Use of metabolic assays to assess thermal and hypoxia stress of freshwater mussel species from central Texas

by

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Abstract

Warming temperatures are a major concern for freshwater mussels as they are limited in range, mobility, and potential to seek refuge from heat stress. Unionid mussels are ectothermic organisms, and the temperature of their immediate environment strongly influences their metabolism. We used metabolic assays at both the organismal and cellular level to assess potential effects of thermal stress and hypoxia on freshwater mussels from central Texas. At the organismal level, as measured via closed respirometry, metabolic rates increased with increasing temperature for all taxa, but the shape and slope of the relationship were variable among species and subpopulations. Sensitivity to hypoxia with rising temperature was only found in two of the taxa tested. Enzymatic thermal optima and potential metabolic activity varied among taxa tested with chronic and acute heat stress. Effects of temperature acclimation on respiratory enzymes were variable as well, revealing potential differences in adaptations between species and subpopulations.

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AICc	bias-corrected Akaike information criterion
AUC	area under curve
DO	dissolved oxygen
DO_{crit}	critical dissolved oxygen level
ETS	electron transport system
HAFW	hard artificial freshwater
PMA	potential metabolic activity
PMA_{max}	maximum PMA
RI	regulation index
RMR_6	resting metabolic rate at 6 mg O_2/L
$T_{breadth}$	thermal enzymatic optimum range
T_{lower}	lower enzymatic thermal optimum
T_{opt}	enzymatic thermal optimum
TPC	thermal performance curve

Chapter 1: Effects of thermal and hypoxia stress vary among mussel species and subpopulations in central Texas

Introduction

Climate change is an emerging threat to freshwater biodiversity (Xenopoulos et al. 2005), adding to other anthropogenic disturbances that may combine to increase water temperatures. Warming temperatures are of special concern since temperature is a primary driver of metabolic rates of aquatic ectotherms (Bayne et al. 1976), as well as availability of oxygen required to maintain metabolic rates (Ganser et al. 2015). In general, metabolic demand of aquatic ectotherms increases with increasing temperature even as oxygen solubility declines (Verberk et al. 2011). Thus, sensitivity to thermal stress is affected both by the degree to which metabolic rate increases, and the ability of a species to obtain sufficient oxygen from the surrounding waters as it becomes less available.

Freshwater unionid mussels comprise a major component of aquatic biodiversity. The United States has the highest diversity of unionids in the world, but populations are in decline (Master et al. 2000). These declines may be further exacerbated by stream temperatures increasing in tandem with rising air temperatures and increasing frequency of low-flow periods due to drought, water withdrawals, and altered flow regimes (Spooner and Vaughn 2008; Payton et al. 2016). Limited mobility and range for these ectotherms leave them vulnerable to thermal stress (Spooner and Vaughn 2008), which may be accompanied by hypoxia events. Evidence suggests unionids are already living close to their upper thermal tolerances in some systems (Pandolfo et al. 2010; Ganser et al. 2015) indicating that both thermal stress and hypoxia events are common for unionids when temperatures peak throughout the year.

The response of metabolic rate to increasing temperatures, as well as the ability to obtain oxygen from ambient waters as dissolved oxygen concentrations decline, has been shown to vary

