A metabolic approach to assessing interactions between climate change, agrochemicals, and freshwater mussel reproduction

by

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Abstract

Unionid mussels are a fundamental part of stream ecology yet are one of the most threatened aquatic organisms due in part to habitat loss, stream fragmentation, and deterioration. The increasingly relevant effects of climate change will further threaten freshwater mussel populations by not only causing a rise in temperature within streams, but by also causing an influx of sedimentation and agrochemicals entering streams due to more strong rainfall events. Due to the unique strategy unionid mussels use to reproduce, assessing the effects of temperature and common agrochemicals have on gravid mussels' metabolic rates and glochidial retention is a necessary step in developing better management practices. To do this, we estimated premature brood expulsion and metabolic stress through the use of closed respirometry by examining the effects of: 1) increasing temperatures and declining dissolved oxygen levels 2) exposed gravid mussels to three common agrochemicals over a 28-day period. Results suggested that temperature, dissolved oxygen levels, and certain agrochemicals affected gravid mussels' metabolic rates as well as their ability to retain glochidia.

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List of Abbreviations

RI	Regulation Index
RMR	Resting Metabolic Rate (mgO_2 / gWW / hr)
DO_{crit}	Critical dissolved oxygen criterion
MO_2	Metabolism
DO	Dissolved Oxygen
AFW	Artificial Freshwater
TAN	Total ammonia nitrogen
SAFRS	South Auburn Fisheries Research Station
CV	Check Valve

Chapter 1: Warming temperatures lead to brood expulsion and respiratory stress by gravid *L. subrostrata*

Introduction

Unionid mussels, or freshwater mussels from the order *Unionida,* include more than 850 species from six families. North America boasts the greatest diversity (306 species) worldwide, with the Mississippi River Basin supporting more than 3-4 times as many mussel species as the Amazon River Basin (Bogan 2008; Haag and Williams 2011). In the past, unionids were heralded for their pearls and thick shells that were used in the button industry. More recently, freshwater mussels are more valued for the ecosystem services they provide. Because of their benthic lifestyle, burrowing behavior used by freshwater mussels to maintain their position within a location allows for the mixture and stabilization of streambeds (Vaughn and Hakenkamp 2001). As mussel beds increase in density, assemblages act as the "livers of the river" by transferring suspended solids and organic materials from the water column to the stream bed (Vaughn et al. 2008). Large assemblages of freshwater mussels can even filter entire reaches of streams as the water flows across them (Haag 2012). Even in death, mussel shells have been shown to be a much-needed source of calcium in low pH waterways (Green 1980). While unionid mussels provide a plethora of ecosystem services, they represent one of the most threatened groups of aquatic organisms (Benz and Collins 2004).

Over the last 100 years, 30 North American freshwater mussel species have become extinct and 65% of the remaining species are considered species of concern (Haag and Williams 2011). Mussel assemblages have been in decline due to drought, sedimentation, systemic habitat destruction, and channelization of streams (Watters 1999; Golladay 2004; Haag 2012). Stream impoundments have long been shown to have negative effects on unionid assemblage both

directly through physical stress and indirectly through changes in habitat, food, and fish-host availability (Haag and Williams 2011; Vaughn and Taylor 1999; Watters 1999). Loss of native fish species from aquatic systems has also negatively impacted mussel species due to their unique reproductive strategy, in which the parasitic larval stage (glochidia) requires a vertebrate host, usually a fish, to complete its development (Barnhart et al. 2008).

Thermal stress is receiving increasing attention as a potential threat to mussels, but effects and tolerance thresholds are still poorly understood. Many species may already be living at or above their thermal tolerance limits (Pandolfo et al. 2010). Multiple drivers of increased water temperatures include urban runoff, industrial discharge, top release dams, modified flow regimes, and climate change (Nelson and Palmer 2007; Mantua et al. 2010; van Vliet et al. 2013). Elevated water temperatures have been shown to shift energetic resources from feeding, growth, and reproduction to the basic metabolic needs for the survival of individuals (Ganser et. al 2013; Ganser et. al 2015). Periodic cessations of respiration can become more frequent as temperature approaches an individual's thermal limit (Anestis et al 2010). While reducing valve gape restricts oxygen intake, which further limits aerobic respiration at high temperatures, previous reports indicate that mussels can alter their physiological behavior to depress metabolic rates needed for survival (Bayne and Newell 1983; Ortmann and Grieshaber 2003).

The relationship between increasing temperature and reduced dissolved oxygen may cause particular hardship for gravid females. Following fertilization, freshwater mussels brood their glochidia in their gills from weeks to months. Because gills are also used for respiration, the brooding period is likely stressful, reducing the ability of brooding females to obtain oxygen from surrounding waters in comparison to non-brooding females and males (Tankersely & Dimock 1993). Mussels have been shown to expel their broods at high temperatures and/or under

hypoxic conditions (Aldridge and McIvor 2003; Gascho Landis et al. 2012). If expelled, broods are non-viable duet to heat stress or fail to attach to a proper host due to altered time of release, mussels may be forced to forego reproduction for that year.

Previous studies that have reported respiration rates of unionid mussels were limited to static closed systems. Several studies involved placing a mussel in a container with no substrate during the day, taking an initial oxygen reading, sealing the container, and after several hours, unsealing the jar to record final DO readings (Patterson 1983; Myers-Kinzie 1998). Several studies introduced a magnetic stirrer to ensure the mixing of water within the chamber, as well as a DO probe to take continuous readings (Aldridge et al. 1987; Tankersly and Dimock 1993; Chen et al. 2001). However, the unnatural positioning of mussels, oxygen consumption associated with the DO probe itself, and the inability to detect valve closure during experimentation presented further limitations. Furthermore, none of the aforementioned studies considered the natural diel cycle that varies among unionids. Several studies have shown that the activity of some mussel species varies diurnally (McCorkle et al. 1997; Englund and Heino 1994; Chen 1998; Rypel 2008), suggesting respiration rates might similarly differ. In this study, I examined the relationship between thermally-induced respiratory stress and brood expulsion by addressing the following objectives:

1) Determine best practices for measuring respiration of *L. subrostrata* in a closed circuit respirometry unit.
2) Determine the effect of temperature on energy demand of brooding females and their ability to obtain oxygen as ambient DO declines.
3) Determine the effect of temperature on valve-closure of brooding females.

4) Determine the relationship between temperature, respiratory stress, and brood expulsion.

Methods

Study animals

Ligumia subrostrata are a sexually dimorphic species that typically inhabit lentic aquatic systems (Williams et al. 2008). Females are long-term brooders that attract host fish by flashing a mantle lure (Corey et al. 2006). Gravid females were collected from an earthen pond located at South Auburn Fisheries Research Station (SAFRS), School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University in March 2016, while water temperatures were 15ºC. All mussels used were naturally propagated in SFAAS ponds to reduce the need to collect individuals from wild populations. After collections, *L. subrostrata* were randomly distributed among six 40L glass recirculating aquariums, filled with artificial freshwater (AFW: 50 mg $CaCO_3$, 25 mg CaCl, 50 mg $NaHCO_3$, and 5 mL 30% salt water/L deionized water) (pH = 8, Total Hardness = 53, Alkalinity = 40) (Gascho Landis et al. 2012). Aquarium temperatures were maintained at 15ºC to ensure the brooding females would retain their glochidia prior to experiments. A 12:12 light:dark cycle was consistently maintained throughout the duration of the experiment. Mussels were fed Reed Mariculture shellfish diet (Reed Mariculture, Campbell, California) every other day at a rate of 0.05 mL/ L (Gascho Landis et. al 2012). Water quality was monitored and recorded weekly for TAN (total ammonia nitrogen), pH, nitrite, and nitrate using Tetra® EasyStrips.

Thermal Acclimation

After acclimating to laboratory conditions at 15ºC for at least one week, water temperatures were adjusted by 1ºC per day until target temperatures of 13, 18, 23, 25, 28, and 32ºC were reached. Mussels were then acclimated to their respective target temperature for at least seven days prior to experiments. Chillers connected to digital temperature controllers (Aqua Logic, Inc.) were used to maintain cool acclimation tanks at 13 and 18 ºC ±1ºC. Titanium heater bars (Finnex, Inc.) connected to digital temperature controllers were used to maintain all three acclimation temperatures (23, 25, and 28 ºC) above 18 ±1ºC.

Oxygen Consumption Measurement

Respirometry experiments were conducted using AutoResp™ 2.3.0 software (Loligo Systems, Inc.) coupled with a four-chamber fiber optic respirometry system (Loligo Systems, Inc.). Fiber optic cables were attached to vertical, acrylic, respirometry chambers (Loligo Systems, Inc). Each acrylic chamber was connected to two Eheim submersible 79 gallon per hour pumps (EHEIM GmbH & Co KG); one to circulate water within the closed circuit during respirometry and one to pump fresh, oxygenated water through the chamber during acclimation or between respirometry cycles (Figure 1).

Preliminary trials were conducted to determine whether *L. subrostrata* acclimated faster to respirometry chambers when allowed to burrow into substrate as opposed to simply lying on their side on a hard surface. For the substrate treatment, respirometry chambers contained a glass jar filled with sand in which a mussel was allowed to burrow such that its foot was anchored in the sand and its posterior siphons were elevated above the sand surface. For the hard surface treatment, contained a glass jar with a hard, plastic lid, and a mussel placed on top of each lid.

Intermittent respirometry (Svendsen et al. 2016) was used to measure respiration rate of each mussel approximately every 0.75 hours for a period of at least 12 hours under normoxic conditions. Respiration rate was regressed against time and time-to-acclimate was defined as the time required for the individual to maintain a stable metabolic rate (Svedsen et al. 2016). Preliminary trials were also conducted to determine whether *L. subrostrata* had a lower metabolic rate during the day versus at night using the same methods.

Closed respirometry was used to estimate the degree of respiratory stress experienced by mussels at different temperatures as DO declined from normoxia to hypoxia. Three endpoints were used as indicators of respiratory stress: 1) Regulation Index (RI) – the ability to maintain a constant respiration rate as dissolved oxygen declines (Mueller and Seymour 2011), 2) Critical DO concentration (DO_{crit}) the dissolved oxygen threshold below which an organism shifts from predominately aerobic to anaerobic metabolism (Mueller and Seymour 2011), and 3) incidents of valve closure which cause metabolic depression and probable shifts from aerobic to anaerobic respiration (Anestis et al. 2010).

After a 24-hour fasting period, gravid mussels were lightly scrubbed with a soft-bristled toothbrush to remove any visible algae that could skew respiration readings. Wet weight was recorded. Mussels were then naturally oriented within a glass jar filled with aquarium sand which was placed within a chamber. One chamber in each run was designated as the “control” chamber (sand, but no mussel) to account for any bacterial built up throughout the run. The mean background oxygen demand from this chamber was then subtracted from the overall respiration rates observed from the other chambers. Mussels were allowed to acclimate to their chamber for two hours prior to experimentation. Valve closure within a run (Figure 2) typically rendered respiration data unusable for RI and DO_{crit} calculations. Closed respirometry experiments ran

overnight and were complete when the DO fell below 1 mg O_2/L. After each experiment, any chambers, jars, sand, tubing and pumps used during the experiment were placed in bleach.

At the termination of the experiment, the water from each chamber was poured through 105 micrometer mesh to collect any expelled glochidia and immediately preserved in 70% ethyl alcohol for later counting (Dodd et. al 2005). The gills from each mussel were also removed and immediately preserved in 70% ethyl alcohol. At a later date, glochidia were flushed from the gills with freshwater into a beaker, diluted to ~1000 ml, and mixed with plunger-type stirrer. Subsamples were enumerated under a microscope until either >100 glochidia had been counted or >60% of the sample had been sampled. Total number of glochidia retained in the gills was calculated as:

$$\text{Total \# Glochidia} = \frac{\text{\# Glochidia Counted}}{\text{Subsample Volume}} \times \text{Sample Volume}$$

Glochidia expelled within the respirometry chamber samples were enumerated in the same manner.

Statistical Analysis

Prior to analysis, all data were tested for normality. If data were not normally distributed, the final analysis was calculated using rank transformed data. Raw metabolic data for RMR, RI, and DO_{crit} were fitted with the curve (linear, quadratic, or 2-parameter hyperbola regression) that had the highest r^2 value for the data set to describe the relationship of metabolic patterns with temperature.

A two-way repeated ANOVA of rank transformed data was used to compare RMR during the day to RMR at night. Effect of substrate type on RMR was assessed using a one-way repeated measures ANOVA. Acclimation time was determined from using a repeated measures

one-way repeated measures ANOVA to analyze intermittent respirometry. Mussels were considered "acclimated" at the point after which the metabolic rate did not significantly change for the remainder of the run. Linear regression was used to determine the relationship between temperature on RMR, RI, and DO_{crit}.

Results

Preliminary Trials to Establish Protocol

Although there were significant differences in RMR amongst the four individual chambers tested ($F_{3,32}$=539.62, $p < 0.0001$; Tukey-Kramer $p<0.0001$ for all pairwise comparisons), there was no significant difference in RMR between daytime and nighttime measurements ($F_{1, 119} = 0.69$, $p = 0.4084$) (Fig. 3) and the interaction between individual and time period was not significant. Thus there is no indication that results of the following respirometry experiments were biased due to being conducted at night.

There was no significant difference in RMR between mussels lying on a hard surface versus mussels oriented in sand ($F_{1, 8}$= 1.59, $p = 0.2424$) over 12 hours (Fig. 4). However, the relationship between RMR and acclimation time differed between surfaces (Fig. 5). When placed in sand, there were no significant differences in RMR ($F_{15, 58} = 1.15$, $p = 0.3343$) among the 16 measurement periods (over a period of 0.8 – 12.3 hours) which indicates that mussels acclimated to test conditions within 1 hour. However, when mussels were placed on a hard surface, there were significant differences in RMR (ANOVA: $F_{15, 57} = 4.04$, $p < 0.0001$) through time. After 3.83 h there were no significant differences in RMR among subsequent measurement periods (Tukey-Kramer $p>0.05$ for all pairwise comparisons after this time period), suggesting mussels took approximately 4 hours to acclimate when held on hard surfaces instead of sand. Based on

these results we conducted respirometry experiments using mussels held in sand with an acclimation period of 2 hrs to account for any variation among individuals that may occur.

