Effects of Thermal Stress on Unionid Glochidia and Rising Salinity on Adult Unionid Mussels

by

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Abstract

Unionid mussels are considered keystone species however, they are the most imperiled group of organisms in North America. Implications from changing thermal and salinity regimes are of particular interest due to potential negative effects on feeding, respiration, and reproduction. In this study, we investigated valve closure behavior of four mussels from central Texas in relation to rising salinity as well as respiratory patterns of *Ligumia subrostrata* glochidia at multiple viabilities and temperatures as dissolved oxygen (DO) declined from normoxic to hypoxic conditions. We found sensitivity to rising salinity varied among species and subpopulations. We found some species had a closure response to rising salinity indicating a cost/benefit associated with early vs delayed closure. Viability was found to have no influence on glochidia respiration rate, Regulation Index (RI), or critical dissolved oxygen criterion (DO_{crit}). However, temperature directly influenced all similar endpoints. We also estimated the percent of brooding female respiration rate comprised of brood oxygen demand could reach 12% depending on DO and temperature.

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List of Abbreviations

CTO	Closed, Transition, Open
EC_{50_open}	The salinity at which number of open individuals declined by 50%
EC_{50_closed}	The salinity at which number of not-closed individuals declined by 50%
DO	Dissolved oxygen
SAFW	Soft artificial freshwater
MO_2	Respiration rate
RI	Regulation Index
DO_{crit}	Critical dissolved oxygen criterion
AUC	Area under the curve

Chapter 1: Effect of rising salinity on valve closure patterns of four mussel species from central Texas, USA

Introduction

Salinization of rivers is a growing ecological concern and is occurring at an increasing rate due to anthropogenic activity and climate change (Williams 2001; Canedo-Arguelles et al. 2013; Herbert et al. 2015). Regions with generally hot dry climates are at particular risk of concentrating ions via evaporation and transpiration (Canedo-Arguelles et al. 2013). Additionally, various anthropogenic activities, such as irrigation, de-icing, mining activity, and impoundments are known to increase salinity within waterways by removal of freshwater or addition of salts (Williams 2001; Hart et al. 2019). Specifically, salinity in rivers in arid and semi-arid regions are increasing due high demand and limited alternative surface water availability (Canedo-Arguelles et al. 2013). Rising salinity is of particular concern for freshwater systems because salinity dictates the distribution of biota, ecosystem metabolism, and food web diversity (Williams 1987; Potapova and Charles 2003; Porter-Goff et al. 2013). Rising salinity that reaches the upper threshold of organisms can affect feeding, respiration, reproduction, and overall maintenance costs (Ercan and Tarkan 2014). High salinity can result in a loss of suitable habitat for many species resulting in extirpation or extinction of a species (Bogan 1993).

Unionid mussels are the most imperiled group of organisms in North America (Williams et al. 1993, Bogan 1993). Unionids are considered keystone species due to their effect on benthic algae and macroinvertebrate communities; therefore, unionid conservation is important to preserve aquatic ecosystems (Blakeslee et al 2013, Hartmann et al. 2016). As filter feeders, mussels are exposed to a myriad of chemicals that are dissolved in the water. Limited mobility

greatly reduces their ability to relocate in order to avoid these stressors. When mussels are exposed to higher saline waters, they do not have any efficient active transport processes to maintain hypotonic body fluid, thus resulting in dehydration or mortality (Hart et al. 2019). In waterways such as the Pecos River, USA, salinities typically range from 6.0 to 12.0 ppt and can periodically reach upwards of 30 ppt which has resulted in death or extirpation of many species to that area (Hoagstrom 2009). In addition to chronic exposures, acute exposure of salinity can occur due to de-icing events (Lob and Silver 2012) or accidental salt spills (Kovacik 1972). Previous studies have examined the effects of salinity on a small number of mussel species. Blakeslee et al. (2013) found evidence of sublethal effects at 2 ppt. At 4 and 6 ppt, the LT_{50} (time in which salinity causes 50% mortality) was 4 and 3 days, respectively for *Elliptio complanata* and >10 and 3.9 days, respectively for *Popenaias popeii* (Hart et al. 2019). Time of death also varied among subpopulations (Hart et al. 2019). These differences in LT_{50} between species and local populations show the importance of determining salinity tolerance at both the species and subpopulation levels.

Since bivalves have limited mobility, escaping an unfavorable environment such as high salinity can be accomplished for short periods of time by closing their valves; unfortunately, they can only grow, respire, and reproduce when their valves are open (Kramer et al. 1989). Tracking, recording, and understanding the valve movement of mussels (valvometry) is gaining popularity as a biological warning system as well as an assessment tool (Zwart et al 1995). Systems such as the MosselMonitor and hull sensors have been used to monitor stressors in fresh and saltwater environments. This technology was first used as an early warning tool that would set off an alarm when mussels exhibited reduced valve gape, indicating sublethal or potentially lethal stress associated with pollutant(s) in the water (Kramer et al. 1989). This process uses

sensors fastened to each valve and estimates the change in distance between the sensors, based on voltage, in real time. This technique has been used as a tool to understand bivalve behavioral responses to a variety of environmental stressors, such as noxious dinoflagellates (Nagai et al. 2006), waterborne arsenic (Liao et al. 2009), and salinity. Hartmann et al. (2016) used hull sensors to measures the effect of salinity on valve gape of *Anodonta anatina*. They found a significant reduction in gape (interpreted as reduced filtration activity) and increased frequency of closure events when exposed to peak concentrations of 7 ppt.

In this study, I examined and compared valve-closure responses of four unionid species to a rapid rise in salinity to levels approaching presumed lethal limits. Major objectives were as follows:

1) Determine the salinity thresholds that induce a valve closure response
2) Determine whether species and/or subpopulations differ in their response to increasing salinity.
3) Identify which species are at higher risk of effects of direct salinity exposure due to a lack of closure, and which species are at higher risk of reduced filtration and respiration due to increased closure.

Methods

Experimental Animals

Initially, this study was focused on three species located in central Texas, USA that were candidates for listing under the endangered species act. As such, two of the species were represented by two subpopulations each (i.e. separate drainages), whereas the third species was represented by a single population due to rarity. However, the taxonomy of the target species

changed during the course of the study. *Cyclonaias houstonensis* was found to be morphologically and genetically indistinguishable from a common and widespread species – *C. pustulosa*. *Cyclonaias petrina* from the Guadalupe River was renamed *C. necki*, whereas the species name was retained for the population in the Colorado River (Johnson et al., 2018; Burlakova et al. 2018). Taxonomy of a third species, *Lampsilis bracteata* remained unchanged. I will use the updated taxonomic classification for the remainder of this study. Study animals (Table 1.1) are therefore represented by *C. pustulosa* (2 subpopulations – Colorado and Navasota Rivers), *C. petrina* (1 population – Colorado River), *C. necki* (1 population – Guadalupe River) and *L. bracteata* (1 population – Llano River).

All species were collected by BIO-WEST, Texas State University, and Auburn University personnel during surveys from May through November as part of a larger project in 2017. Each collection day, mussels were placed in coolers between moist cotton towels. Ice-packs were added above and below the toweling to try to maintain a shipping temperature intermediate between collection temperature in Texas and holding temperature (18 °C) at Auburn. All coolers were shipped overnight via FedEx. Upon arrival, mussels were tagged (Hallprint, Hindmarsh Valley, Australia) their length was measured, then they were placed in upwellers containing ~70 L of hard artificial freshwater (HAFW: 0.192 g $NaHCO_3$, 0.10 g $CaSO_4 \cdot H_2O$, 0.10 g $CaCl_2$, 0.06 g $MgSO_4$, and 0.008 g KCl per liter of reverse osmosis/deionized water; modified from Smith et al. 1997) at 18 °C. Water chemistry parameters were as follows: pH = 8.35, total hardness = 197.5 mg/L $CaCO_3$, and total alkalinity = 120 mg/L $CaCO_3$. Upwellers were made from two plastic containers that fit within another. A submersible Resun King-2 pump (1000 L/h) (Shenzhen Xing Risheng Industrial Co., Shenzhen, China) was used to pump HAFW from the smaller internal container to the larger external container. The smaller

internal container's bottom was removed and replaced with 6.35 mm mesh lined with aquarium pea gravel. The gravel acted as a substrate for the mussels as well as a biofilter. Upwellers were placed in a large trough that acted as a water bath controlled via 0.25 kW chiller/heater unit (TECO, São Paulo, Brazil). Upweller biofilters were allowed to establish for > 2 weeks prior to arrival of experimental mussels. Water quality parameters (ammonia, nitrites, and nitrates) were measured 3 times/week using either Tetra 6-in-1 and Ammonia Aquarium Test Strips or API 5 in 1 and Ammonia Test Strips (TETRA Technologies, Inc., The Woodlands, TX, USA). Ammonia and nitrites remained at undetectable levels (< 0.5 mg/L) throughout the study. Nitrates were consistently detected. Water exchanges were performed when nitrate concentrations exceeded 20 mg/L.

Due to collection permit limitations arising from all species being considered candidates for listing under the Endangered Species Act at the beginning of the study, individuals were used for sequential experiments. The 24 *C. pustulosa,* 16 *C. petrina,* 8 *C. necki, and* 8 *L. bracteata* used in this study had been previously utilized for previous temperature/respirometry and suspended solids/valvometry studies. They were then allowed to recover for > 4 weeks at 18 °C in the previously described upwellers. Following the recovery period, water temperature was raised by 1 °C/day to a target temperature of 28 °C and then acclimated to this temperature for ≥ 1 week prior to initiation of experiments. Mussels in each upweller were fed 2 mL Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, CA, USA) in the morning and 1 mL in the afternoon on a daily basis and held at a 12 h light: 12 h dark cycle.

Experimental Protocol

I used a MosselMonitor® system (Flow through version, AquaDect B. V, Brouwershaven, Netherlands) coupled with PresentIT™ 3.0 software to monitor mussel valve movement during

this study. The MosselMonitor and a connected cone tank were filled with ~70L of HAFW and held at 28 °C with a titanium 300-W heater (Finnex, Chiciago, USA) and aqualogic® digital temperature controller (Aqua Logic Inc., San Diego, CA; Fig. 1.1). Eight plastic baskets (8 x 5 x 4 cm) with 6.35 mm mesh bottom were individually fastened to the MosselMonitor's Polyvinyl chloride (PVC) mussel holder using two stainless steel screws.

For a given run, the side of each plastic basket, as well as the valves of eight experimental mussels, were lightly sanded with 80-grit sandpaper to improve adhesion. One valve of each mussel was glued (Unifast Trad Methylmethacrylate two-part Resin GC America Inc. Alsip, IL, USA) to the plastic basket wall parallel to the side of the MosselMonitor. This ensured the mussel remained at a fixed distance from an electromagnetic sensor behind the MosselMonitor wall. A second sensor, wrapped in water-resistant mesh tape (3M™,St. Paul, MN, USA), was glued directly to the other valve of the mussel at a position where the MosselMonitor real-time reading was between 150 and 200 - which converts to ~20 mm between sensors when the mussel is closed, as per manufacturer instructions. Aquarium pea gravel was then added to the basket until half of the mussel was embedded in the substrate. At this point, all mussels and sensors were lowered and secured tightly to the designated positions inside the MosselMonitor. Lastly, within the PresentIT™ 3.0 control panel, a system soft reset was performed at 09:00 hours (day 1) to indicate the attachment of new mussels; this initiated a three day acclimation period within which mussels acclimated to the system and software monitored distance between sensors and calculated the baseline maximum and minimum valve opening exhibited by each mussel. Following acclimation, percent gape was based on the baseline values. Mussels exhibiting a distance between sensors that was equal to the minimum baseline value recorded during the 3-day acclimation period received a score of 0% gape, whereas a gape

that was equal to the maximum baseline value received a score of 100% gape. Percent gape was recorded every 10 seconds. Day 4 was considered an internal control period during which % gape was monitored while no salt was added to the system. Day 5 was considered the experimental period during which % gape was monitored while salt was added to the system (Fig. 1.2). During the entire run (Days 1-5), mussels were fed Shellfish Diet (2 mL at 9:00 and 1 mL at 14:00) and were on a 12 : 12 h light-dark cycle (light: 08:00-20:00 h; dark: 20:00-08:00 h).

During Day 5, Diamond Crystal pool salt (Cargill Inc., Minneapolis, MN, USA) was added to a cone tank via a belt feeder (Fig. 1.1). The cone tank was connected to the MosselMonitor via flexible tubing. A submersible Resun King-2 pump transferred water to the MosselMonitor at a constant rate of 360 L/hr and tubing returned water to the cone tank at the same rate via ambient head pressure. The belt feeder was calibrated in 1 hr increments and sufficient salt (300 g) was evenly spread over the appropriate belt length (i.e. time) to bring salinity of the MosselMonitor system up to 4 ppt over an 11 hour period. Addition of salt was initiated at 09:00 h on Day 5, one hour after the lights came on and continued until just before the lights went out at 20:00 h. The experiment was then terminated to avoid extended exposure to high salinity and avoid potential lethal effects to the mussels.

A Pinpoint® Salinity Monitor (American Marine Inc., Ridgefield, CT, USA) was used to measure salinity immediately after mussel attachment (day 1), at 09:00 h during the internal control period (day 4), and every hour from 09:00 to 20:00 h of the experimental period (day 5). Salinity was measured from the cone tank to allow sampling without disturbing mussels. Preliminary experiments confirmed that salinity inside the MosselMonitor was equal to that of the cone tank.

Although Day 4 served as an internal control for all runs, there was still a question as to whether any valve closure patterns we observed on Day 5 were due to rising salinity, or simply a function of time, regardless of salinity. To address this issue we conducted two extra runs using our most abundant species – *C. pustulosa* (Colorado River) and *C. petrina*. During these runs, no salt was added during Day 5 so that valve closure patterns could be compared between days in the absence of salt. These are referred to as our independent control runs, as opposed to our internal control periods (i.e. Day 4 within salt runs).

