to a fiber optic cable and placed in a closed loop with the chamber and the recirculation pump (Fig. 1). Each fiber optic cable for each individual chamber was attached to a Witrox system (one system per respirometry tank for a total of two Witrox systems) that communicated dissolved oxygen data to the AutoResp software via Bluetooth. Each Witrox system had a temperature probe that was placed in the 50 gallon seawater tank, which adjusted the oxygen readings from the sensors according to temperature. The water in the respirometer was maintained at 17 ºC during each experiment using a chiller.

Using the AutoResp software each oxygen sensor was calibrated using a two-point calibration system. For 100% O_2 saturation each oxygen probe was placed in a beaker of air saturated seawater achieved through bubbling with an air-stone until a constant oxygen reading was observed in the AutoResp software. For 0% O_2 saturation each sensor was flushed with 100% nitrogen gas until a constant oxygen reading was observed in the AutoResp software. Calibration settings were established prior to each experiment.

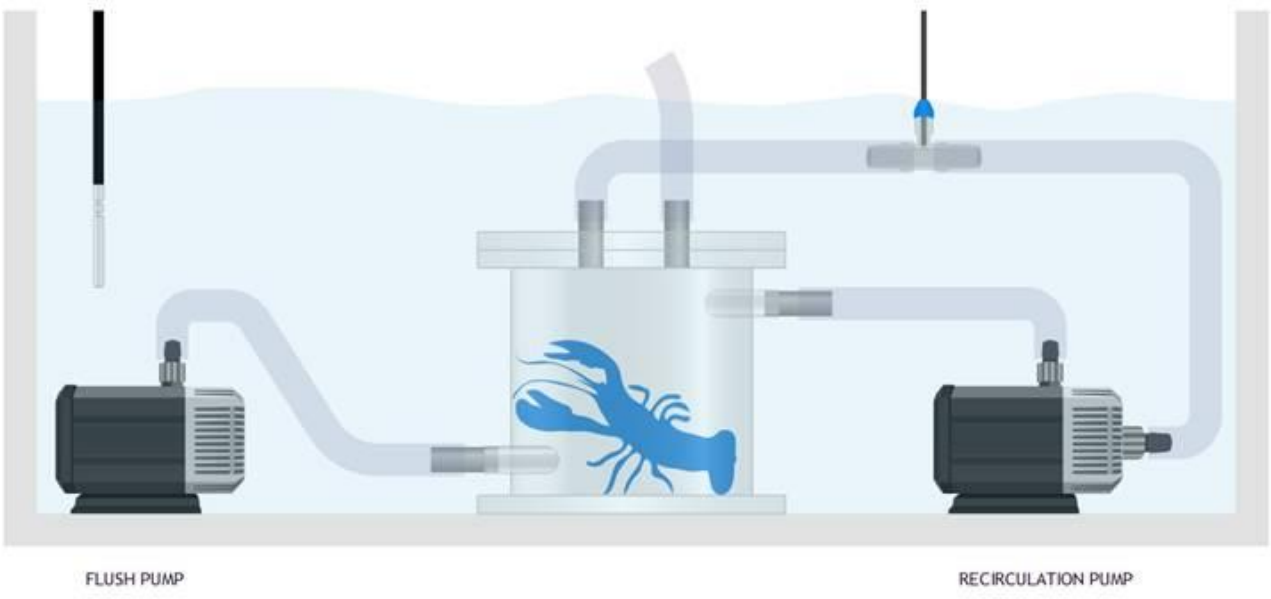

Figure 1. Respirometry setup, with the animal enclosed in a respirometry chamber attached to two pumps. The recirculation loop includes the chamber, the recirculation pump and the oxygen sensor (Loligo Systems).

Experimental Design

Once sealed in the respirometry chamber, mussels were allowed to acclimate to the experimental salinity condition for 45 minutes while ambient water was flushed through the chamber to maintain an oxygenated environment, controlled by the AutoResp software. The respirometer was then closed using the AutoResp software, placing each chamber in a closed loop. The water in the chamber recirculated for at least 45 minutes while the mussels depleted the oxygen through respiration. AutoResp programming collected and stored data on oxygen content of the water in each respirometry chamber.

Three salinity conditions were used: 35 ppt as a control and 28 and 25 ppt as hyposaline treatments. The proper salinity was created in the tank and measured using a refractometer prior to experimentation. 35 ppt was chosen as a control condition as normal seawater tends to have about 35 ppt salinity, while 28 ppt was chosen as a hyposaline condition and 25 ppt was chosen to indicate severe hyposaline conditions. 25 ppt was selected as the lowest salt content to ensure mussel survival while representing severe hyposaline stress. Environmentally relevant salinity conditions were chosen that represent salinities found within the species' range (Braby and

Somero, 2006). Of the total N=39 mussels used in the experiment, N=11 were used in 35 ppt salinity, N=16 mussels were used in 28 ppt salinity and N=12 mussels were used in 25 ppt salinity.