Temperature Trials

For each 1°C increase in temperature, I observed a 53.2×10^{-5} increase in RMR ($p < 0.0001$; $r^2 = 0.6451$) (Fig. 6). RI decreased by 0.027 for each 1°C increase in temperature ($r^2 = 0.216$, $p = 0.022$) (Fig. 7). RI was not affected by mussel mass ($F_{1,13} = 0.90$, $p = 0.3604$). There was no significant relationship between temperature and DO_{crit} ($p < 0.05$), nor was DO_{crit} affected by mussel mass ($F_{1,10} = 1.23$, $p = 0.2944$) (Fig. 8). As temperatures increased, mussels exhibited a significant positive relationship between valve closure and temperature ($r^2 = 0.7069$, $p = 0.0470$) (Fig. 9).

Brood retention under normoxic conditions during the acclimation period was negatively affected by temperature ($r^2 = 0.4150$, $p < 0.001$) (Fig. 10). Individuals held at 23 ºC ($p = 0.046$) and 28 ºC ($p < 0.001$) retained significantly fewer glochidia than individuals held at 13 ºC. When exposed to declining DO during respirometry, an increasing proportion of gravid *L. subrostrata* broods were expelled as temperatures increased above 18 ºC (Fig. 11). We observed a significant expulsion of the brood, a shift to net conformation, and a sharp increase in valve closures between 18ºC and 23ºC (Figure 12).

Discussion

Our preliminary respirometry trials allowed us to address several issues that could occur during a respirometry experiment that, to our knowledge, have not yet been examined for unionid mussels. When averaged over a twelve-hour period, there was no significant difference

in respiration rates between mussels lying on a hard surface and mussels oriented naturally in sand. However, while mussels in sandy substrate acclimated to chambers within an hour, respiration rates of mussels on hard substrate were initially elevated and took approximately 4 hours to stabilize. After 6 hours, all respiration estimates were significantly lower than the initial respiration rate. Thus, respiration studies that place mussels on a hard surface and estimate respiration based on a single initial and a single final measurement period without allowing for sufficient acclimation time may be overestimating respiration rates. In contrast, respiration rates based on single initial and final measurement periods would underestimate respiration rates if mussels were periodically closing valves and ceasing aerobic respiration as observed in this study. From our preliminary studies, we can assume that the use of simple initial/final respirometry techniques increases the chances of respiration rates being affected by methodological artifacts. Incorporation of sediment for natural orientation and use of professional respirometry equipment that allows for frequent estimates of respiration rates during a trial are of great use in minimizing these issues. However, a side-by-side comparison of methodologies would need to be performed to test for significant differences.

Although respiration rates varied significantly among individuals held under the same conditions, we found no evidence for differences in respiration rates between day and night. Thus, we do not expect that our results would have been different if we had conducted them during daylight as opposed to night time hours. However, diurnal activity differs between species (McCorkle et al. 1997; Englund and Heino 1994; Chen 1998; Rypel 2008) and should be considered on a case-by-case basis when running respirometry.

Metabolic demand required for basic maintenance, as indicated by RMR, more than doubled as temperatures rose from 13 to 28ºC. In natural systems, gravid females would then

need to extract increasing amounts of oxygen to maintain basic metabolic function, even as the availability of dissolved oxygen decreases as ambient water temperature rises. Although DO_{crit} did not increase as temperatures rose and remained below 3 mg O_2/L, the ability of gravid females to regulate oxygen consumption decreased with increasing temperature. As temperatures rose, gravid females were less able to extract oxygen from surrounding waters as DO declined as demonstrated in the be the switch from regulating their oxygen intake to net conformation between 18ºC and 23ºC. This put gravid females under increasing respiratory stress, particularly as conditions approached hypoxia. In addition to a decreasing RI, gravid females exhibited increasing frequency of valve closure with increasing temperature, further compromising their ability to extract oxygen from the environment.

Previous studies have shown that brooding females will expel broods in response to rapid and gradual increases in temperatures (Matteson 1948; Gascho Landis et al. 2012). Females brood glochidia in their gills, and previous studies have suggested that glochidial broods reduce the respiration rates of females (Tankersely and Dimock 1993). In particular, Gascho Landis et al. (2012) observed that *L. subrostrata* began expelling broods between 15ºC and 25ºC. Our results suggest that brood expulsion is a response to respiratory stress. As temperatures rise, oxygen demand increases, but the ability of brooding females to obtain oxygen decreases as DO declines. This scenario is supported by Tankersely and Dimock (1993) who suggested that the presence of glochidia in the gills inhibited the respiration rates of brooding females. Release of broods would presumably help alleviate respiratory stress. We observed decreasing retention of broods as females were acclimated to increasing temperatures under normoxic conditions. When brooding females were exposed to short-term declines to hypoxia in respirometry chambers,

brood expulsion within the chambers increased with increasing temperature, particularly at 23°C and 28°C.

In natural systems, rising temperatures from anthropogenic causes may induce premature brood expulsion. Brooding females may be especially sensitive to warming temperatures if DO is reduced. Even short-term (i.e. < 2 hrs) hypoxia events appeared to initiate brood expulsion between 18°C and 23 °C. These results suggest natural resource managers and regulatory agencies may want to pay particular attention to dissolved oxygen guidelines during the summer months.

In this study, response of brooding females to increasing temperature and hypoxia was highly variable, suggesting some individuals within a given population may be particularly sensitive to rising temperatures, whereas others are more tolerant. Additional research examining the factors leading to increased sensitivity and/or tolerance of brooding females to thermal and respiratory stress is warranted. Additional information is also needed to determine whether prematurely expelled broods represent a reproductive loss or if they are still viable and have a reasonable chance of attaching to an appropriate host. Tuabert et al. (2014) found that metamorphosis success of *Unio crassus* declined with increasing temperatures between 17 and 23°C, suggesting premature expulsion may be linked to decreasing viability. However, Corey et al. (2006) and Gascho Landis et al. (2012) found that even though *L. subrostrata* is a mantle displaying specialist, broods were often expelled as tear shaped conglutinates, which may represent a secondary host attraction strategy for expelled broods if they still contain viable glochidia. Successful management of unionid populations will require not only an understanding of the stresses imposed by rising temperatures, but also the physiological and behavioral strategies of brooding females to deal with these stresses. As water temperatures continue to rise

in many parts of the world (van Vliet et al. 2013) due to climate change and other anthropogenic factors, understanding the complex relationships between physiology, temperature, and reproduction of declining freshwater mussel populations will assume ever-increasing importance.

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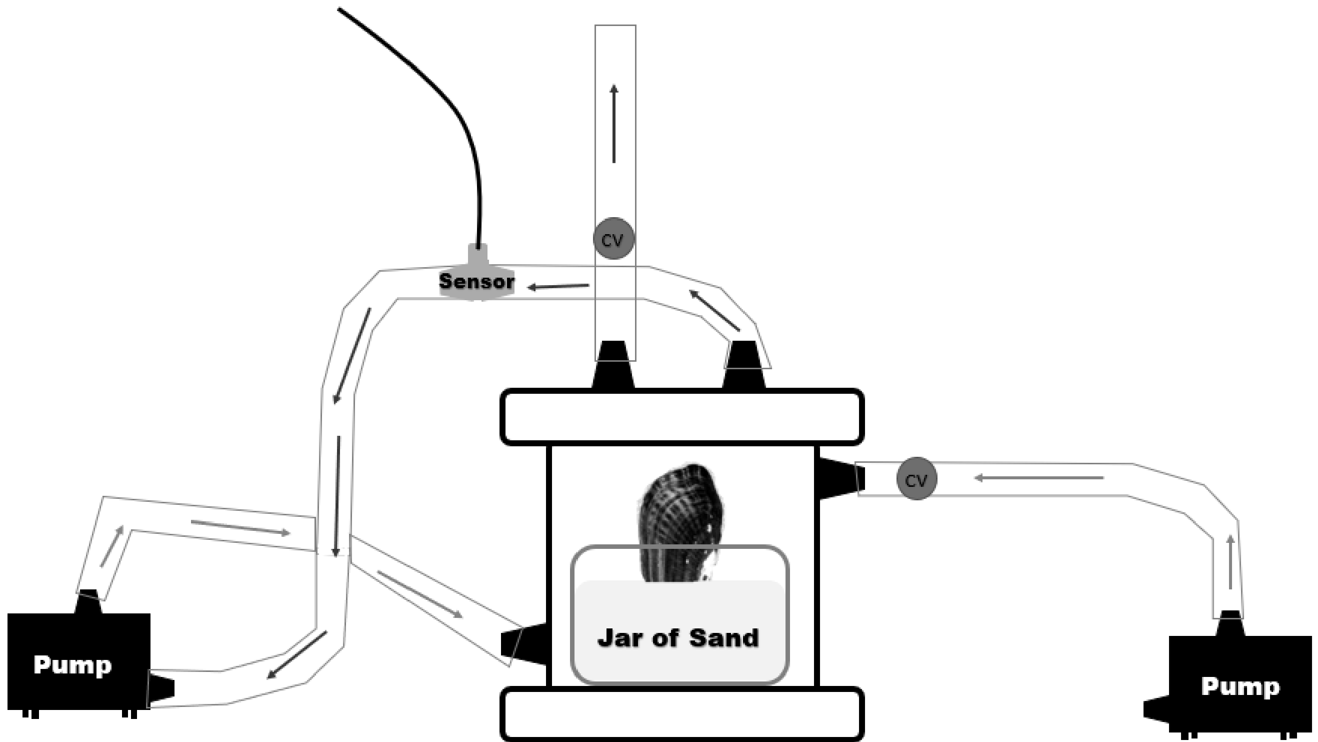

Figure 1: The respirometry set-up used for closed circuit respirometry. Mussels were placed in a glass jar filled with aqurium sand that was placed within an acrylic respirometry chamber. During the initial 2-hr acclimation, water flowed through the pump (right) and out the top of the chamber, maintaining oxygen levels within the chamber. Once experimentation began, water flowed through the pump (left), into the chamber, out of the chamber past the oxygen sensor, and back through the pump (left), to complete a closed loop. Check valves (CV) were placed on the flow-through circuit to ensure no water exchange occurred through this loop while measurements of respiration were recorded.

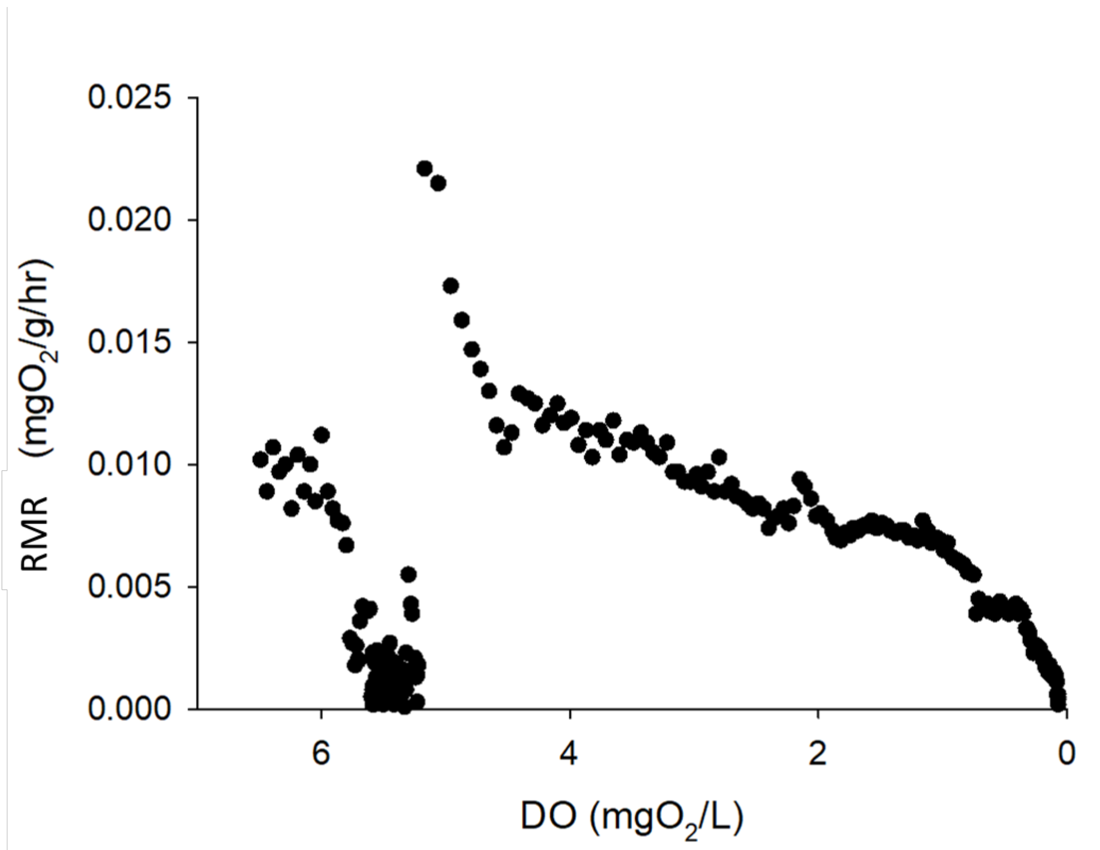

Figure 2: Change in RMR with declining DO concentration. Aa rapid decline in RMR to zero, followed by a rapid increase in RMR indicates an incidence of valve closure and re-opening between 6 and 5 mgO_2/L.