In total, I conducted five salt runs and two independent control runs. Species and number of individuals per run are shown in Table 1.2. Species used in each run was determined by species availability at that time. Within a species, mussels were chosen randomly from the available pool of individuals and mussel location in the MosselMonitor was chosen randomly.

Data Analysis

Salinity

A one way repeated measures analysis of variance (ANOVA) on rank-transformed data (SigmaPlot 13.0) was used to test for significant differences in salinity measurements among runs.

To estimate salinity at each 10-second interval at which % gape was measured, a linear regression was fit to measured salinity data vs time plots for each run and the resultant prediction equation was used to estimate salinity at the time of each corresponding % gape measurement.

Defining closed, transition, open categories

In order to characterize valve behavioral patterns in the absence of salt exposure, gape data during the internal control period and both days from the independent control runs were

discretized into 10 bins from 0 to 100% gape. A log-linear model and a posthoc Tukey-adjusted multiple comparisons test were used to compare the frequency of gape recordings among the 10 different bins. Results were used to determine the % gape thresholds which defined "closed", "transition", and "open" gaping behavior (CTO). Starting from the 0-10% gape bin, the point at which we observed the first significant difference between adjacent bins was defined as the cutoff between "closed" and "transition". The next significant difference between adjacent bins was defined as the cutoff between "transition" and "open".

Independent control runs

The independent control results were analyzed to determine whether gaping patterns in the experimental run might have been simply an effect of days in the MosselMonitor rather than salinity. A generalized linear mixed model (GLMM) was used to compare count data in the CTO categories on day 4 (no salt) and day 5 (no salt) during the independent control run to determine if there was an effect of day on gape. Since the data was not normally distributed, a log-linear model, followed by a Tukey-Kramer multiple comparisons test, was used to determine the effect of gape category (CTO), day, species-location, and the interactions between them.

Internal control runs

To test whether gaping patterns observed during rising salinity were simply a function of hour rather than salinity, a log-linear mixed model was used to determine whether count data in each gape category exhibited a significant linear relationship with time (hour) on day 4 (no salt) and on day 5 (salt).

Effect of salinity on gaping behavior

Percent gape measurements recorded were used at each 0.1 ppt salinity increment (i.e. % gape at 0.5 ppt, 0.6 ppt, and so on). The number of individuals within each CTO categories at each salinity increment was then calculated based on % gape readings. To estimate the salinity at which the initial number of open individuals decreased by 50%, I used the US Environmental Protection Agency's Toxicity Relationship Analysis Program (TRAP v. 1.3a) was used to fit logistic regressions through plots of number of individuals open vs salinity for each species and subpopulation tested, and to calculate confidence intervals around resultant Effective Concentration (EC_{50}) estimates. To estimate the salinity at which the initial number of not-closed individuals decreased by 50%, TRAP software was used to perform the same analysis on number of individuals not-closed (i.e. open plus transition categories) vs salinity plots. Significant differences in EC_{50} estimates among species and subpopulations were assumed when 95% confidence intervals (CI) did not overlap.

Results

While mussels were held in holding tanks and throughout the runs, nitrite and ammonia were undetectable (<0.5 mg/L). Nitrates were constantly detected in the upwellers but never detected in the MosselMonitor. A water exchange was performed when nitrate levels were found above 20 mg/L. During the trials, there were three incidences of mortality discovered at the end of the experiment. One *C. petrina* died during the independent control run, where salt was never added to the system. Two *L. bracteata* died during run 5. Additionally, Runs 6 and 7 had a sensor malfunction: one *C. pustulosa* from the Colorado River and one *C. petrina* were affected. All of these data were discarded from the final analysis. Therefore, a total of 23 *C.*

pustulosa (15 from Colorado River and 8 from Navasota River), 14 *C. petrina,* 8 *C. necki, and* 6 *L. bracteata* were utilized in this study (Table 1.1).

In the five runs where salinity was increasing, salinity increased linearly with time (Fig. 1.3 and 1.4). Salinity did not differ significantly among runs ($X^2_{(4)} = 7.109$, $p = 0.130$).

Defining Closed, Transition, Open Categories

When salt was not added to the system, mussels exhibited a bimodal pattern in number of measurements (i.e. time) within % gape bins. Mussels spent most of their time either gaped wide open or gaped minimally, with the least amount of time spent in intermediate bins (Fig. 1.5 and 1.6). Number of measurements differed significantly among % gape bins $F_{(9, 397)} = 38.36$, $p < 0.0001$). The 10-20% gape bin was significantly higher than 20-30% gape ($t_{(397)} = 6.07$, $p < 0.0001$), and the 50-60% gape bin was significantly lower than the 60-70% gape bin ($t_{(397)} = -4.52$, $p < 0.0001$). Based on these results, the CTO categories were defined as follows: closed = less than 20% gape, transition category = greater or equal to 20% but less than 60% gape, and open = greater than or equal to 60% gape (Fig. 1.5).

Independent Control Run

Number of counts (i.e. time) differed significantly among the three gape categories (CTO) regardless of day and species-location ($F_{(2, 66)} = 19.32$, $p < 0.0001$). Significantly more time was spent in the open category than transition ($t_{(66)} = -4.67$, $p < 0.0001$) or closed ($t_{(66)} = -4.29$, $p = 0.0002$) categories. There was no significant interaction between gape category and day ($F_{(2, 65)} = 2.15$, $p = 0.1243$). No significant differences were found between day 4 and day 5

in terms of time spent within open ($t_{(65)}$ = -0.10, p =1.000), transition ($t_{(65)}$ = -1.41, p = 0.7220), or closed ($t_{(65)}$ = 1.50, p = 0.6635) categories.

Internal Control Run

During day 4 (no salt), there was no significant change in number of open or closed measurements through time ($t_{(303)}$ = 0.63, p = 0.5308; $t_{(59)}$ = 0.84, p = 0.4019, respectively). Number of transition measurements decreased with time ($t_{(78)}$ = -2.18, p = 0.0324). In contrast, during day 5, there was significant decrease in number of open measurements through time ($t_{(230.3)}$ = -7.65, p < 0.0001), a significant increase in number of transition measurements ($t_{(209)}$ = 7.65, p < 0.0001), and no significant change in number of closed measurements ($t_{(162)}$ = 1.19, p = 0.2340).

Effect of salinity on gaping patterns

The salinities at which number of open individuals declined by 50% (EC_{50_open}) and the salinities at which number of not-closed individuals declined by 50% (EC_{50_closed}), relative to initial conditions, differed significantly among species and between subpopulations. *C. petrina* exhibited the lowest EC_{50_open} at 2.45 ppt whereas *C. pustulosa* (Colorado River) and *L. bracteata* exhibited the highest EC_{50_open} values at 3.40 and 3.37 ppt, respectively. The *C. pustulosa* (Navasota River) subpopulation had a significantly lower EC_{50_open} (2.95 ppt) than the *C. pustulosa* (Colorado River) subpopulation (Table 1.3, Fig. 1.7). Similarly, *C. petrina* exhibited the lowest EC_{50_closed} (3.12 ppt) whereas *L. bracteata* and *C. necki* exhibited the highest EC_{50_closed} at 3.45 and 3.48 ppt, respectively. However, EC_{50_closed} did not differ between the *C.*

pustulosa subpopulations, neither of which exhibited sufficient closures to calculate an EC_{50_closed} as salinities increased to 4 ppt (Table 1.3, Fig. 1.8).

Discussion

Salinization of freshwater ecosystems has the potential to lead to sublethal stress, mortality, and extirpation of native species (Williams 1987; Potapova and Charles 2003; Rahel 2010). The threat to unionid mussels from rising salinity is increasingly important, especially when located within arid and semi-arid climates, given that unionids lack the ability to efficiently maintain hypotonic fluids within their tissue and efficiently seek refuge (Gainey and Greenberg 1977; Hart et al. 2019; Randklev et al. 2018). The inability to regulate internal body conditions may increase overall sensitivity to contaminants like salinity (Hartmann et al. 2016). Mean salinity of rivers from which experimental mussels were collected is typically low (i.e. ≤ 0.6 ppt) but can fluctuate, with salinity in the lower Colorado River exceeding 4.9 ppt within the last 30 years (Lower Colorado River Authority; Guadalupe-Blanco River Authority). Adjacent streams like the Pecos River can reach salinity of upwards of 30 ppt (Hoagstrom 2009). The risk of salinity exposure in the natural environment is a potential threat to mussels, and may partially explain distribution patterns (i.e. no mussels in high salinity reaches of the Pecos River). Information on how effects of salinity may differ among mussel species and subpopulations is necessary to understand current distribution patterns and to assess the degree of threat posed by future high salinity events.

When salinity was low (i.e. < 1ppt), mussel gaping behavior was bimodal with the majority of time spent either open (> 60% gape) or closed (< 20% gape) with very little time spent in the transition phase (between 20 and 60% gape). When mussels are open, they actively

drawing water in and out of the mantle cavity, allowing for feeding, respiration, reproduction and removal of wastes. However, they are directly exposed to any chemicals dissolved in the water. Bivalves may close in response to presence of chemicals (Kramer et al. 1989; Liao et al. 2009), presumably reducing their exposure, but incurring costs with regards to reduced feeding, respiration, and reproduction.

Gaping behavior response to increasing salinity differed among species and subpopulations and did not appear to be an artifact of number of days spent in the MosselMonitor (see independent control results) or an artifact of diurnal cycles (see internal control results). *C. petrina* behavior appeared to be the most sensitive to increasing salinity with number of open individuals decreasing by 50% at a salinity of ~2.5 ppt and number of not-closed individuals decreasing by 50% at ~3.1 ppt. In contrast, *L. bracteata* appeared to among the least sensitive species with number of open individuals decreasing by 50% at a salinity of ~3.4 ppt and number of not-closed individuals decreasing by 50% at a higher salinity of 3.5 ppt. Thus, sublethal effects of salinity related to reductions in filtration, aerobic respiration, and reproduction arising from alteration of gaping behavior would be expected to become apparent across all species as salinities increased from 2.5 to 3.5 ppt with *C. petrina* likely to show the earliest onset of effects.

Subpopulations within species exhibited differences in terms of effects of salinity on number of individuals remaining open, but similarities in closure response. The *C. pustulosa* from the Navasota River exhibited a 50% reduction in number of individuals remaining open at a lower salinity (2.9 ppt) than did the subpopulation from the Colorado River (3.4 ppt). However, neither species exhibited a 50% decreased in number of not-closed individuals even when salinities rose to 4 ppt. Thus the decrease in number open mussels appeared to be due to an

increase in the transition phase rather than an increase in valve closure. This response is similar to Kramer et al. (1989), who found low concentrations of copper induced a reduction of valve gape without complete closure. Sublethal effects of salinity related to reductions in filtration, aerobic respiration, and reproduction would likely become apparent at lower salinities for the Navasota River subpopulation. However, these effects would likely be reduced for *C. pustulosa*, regardless of subpopulation, because most individuals remained > 20% open even as salinities rose to 4 ppt.

Direct exposure to salinity was higher for *C. pustulosa* relative to other species due to greater gape, and presumably higher ventilation rates of water moving through the mantle cavity. It is possible that *C. pustulosa* exhibited a significantly weaker closure response than other species because they are less sensitive to direct effects of salinity. It is also possible that they would show the highest mortality rates under prolonged exposure to high salinity, and the strategy of remaining open is only beneficial during short pulses of salinity. In this study, we halted the experiment shortly after salinities reached 4 ppt because prolonged exposure at 4 ppt has been reported to result in high mortality for some mussel species. We did not want to risk inducing mortality because *C. pustulosa* from both subpopulations were considered to be rare, candidate species (*C. houstonensis*) when experiments were underway.

Results of this study demonstrate that mussel species and subpopulations can differ in their behavioral responses to increasing salinity, with important implications for exposure regimes, sublethal, and lethal effects. Additional research examining the costs/benefits associated with early vs delayed closure in response to rising salinity will be of great help in determining whether different taxa need to be managed differently in aquatic systems at risk of high salinity events.

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Tables and Figures

Table 1.1: Revised taxonomy, collection data, sampling locations, mean length, and range of length used in the study

Species	Date	Drainage	Collection site	Length (mm) Mean (*SE*)	Range
C. pustulosa	5/17/2017	Colorado River	Altair	49.36 (*1.70*)	38.9-57.9
	7/17/2017	Navasota River	Easterly	45.59 (*1.36*)	37.7-50.6
C. petrina	5/17/2017	Colorado River	Altair/Lometa	63.63 (*1.88*)	53.4-79.1
C. necki	11/01/17	Guadalupe River	Gonzales	49.89 (*1.69*)	44.1-57.4
L. bracteata	11/01/17	Llano River	Robinson Lake	59.63 (*3.18*)	51.9-69.2

Table 1.2: All MosselMonitor runs, type, species and number of individuals per run and date of run.

Run	Type	Species location (number of individuals)	Date
1	Independent Control (no salt)	*C. pustulosa* Colorado River (4) *C. petrina* Colorado River (4)	12/13/17
2	Independent Control (no salt)	*C. pustulosa* Colorado River (4) *C. petrina* Colorado River (4)	1/3/18
3	Salt	*C. pustulosa* Navasota River (4) *C. necki* Guadalupe River (4)	1/29/18
4	Salt	*C. pustulosa* Navasota River (4) *C. necki* Guadalupe River (4)	2/5/18
5	Salt	*L. bracteata* Robinson Lake (8)	2/19/18
6	Salt	*C. pustulosa* Colorado River (4) *C. petrina* Colorado River (4)	2/26/18
7	Salt	*C. pustulosa* Colorado River (4) *C. petrina* Colorado River (4)	3/4/18

Table 1.3: The salinity where Individuals open and not-closed decreased by 50% (EC_{50_open} and EC_{50_closed}, respectively) and the 95% CI for each species and subpopulation. Letters indicate significant differences based on 95% CI.