among unionid species (Chen et al. 2001; Ganser et al. 2015). Resting metabolic rate (RMR) represents the oxygen demand of organisms while at rest and approximates the energy required for basal maintenance (Burton et al. 2011). In ectotherms, RMR typically increases with increasing temperature under normoxic conditions, but may level off or decline at extremely high temperatures in some species – a phenomenon termed metabolic depression or compensation (Brown 1989; Crocker and Cech 1997; Walsh et al. 1997; Anestis et al. 2007). The ability of organisms to obtain oxygen as it declines in surrounding waters is related to their oxygen regulation ability and critical dissolved oxygen concentrations. At a given temperature, perfect oxygen regulators maintain a constant metabolic rate independent of declining dissolved oxygen levels whereas perfect conformers exhibit declining metabolic rates with declining dissolved oxygen from normoxic to hypoxic conditions. In reality, most organisms fall somewhere in between perfect regulation and perfect conformation (Mueller and Seymour 2011; Fig. 1.1a,b,c). Below a critical dissolved oxygen threshold (DO_{crit}), organisms (including unionids) are unable to obtain sufficient oxygen to maintain aerobic respiration and switch to primarily anaerobic respiration (Fig. 1.1b,c). This is generally characterized by a rapid decline in respiration rate. Dissolved oxygen concentrations below the DO_{crit} are thought to be highly stressful to aquatic organisms, where lower relative DO_{crit} concentrations indicating a higher hypoxia tolerance (Rogers et al. 2016). The ability of adult unionids to regulate oxygen consumption may decrease with increasing temperature, but strength and presence of temperature effects vary among species (Chen et al. 2001).

Altered metabolic patterns are likely to affect the amount of energy available for maintenance, growth and reproduction (Ganser et al. 2015). Therefore, assessment of species-specific differences in the relationship between temperature, declining oxygen, and metabolic

patterns may be of great use in predicting effects of rising temperatures on unionid communities and assemblages. Testing for differences in these relationships among species may be particularly important when trying to determine whether sympatric species need to be managed as separate conservation units and, similarly, whether subpopulations within a species need to be managed separately.

Cyclonaias houstonensis and *C. petrina* were considered to be rare species, and candidates for listing under the Endangered Species Act when this study was initiated (Johnson et al. 2018). Both species are endemic to watersheds of central Texas– a region frequently subjected to hot (i.e. > 30 °C) summer water temperatures due to low latitudes and altered flow regimes from dams, irrigation, and other anthropogenic activities. While under consideration for protection, *C. houstonensis* was found to be genetically and morphologically indistinguishable from the common and widespread *C. pustulosa* species complex, with only weak evidence for inter-drainage population structure (Johnson et al. 2018). In contrast, *C. petrina* was found to contain a cryptic lineage – *C. necki* – which is now considered to be a distinct species, with *C. petrina* endemic to the Colorado River drainage and *C. necki* endemic to the Guadalupe River drainage (Johnson et al. 2018; Burlakova et al. 2018). Hereafter, I will use the updated taxonomy with *C. pustulosa* replacing *C. houstonensis* collected from the Colorado and Navasota rivers, *C. petrina* retained for individuals collected from the Colorado River, and *C. necki* replacing *C. petrina* collected from the Guadalupe River.

The main objectives of this study were to 1) determine the degree to which energy demand, and ability to obtain oxygen, change with increasing temperature for one common species (*C. pustulosa* – two subpopulations) and two rare species (*C. petrina* and *C. necki*), and 2) determine whether the primary mechanisms driving sensitivity to thermal stress and hypoxia

differ among species and subpopulations, and 3) predict which species and/or subpopulations are likely to be most sensitive to thermal stress and hypoxia.

Methods

Mussel Collection and Laboratory Acclimation

In 2017, *Cyclonaias pustulosa* were collected from the lower Colorado River near Altair, TX on April 28 and May 17, and from the Navasota River near Easterly, TX on July 17. *Cyclonaias petrina* were also collected from the lower Colorado River near Altair on April 28 and May 17, as well as from an additional site near Lometa, TX. *Cyclonaias necki* were collected from the Guadalupe River on August 17 and November 11 (Table 1.1).

Shipping methodology was adapted from the recommendations of the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM 2013). Mussels were placed in coolers between layered moist cotton towels at field sites in preparation for shipments. Sufficient ice packs were added above and below the toweling to attempt to maintain a shipping temperature intermediate between collection temperature in Texas and holding temperature of 18 °C at the receiving lab. All coolers were shipped overnight to the South Auburn Fisheries Research Station (SAFRS), Auburn University, Auburn, AL.