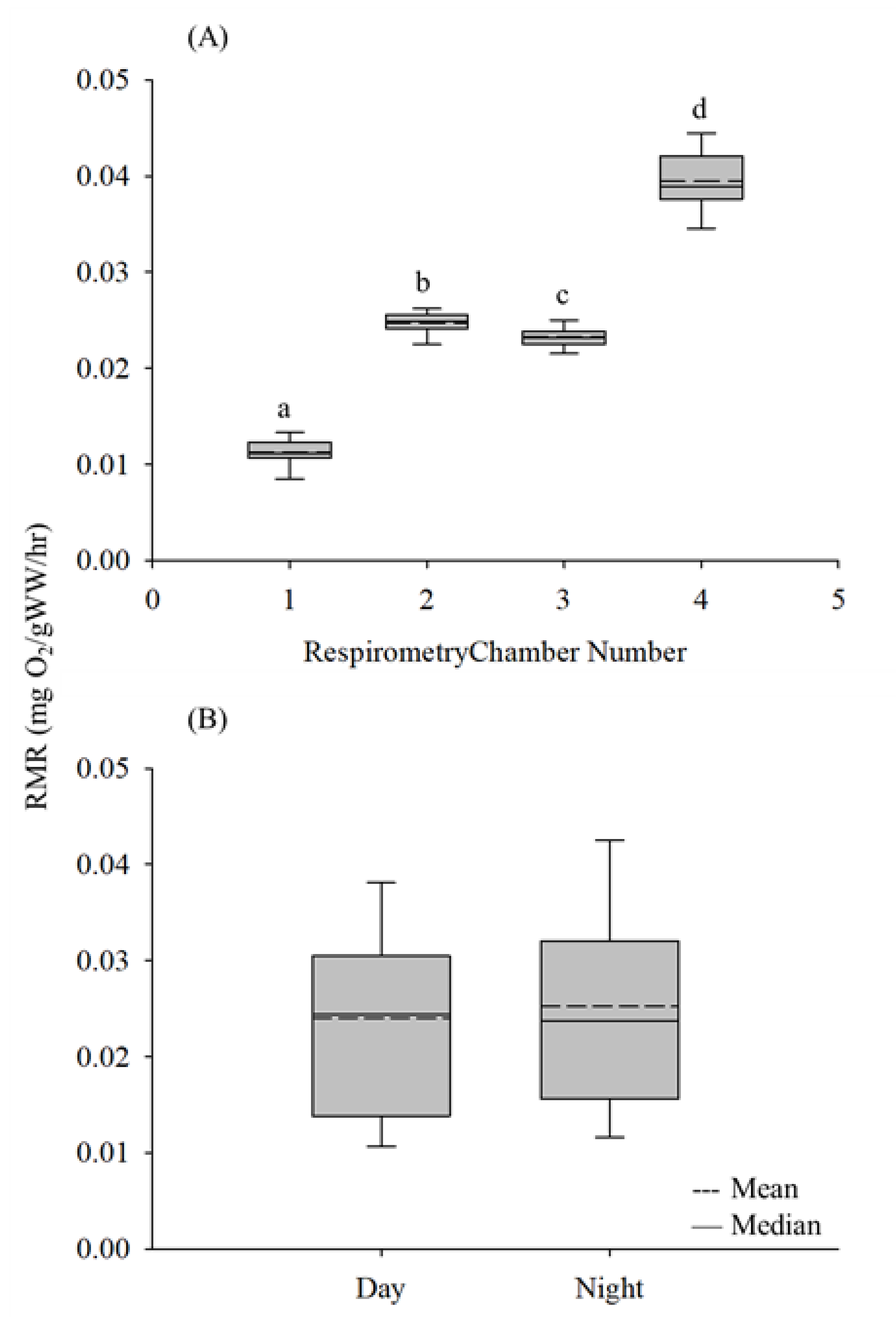

Figure 3: A) Box plot of mean RMR estimates for four individuals averaged across day and night . B) Mean RMR for all individuals during the daytime and during the nightime. Letters above each box represent significant differences among individuals. No significant differences were found between day and night. Boxes represents the upper and lower quartiles of data. Outliers are not shown.

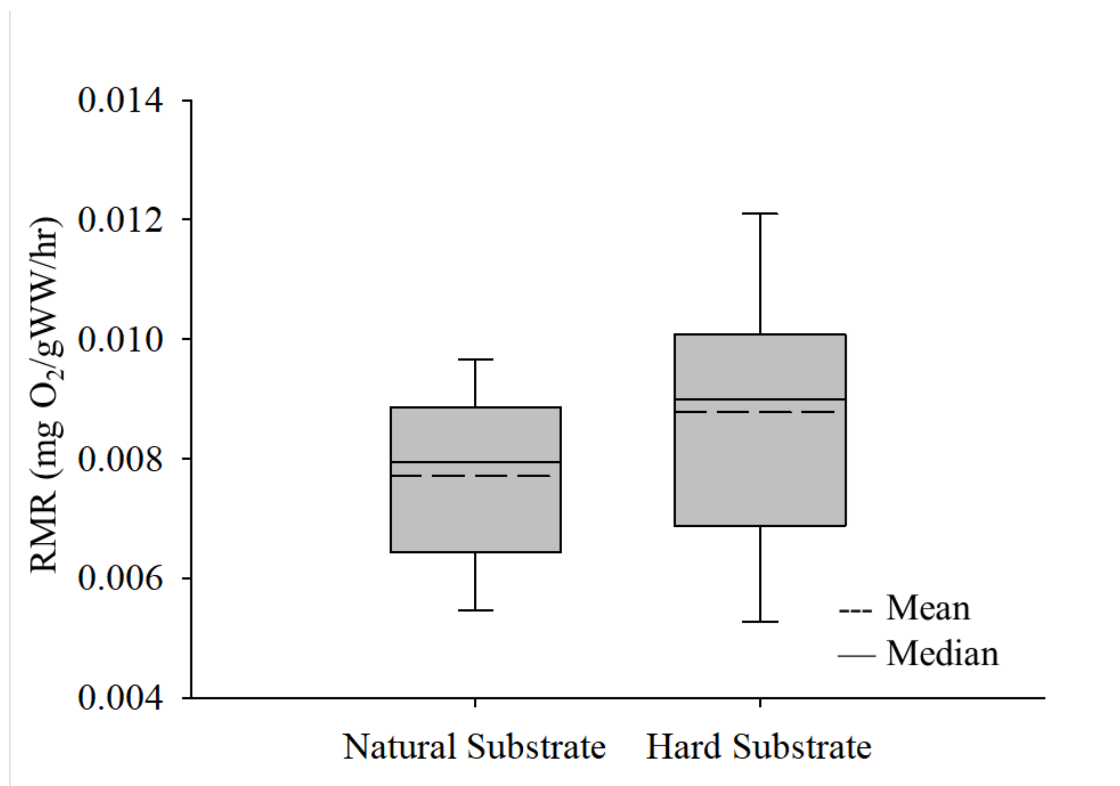

Figure 4*:* A comparison of respiration rates of mussels positioned on a hard, unnatural substrate vs. in a sand substrate (natural). Box represents the upper and lower quartiles of the data set. There was no significant difference in RMR between substrate type. Outliers are not shown.

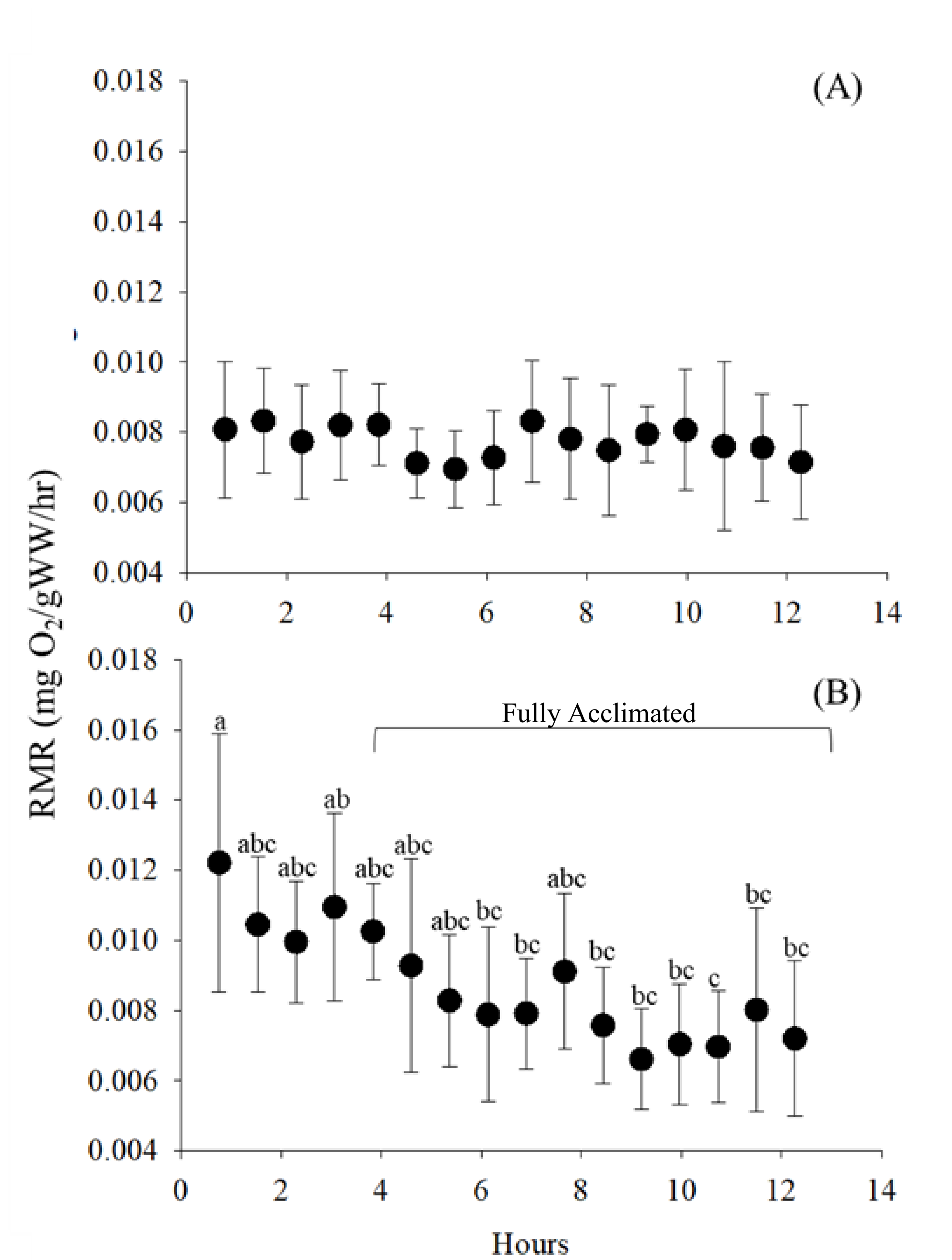

Figure 5*:* Mean RMR of mussels through time when A) naturally oriented in sand and B) laying on side on hard surface. Letters represent significant differences in RMR among hours. Error bars represent ± 1 SE.

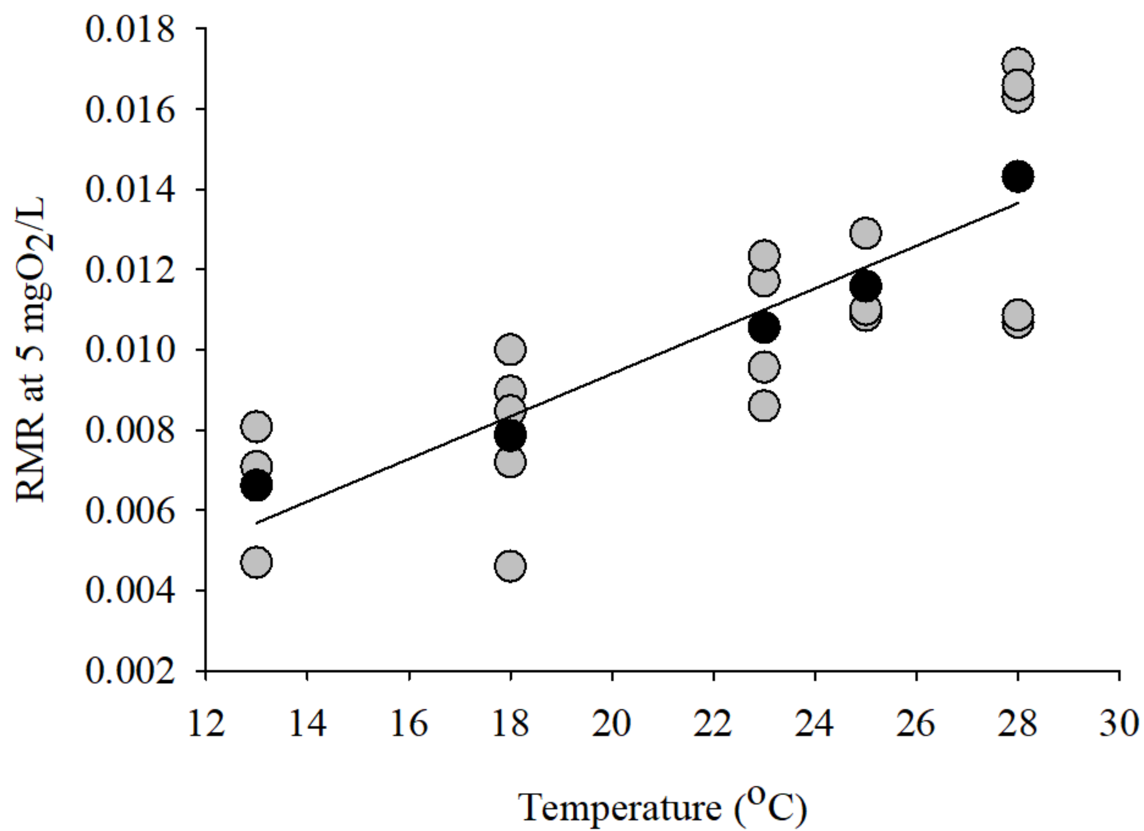

Figure 6: RMR estimated at an ambient DO of 5 mgO_2/L for mussels acclimated to a range of temperatures. Grey circles represent RMR of individual mussels. Black circles represent the mean RMR for that temperature. Solid line represents a linear regression through the individual data.