Species	**EC_{50_open} Salinity ppt**	**EC_{50_open} 95% CI**	**EC_{50_closed} Salinity ppt**	**EC_{50_closed} 95% CI**
C. necki	2.833[b]	2.759 – 2.907	3.482	3.399 - 3.564[b]
C. petrina	2.449[a]	2.385 – 2.514	3.127	2.991 - 3.262[a]
C. pustulosa (Colorado River)	3.400[c]	3.300 – 3.500	N.A.	N.A.
C. pustulosa (Navasota River)	2.950[b]	2.882 – 3.018	N.A.	N.A.
L. bracteata	3.369[c]	3.284 – 3.454	3.453	3.368 – 3.539[b]

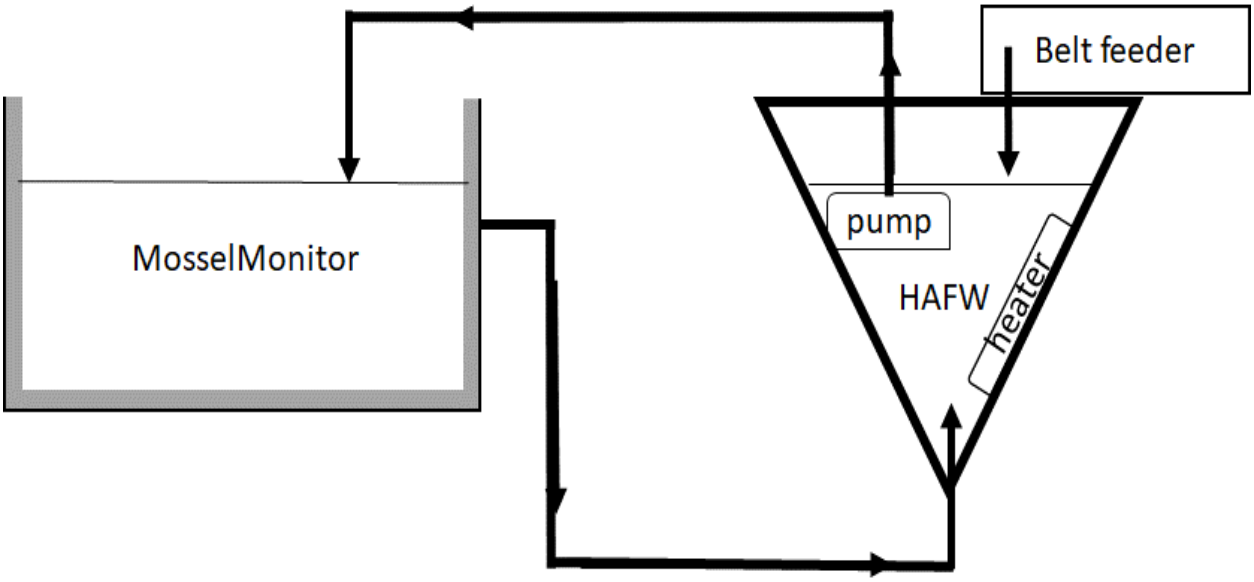

Figure 1.1: Schematic of the MosselMonitor system with attached cone tank and belt feeder.

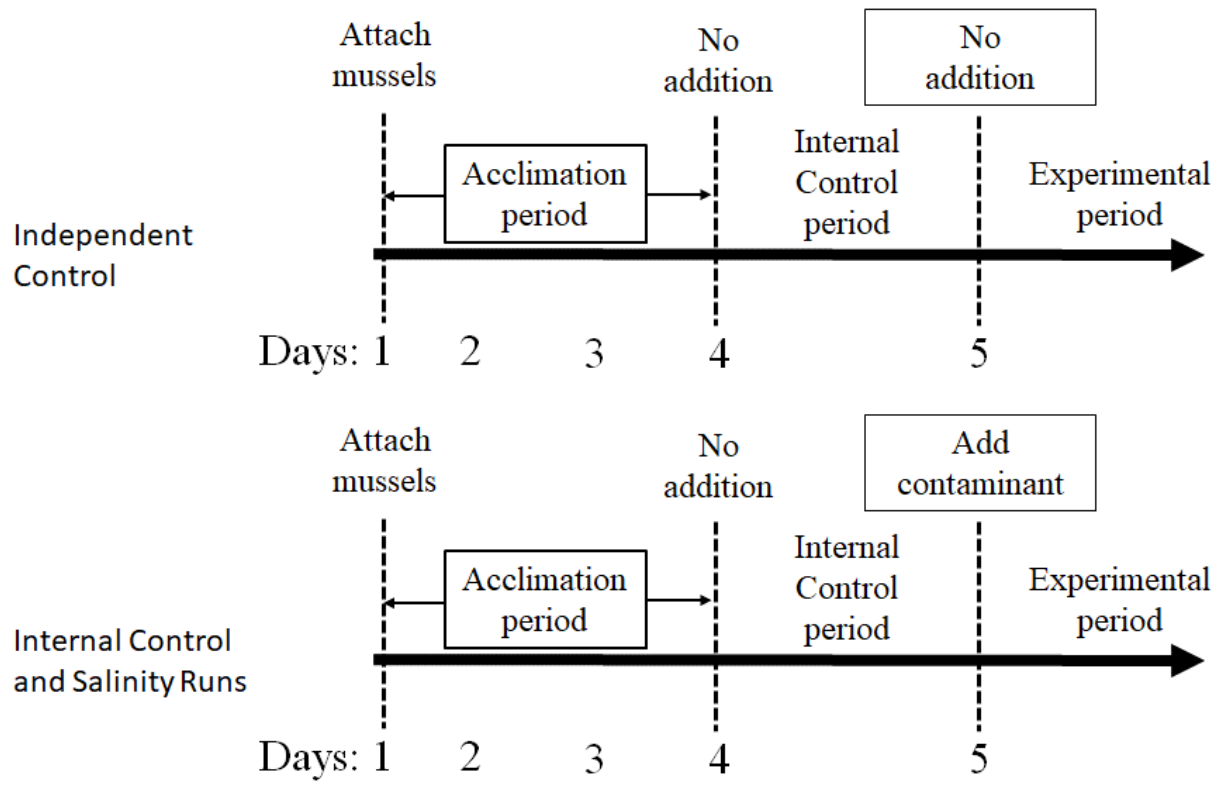

Figure 1.2: Experimental design for independent controls, internal control, and salinity runs.

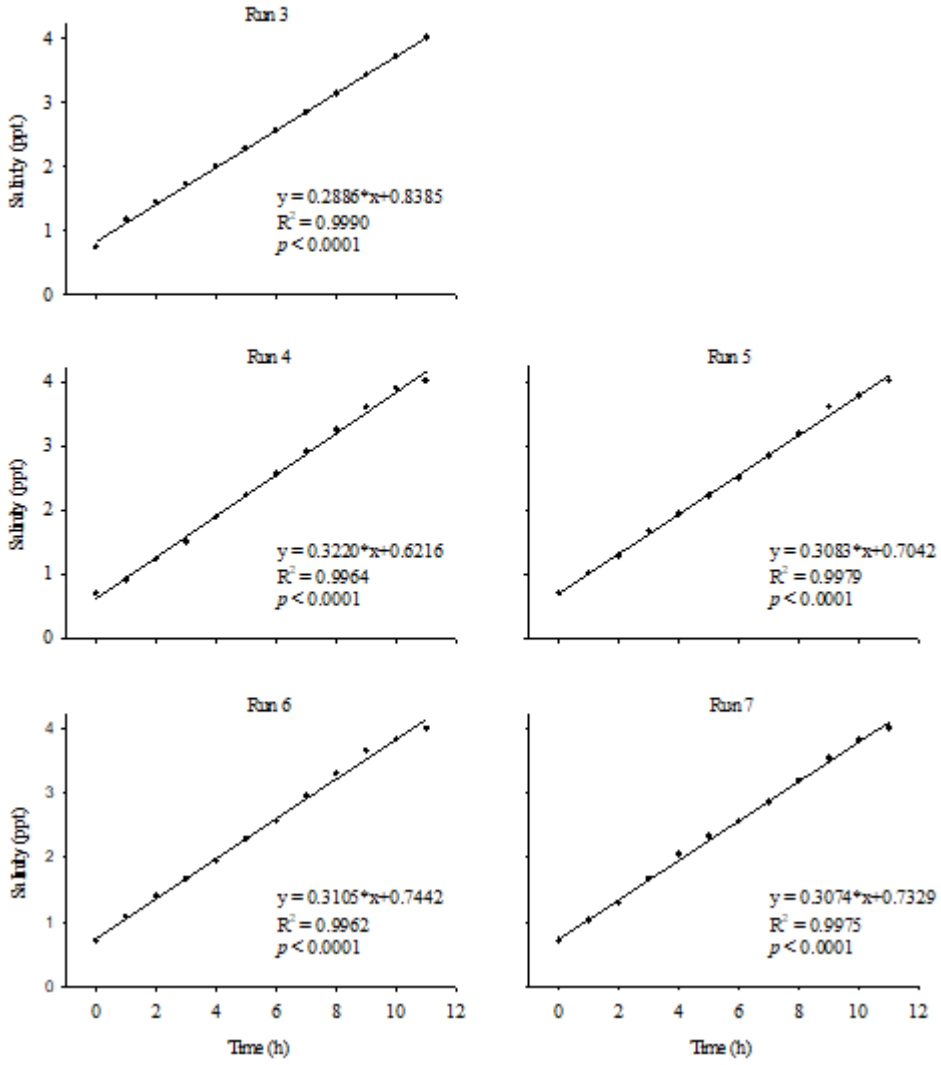

Figure 1.3: Salinity readings throughout all 5 runs where salt was added over time with linear regression. All regressions were statistically significant with *p*-values <0.0001 and R^2 ranged between 0.9962 and 0.9990

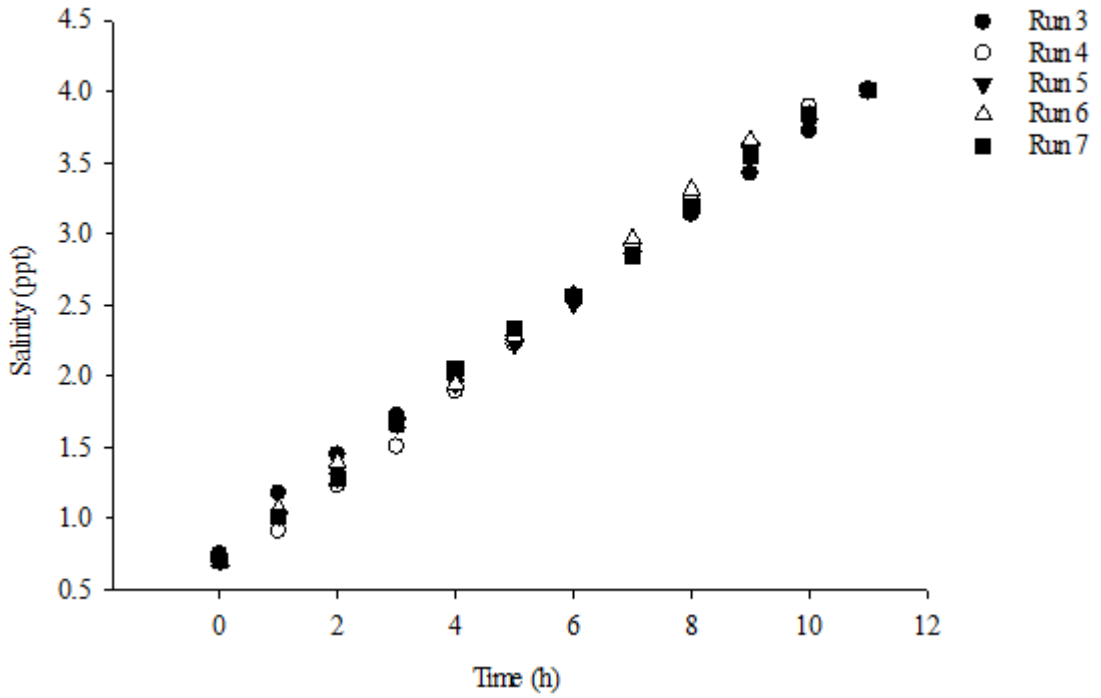

Figure 1.4: Salinity readings throughout all 5 runs over time. All runs were not significantly different from each other ($X^2_{(4)} = 7.109$, $p = 0.130$).