After respirometry, the shell length (cm) of each mussel was obtained using calipers. The mussels were then dissected and the mass (g) of the soft tissue was recorded for each.

Statistical Analysis

The respirometry data was expressed as dissolved oxygen concentrations (mg O_2/L) for each chamber. These data were imported into Excel from the AutoResp software and regression lines were generated for each mussel from the change in oxygen over time (mg O_2/L*min). Mussel data was excluded if a decrease in oxygen did not occur during the respirometry experiment and a linear regression had an R^2 value of less than 0.9. This data was used to find the change in oxygen for each mussel over time.

Using the respirometry data, metabolic rate for each mussel was calculated using Equation 1.

$$MO_2\left(\frac{mg}{hr*kg}\right) = \frac{chamber\ volume*\Delta O_2}{\Delta time*body\ mass\ (g)}.$$

Equation 1. Total chamber volume was calculated by adding the chamber volume (500 mL) and the tube volume (65.497 mL) and subtracting the mussel volume which was equal to the tissue mass. The changes in oxygen and time were unique to each individual and based on the respirometry data.

A One-way ANOVA was used to compare the average metabolic rate for mussels in each salinity treatment (α=0.05) and all analyses were completed using JMP Pro (V. 14.0).

RESULTS

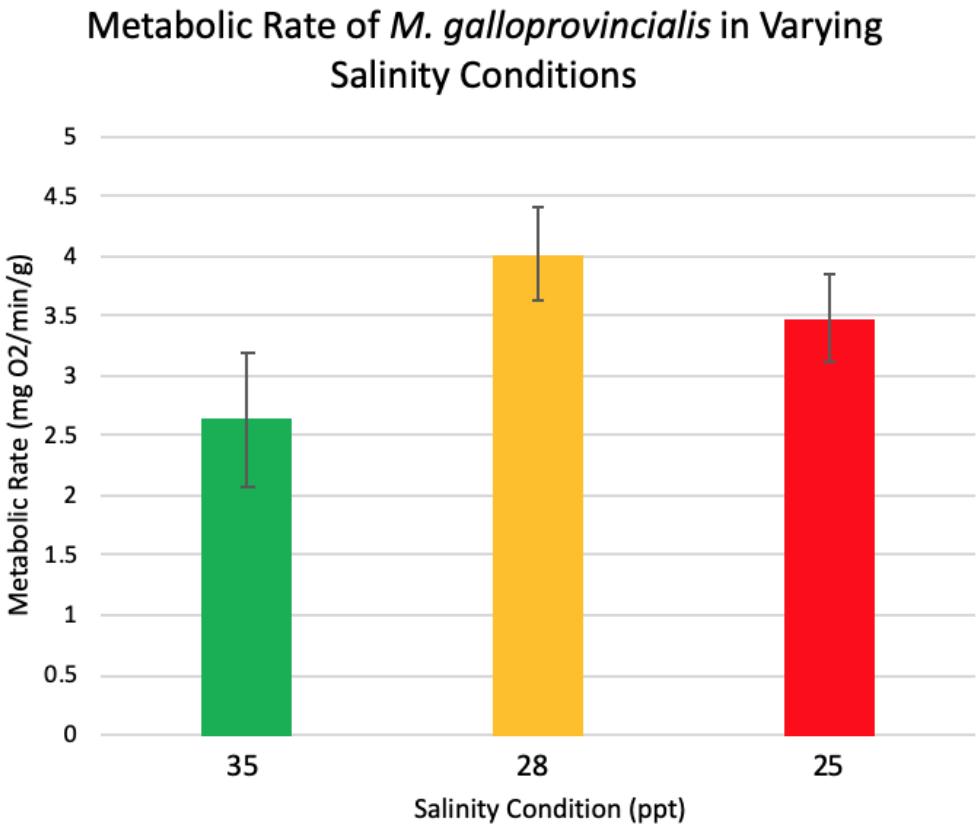

Figure 2. Average metabolic rate (mg O_2/min/g) of *M. galloprovincialis* mussels (n=11-16 per treatment) under each salinity condition (35, 28 and 25 ppt). There was no significant change in metabolic rate due to salinity exposure (One-way ANOVA, P=0.0975, α = 0.05). Error bars indicate ± S.E.M.

Table 1. Number of replicate mussels (n), *M. galloprovincialis*, and average metabolic rate (mg O_2/min/g) for each salinity treatment.