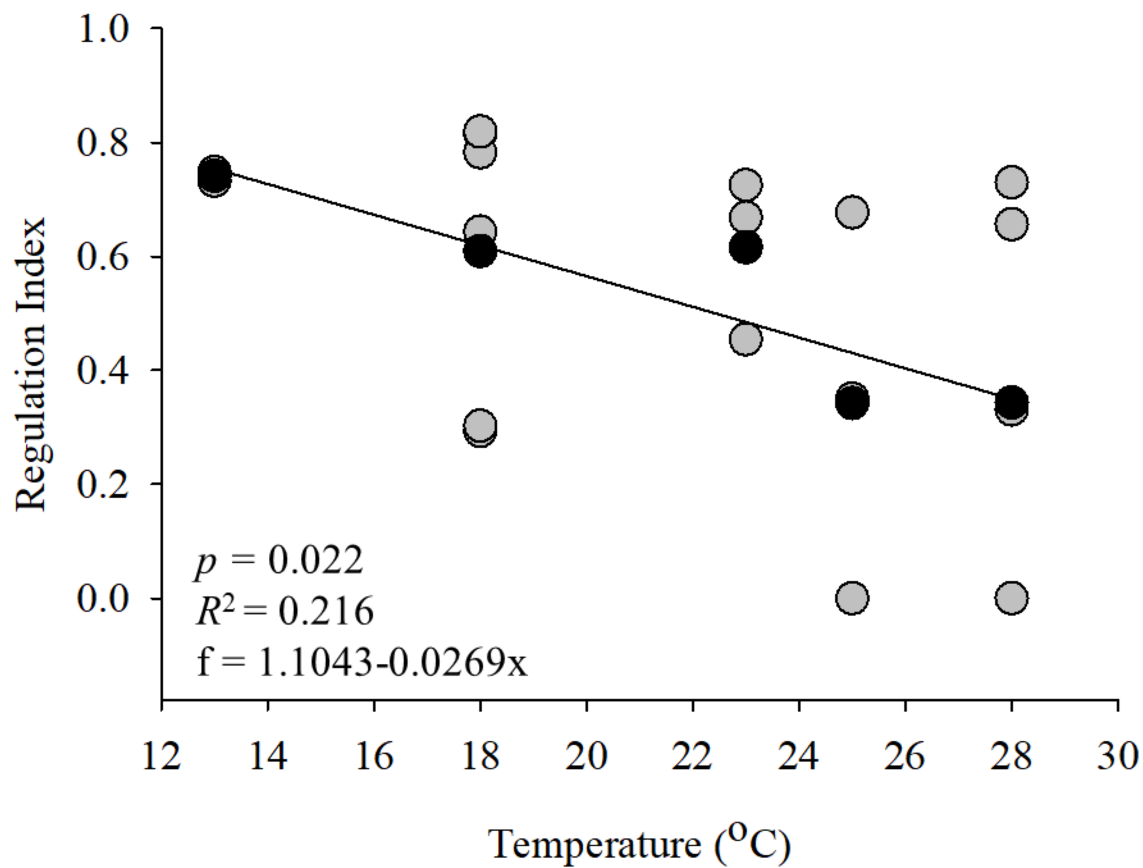

Figure 7: Relationship between regulation index and temperature, in which 1 is considered "prefect" regulation and 0 is considered "perfect" conformation. Grey circles represent raw data. Black circles represent the mean. Solid line represents a linear regression through the raw data.

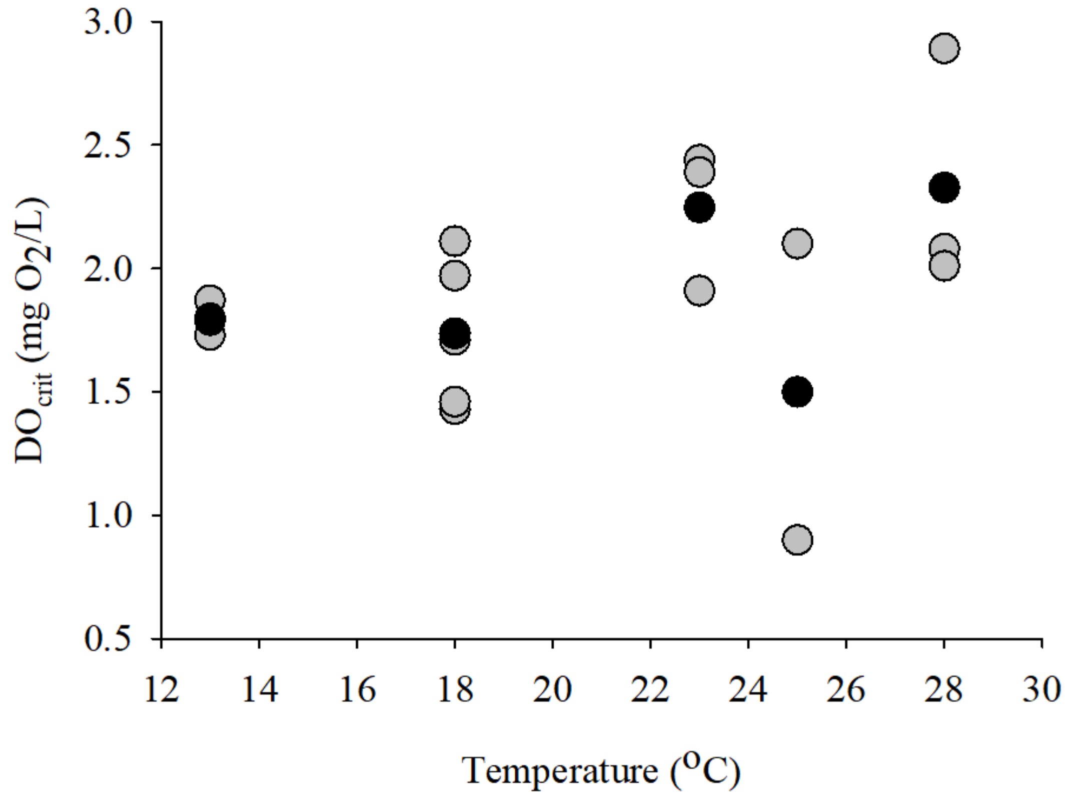

Figure 8: Relationship between DO_{crit} and temperature. Grey circles represent raw data. Black circles represent mean $DO_{crit.}$

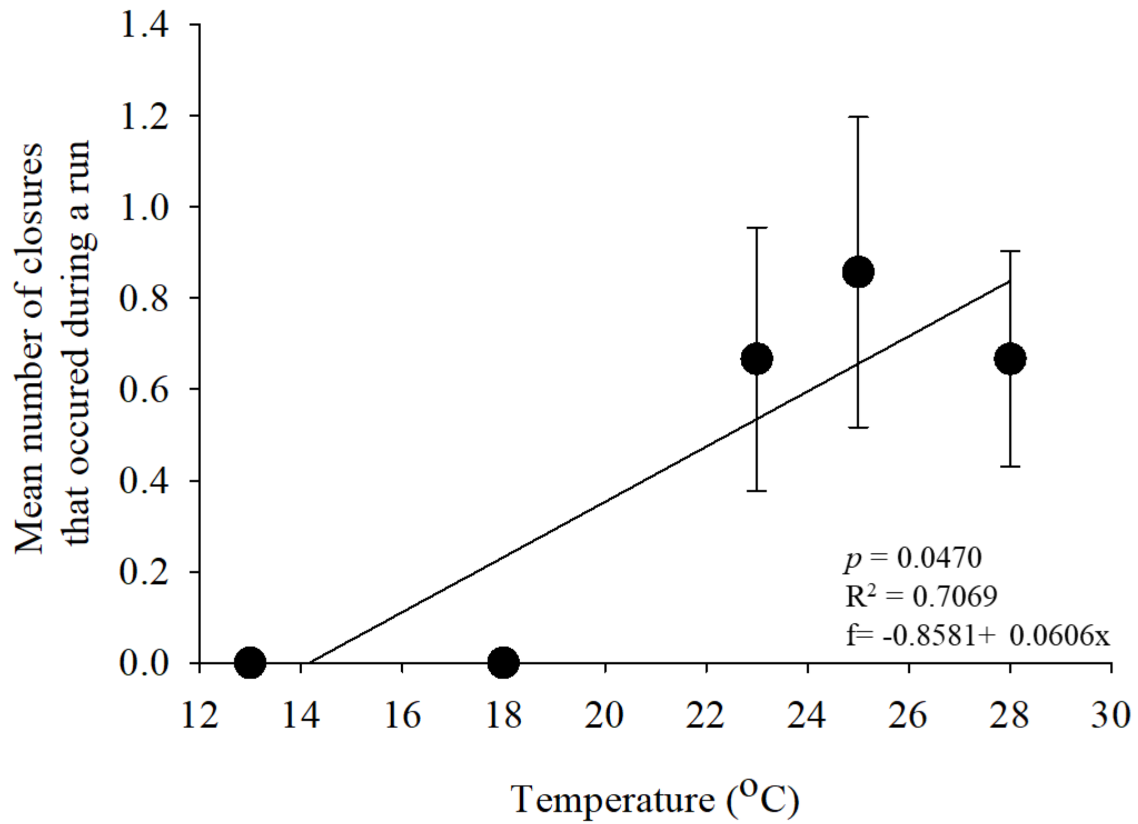

Figure 9: The mean number of closures that occurred during respirometry trials at each temperature. Solid line represents a linear regression through the data. Error bars represent ± 1 SE.

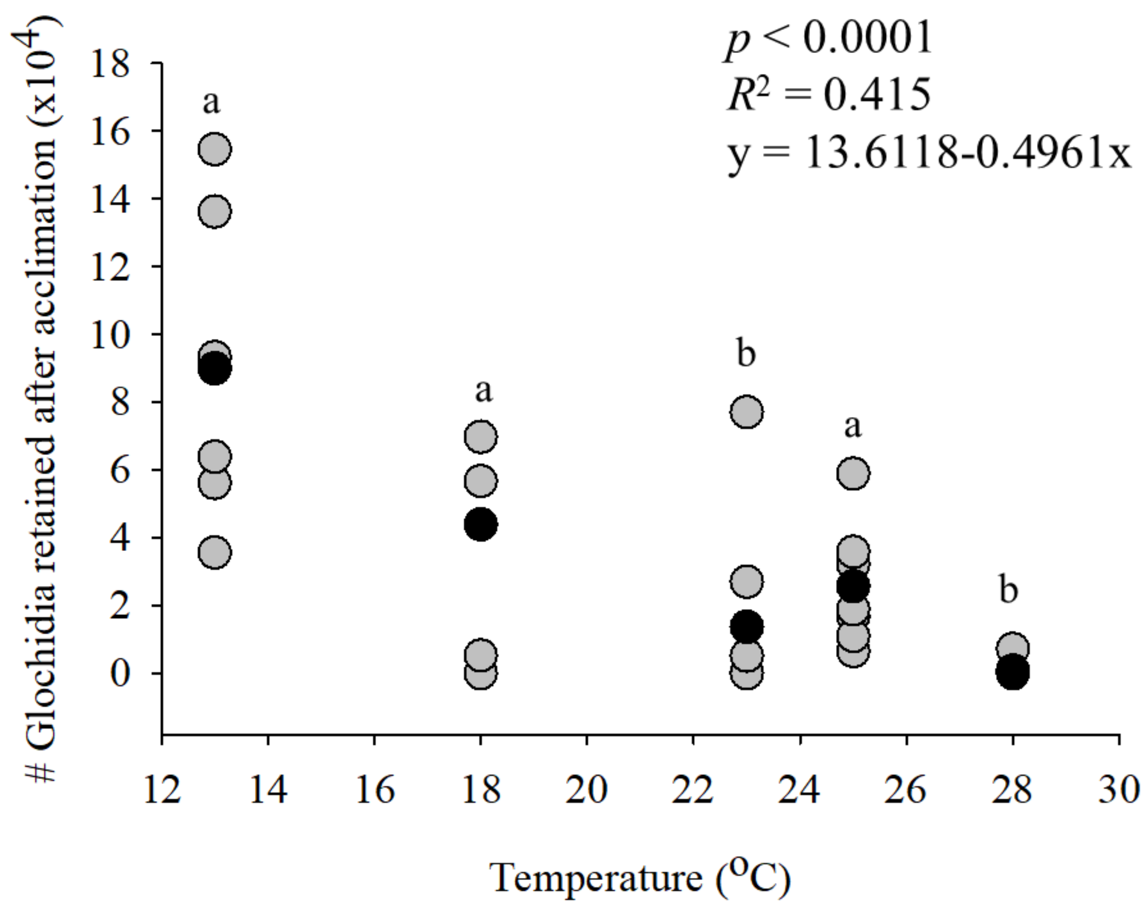

Figure 10: Relationship between temperature and glochidial retention during a 2-week normoxic acclimation period. Asterisks denote a significant difference in glochidial retention compared to the lowest temperature tested. Solid line represents a linear regression through the individual data.

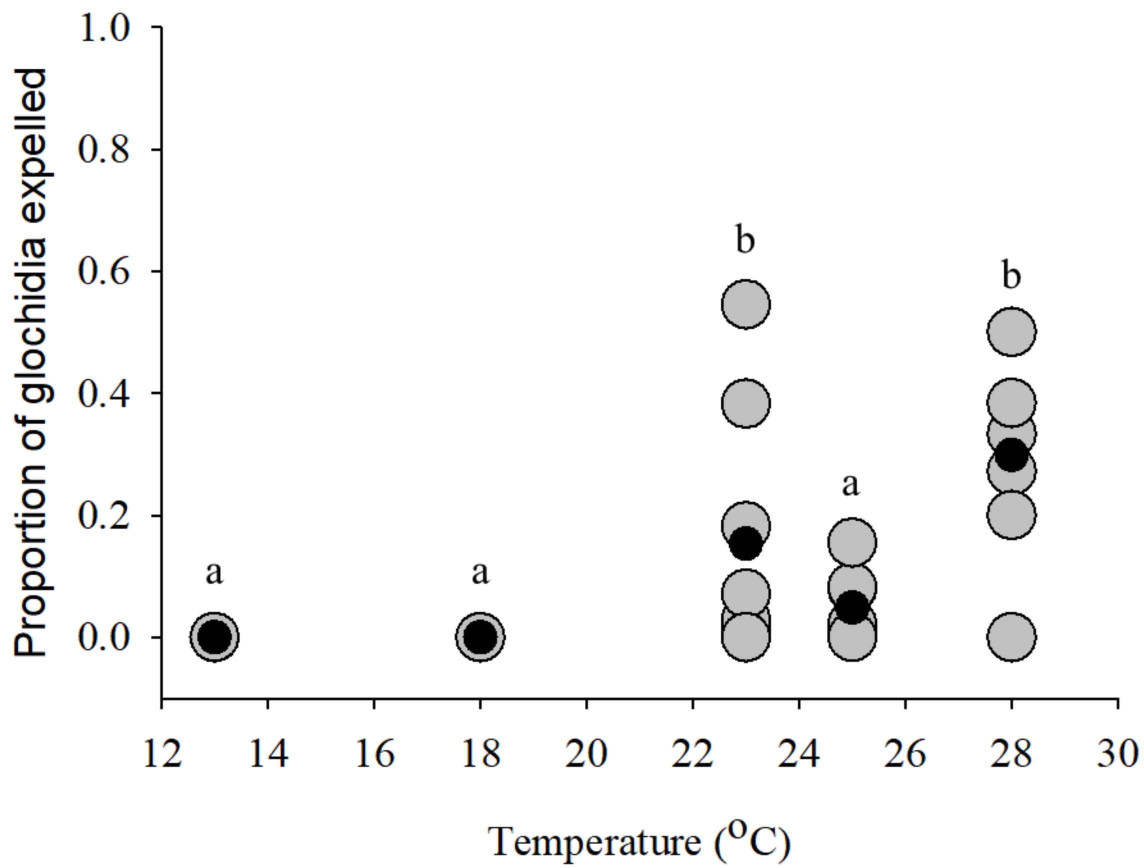

Figure 11: Change in proportion of glochidia expelled with increasing temperature under conditions of declining DO in respirometry chambers. Asterisks denote significant differences in glochidia expulsion compared to that observed at the lowest temperature tested.