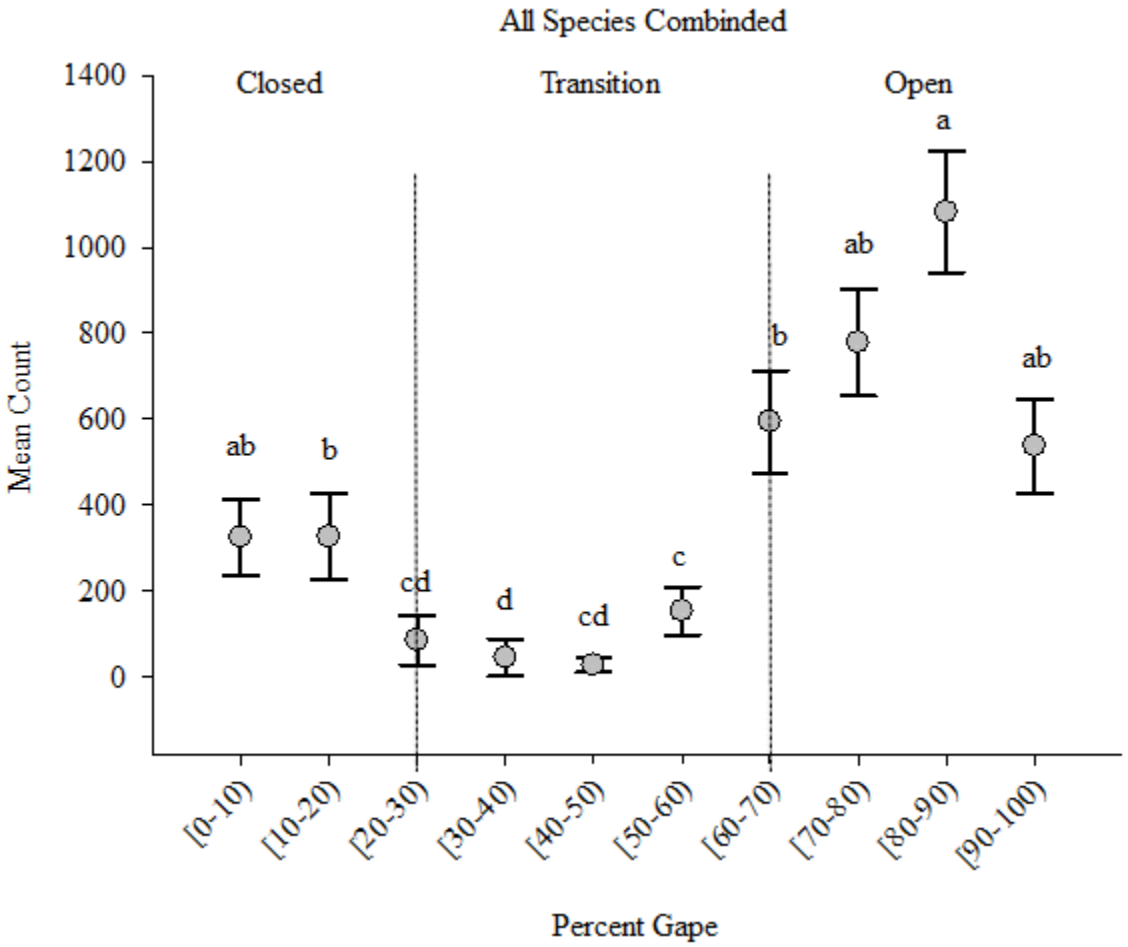

Figure 1.5: The mean count of all species in each of the 10 percent gape bins demonstrating the bimodal pattern when salt was not added. Error bars represent standard error. Brackets indicate including when parentheses indicate excluding. The letters represent significant differences ($p < 0.05$). The dotted lines indicate the point at two consecutive points are significantly different, thus resulting in Closed, Transition, and Open (CTO) categories.

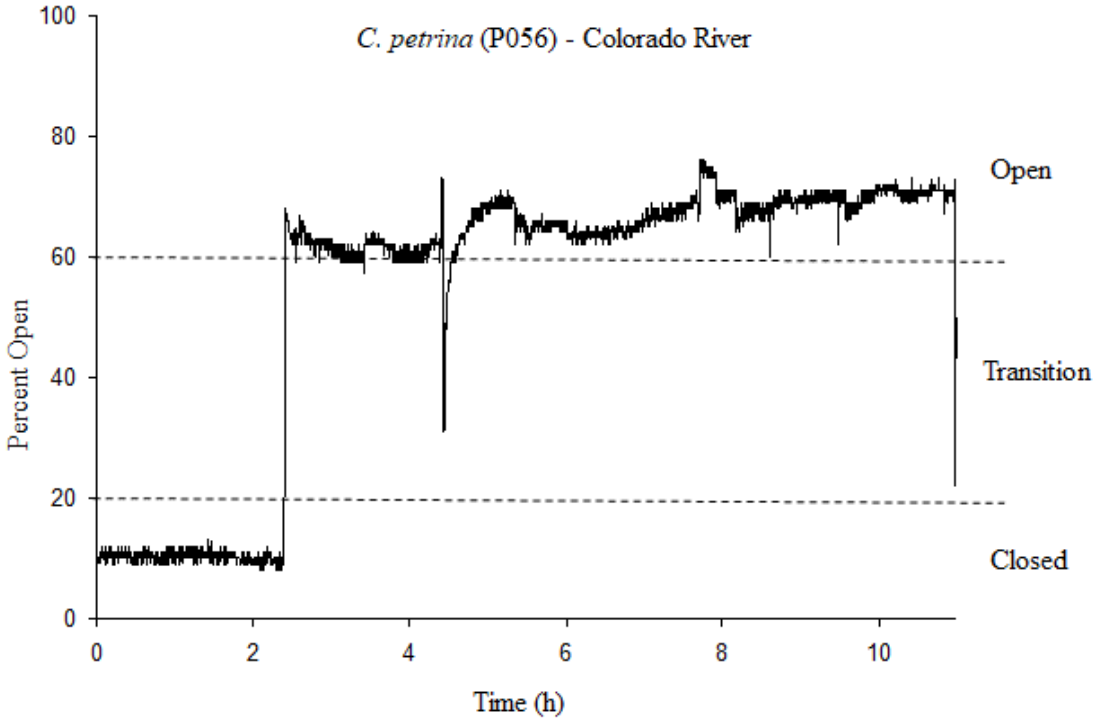

Figure 1.6: One *C. petrina* raw percent open data over time. Dashed lines indicated the cutoff limits between the Closed, Transition, and Open categories.

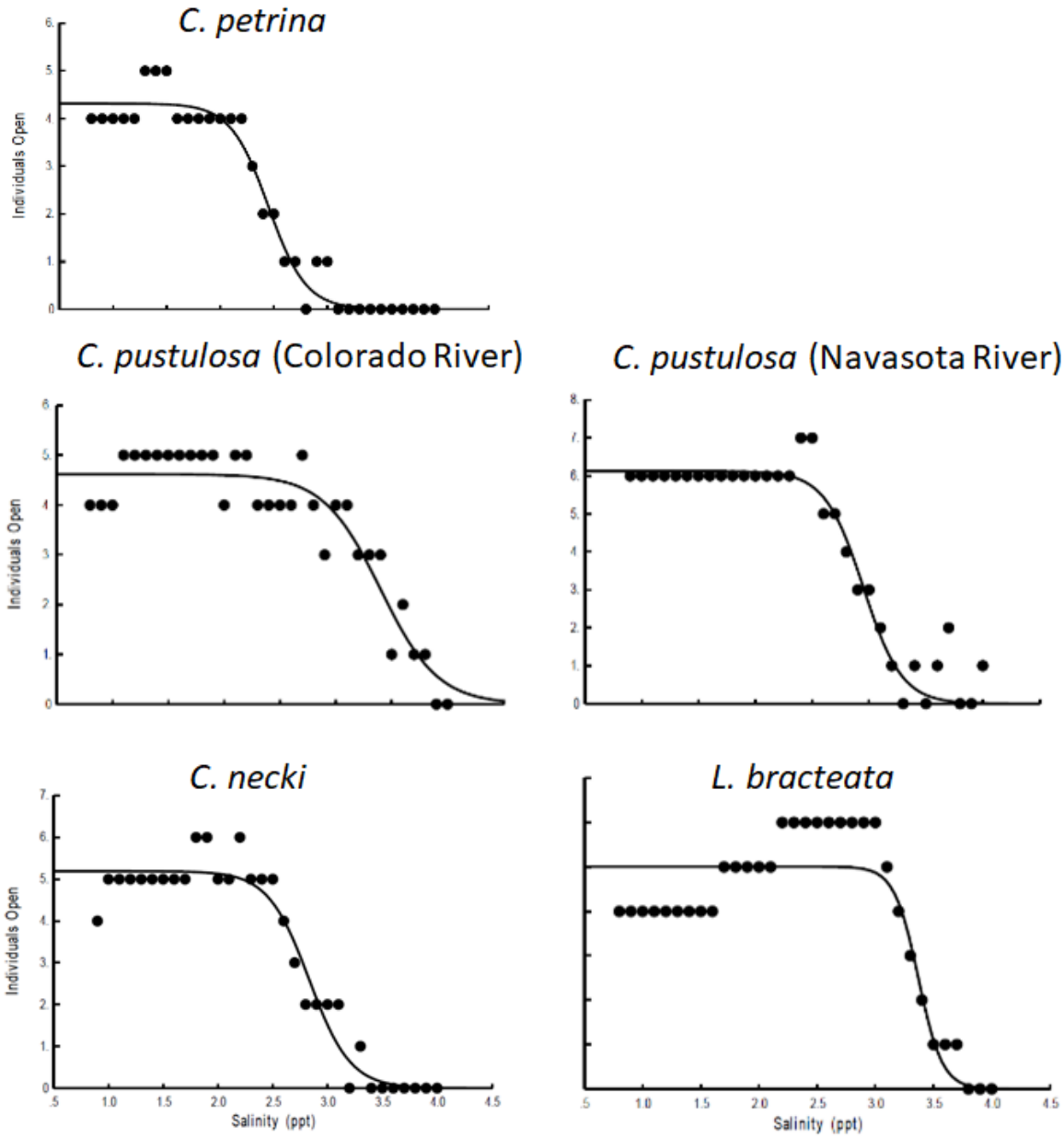

Figure 1.7: The raw number of individuals open (>60% gape) for each species as salinity rises.

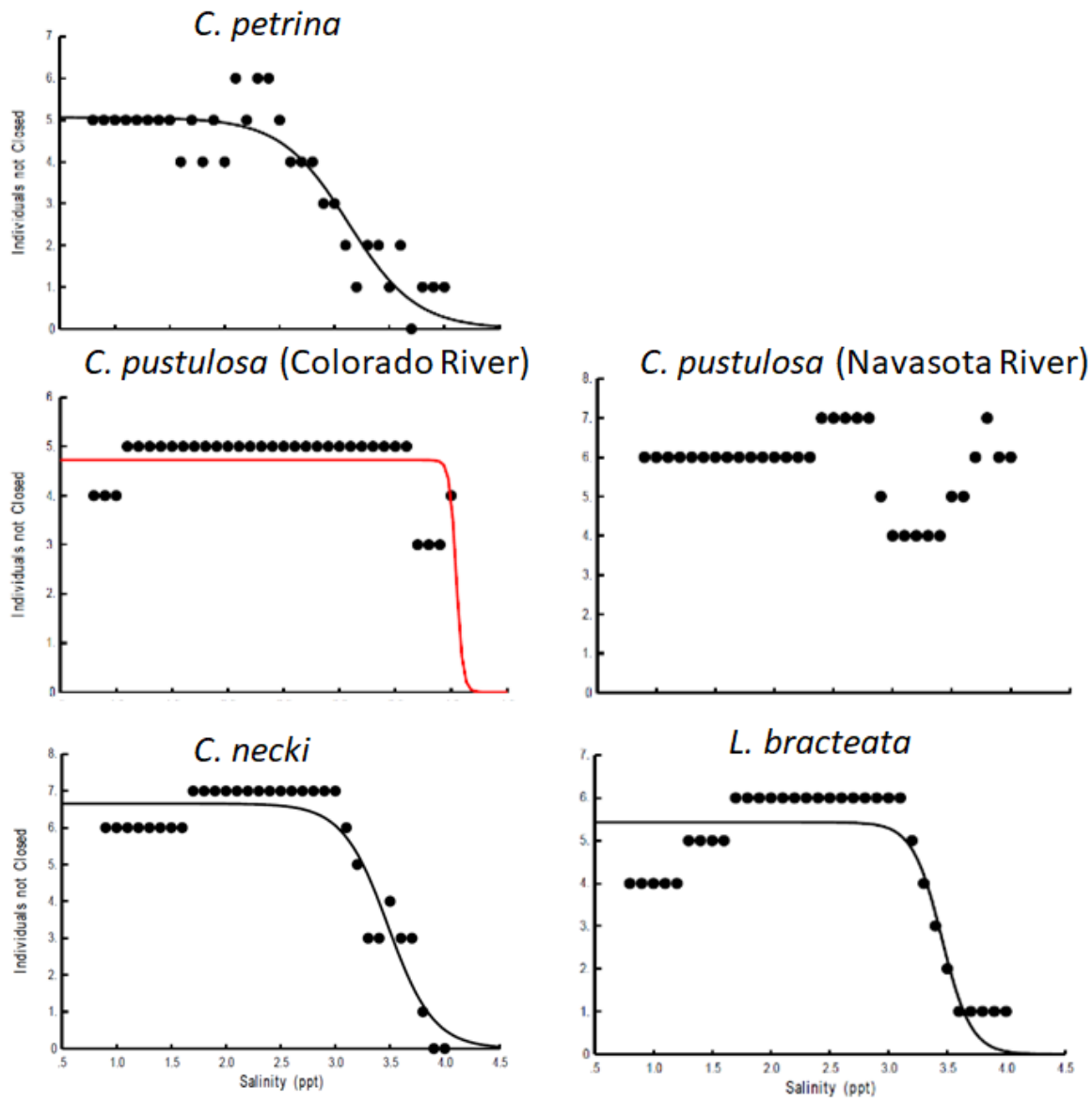

Figure 1.8: The raw number of individuals not-closed (>20% gape) for each species as salinity rises.

Effect of Brood Viability and Rising Temperature on Oxygen Consumption of *Ligumia subrostrata* Glochidia

Introduction

Water temperature in freshwater ecosystems drives overall health and distribution of biota (Caissie 2006). Rising temperature is of great ecological concern for the present and future of freshwater ecosystems. Temperatures have been known to increase due to anthropogenic activity as well as climate change, which greatly impacts these environments (Walther et al. 2002). Altered flow regimes, riparian clearing, and thermal discharge can directly or indirectly increase water temperatures in lakes and streams (Spooner and Vaughn 2008). Additionally, on a global scale, climate projection models show that the earth will warm 0.2 °C every decade, while most land regions increase at a higher rate (Allen et al. 2018). This increasing trend in temperature can induce various biotic responses, many of which are unfavorable (Caissie 2006). Aquatic systems are at particular risk because the amount of dissolved oxygen (DO) available is inversely proportional to temperature.