Salinity Treatment (ppt)	Replicates (n)	Average Metabolic Rate (mg O_2/min/g)
35	11	2.64
28	16	4.02
25	12	3.48

The average metabolic rate for mussels exposed to 28 ppt salinity was the highest at nearly 2-fold higher than control, followed by mussels exposed to 25 ppt salinity, which was nearly 1.5-fold greater than control. Mussels exposed to 35 ppt salinity, the control condition, had the lowest average metabolic rate of 3.48 mg O_2/min/g (Table 1, Fig. 2). This indicates a trend towards increasing metabolic rate under moderately hyposaline conditions. However, the difference between the average metabolic rate of mussels under each salinity condition was not statistically significant (One-Way ANOVA, P=0.0975).

DISCUSSION

The purpose of this study was to examine the effect of hyposaline stress on metabolic rate of *M. galloprovincialis* mussels. The results of this experiment indicate that *M. galloprovincialis* mussels show a trend towards increasing metabolic rate under hyposaline conditions as compared to mussels under control conditions, consistent with initial expectations. However, we did not identify a significant difference between salinity exposures.

A previous study on *M. galloprovincialis* acclimated the mussels to 14, 28 or 35 ppt salinity at pH 7.8 for 28 days before freezing the organisms and performing biochemical tests to measure their response (Freitas *et al.*, 2017). Glycogen and lipid content of the tissues was measured, antioxidant assays were performed to identity superoxide dismutase and catalase activity, and electron transport chain activity was measured through the absorbance of formazan (Freitas *et al.*, 2017). They found that mussels exposed to 14 and 28 ppt salinity had significantly greater electron transport chain activity and significantly less glycogen content than mussels at 35 ppt salinity, indicating a response to hyposaline stress involving an increased consumption of energy (Freitas *et al.*, 2017). Thus, previous research indicates that hyposalinity stress results in greater energy use, which was not consistent with our findings. Perhaps a longer acclimation time may be key in analyzing the physiological response of mussels to hyposaline stress. In our study the mussels were acclimated to their salinity condition for 45 minutes before respirometry was performed to measure the acute response to hyposaline stress and this may not have been a long enough duration to elicit a measurable response.

The manipulation of only a single stressor could have also contributed to the lack of significance in our results. A review paper by Gunderson *et al.* (2016) highlighted the importance of multiple stressor studies in marine environments as these studies emulate

conditions in the natural world, where multiple factors are changing at once. Additionally, this review noted the lack of quality multiple stressor studies in the literature, with the majority of stressor studies on marine life focusing on the effects of the interaction between temperature and salinity on marine organisms. Many other studies have assessed the effects of multiple stressors on marine invertebrates, such as the two aforementioned studies by Freitas *et al.* (2017) and Shumway and Koehn (1982), as well as studies by Cole *et al.* (2016) and Dickinson *et al.* (2012). One study on flat oysters (*Ostrea angasi*) showed that single stressors (elevated pCO2, elevated temperature, hyposalinity or reduced food availability) did not produce statistically significant differences in physiology, but the combined effects of two or more of these stressors were enough to delay development, reduce larval size and increase mortality in oysters (Cole *et al.*, 2016). Unlike the experiment by Cole *et al.* in 2016, our experiment only manipulated salinity and held all other variables constant, therefore it is possible that the stress caused by hyposaline conditions was not enough to produce a significant result. However, if salinity stress had been combined with some other stressor, such as temperature, perhaps statistically significant results could have been found. In fact, this experiment has already been used to inform the creation of a follow-up experiment investigating the combined effects of hyposaline stress and heat stress on *M. galloprovincialis* mussels to investigate this further.

Changes in metabolic rate are not the only physiological response to hyposaline stress in mussels. Previous studies investigating changes in the mussel proteome to hyposaline stress indicate an increased abundance of ATP synthase and decreased abundance of oxidative stress proteins in *M. galloprovincialis* mussels 24 hours after a 4 hour exposure to 29.8 ppt salinity, a moderately hyposaline condition (Tomanek *et al.*, 2012). These changes are consistent with a greater demand for energy in the form of ATP and greater energy consumption in mussels exposed to hyposaline stress. These results are consistent with the results of our experiment where metabolic rate increases at the cellular level and at the organismal level in *M. galloprovincialis* exposed to moderate salinity stress.

As climate change progresses and mussels are exposed to a variety of stressors in their natural environment, further studies are needed to predict the physiological response of *M. galloprovincialis* mussels to changing climate conditions like increased temperature, ocean acidification and decreased salinity. Nevertheless, the results shown in this experiment are helpful in predicting the response of *M. galloprovincialis* to hyposaline stress and indicate that

mussels are more likely to have a higher metabolic rate when exposed to moderately hyposaline conditions. An increase in metabolic rate will likely occur in wild *M. galloprovincialis* populations in the near future if the effects of climate change continue to worsen.

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