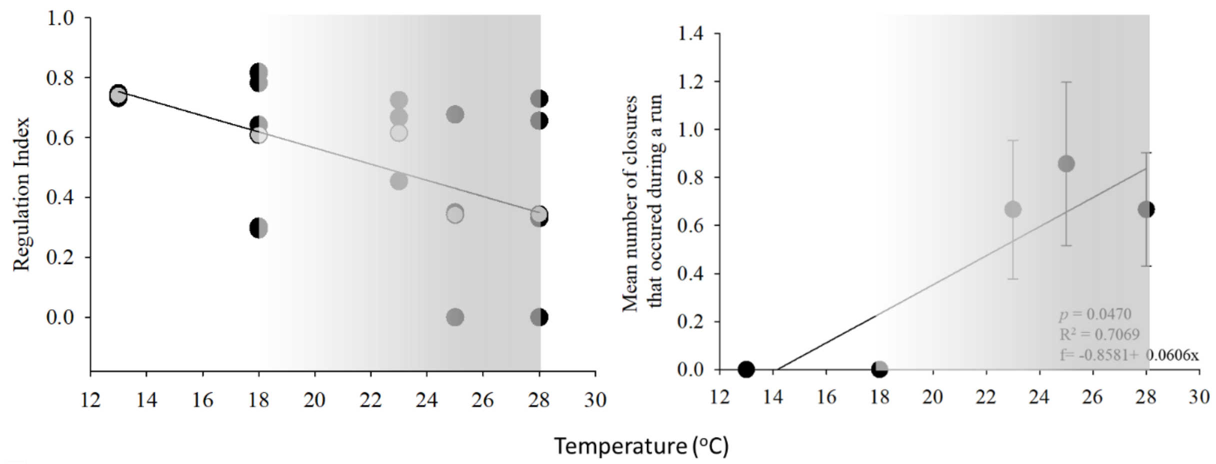

Figure 12: Illustration showing the potential connection between brood expulsion and respiratory stress. The grey gradient denotes the occurance of brood expulsion that occurred between 18ºC and 28 ºC (Figure 10).

Chapter 2: Effects of common agrochemicals on brood expulsion and respiratory ability in gravid female mussels

Introduction

Freshwater mussels, or unionids, represent a major component of aquatic biodiversity. They provide a multitude of ecosystem services such as biofiltration (Eversole et al. 2008; Haag 2012), stabilizing stream beds (Haag 2012), and influence surround benthic communities. Yet, they are one of the most threatened groups of aquatic organisms (Benz and Collins, 2004). The Southeastern United States boasts the greatest biodiversity of freshwater mussels in the world, with Alabama having the highest mussel diversity of all the southeastern states. However, these populations are in decline (Haag and Williams 2014).

While the Southeastern United States hosts hotspots of aquatic biodiversity (Folkerts, 1997; Taylor et al. 2007), it also contains diverse agriculture production systems (Ingram et al. 2013). Overall diversity within aquatic systems can enhance a waterway's ability to sequester excess nutrients and increase resistance towards invasive species. However, water quality and overall health of aquatic systems is often negatively correlated with agricultural activities. Suspended solids, fertilizers, pesticides, and herbicides from agricultural fields have all been shown to have negative impacts on aquatic fauna (Farris and Van Hassel 2007). Understanding the adverse effects that agricultural practices can have on aquatic systems will help us develop best agricultural management practices and effective regulations of agrochemicals.

Interactions between climate change and agrochemicals have the potential to further depress populations of freshwater mussels. In the southeast, the changing climate is expected to result in warmer temperatures, stronger pulses of rainfall, but more frequent drought. Climate

change scenarios have also predicted an increased use of pesticides and herbicides to combat weed growth (Ingram et al. 2003). Strong rainfall pulses and anthropogenic destruction of riparian zones will inevitably move chemicals from fields into nearby bodies of water. When combined with the thermal stress caused by low flow events, this influx of chemicals could further imperil mussel populations. The presence of chemicals in water have also been shown to decrease valve gape width (Salanki et al 2003), which reduces the amount of oxygenated water moving across the gills. This is of particular importance due to the unionid mussel's unique life history strategy. Following fertilization, freshwater mussels brood their developing larvae, called glochidia, for weeks to months within their gills. Because gills are also used for respiration, the brooding period is stressful and likely reduces the ability of mussels to obtain oxygen from surrounding waters (Tankersely and Dimock 1993). Under thermal and hypoxic stress, mussels will expel their broods (Aldridge and McIvor 2003; Gascho Landis et al. 2012). Hypoxic conditions during the brooding season may cause mussels to forego reproduction for that year due to ejection of immature broods to relieve respiratory stress (Aldridge and McIvor 2003). Increasing temperatures due to climate change will increase the frequency and duration thermal and hypoxic stress on freshwater mussels. Chemicals widely used in large-scale agricultural production as well as urban application may further exacerbate this problem due to its increased usage in the spring and summer growing periods coinciding with mussel reproduction. In this study, we examined the effects of three common agrochemicals (atrazine, glyphosate, nitrate) that we could expect to increase in usage as climate change progresses.

The herbicide atrazine of the triazine class is commonly used to prevent broadleaf weeds and is the most commonly detected pesticide in U.S. drinking water (Gilliom et al. 2006). Atrazine has widely been reported in most aquatic systems even though it is suspected to be a

teratogen and endocrine disruptor (Mizota and Ueda 2005). In unionid mussels, atrazine has been shown to cause DNA damage in glochidia in acute tests (Conners & Black 2004) and reduced viability in chronic toxicity tests at 4.3 mg/L (Bringolf et. al 2007). Unionids are also less likely to aggregate at atrazine concentrations above 15 µg/L (Flynn & Spellman 2009). Because unionids rely on broadcasting sperm to fertilize broods, areas of low mussel densities could result in unsucceful reproductive years. Low levels of atrazine (0.08 – 1 µg/L) have been shown to bioaccumulate within tissues located in the visceral mass, foot, and mantle/siphon within one hour (Jacomini et al. 2006). With absorbtion primarily occuring within the gills (Jacomini et al 2006), viability of broods are put at an increased risk. Endocrine disruption caused by atrazine exposure could also decrease the successful transmission of broods onto host fish if mussels are displaying mantle lures at inappropriate times.

Glyphosate is a widely used broad-spectrum herbicide intended for killing a variety of weeds, the most popular of which is RoundUp®, a product of the Monsanto Corporation created in the 1970s. For large scale productions, the recommend application varies between 398 – 797 mg RoundUp®/ m^2 depending on season. While glyphosate alone is generally considered to be a low risk to aquatic organisms, an increasing number of studies have shown surfactants within the glyphosate formulations in commercial herbicides such as RoundUp® are substantially more toxic to aquatic organisms (Folmar et al. 1979; Relyea 2005; Bringolf et al. 2007). Thus far, studies on the impacts of glyphosate formulations in aquatic systems have primarily been focused on *Daphnia*, amphibians, and fishes. The impact that glyphosate formulations have on mussels, especially on adults, is limited. Presently, Bringolf et al. (2007) has determined that chronic exposure to 6 mg RoundUp®/L caused the termination of foot movement in 50% of early juvenile *Lampsilis siliquoidea*. In the same study, glochidia were shown to be more sensitive to

glyphosate than early juveniles. Bringolf et al. (2007) determined the EC50 for glochidia exposed to RoundUp® for 48 hours was 2.9 mg/L while early juveniles were able to persist up at concentrations to 5.9 mg/L. Similar to atrazine, glyphosate formulations have been shown to cause degradation to gill tissues in *C. fluminea* (dos Santos & Martinez 2013) at 10 µg/L, which is below the Canadian aquatic life standards of 65 µg/L (Battaglin et. al 2009) as well as the 2013 U.S. EPA's maximum allowed concentration for glyphosate in drinking water (0.7 mg/L). Because glochidia are brooded in the gills, chronic exposure to glyphosate formulations could lead to the premature expulsion of broods as the gill tissue degrades. More alarmingly, a study conducted on the marine mussel, *Perna perna*, demonstrated that exposure to glyphosate inhibited cholinesterase activity, leading to neurotransmission interruption (Sandrini et al. 2013). Glyphosate is already regularly detected in streams (Giesy et al. 2000) and its usage is only expected to increase as the development of genetically modified crops resistant to glyphosate becomes more widespread. Due to there being little information on the effects of glyphosate on adult mussels, especially in relation to reproduction, more research is needed to understand the impacts of this commonly used herbicide.

Nitrate is commonly used as a fertilizer in both large scale agricultural productions and urban gardens to support growth and health of plants because it is the most readily assimilated form of nitrogen by plants. It is also the most common form of nitrogen exported to freshwater systems from terrestrial ecosystems. Not only is excess nitrogen availability within aquatic systems associated with eutrophication, but studies have shown the relationship between impaired mussel populations and elevated nitrate concentrations (Köhler 2006; Douda 2007; Zettler and Jueg 2007). More recently, areas in central Europe with increased NO_3 levels exhibited a lower probability of occurrence of species (Douda 2010). Douda (2010) also

predicted 50% mortality of juveniles around 1000mg/L N-NO^-_3 when exposed for 96hr-LC50. A similar 96hr-LC50 at 20ºC recorded lethal effects when early stage juveniles were exposed to 357mg N-NO^-_3/L – 937mg N-NO^-_3/L (Soucek & Dickinson in 2012). To my knowledge, no studies have been performed to assess the direct effects that nitrates could have on adult unionid mussels.

Understanding how to maintain healthy mussel populations will require studying the interactions between brood expulsion, temperature, agrochemicals, and dissolved oxygen. In Chapter 1, I showed that respiratory stress as a product of increased temperatures is likely a mechanism behind brood expulsion. In this study, I examine whether the introduction of commonly used agrochemicals also causes respiratory stress and further exacerbates brood expulsion. I will assess the effects of chronic exposure to atrazine, glyphosate, and nitrate on the reproductive success of gravid unionids by evaluating 1) mantle lure display behavior, 2) respiratory stress, by using RI and DO_{crit} as proxies for "stress", 3) brood expulsion. The first 11 days will be dedicated to evaluating the effects of incremental increases in temperature (warming stage), followed by a 10-day period in which the temperature is maintained (constant stage) based on results from chapter one.

Methods

Study animals

Ligumia subrostrata is a sexually dimorphic species that typically inhabits lentic aquatic systems (Williams et al. 2008) Females are long-term brooders that attract host fish by flashing a mantle lure (Corey et al. 2006). Gravid females were collected from an earthen pond located at South Auburn Fisheries Research Station (SAFRS), School of Fisheries, Aquaculture, and

Aquatic Sciences, Auburn University in March 2017, while water temperatures were 13ºC. All mussels used were naturally propagated in SAFRS ponds to reduce the need to collect individuals from wild populations. After collections, *L. subrostrata* were randomly distributed among six recirculating aquariums, filled with artificial freshwater (AFW: 50 mg $CaCO_3$, 25 mg CaCl, 50 mg $NaHCO_3$, and 5 mL 30% salt water/L deionized water) (pH = 8, Total Hardness = 53, Alkalinity = 40) (Gascho Landis et al. 2012). Aquarium temperatures were maintained at $13^{O}C \pm 1^{o}C$ to ensure the brooding females would retain their glochidia prior to experiments. A 12:12 light:dark cycle was consistently maintained throughout the duration of the experiment. Mussels were fed Reed Mariculture shellfish diet (Reed Mariculture, Campbell, California) every other day at a rate of 0.05 mL/L (Gascho Landis et. al 2012). Water quality was monitored and recorded weekly for TAN (total ammonia nitrogen), pH, nitrite, and nitrate using Tetra® EasyStrips.

Test chemicals

RoundUp Ultramax ™ (active ingredient: 50.2% glyphosate IPA salt) and Hi-Yield Nitrate of Soda Plant Food (Fertilizer Analysis: 16-0-0) were purchased from a local retailer. Drexel Atrazine 4L Herbicide (active ingredient: 42.2% atrazine) was purchased from Seed World © (2019). Atrazine and glyphosate are two of the most widely used herbicides in the United States (Woodburn 2000). Nitrate is a common commercial fertilizer used in agriculture as well as urban landscapes worldwide (Lu and Tian 2017). All chemicals are water soluble and were mixed with AFW until the desired concentration was reached.

Gravid chronic toxicity test

To measure the influence of agrochemicals on brood expulsion, we exposed adult gravid *L. subrostrata* to RoundUp ® (glyphosate), Nitrate, or Drexel Atrazine for 21 days. All assay exposures were run concurrently with exposure concentration based on the active ingredient within each agrochemical. Because information on the effects of each toxicant on adult mussels was limited, concentrations were based on chronic EC50 glochidia and early stage juvenile studies mentioned in the introduction. A working solution of glyphosate (6 mg/L) was prepared by mixing 9mL of RoundUp® in AFW to a final volume of 10L. A working solution of atrazine (4 mg/L) was prepared by mixing 9.4mL of Drexel Atrazine in AFW to a final volume of 10L. Because no chronic studies were found for nitrates, initial nitrate concentrations were based on an acute study by Douda (2010) in which reduced viability of juveniles was shown at a nitrate concentration of 1000 mg/L over 96 hours. To correct for length of study, I applied an application factor of 0.1 to get a final nitrate concentration of 100 mg/L for this chronic study. This technique is consistent with many studies that suggest chronic toxicity studies are generally one order of magnitude lower than the acute toxicity concentrations and was reconfirmed by Mummert et al. (2003). A working solution of nitrate (100mg/L) was prepared by mixing 6.25g of Hi-Yield Nitrate of Soda Plant Food into AFW to a final volume of 10L.