Unionid mussels are the most imperiled group of organisms in North America (Williams et al. 1993, Bogan 1993). Unionids are considered keystone species due to their effect on benthic algae and macroinvertebrate communities; therefore, unionid conservation is important to preserve aquatic ecosystems (Spooner and Vaughn 2006). Mussels lack the ability to seek refuge from unfavorable water conditions, and as ectotherms, their metabolic processes are strongly dependent on ambient water temperatures - leaving them particularly sensitive to thermal stress (Spooner and Vaughn 2008). The dependence of metabolic rates on temperature, combined with the inverse relationship between temperature and available oxygen, make it particularly important to understand how unionid respiratory patterns change with temperature.

Metabolic patterns of aquatic organisms, including unionids, vary among species based on temperature as well as DO (Chen et al. 2001, Wood 2018). Traditionally, oxygen consumption patterns of organisms were divided into two categories; oxygen regulation or conformation (Cobbs and Alexander 2018). An oxygen regulator maintains a constant respiration rate (MO_2) as DO decreases; conversely, the MO_2 of oxygen conformers decreases proportionally to DO. However, most organisms fall somewhere between true regulation and conformation. To address this issue, Mueller and Seymour (2011) developed a methodology to quantify the regulatory ability – termed the regulation index (RI) of an organism as DO declines. This methodology also allows endpoints such as the critical DO value (DO_{crit}), the dissolved oxygen concentration below which an organism switches from primarily aerobic to primarily anaerobic respiration to be easily defined and calculated (Mueller and Seymour 2011). A lower DO_{crit} and a higher RI potentially indicate a higher tolerance to hypoxia (Chen et al 2001).

The life cycle of unionid mussels is complex. There is a large diversity among life histories (Haag 2012), however, they all have a parasitic larval stage referred to as glochidia that use a fish, frog, or salamander as a host following release from a brooding female. Prior to release, the glochidia are brooded in the gills, which are also used for respiration. Brooding females may exhibit increased sensitivity to environmental stress and a reduction in respiration rates while glochidia within the gills exert an additional oxygen demand. Oxygen demand of a brood has been estimated at ~10% of combined (female plus glochidia) brooding female respiration at ~10 °C (Tankersley and Dimock 1993). To the best of my knowledge, the effect of temperature on respiration rate, regulatory ability and DO_{crit} of glochidia has not been examined in the published literature. Similarly, the effect of rising temperatures on the proportion of total

brooding female oxygen demand that is comprised of glochidial respiration has not been reported.

In this study, the respiration rate of *Ligumia subrostrata* glochidia was analyzed using a closed microplate respirometry system. My first objective was to determine whether glochidia MO_2, RI and DOcrit are affected by brood health- as measured by brood viability (Fritts et al. 2014). My second objective was to examine the relationships between glochidial MO_2, RI, DO_{crit}, and temperature. My third objective was to estimate the proportion of brooding female respiration that is comprised of glochidial respiration and how this changes with increasing temperature and declining DO.

Methods

Experimental Animals

Ligumia subrostrata is a long-term brooder found in lentic habitats throughout southcentral to central North America (Corey et al. 2006). *L. subrostrata* typically spawns in the fall, glochidia are brooded over winter, then released to attach to host fish in spring or early summer (Gascho Landis and Stoeckel 2015) when they are exposed to warming and potentially stressful temperatures while brooding. Brooding *L. subrostrata* were collected by hand from an earthen pond at the South Auburn Fisheries Research Station of Auburn University in March and November 2018. The day of collection, mussels were placed in a cooler between moist cotton towels. Ice-packs were added above and below the toweling to try to maintain a transport temperature intermediate between collection temperature and holding temperature (18 °C) in the lab at North Auburn Fisheries Research Station of Auburn University. Mussels were placed in upwellers containing ~70 L of soft artificial freshwater (SAFW: 50 mg $CaCO_3$, 25 mg $CaCl_2$, 50

mg $NaHCO_3$, and 5 mL 30% salt water/L deionized water) and maintained at 18 °C to maximize glochidia retention (Gascho Landis et al. 2012). Upwellers were made from two, nesting, plastic containers. A submersible Resun King-2 pump (1000 L/h) (Shenzhen Xing Risheng Industrial Co., Shenzhen, China) was used to pump SAFW from the smaller internal container to the larger external container. The smaller internal container's bottom was removed and replaced with 6.35 mm mesh covered with pea gravel. The gravel acted as a substrate for the mussels as well as a biofilter. Upwellers were placed in a large trough that acted as a water bath controlled via 0.25 kW chiller/heater unit (TECO, São Paulo, Brazil). Upwellers were allowed to establish for > 2 weeks prior to arrival of experimental mussels. Water quality (ammonia, nitrites, and nitrates) was measured 3 times/week using either Tetra 6-in-1 and Ammonia Aquarium Test Strips or API 5-in-1 and Ammonia Test Strips (TETRA Technologies, Inc., The Woodlands, TX, USA).

Effect of brood viability on glochidial respiration

Fourteen brooding females were randomly assigned to one of six upwellers and acclimated to laboratory conditions for ≥ 2 months at 18 °C prior to viability experiments. During this time, females were fed 2 mL Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, CA, USA) in the morning and 1 mL in the afternoon on a daily basis and held at a 12 h light: 12 h dark cycle. Mussels were not fed 24 hours prior to initiation of respirometry runs.

To initiate experiments, 4-5 females were randomly chosen from two upwellers. Both gills were removed from a given female, and placed into a crystalline dish, which was floating in the beaker water bath (Fig. 1) filled with SAFW at 18 °C. Using a 3 mL syringe with a 22 gauge BD PrecisionGlide® needle and SAFW from the beaker water bath, all glochidia were flushed from each gill. The gill tissue was removed from the dish and the glochidia were transferred to a

100 mL beaker set in the beaker water bath. Water from the water bath was added to the beaker to reach a final volume of 70 - 80 mL. The glochidia solution was then mixed with a plunger to ensure even distribution of glochidia in the solution, and 0.25 or 0.5 mL subsamples transferred to a counting tray and enumerated under cross-polarization at 30x magnification until at least 100 individuals had been counted. The concentration of glochidia in the beaker was calculated as the sum of glochidia in all subsamples combined divided by the combined subsample volume. Brood viability was determined using the salt test following the methodology of Fritts et al. (2014).

Respirometry was conducted using an optical, 24-well microplate system (Loligo Systems®, Viborg, Denmark). Preliminary runs indicated an optimal load of 2,000 glochidia per each 1 mL well which resulted in a fairly even layer of glochidia on the bottom of each well and a run time of ~ 24 hrs at 18 °C for glochidia to bring DO from 100% saturation to >0.5 mg O_2/L. Increasing the number of glochidia above 2,000 decreased the run time but resulted in glochidia piling on top of each other at the bottom of the well.

To initiate a run, the sensor plate was placed in the beaker water bath (18 °C) for >45 min to ensure that the optical DO sensors at the bottom of each well were fully saturated. Based on the glochidial concentration in the brood beakers (see previous section), a sufficient volume was pipetted from a given beaker to transfer an estimated 2,000 glochidia into a test tube. Glochidia settled at the bottom of the test tube were then withdrawn with a 100 µl pipette and transferred into their respective well in the submersed sensor plate. Glochidia immediately settled to the bottom of each well. This process was repeated for 4-5 broods per plate with three replicate wells filled per brood (i.e. a total of 6,000 glochidia tested per brood). The remaining wells were used as controls. Well assignment was determined randomly. Once all wells were loaded, the sensor

plate was gently lifted from the beaker water bath and a 3 ml syringe used to gently flush out and replace the water in each well in order to ensure that all wells were starting with fresh, DO-saturated SAFW. Wells were then sealed by rolling a sheet of parafilm across the top of the sensor plate, avoiding entrapment of air bubbles, followed by a silicon gasket and a Polyvinyl chloride (PVC) block. The entire assembly was then transferred to a specialized microplate water bath (Loligo Systems®). Foam spacers were placed on top of the PVC block, so that when the lid was fastened onto the water bath, it pushed on the spacers, adding pressure to the assembly and assuring a tight seal. The water bath was then placed on a microplate reader on a Waverly Sk-D3309-Pro hula table (BioMed, Lake Forest, CA, USA; Fig 2.1) and gently rocked throughout the entire trial to ensure mixing of water within each individual cell. The entire system was attached to a 66 L cooler outfitted with a TECO 0.25 kW chiller/heater unit that helped keep a constant temperature flowing through the microresp water bath (Fig 2.1). Dissolved oxygen was measured and logged for each well every 15 seconds using MicroResp™ software. A given run was terminated when DO in each well was drawn down to < 0.5 mg O_2/L. Respiration rates (MO_2) were calculated using the following formula:

Respiration (mg O_2/2000 glochidia/h) = ($[O_2]t_0 - [O_2]t_1$) x (V/t)

Where:

$[O_2]t_0$ = oxygen concentration at t_0 (mg O_2/L)

$[O_2]t_1$ = oxygen concentration at t_1 (mg O_2/L)

V = respiration volume (L)

$t = t_1 - t_0$ (hour)

Time increment ($t_1 - t_0$) used to calculate respiration rates ranged from 15 to 60 min depending on temperature, with longer increments used at cooler temperatures. Small temperature fluctuations during the loading process can affect the DO reading therefore, initial DO readings were deleted if the control DO readings were not stable (i.e. varied by > ± 0.2 mg O_2/L). If temperature was stable during loading process then the first MO_2 reading of all experiments was omitted to account for potential loading effects.

Correction for background (bacterial) oxygen demand

To reduce background oxygen demand, the entire system was bleached with a 3% solution then rinsed two times with reverse osmosis deionized (RO/DI) water prior to every experiment to reduce bacteria in the sensor plate wells, tubing, and water bath. To account for background respiration of remaining bacteria, we ran control wells during each run, which did not contain glochidia. The mean control respiration was then divided by the mean respiration of each experimental well at normoxia to determine the ratio (correction factor) of background respiration to glochidia respiration. Assuming the ratio remained constant as anoxic conditions are approached, we corrected our data in each well by multiplying the observed respiration by 1 minus the correction factor.

Effect of temperature on glochidial respiration

To examine the effect of temperature on respiratory patterns of glochidia, 20 brooding females were collected (11/29/2018) and acclimated to laboratory conditions for ≥ 1 week at 18 °C. Mussels were then randomly assigned to one of two upwellers per experimental temperature. Water temperature of each upweller was changed by 1°C/day to reach the experimental

temperatures of 13, 18, 23, 28 °C. Brooding mussels were acclimated to experimental temperature for ≥ 1 week prior to initiation of experiments. Respiration rates of glochidia were measured at each experimental temperature using the previously described methodology, with 4-5 broods per temperature and three replicate wells per brood. Glochidia from a given brood were only tested at a single experimental temperature.

Data analysis

For each experimental well, MO_2 (mg O_2/2,000 glochidia/h) corrected for background respiration was plotted against DO (mg O_2/L). To determine the relationships between MO_2, DO, and brood viability at progressively lower dissolved oxygen conditions, I first calculated MO_2 at DO 6, 5, 4, and 3 mg O_2/L for each well as the mean of MO_2 estimates falling within ± 0.5 mg/L of each target DO (i.e. MO_2 at 3 mg O_2/L = mean of all MO_2 estimates when DO was between 2.5 and 3.5 mg O_2/L). The estimates for all 3 replicate wells/brood were then averaged and the resulting dataset analyzed via a repeated measures analysis of covariance (ANCOVA) with viability as the covariate and DO as the grouping variable. A Tukey-Kramer multiple comparisons was used to test for significant differences in MO_2 among viabilities and DO levels. The same approach was used to analyze data for the temperature experiments with temperature as the covariate and DO as the grouping variable. Additionally, a repeated measures ANCOVA with a Tukey-Kramer multiple comparison test was used to compare between MO_2 and temperature.

The ability of glochidia to regulate oxygen consumption was quantified as a regulation index (RI) according to the methods of Mueller and Seymour (2011). For each well, MO_2 (mg O_2/2,000 glochidia/h) corrected for background respiration was plotted against DO (mg O_2/L) for

each well and fitted with the curve (3-parameter exponential rise to maximum, quadratic, 2-parameter hyperbola, or 2-segment piecewise regression) that exhibited the lowest Akaike information criterion adjusted for small sample size (SigmaPlot 13.0). Each curve was anchored at 6.0 mg O_2/L to avoid bias due to variation in initial DO across experimental temperatures (Mueller and Seymour 2011). I used the SigmaPlot area under the curve (AUC) macro to calculate AUC for 1) the observed data, 2) a horizontal line that represented perfect regulation, and 3) a linear decrease that represented perfect conformation. RI was calculated as (Observed AUC-Conformation AUC)/(Regulation AUC-Conformation AUC). The RI provided a quantitative measure of the degree to which glochidia were able to regulate oxygen consumption as ambient DO declined from 6 to < 0.2 mg O_2/L, with the range of RI values from 1 to 0 representing a range from perfect regulation to perfect conformation, respectively. DO_{crit} was calculated as the dissolved oxygen concentration showing the greatest distance between the observed MO_2 and the perfect conformation line (Mueller and Seymour 2011).

Mean RI and DO_{crit} per brood were plotted against brood viability and data fit with a linear regression to test for a significant ($p < 0.05$) relationship between these endpoints and brood viability at 18 °C.

A One Way ANOVA with Tukey multiple comparisons was used to test for significant differences in RI and DO_{crit} among temperatures for normally distributed data (SAS® version 9.4; SAS 2013, Cary, NC, USA).

Contribution of brood to brooding female oxygen demand

Brooding female respiration rates (female plus brood), as DO declined to < 0.5 mg/L, at 13, 18, 23, and 28 °C were obtained from Gibson (2019). Respiration rates were converted from

a mass-specific rate (mg O_2/gWW/h) to respiration per a standardized individual mussel by multiplying mass specific values by 30.25 g (the mean mass of brooding females used by Gibson, 2019). For each temperature and DO combination, MO_2 of 2,000 glochidia was multiplied by 37.5 in order to estimate the MO_2 of an average sized brood of 75,000 glochidia per female (Haag 2013).

To estimate the relationship between MO_2 and temperature at progressively lower DO for broods and for brooding females, a linear regression was fitted to MO_2 vs temperature at each DO. A Q_{10} was calculated for MO_2 at each DO based on the following formula:

$$Q_{10} = (MO_2^{t2} / MO_2^{t1}) \exp(10/(t_2 - t_1))$$

Where:

MO_2^{t1} = MO_2 at Lower Temperature

MO_2^{t2} = MO_2 at Higher Temperature

t1 = Lower Temperature

t2 = Higher Temperature

To calculate the percent of brooding female respiration rate that was comprised of the brood respiration rate, brood MO_2 was divided by brooding female MO_2 at each DO and multiplied by 100. Percent brood oxygen demand was then plotted against temperature and fitted with either a linear, or quadratic equation – whichever showed the lowest Akaike information criterion adjusted for small sample size.

Results

While mussels were held in upwellers prior to each run, nitrite and ammonia were undetectable (< 0.5 mg/L). However, nitrates were constantly detected in the upwellers and a water change was performed when nitrate levels were found above 20 mg/L. There was no mortality observed in the holding upwellers prior to either the brood viability or temperature experiments.

Viability Trials

Brood viability ranged from 46.5-94.0%. Neither RI ($p = 0.5419$) nor DO_{crit} ($p = 0.0667$) of glochidia showed a significant linear relationship with viability (Fig 2.2). MO_2 of glochidia was not significantly influenced by viability ($F_{(1, 13)} = 0.99$, $p = 0.3387$) or DO concentrations ($F_{(4, 35)} = 2.41$, $p = 0.0681$). There was no significant interaction between viability and DO ($F_{(3, 35)} = 0.86$, $p = 0.4705$; Fig. 2.3).

Temperature Trials

In temperature experiments viability of all broods was > 80%. At 13 °C, the relationship between glochidia MO_2 and DO in many wells showed a distinctive pattern where MO_2 increased rapidly to peak rates near the apparent DO_{crit} (Fig. 2.4). This pattern was rare to non-existent at higher temperatures and precluded the quantification of RI and DOcrit following the methodology of Mueller and Seymour (2011). Excluding the 13 °C data, there were significant differences in DOcrit ($F_{(2, 11)} = 75.66$, $p < 0.0001$) among temperatures with DO_{crit} significantly increasing from 18 to 23 °C ($t_{(11)} = - 6.60$, $p = 0.0001$) and from 23 to 28 °C ($t_{(11)} = - 6.05$, $p = 0.0002$). There were also significant differences in RI ($F_{(2, 11)} = 31.07$, $p < 0.0001$) with

temperature. RI was significantly higher at 18 than 23 °C ($t_{(11)} = 6.42$, $p = 0.0001$) or 28 °C ($t_{(11)} = 7.37$, $p < 0.0001$), but did not differ between 23 and 28 °C ($t_{(11)} = 1.01$, $p = 0.5849$; Fig 2.5).

13 °C data was retained for glochidial MO_2 analysis because the unusual patterns (Fig 2.4) did not interfere with estimating MO_2 at each DO concentration. MO_2 changed significantly with temperature ($F_{(1, 18)} = 174.5$, $p < 0.0001$) and DO ($F_{(4, 50)} = 12.74$, $p < 0.0001$). The interaction between temperature and DO was significant ($F_{(3,50)} = 6.14$, $p = 0.0012$). At all temperatures > 18 °C, MO_2 at a DO of 3 mg O_2/L was significantly lower than DO concentrations of 4, 5, or 6 mg O_2/L ($p < 0.05$; Table 2.1; Fig 2.7). MO_2 was significantly different with temperature at all DO concentrations: 3 ($F_{(3, 18)}=32.1$, $p < 0.0001$), 4 ($F_{(3, 18)} = 43.09$, $p < 0.0001$), 5 ($F_{(3, 18)} = 93.49$, $p < 0.0001$), 6 mg O_2/L ($F_{(3, 18)} = 66.35$, $p < 0.0001$; Fig 2.6).

The Q_{10} of the brooding females ranged from 1.6 to 1.7 while the glochidia ranged from 3.5 to 4.2. At all DO concentrations, Q_{10} of an average brood was higher than that of an average-size brooding female (Table 2.4).

Contribution of brood to cumulative brooding female respiration.

At all four DO concentrations examined, the relationship between glochidial MO_2 and temperature was a better fit with linear regression, while the relationship between brooding female MO_2 and temperature was a better fit with quadratic regression (Fig 2.7 a, b). Percent contribution of brood to cumulative brooding female respiration (i.e. brood MO_2/brooding female MO_2 x100) ranged from 2.88 to 12.38% across all DO concentrations. Percent brood contribution vs. temperature plots were best fit by a quadratic equation for all four DO concentrations, increasing and then leveling off with rising temperature (Table 2.2, Fig. 2.7 c). At the highest temperature tested (28 °C) brood respiration comprised 9.86% of brooding female

respiration at a DO of 6 mg O_2/L and 12.38% of brooding female respiration at a DO of 3 mg O_2/L (Table 2.3).

Discussion

Brooding females must obtain enough oxygen from filtered water to meet their own oxygen demand, while glochidia brooded within their gills are also extracting oxygen from the water. The proportion of total brooding female oxygen demand (adult mussel plus glochidia) that is comprised of the oxygen demands of the brood is likely affected by multiple factors including brood viability, temperature, and dissolved oxygen concentrations in surrounding water. Understanding how these factors affect oxygen demand and respiratory patterns of glochidia can provide valuable insight as to the effects of thermal and hypoxia stress on glochidia as well as the degree to which glochidia are likely to impose respiratory stress on brooding females.

Brood viability is a behavioral endpoint that is used as a general assessment of brood development stage and/or brood health with low viability indicating immature and/or unhealthy glochidia (i.e. Fritts et al. 2014). Brood viability within the ~45-95% range had little to no effect on oxygen demand of glochidia, suggesting that changes in glochidia maturity and/or condition are unlikely to affect respiratory stress of brooding females. Similarly, susceptibility of glochidia to hypoxia stress is unlikely to change dramatically as they mature or exhibit declines in condition, as evidenced by a lack of relationships between RI, DO_{crit}, and viability. The low DO_{crit} recorded for glochidia in majority of broods (<1.5 mg O_2/L) was equal to or lower than that reported for most adult mussels (Gibson 2019), suggesting that glochidia do not necessarily require higher DO concentrations than adults. However, RI appeared to decrease while DO_{crit}

and MO_2 appeared to increase at a viability of ~45% relative to values > 60% viability. This suggests that energy demand and sensitivity to hypoxia may increase as brood viability drops below 60%, but more data at viabilities < 60% is required to determine whether this is a general trend.

Unlike viability, temperature had strong effects on glochidial respiration. Interestingly, at 13 °C, we observed an unusual pattern where MO_2 frequently exhibited a dramatic increase at low DO concentrations followed by a sharp decrease as DO concentrations declined further. Similar patterns have occasionally been reported for other aquatic species (Wood 2018) and methodology has been recently proposed for analyzing these types of data sets (Cobbs and Alexander 2018). However, at this time, it is unknown what drives this pattern and how it relates to hypoxia tolerance. The MO_2 spike at low DO in the glochidial wells was not mirrored by a spike in MO_2 in the control wells, so it appears to be a real pattern and not simply an artifact of a temporary malfunction of the respirometry apparatus. Additional research investigating the mechanism behind this pattern and why it appears to only occur at cool (13 °C) temperatures for glochidia is warranted.

The relationship between glochidial MO_2 and temperature showed similarities and differences relative to that of brooding females. At all four DO concentrations examined, MO_2 increased linearly with temperature for glochidia. Conversely, the relationship between MO_2 and temperature was best described by a quadratic regression for brooding females. As evidenced by Q_{10} estimates, oxygen demand of glochidia increased at more than twice the rate than brooding females as temperatures increased, regardless of DO. The disparity between Q_{10} of glochidia and brooding females resulted in a rapid increase in the proportion of brooding female oxygen demand that is represented by oxygen demand of the brood. At cool (i.e 13 °C) temperatures, the

brood represented less than 4% of the brooding female oxygen demand, but this demand quickly rose and then leveled off at approximately 10% as temperatures increased further to 23-28 °C. The estimate of 4% oxygen demand contradicts those of a previous study (Tankersley and Dimock 1993) that measured a 10% oxygen demand of *Pyganodon cataracta* brooding females and their removed gills containing glochidia at cooler temperatures, which might have been overestimated due to the addition of the gill tissue. The degree of stress that broods might afflict on the brooding female by consuming 10% of the total oxygen acquired by the brooding female was not examined in this study. However, a previous study (Gascho Landis et al., 2012), found that temperatures ≥25 °C induced brood release by *L. subrostrata*. This coincides with the temperature range (23-28 °C) in the current study where % oxygen demand reached a maximum of ~10% - suggesting that the increased oxygen demand of broods might be a mechanism driving release of broods during prolonged exposure to warm temperatures.

In addition to potential stress inflicted on the brooding female, the higher Q_{10} of glochidia suggests that they burn through their energy stores at a much faster rate than brooding females. While brooding females can at least partially offset increasing energy demand by acquiring additional energy via feeding, glochidia are not known to actively feed and are presumably dependent upon their energy stores. Due to increased rates of energy depletion with temperature, it is likely that brood viability decreases at a faster rate when brooding females are exposed to warming temperatures. Thus increases in aquatic thermal regimes over time are likely to decrease the length of time that brooding females have to transfer glochidia onto an appropriate host.

There was some evidence that hypoxia would decrease the rate of energy reserve depletion as MO_2 of glochidia was significantly lower at 3 mg O_2/L than at higher DO

concentrations as temperatures rose above 18 °C. However, this benefit appeared to be partially offset by an increase in DOcrit and a decrease in RI as temperatures increased from 18 to 28 °C. In general, increases in DOcrit and decreases in RI are considered to represent increasing sensitivity to hypoxia (Chen et al 2001), although the usefulness of DO_{crit} as a sole indicator of hypoxia tolerance has been recently challenged (Wood 2018).

In summary, we found that brood viability had little to no effect on MO_2 regardless of DO concentration, RI, and DO_{crit}. However, further investigation of viability <60% should be completed to further understand the impact of low viability. On the other hand, temperature had a direct influence by increasing MO_2 and DO_{crit} as well as decreasing RI. In general, we found that glochidia have a fairly low DO_{crit}, indicating reduced susceptibility to hypoxia compared to adults. However, at a higher temperature (>18 °C) the MO_2 of glochidia at lower DO concentrations is lower than higher DO concentrations. The reproductive strategy of brooding glochidia within the gills of the female potentially adds an additional layer of stress since the Q_{10} of glochidia was higher than the adult thus, resulting in upwards of 12% of the total oxygen consumption at warmer temperatures. In order to successfully manage mussel populations, it is important to understand how temperature regimes influence the physiological performance of both the adult as well as the developing larvae. As temperature continues to rise to unforeseen levels, the physiological and reproductive effect from higher temperature is of upmost importance when managing unionid populations.

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Tables and Figures

Table 2.1: Repeated measures ANCOVA and Tukey-Kramer multiple comparison test for MO_2 and temperature at various DO concentrations. Table focuses on comparison of MO_2 estimates at 3 mgO_2/L compared to those at 4, 5 and 6 mgO_2/L. All comparisons reported are significantly different.

Temperature	DO (mgO2/L)	DO (mgO2/L)	Estimated difference in MO_2	Standard Error	DF	t Value	Pr > |t|	Adj P
18	3	4	-0.00005	0.000018	50	-2.72	0.009	0.043
	3	5	-0.00006	0.000018	50	-3.41	0.0013	0.0069
	3	6	-0.00006	0.000018	50	-3.18	0.0025	0.0131
19	3	4	-0.00006	0.000017	50	-3.56	0.0008	0.0044
	3	5	-0.00007	0.000017	50	-4.13	0.0001	0.0008
	3	6	-0.00006	0.000017	50	-3.67	0.0006	0.0032
20	3	4	-0.00007	0.000017	50	-4.39	<.0001	0.0003
	3	5	-0.00008	0.000017	50	-4.8	<.0001	<.0001
	3	6	-0.00007	0.000017	50	-4.1	0.0001	0.0008
21	3	4	-0.00009	0.000017	50	-5.12	<.0001	<.0001
	3	5	-0.00009	0.000017	50	-5.36	<.0001	<.0001
	3	6	-0.00007	0.000017	50	-4.43	<.0001	0.0003
22	3	4	-0.0001	0.000017	50	-5.68	<.0001	<.0001
	3	5	-0.0001	0.000017	50	-5.75	<.0001	<.0001
	3	6	-0.00008	0.000017	50	-4.63	<.0001	0.0002
23	3	4	-0.00011	0.000018	50	-6.05	<.0001	<.0001
	3	5	-0.00011	0.000018	50	-5.96	<.0001	<.0001
	3	6	-0.00008	0.000018	50	-4.68	<.0001	0.0001
24	3	4	-0.00012	0.000019	50	-6.25	<.0001	<.0001
	3	5	-0.00012	0.000019	50	-6.02	<.0001	<.0001
	3	6	-0.00009	0.000019	50	-4.64	<.0001	0.0001
25	3	4	-0.00013	0.000021	50	-6.33	<.0001	<.0001
	3	5	-0.00012	0.000021	50	-5.98	<.0001	<.0001
	3	6	-0.00009	0.000021	50	-4.53	<.0001	0.0002
26	3	4	-0.00014	0.000023	50	-6.32	<.0001	<.0001
	3	5	-0.00013	0.000023	50	-5.87	<.0001	<.0001
	3	6	-0.0001	0.000023	50	-4.38	<.0001	0.0003
27	3	4	-0.00016	0.000025	50	-6.27	<.0001	<.0001
	3	5	-0.00014	0.000025	50	-5.74	<.0001	<.0001
	3	6	-0.00011	0.000025	50	-4.22	0.0001	0.0006
28	3	4	-0.00017	0.000027	50	-6.18	<.0001	<.0001
	3	5	-0.00015	0.000027	50	-5.6	<.0001	<.0001
	3	6	-0.00011	0.000027	50	-4.07	0.0002	0.0009

Table 2.2: Statistics for quadratic regressions fit through plots of (Brood MO_2/Brooding Female MO_2)*100 versus temperature at each DO concentration.