Fifteen gravid mussels were randomly assigned to each treatment. Briefly, there were three replicate enclosures dedicated to each test chemical plus a control. Each replicated enclosure contained five mussels that were individually housed within a modified 1L polypropylene beaker that allowed passage of water and food while simultaneously retaining expelled glochidia. Thus, there were 15 mussels per treatment, each housed within an individual container, distributed among three treatment tubs. A Homasy 80gph submersible pump was

placed in each replicate enclosure to ensure the proper mixing and oxygenation of water. An external Zacro LCD Digital Thermometer was placed on each enclosure. On day one, all replicate enclosures were placed within a single 13ºC ± 1ºC water bath to ensure temperatures were the same across the treatments. The temperature was adjusted by 1ºC per day until the constant stage test temperature (21ºC) was reached, where the temperature was held for seven days. The final test temperature of 21ºC was decided based on results from chapter 1. Temperature was maintained using a TECO TK-1000 heater/chiller.

Every two days, 1L beakers were removed from their replicate enclosure. Glochidia were collected from the 1L beakers, preserved in 70% ethanol, and stored for counting later. Once glochidial collections were complete, mussels were immediately place back in their beaker. A 100% water change was performed every two days to ensure chemical concentrations and water quality remained relatively constant. Once replenished, each treatment container was shifted by one unit to the right to minimize the potential for location effects. Shellfish diet (0.05 ml/L) was then added to each treatment container. Mortality was recorded every other day. Display behavior within each treatment was visually assessed by counting the number of mussels actively displaying in a 10 second period prior to disturbance.

Oxygen Consumption Measurement

Respirometry experiments were conducted using AutoResp™ 2.3.0 software (Loligo Systems, Inc.) coupled with a four-chamber fiber optic respirometry system (Loligo Systems, Inc.). Fiber optic cables were attached to vertical, acrylic, respirometry chambers (Loligo Systems, Inc). Each acrylic chamber was connected to two Eheim submersible 79 gallons per

hour pumps (EHEIM GmbH & Co KG). Orientation and function of respirometry system was the same as in Chapter 1.

Closed respirometry was used to estimate the degree of respiratory stress experienced by mussels at different temperatures as DO declined from normoxia to hypoxia. Three endpoints were used as indicators of respiratory stress: 1) Regulation Index (RI) – the ability to maintain a constant respiration rate as dissolved oxygen declines (Mueller and Seymour 2011), and 2) critical DO concentration (DO_{crit}) the dissolved oxygen threshold below which an organism shifts from predominately aerobic to anaerobic metabolism (Mueller and Seymour 2011), and 3) incidents of valve closure which cause metabolic depression and probable shifts from aerobic to anaerobic respiration (Anestis et al. 2010).

After a 24-hour fasting period, gravid mussels were lightly scrubbed with a soft-bristled toothbrush to remove any visible algae that could skew respiration readings. Wet weight was recorded. Mussels were then naturally oriented within aquarium gravel placed within a chamber. Aquarium gravel was used as substrate instead of sand to ensure no build up of chemicals occurred within the substrate during experimentation. Based on results from Chapter 1, mussels were allowed to acclimate to their chamber for two hours prior to experimentation. Closed respirometry experiments ran overnight and were complete when the DO fell below 1 mg O_2/L. After each experiment, any chambers, jars, aquarium gravel, tubing and pumps that were used during the experiment were placed in bleach for at least three hours.

At the termination of the experiment, the water from each chamber was poured through 105 micrometer mesh to collect any expelled glochidia and immediately preserved in 70% ethyl alcohol for later counting (Dodd et. al 2005). The gills from each mussel were also removed and immediately preserved in 70% ethyl alcohol. At a later date, glochidia were flushed from the

gills with freshwater into a beaker, diluted to ~1000 ml, and mixed with plunger-type stirrer. Subsamples were counted under a microscope until either >100 glochidia had been counted or >60% of the sample had been sampled. Total # glochidia retained in gills was calculated as:

$$\text{Total \# Glochidia} = \frac{\text{\# Glochidia Counted}}{\text{Subsample Volume}} \times \text{Sample Volume}$$

Expelled glochidia in respirometry chamber samples were enumerated in the same manner.

Statistical Analyses

A. Glochidia Expulsion

Two-way repeated measures analysis of covariance (ANCOVA) was used to compare between proportions of glochidia expelled during or retained after the 21-day period among treatment groups. Treatments and expelled/retained variable were the two grouping variables, while mussel's body weight was a covariate. To account for variations in glochidial expulsion/retention proportions that could be attributed to variation in mussel's body weight (covariate), multiple mixed-model ANCOVA were used separately to compare the glochidial expulsion/retention proportions during the initial warming stage, secondary constant temperature stage, and retention after 21 days among treatment groups (control, nitrate, atrazine, and glyphosate). Results of ANCOVA analyses for main effects and interactions of treatment, mussel mass (gWW), total brood per mussel, total brood retained after 21 days can be found in Table 1.

B. Metabolic Patterns

Acclimation time was based on data reported in Chapter 1. Aquarium gravel did not significantly affect acclimation time (Haney and Stoeckel, unpublished data). In order to describe the relationship between metabolic patterns and water temperature, different regression curves (linear, quadratic, or 2-parameter hyperbola regression) were fitted through raw data of RMR,

RI, and DO_{crit}. The model with the highest r^2 value was selected. To account for variations in metabolic pattern parameters that could be attributed to variation in model covariates (mussel's body weight, length, total brood count, and proportion displaying), multiple mixed-model ANCOVAs were used separately to compare the metabolic pattern parameters (RMR, RI and DO_{crit}) among treatment groups (control, nitrate, atrazine, and glyphosate).

The least-squares adjusted means were used to compare the true effects of the treatment groups, unbiased by differences in covariates. Prior to all statistical analyses, the Shapiro–Wilk test was used for normality analysis of the variables. For non-normally distributed data, ANCOVA was performed on rank-transformed data. If there were significant differences, the Tukey's Studentized Range (HSD) test was used for post-hoc analysis. Statistical significance was set at $p < 0.05$, and all data were presented as the mean ± standard error (*SE*). Analyses were performed with SAS® version 9.4 (SAS, 2013).

Results

Glochidial Expulsion over 21-d exposure period

Across both temperatures stages and all treatments, gravid mussels retained more glochidia over the 21-day period than they expelled ($F_{1, 21} = 6.34$, $p = 0.02$; Fig. 1). The number of glochidia expelled during the warming period was not significantly different than the number released during the constant period ($t_{(29)} = 1.34$, $p = 0.3840$; Fig. 2). Among treatments, glochidial expulsion throughout the warming period was lower for mussels exposed to glyphosate than those in the control treatment ($t_{(76)} = 2.75$, $p = 0.0368$; Fig. 3). Significantly fewer glochidia were expelled during the constant stage for mussels exposed to glyphosate ($t_{(76)} = 2.99$, $p = 0.0192$) and atrazine ($t_{(76)} = 2.94$, $p = 0.0224$) when compared to the control

group (Fig. 4). Mussels exposed to atrazine actively displayed significantly more than the control ($t_{(32)} = 2.78$, $p = 0.0430$; Fig. 5).

Over the entire 21-day experimental period, no treatment significantly affected retention of glochidia when compared to the control (Atrazine: $t_{(76)} = 1.12$, $p = 0.6809$; Glyphosate: $t_{(76)} = -2.57$, $p = 0.0573$; Nitrate: $t_{(76)} = -0.38$, $p = 0.9807$; Fig. 6).

Metabolic Patterns

Regulatory ability was not affected by treatment (Fig. 7). Effect of treatment on DO_{crit} was not significantly different than the control (Fig. 8). However, DO_{crit} was marginally higher than the control for mussels exposed to atrazine ($t_{(11)} = 2.92$, $p = 0.057$). RMR at 5 mg O_2/L was significantly higher than the control across all treatments (Atrazine: $t_{(52)} = 3.20$, $p = 0.0120$; Glyphosate: $t_{(52)} = -5.12$, $p < 0.0001$; Nitrate: $t_{(52)} = -3.73$, $p = 0.0026$; Fig. 9).

Discussion

To my knowledge, this is the first study that attempts to examine the effects of atrazine, glyphosate, or nitrate on overall brood retention, respiratory ability, and fitness of gravid unionid mussels. Chronic exposure to atrazine, nitrate, or glyphosate significantly increased RMR under normoxic conditions. If RMR is already inflated due to high ambient water temperature (Chapter 1), the presence of agrochemicals would further exacerbate populations that in these streams. Due to the limited available energy that can be distributed among maintenance, growth, and reproduction (Burton et al. 2011), the combination of high temperatures and agrochemical exposure could decrease fitness within an individual as energy resources are shifted from growth and reproduction to maintaining basal matablic demand required for survival (Ganser et al.

2015). High individual variation within treatments also suggests that a portion of the population is less tolerant to exposures, which could result in a decrease in recruitment success as adults forgo a reproductive year to survive.

While all three chemicals led to increased respiration rates, the three toxicants, to our surprise, had no effect on regulatory ability or DO_{crit}. However, DO_{crit} of individuals exposed to atrazine was only marginally insignificant ($p = 0.057$) when compared to the control group. Gravid mussels exposed to atrazine also actively displayed their mantle lure significantly more often than other treatments. When exposed to atrazine, endocrine disruption has been widely seen in both fish (Bringolf et al. 2004; Rohr and McCoy 2009; Tillitt et al. 2010) and amphibians (Hayes et al. 2003; Rohr and McCoy 2009; Hayes et al. 2010). While the relationship between atrazine and endocrine disruption has yet to be studied in mussels, Bringolf et al. (2010) demonstrated that chemicals have the potential to disrupt several aspects of freshwater mussel reproduction. A disruption that affects the timing of mantle lure display could cause mussels to unnecessarily allocate energy for reproduction when acceptable hosts are not available or, if hosts are available, to prematurely expel glochidia regardless of temperature cues or prior fertilization. Missing important reproductive cues could be of particular importance for species that do not have secondary reproductive strategies.

Overall, I found no evidence that atrazine, glyphosate, or nitrate increase brood expulsion. Surprisingly, gravid mussels across all treatments retained more glochidia on average than they expelled during the 21-day experiment. There was evidence of weak effect of treatment, but data suggests that certain chemicals actually improved retention regardless of exposure or temperature. During the initial warming stage, glyphosate suppressed the amount of glochidia expelled in comparison to the control. Suppression was also observed during the

constant temperature stage by mussels in both the atrazine and glyphosate treatments. In previous studies, atrazine has been shown to cause damage to gills (Dos Santos and Martinez 2013), ovaries, and testes (Zupan and Kalafatic 2003) in Unionida. Gill damage (Dos Santos and Martinez 2013) and significantly reduced pedal movement (Bringolf et al. 2007) has been shown when mussels are exposed to glyphosate. Glyphosate has also been shown to interupt neurotransmission in marine mussels (Sandrini et al. 2013). Because of this, significantly lower cases of brood retention could be a sign that the mechanism controlling glochidial expulsion is damaged. Both glyphosate and atrazine should be considered for further studies to better understand their overall effects on mussel reproduction.

Chronic exposure to high levels of nitrate seemed to have little effect on gravid *L. subrostrata*, other than increasing resting metabolic rate. While *L. substrostrata* was relatively unaffected by nitrate, Soucek and Dickinson (2012) showed that sensitivity to nitrate likely depends on the species. However, the only information we really have on the effects of nitrates on mussels has only been focused on early life stages (Douda 2010; Moore and Bringolf 2018). With nutrient pollution quickly becoming one of the most prevalent causes in the degredation of water quality, understanding the impacts nitrates have onn mussel populations will become more critical. More exposure studies across all unionid life history stages and strategies are needed to further our knowledge on how to develop best management strategies for restoring unionid populations and prevent further declines.

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Figures

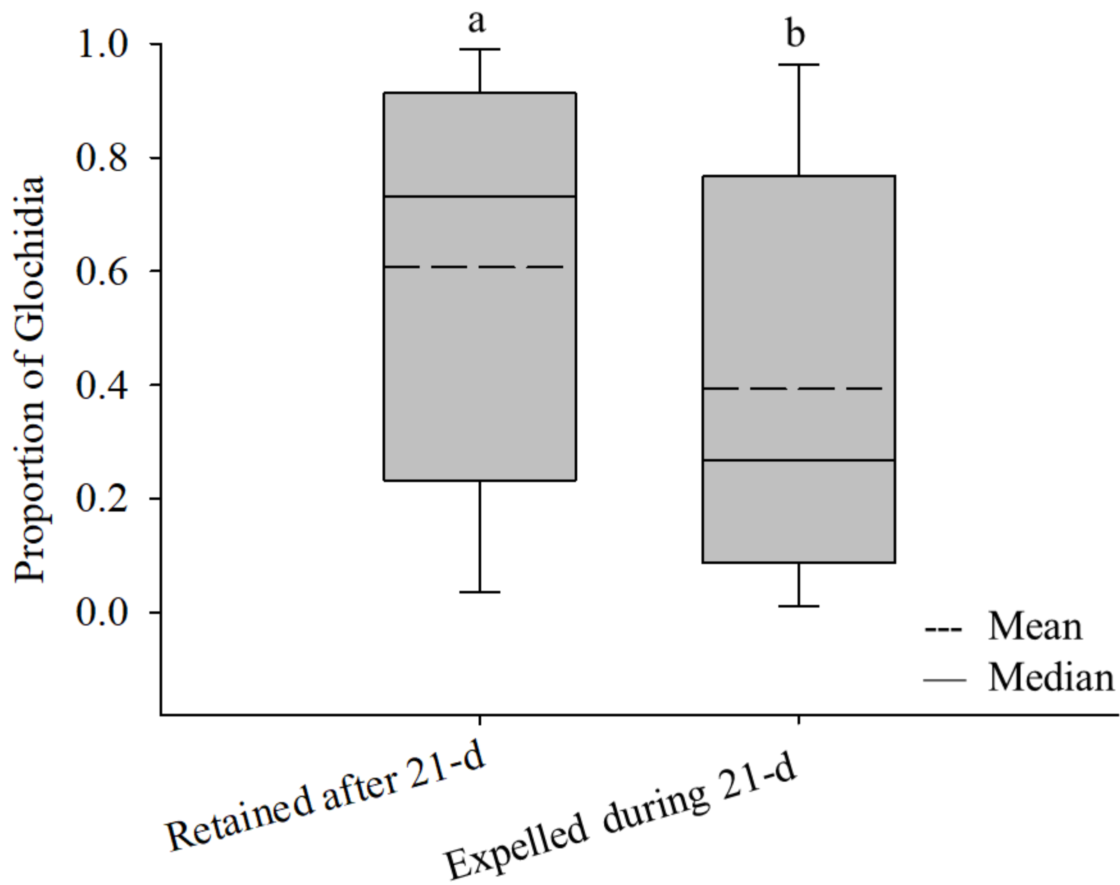

Figure 1: Comparison of the proportion of glochidia retained after or expelled during the 21-d chronic toxicity trial. Different letters represent significant differences identified by two-way repeated measures ANCOVA model (grouping variables: treatments and expelled/retained; covariate: wet mass). Box limits represent the upper and lower quartiles of the data set.