DO (mgO_2/L)	R^2	Quadratic Equation	*p*	Shapiro-Wilk Normality Test
6	0.9983	Y=(2.6824*x)+(-.0549*x^2)-22.2887	0.0412	0.9719
5	0.9986	Y=(2.4339*x)+(-0.0463*x^2)-20.7341	0.0214	0.9719
4	0.9982	Y=(2.7687*x)+(-0.0541*x^2)-24.2472	0.0248	0.9719
3	0.9999	Y=(2.1257*x)+(-0.0364*x^2)-18.5886	0.0046	0.9719

Table 2.3: The percent of brooding female respiration rate comprised of brood oxygen demand at each temperatures and DO concentration tested.

	Brood RMR/ Brooding Female RMR (%)			
Temperature (°C)	DO_6	DO_5	DO_4	DO_3
28	9.86	11.18	10.91	12.38
23	10.23	10.69	10.71	11.01
18	8.37	8.18	8.18	7.89
13	3.26	3.06	2.57	2.88

Table 2.4: Estimated Q_{10} values for MO2 for an average-sized brood and brooding female at each DO concentrations as temperatures rose from 13 to 28 °C.

DO (mg/L)	Q_{10} Brood	Q_{10} Gravid Female
6	3.5	1.7
5	3.7	1.6
4	4.2	1.6
3	3.7	1.4

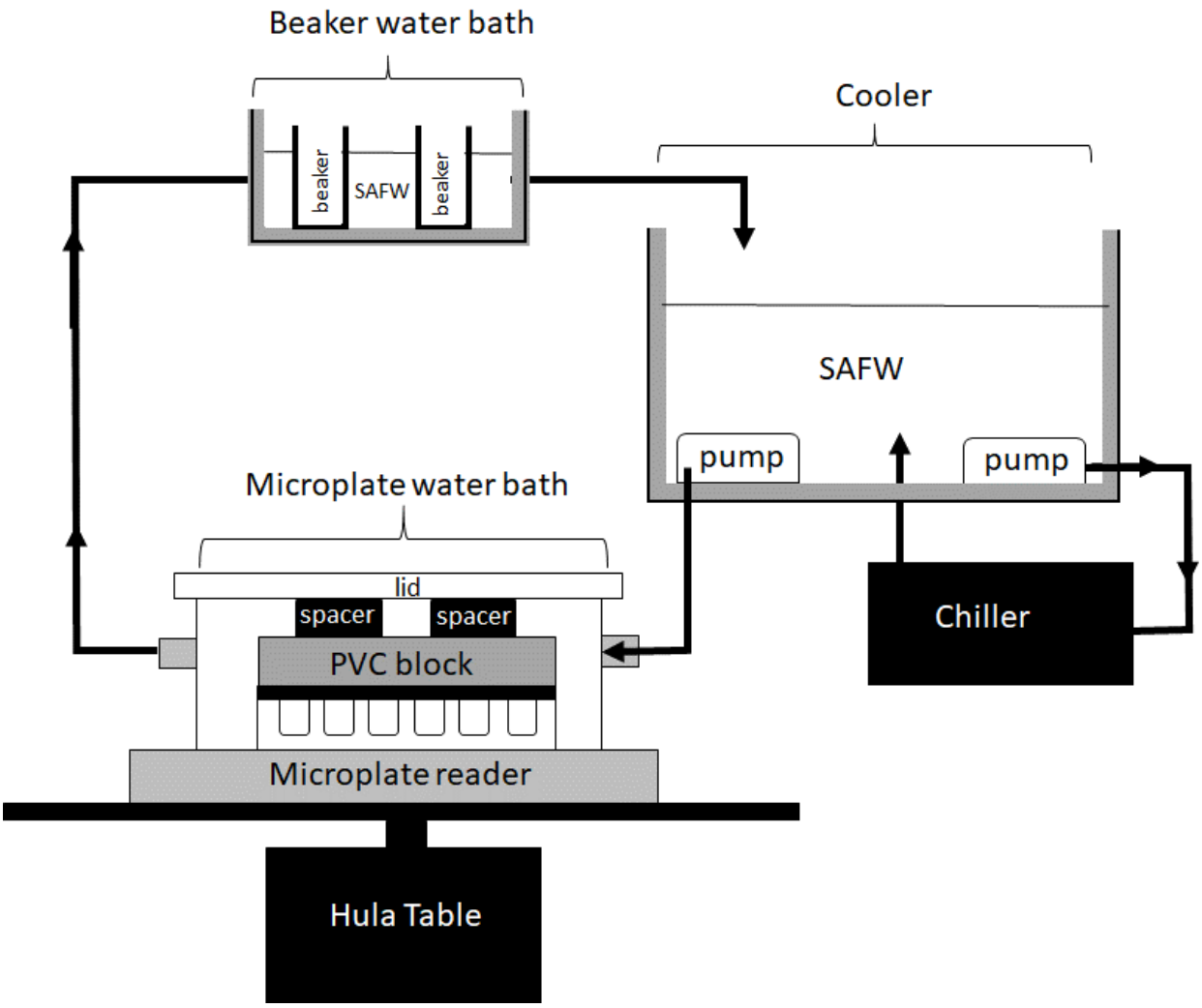

Figure 2.1: Schematic of the MicroResp^{TM} system with attached cooler and waterbath systems.

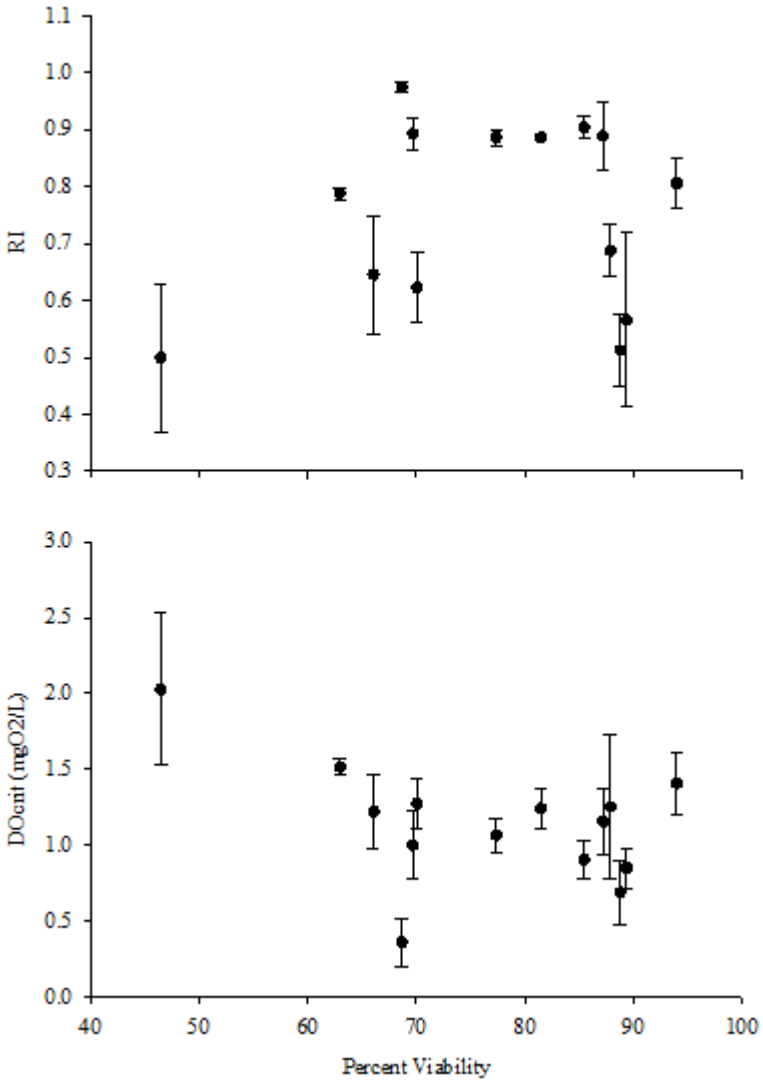

Figure 2.2: Relationship between A) RI and percent brood viability and B) DOcrit and percent brood viability estimated from three replicate batches of 2,000 glochidia per brood. Error bars represent ± 1 *SE*. Neither RI ($p = 0.5419$) nor DO_{crit} ($p = 0.0667$) showed a significant linear relationship with viability.

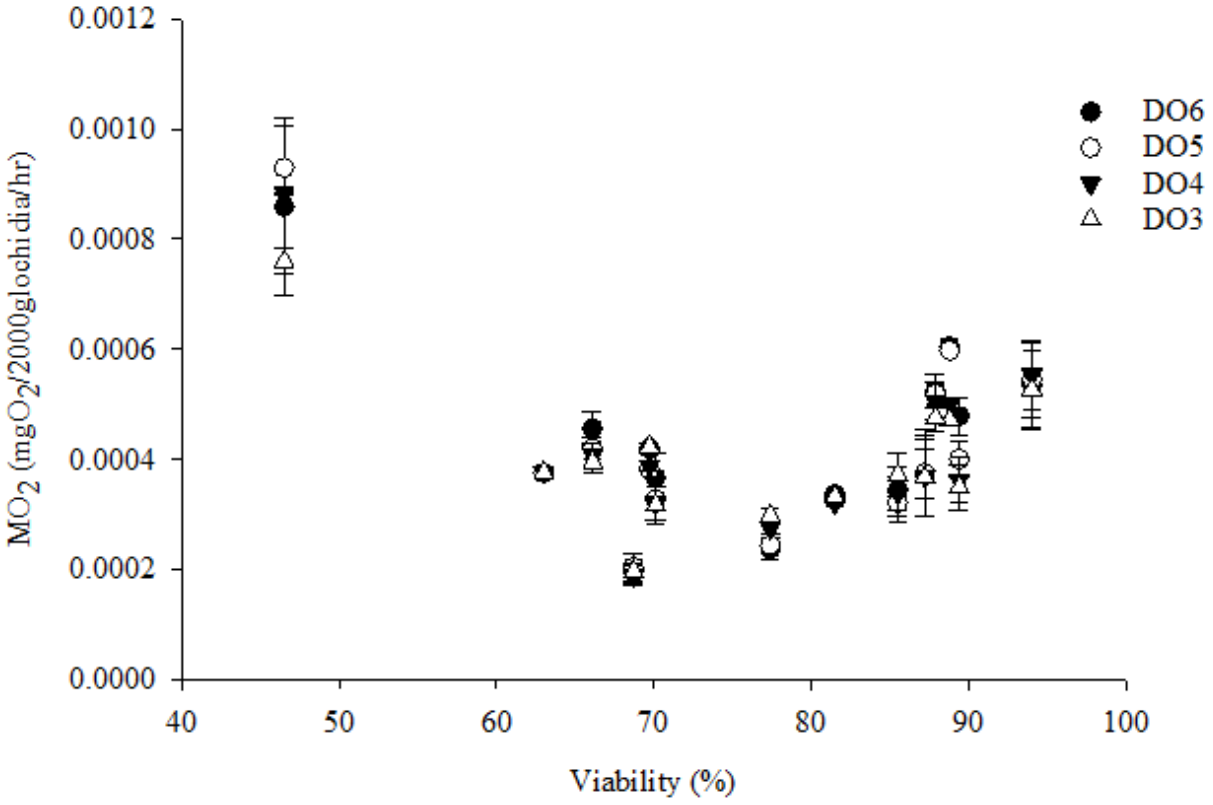

Figure 2.3: Relationship between MO2 of glochidia and brood viability at four DO concentrations. Each data point represents the mean of three replicate estimates from the same brood. Error bars represent ± 1 *SE*. MO_2 was not significantly influenced by brood viability ($F_{(1, 13)} = 0.99$, $p = 0.3387$).

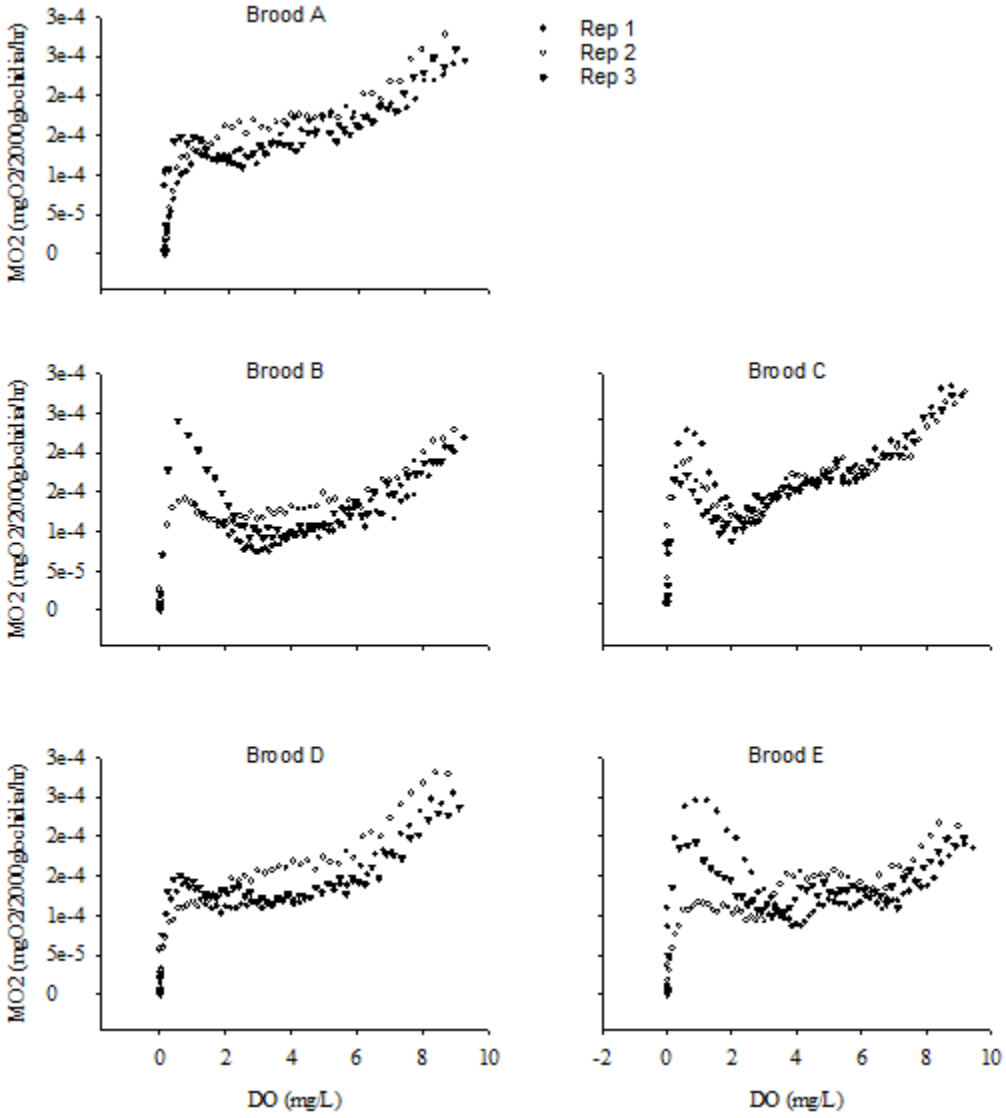

Figure 2.4: Relationship between MO_2 and DO at 13 °C. Each panel shows data from three replicate wells for a given brood. Many broods and replicates exhibited a pattern of an increase in MO_2 at low DO followed by a sharp decrease in MO_2 when approaching anoxia. This pattern was not observed at higher temperatures and prevented calculations of RI and DOcrit at 13 °C

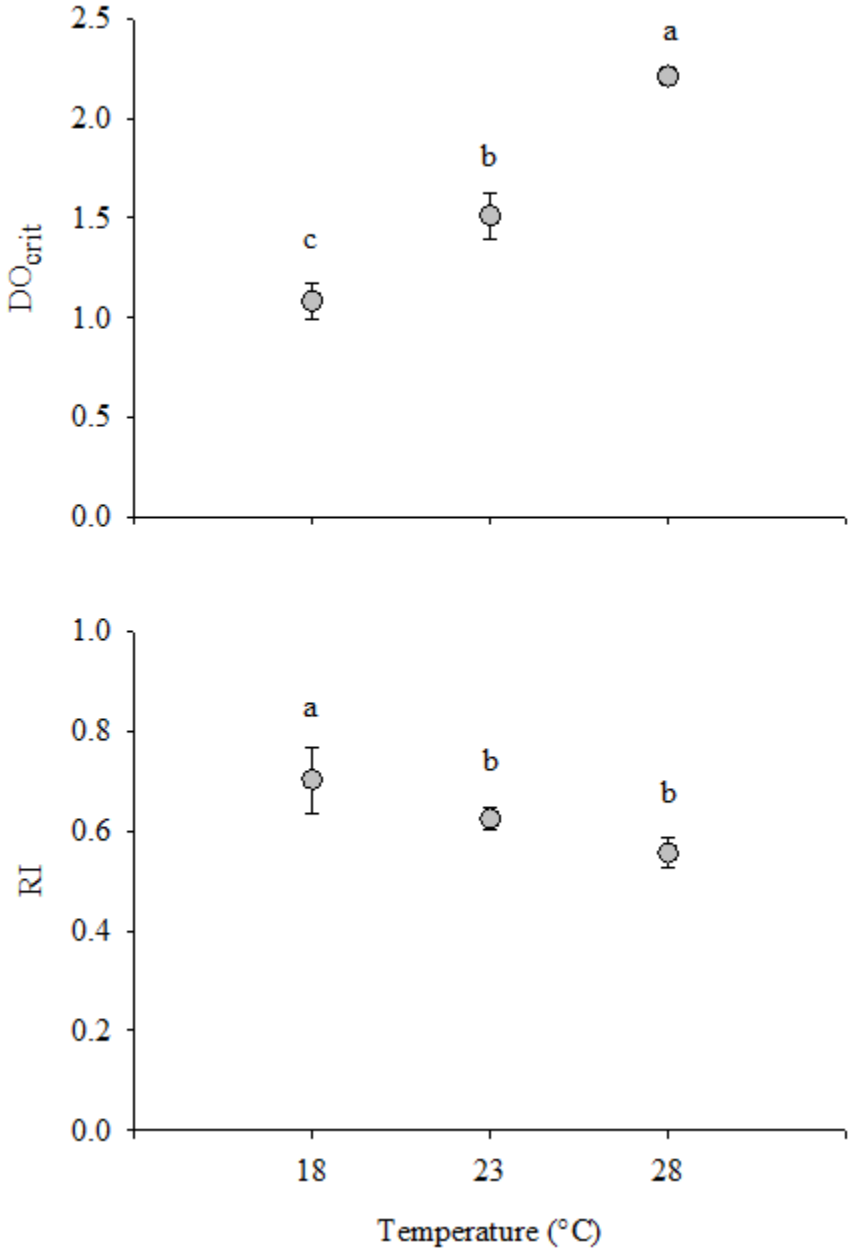

Figure 2.5: A) Mean DOcrit and B) mean RI of *L. subrostrata* glochidia at three different temperatures. Error bars represent ± 1 *SE*. Letters indicate significant differences among temperatures ($p < 0.05$).

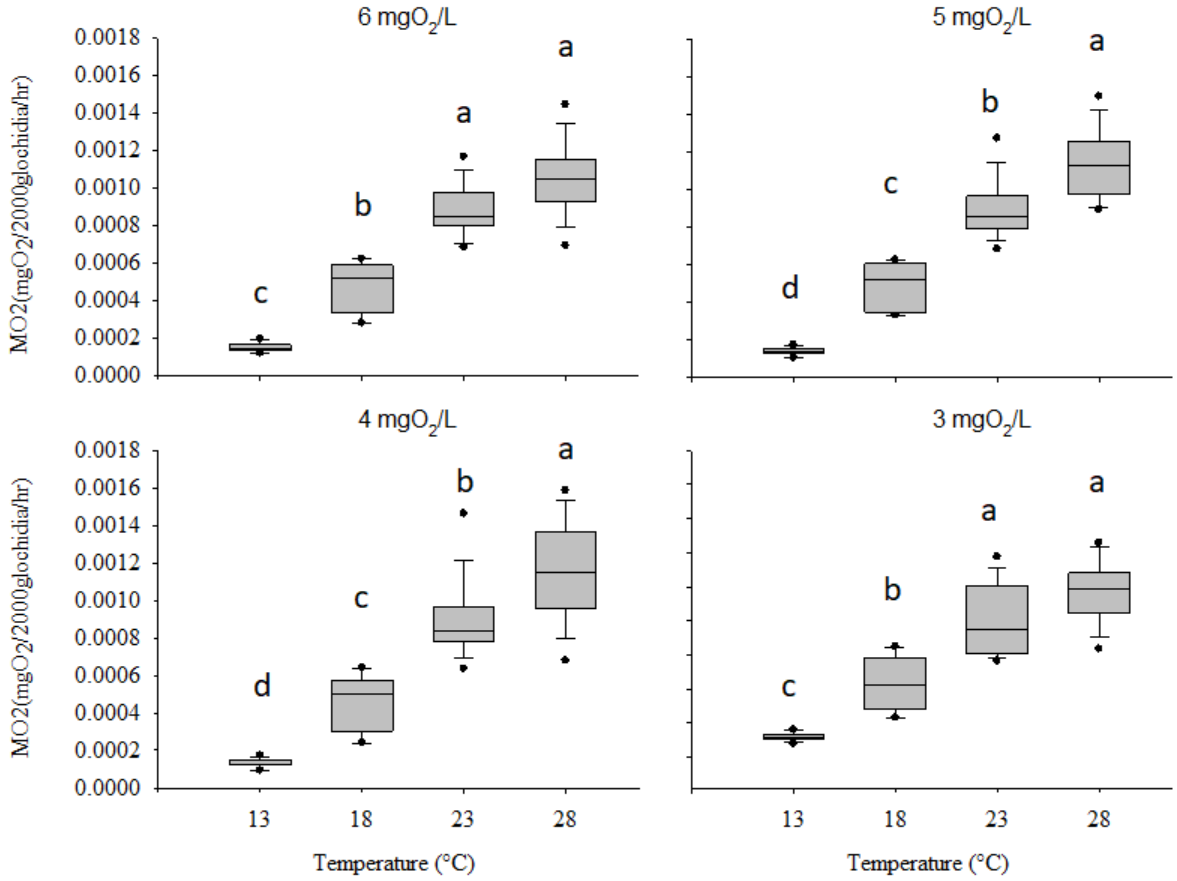

Figure 2.6: MO_2 of glochidia at four different temperatures at four different DO concentrations. Height of box plots represent the upper (q3) and lower (q1) quartiles, and the solid horizontal line represents the median. Vertical lines represent the (1.5 * interquartile range) of data (interquartile range = q3-q1) and solid circles represent data that fall outside of the 1.5* interquartile range. Letters indicate significant differences between MO2 among temperatures at each DO concentration ($p < 0.05$).

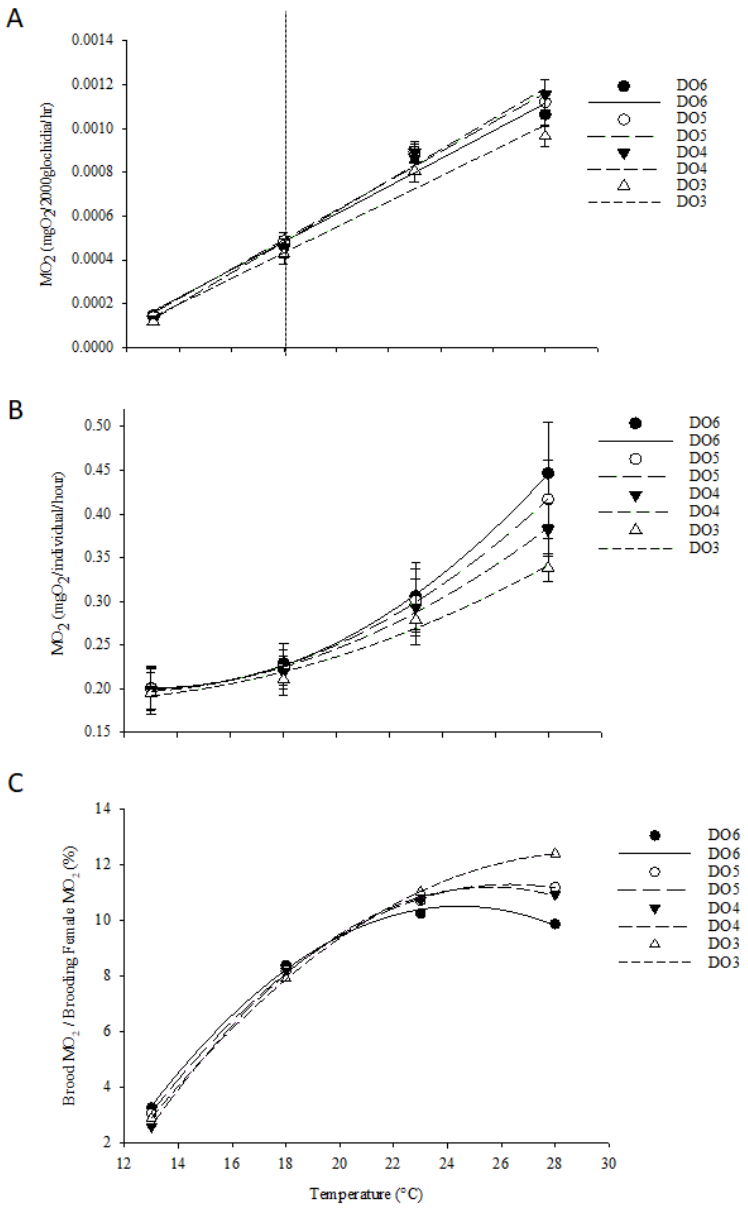

Figure 2.7: A) Relationship between MO_2 of an average sized brood of 75,000 glochidia and temperature, B) relationship between MO_2 of an average-sized brooding female (30.25 g) and temperature, and C) relationship between the proportion of brooding female MO_2 comprised of glochidial MO_2 and temperature. Lines represent best-fit regressions (linear, A; quadratic, B, C) through MO_2 data at each DO concentration. Vertical line in (A) represents the temperature threshold beyond which MO_2 at 3 mg O_2/L remains significantly lower than MO_2 at higher DO concentrations.