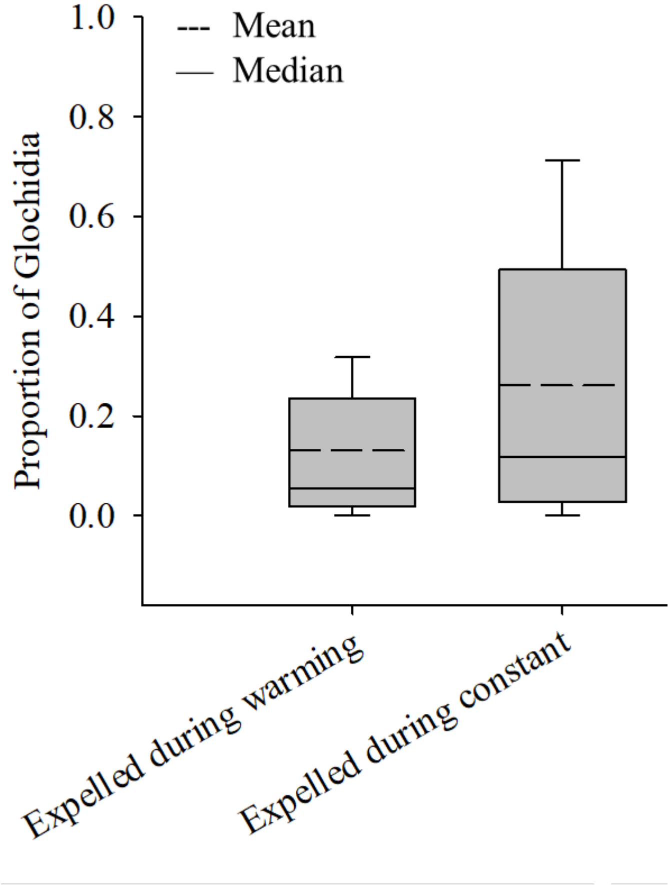

Figure 2: The proportion of glochidia expelled during the initial warming period (day 0 – 12) and the constant temperature period (day 13 – 21). Box limits represent the upper and lower quartiles of the data set.

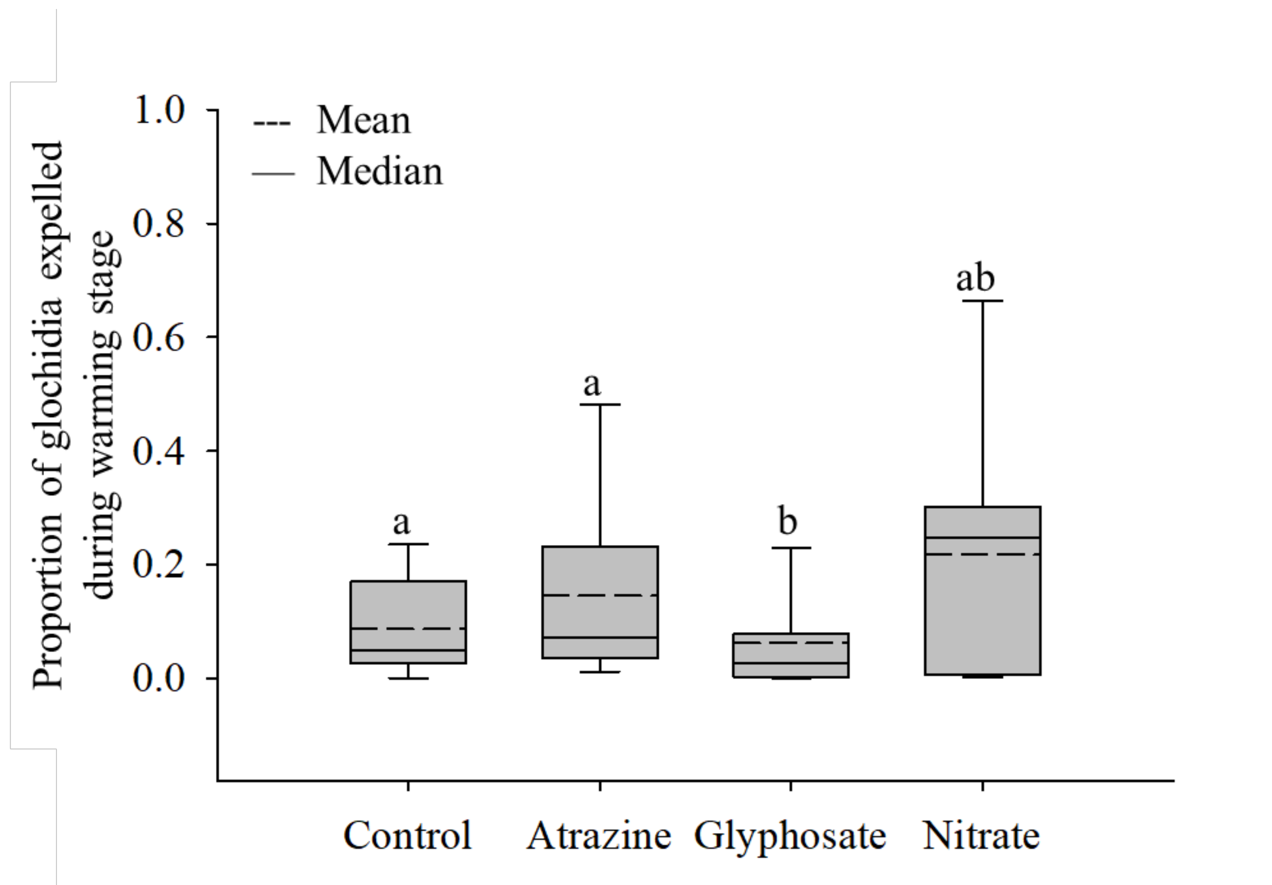

Figure 3: The proportion of glochidia expelled during the initial 12-d warming period (13 – 20 ºC) in different treatments. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after one-way ANCOVA on ranks (grouping variable: treatments; covariate: wet mass). Box limits represent the upper and lower quartiles of the data set.

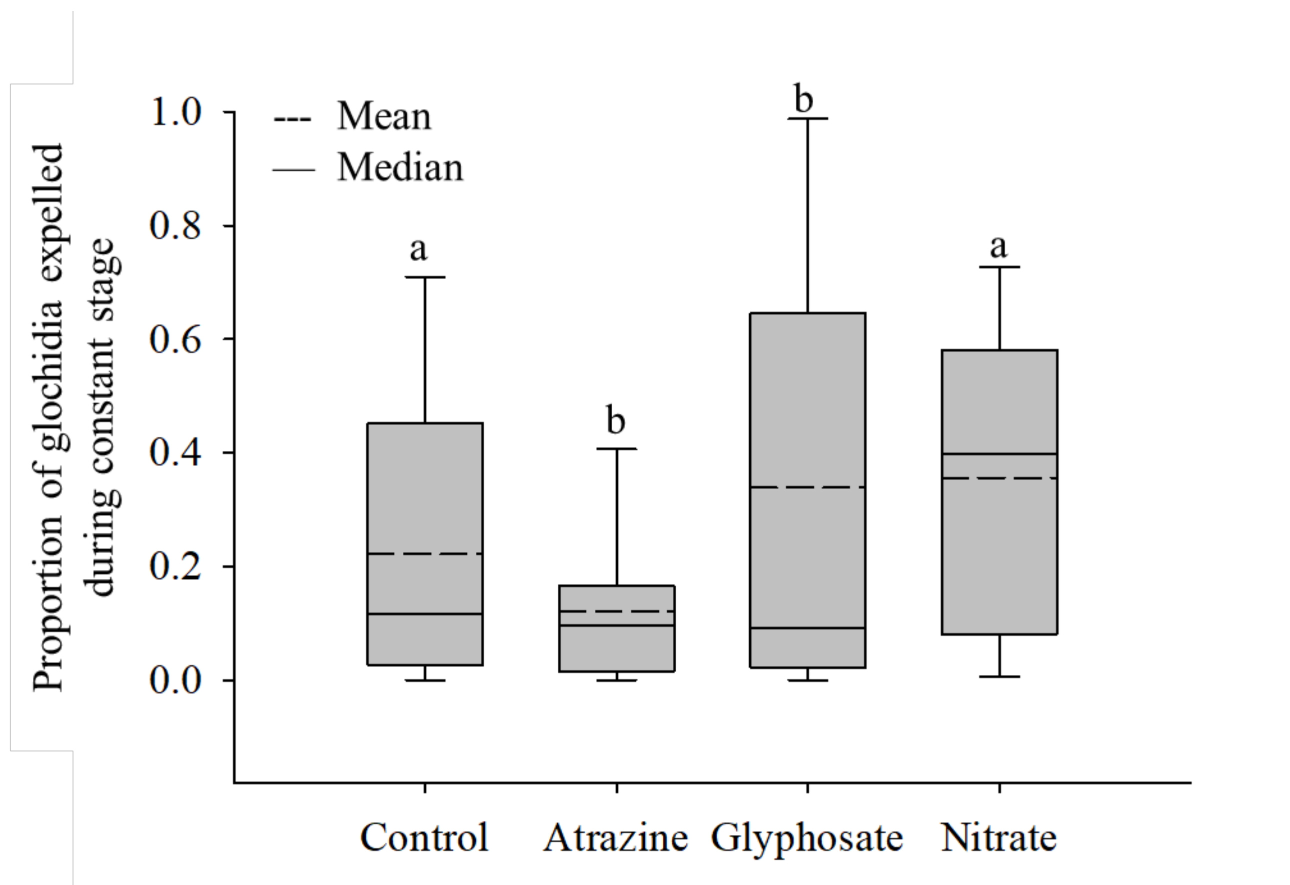

Figure 4: The proportion of glochidia expelled during the final 10-d constant period (21ºC) in different treatments. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after one-way ANCOVA on ranks (grouping variable: treatments; covariate: mussel's body weight). Box limits represent the upper and lower quartiles of the data set.

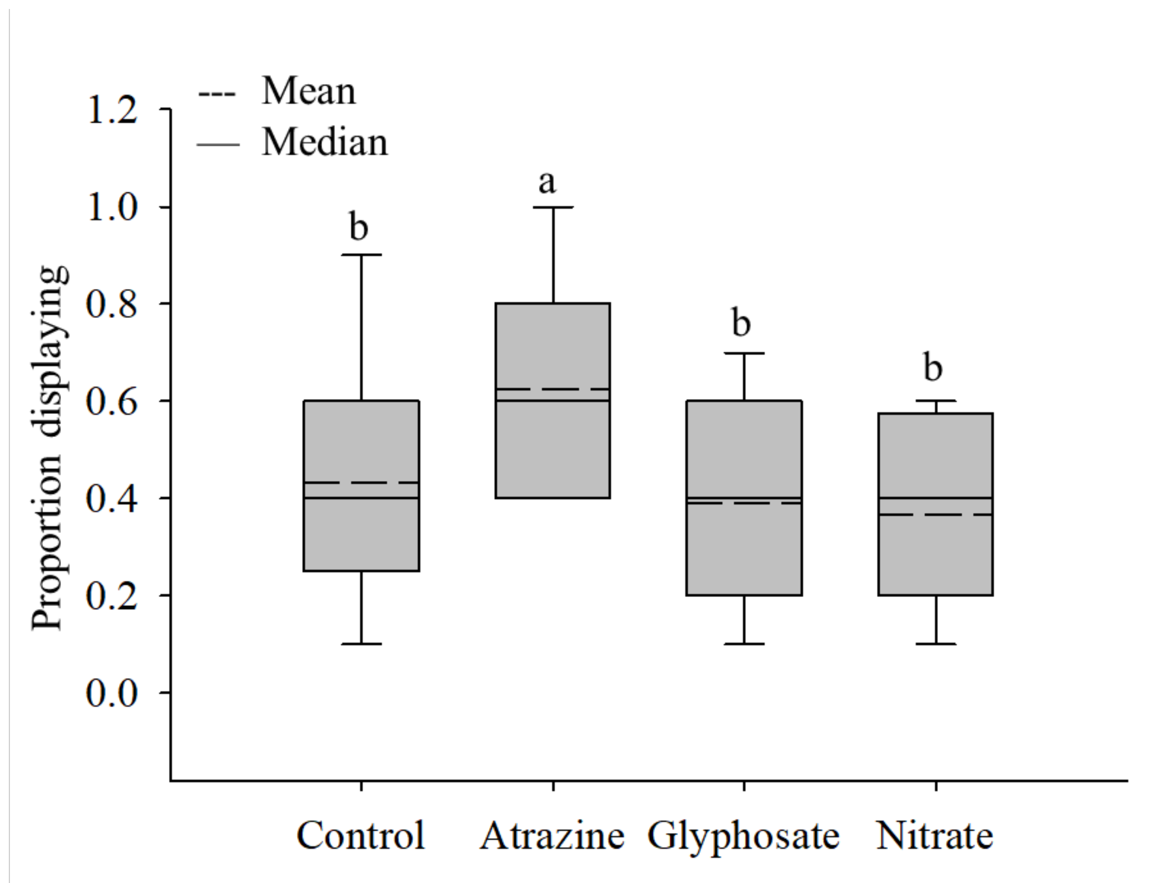

Figure 5*:* Comparison of the proportion of gravid females actively displaying their mantle lure across treatments. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after one-way repeated measures ANOVA (grouping variable: treatments). Box limits represent the upper and lower quartiles of the data set.

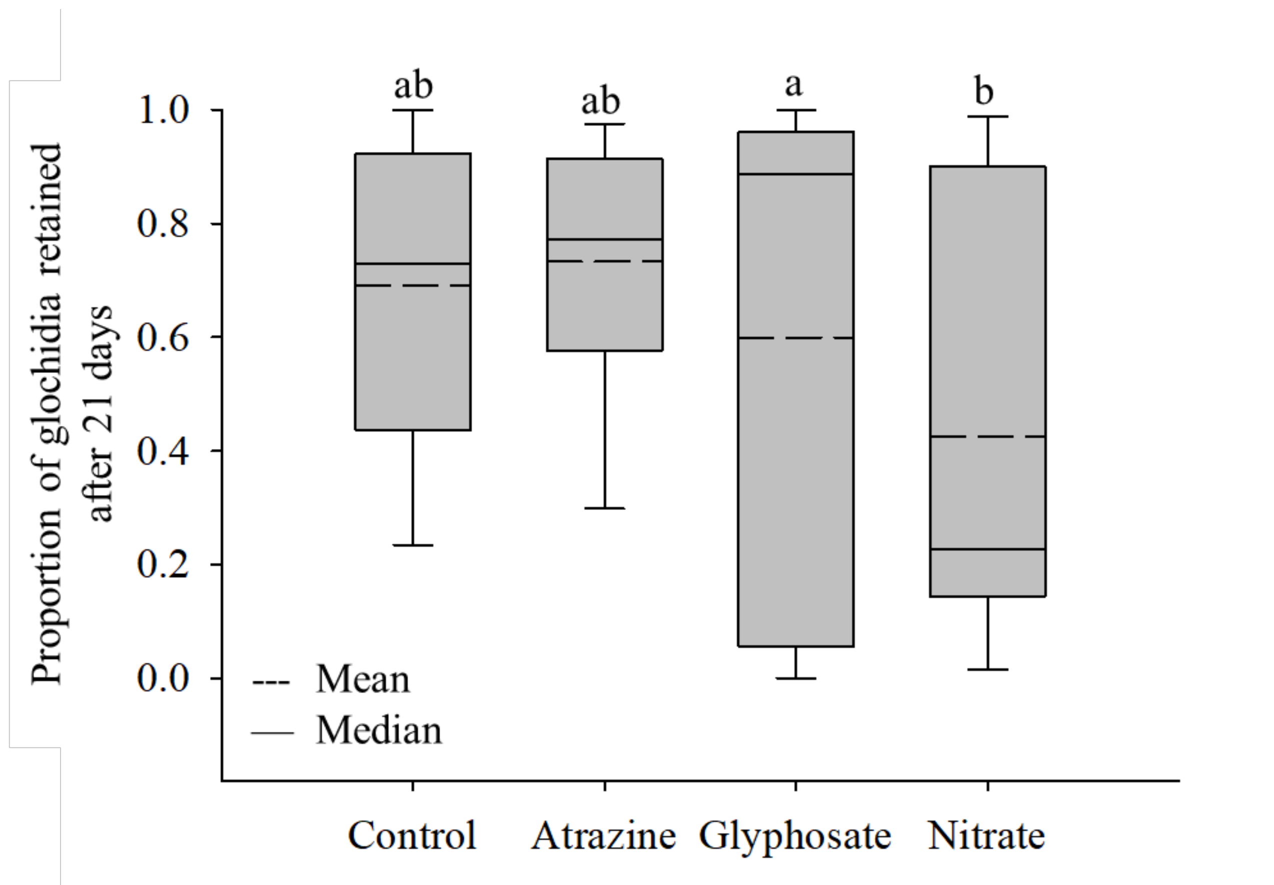

Figure 6*:* The proportion of glochidia retained after the 21-d chronic toxicity study across treatments. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after one-way ANCOVA on ranks (grouping variable: treatments; covariate: wet mass). Box limits represent the upper and lower quartiles of the data set.

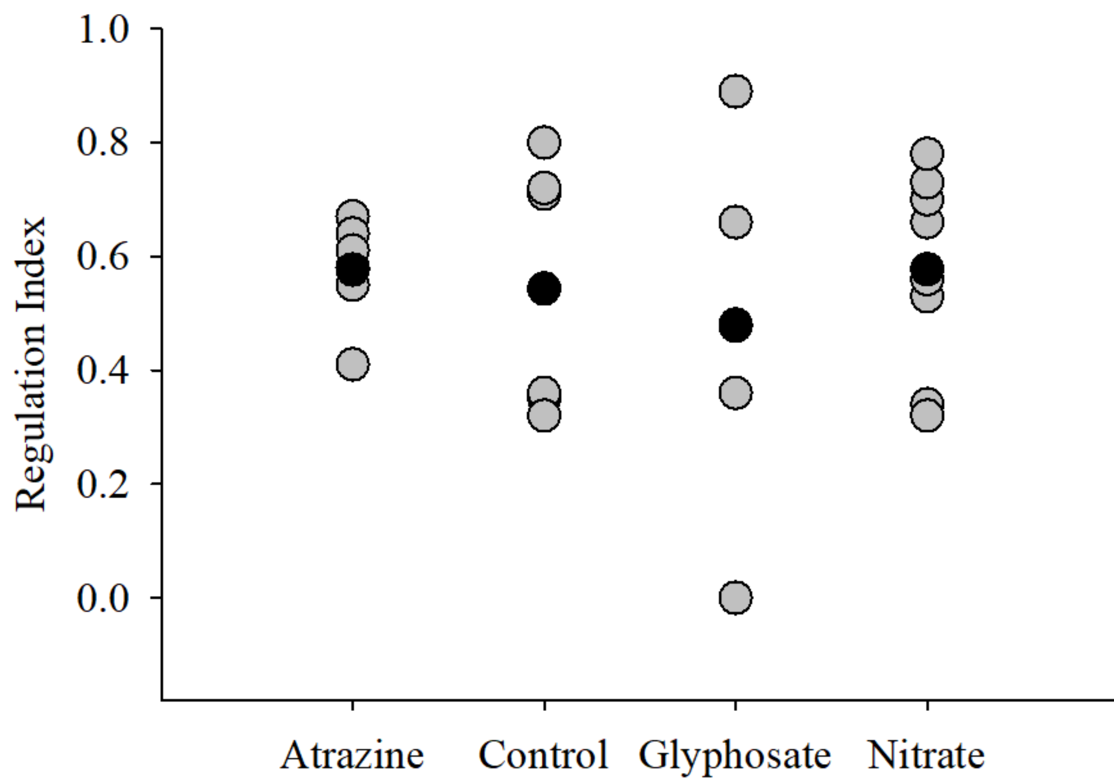

Figure 7*:* Comparison of regulation index among treatments. Grey circles represent raw data. Black circles represent the mean. A regulation index of 1 indicates perfect regulation. A regulation index of zero indicates perfect conformation.

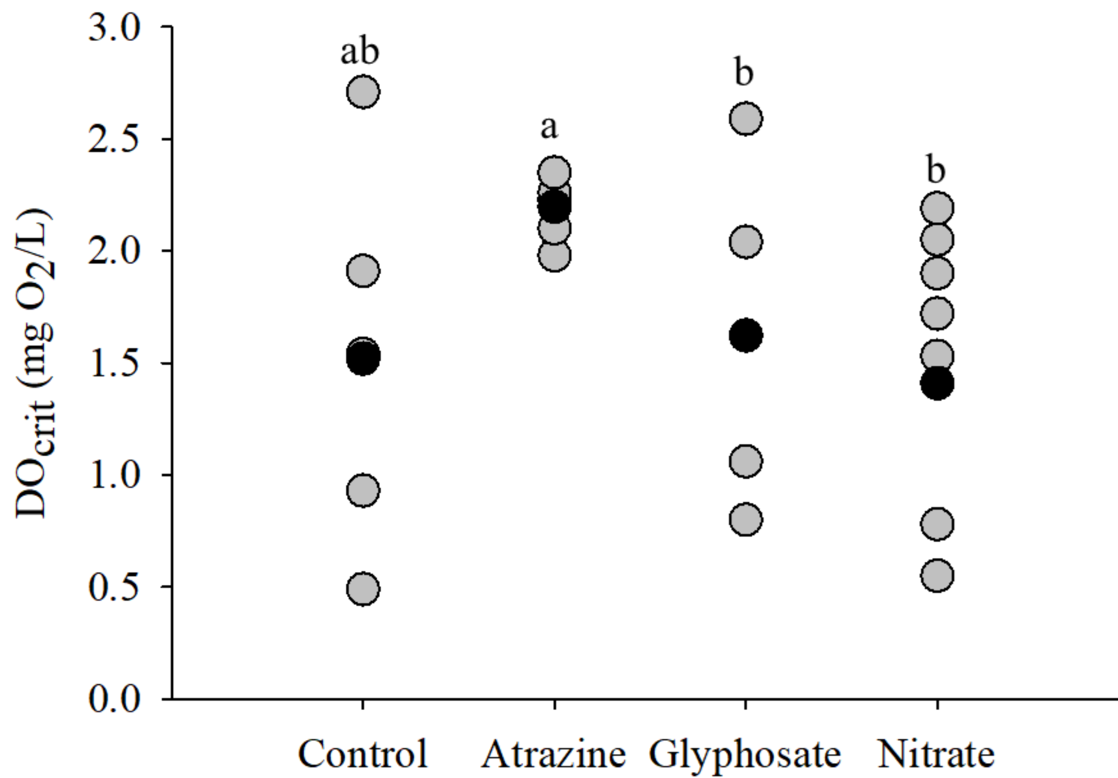

Figure 8: The critical dissolved oxygen level (DO_{crit}) for individual mussels (grey) and the mean (black) DO_{crit} across treatments. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after ANCOVA (grouping variable: treatments; covariates: wet mass, shell length, proportion display, total brood count, total brood count per gWW, total brood count per mm).

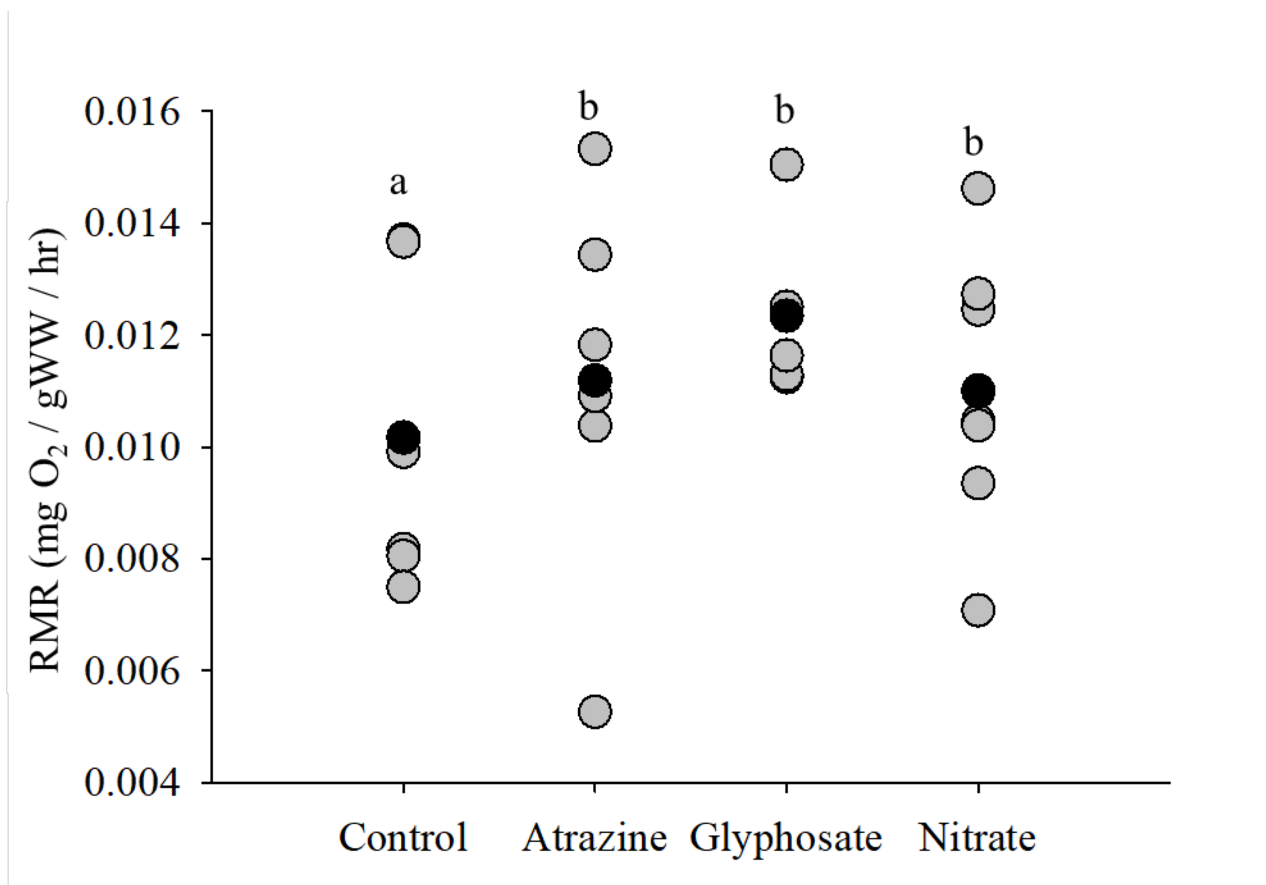

Figure 9: Comparison of RMR among treatments. Individual (grey) and mean (black) RMR for gravid mussels at 5 mgO_2/L across treatment groups. Different letters represent significant differences identified by Tukey HSD post-hoc comparisons after ANCOVA (grouping variable: treatments; covariates: wet mass, shell length, proportion display, total brood count, total brood count per gWW, total brood count per mm).

Tables

Table 1: Results of ANCOVA analyses for main effects and interactions of treatment, mussel mass (gWW), total brood per mussel, total brood retained after 21 days. Initial length (mm), and proportion of mussels displaying at a time on resting metabolic rate (RMR), regulation index (RI) and critical dissolved oxygen concentration.

Effect	RMR			RI			DO_{crit}		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	4	10.91	**<.0001**	4	10.80	**<.0001**	4	9.76	**0.0013**
gWW	1	11.82	**0.0012**	1	10.53	**0.0020**	-	-	-
Total brood	1	11.43	**0.0014**	1	16.04	**0.0002**	1	7.87	**0.0171**
Total brood / mm	-	-	-	1	16.50	**0.0002**	1	8.25	**0.0152**
Total brood / gWW	1	5.81	**0.0195**	-	-	-	1	9.95	**0.0092**
Retained after 21-d	-	-	-	-	-	-	-	-	-
Retained after 21-d / gWW	1	13.15	**0.0007**	1	10.28	**0.0023**	1	8.68	**0.0133**
Retained after 21-d / mm	1	27.37	**<.0001**	1	9.08	**0.0039**	-	-	-
Initial Length	1	16.24	**0.0002**	-	-	-	1	9.09	**0.0117**
Proportion Displaying	-	-	-	-	-	-	1	6.20	**0.0301**
Proportion retained after 21-d	1	13.18	**0.0006**	-	-	-	-	-	-