Upon arrival, mussels were tagged with 8 x 4 mm external Hallprint shellfish tags (Hallprint, Hindmarsh Valley, South Australia), measured in length, and then placed in upwellers containing 80 L of hard artificial freshwater (HAFW: 0.192 g $NaHCO_3$, 0.10 g $CaSO_4 \cdot 2H_2O$, 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); pH = 8.35, total hardness = 197.5 mg/L $CaCO_3$, and total alkalinity = 120 mg/L $CaCO_3$). Bacterial biofilters in upwellers were established for > 2 weeks

prior to arrival of experimental mussels using local mussels from ponds at the research station. Mussels in each upweller were fed 2 mL of Shellfish Diet 1800 (Reed Mariculture Inc., Campbell, CA, USA) in the morning and 1 mL in the afternoon daily. Water quality (ammonia, nitrites, and nitrates) was measured 3 times/week using either Tetra 6-in-1 and Ammonia Aquarium Test Strips (Spectrum Brands, Inc., Blacksburg, VA, USA) or API 5 in 1 and Ammonia Test Strips (MARS Fishcare, Inc., Chalfont, PA, USA).

All newly arrived mussels were acclimated to the same holding conditions (18 °C and 12:12 L:D cycle) for > 2 weeks before being acclimated to experimental temperatures in order to minimize any differences associated with collection season. Following the laboratory acclimation, mussels were assigned with random number generation to one of six temperature treatments (15, 17, 23, 28, 32, and 36 °C). Assigned mussels were placed in insulated upwellers (70 L) equipped with chillers (AquaEuroUSA, Gardena, CA, USA) and/or heaters (Finnex TH-300, Illinois, USA) with temperature control (4 mussels/species/cooler, 2 coolers per temperature treatment). Temperatures were adjusted up or down at a rate of 1 °C/day until the target temperature was reached, and mussels subsequently acclimated to the target temperature for > 1 week (Chen et al. 2001). During the temperature acclimation period, mussels were fed Shellfish Diet 1800 twice daily (2 mL morning, 1 mL afternoon per ~70 L upweller) and held at a 12h light: 12h dark cycle.

Respirometry

Respirometry experiments were conducted in 8-chamber fiber-optic respirometry systems using AutoResp™ 2.3.0 software (Loligo® Systems, Viborg, Denmark). Chambers were made of acrylic and ranged in volume from ~200 – 700 mL. Each chamber was connected to two Eheim

(EHEIM GmbH & Co., Deizisau, Germany) submersible 300 L/h pumps: one pump circulated fresh, oxygenated water through the chamber during initial chamber acclimation, and the other circulated constant water through the chamber during experiments. A flow-through oxygen cell with an optical dissolved oxygen (DO) sensor was inserted in the recirculation tubing of each chamber (Fig. 1.2). Respirometry chambers, associated pumps, and sensors were submerged in a ~300 L rectangular tub filled with HAFW. Water was aerated within the tub to maintain near 100% saturation. Temperature was controlled by a TECO TK-2000 chiller/heater unit (TECO, USA). To reduce ambient oxygen demand from bacteria, chambers, tubing, and gravel associated with the respirometry setup were chlorinated with 5 mL of bleach per gallon of tap water before each trial and then rinsed with tap water.

Intermittent respirometry was used to determine the amount of time needed to acclimate mussels to respiration chambers. We placed *C. pustulosa* and *C. petrina* (4 mussels/species, 1 mussel/chamber) in mesh-bottomed PVC cups filled with pea gravel within each respiration chamber. All acclimation trials were performed at ~21 °C, under normoxic conditions (> 6 mg O_2/L), wherein mussels were allowed to draw oxygen down for 20 minutes to ~80% air saturation, followed by a flushing period to restore DO to near 100% saturation. This cycle was then repeated for roughly 12 hours.

Closed respirometry was used to estimate RMR of *C. pustulosa*, *C. petrina*, and *C. necki* at six temperatures as DO decreased from approximately 100% to near 0% saturation. We first measured RMR of 8 randomly selected individuals per temperature for *C. pustulosa* and *C. petrina* from the Colorado River. Each respirometry experiment consisted of 4 individuals per species per run with 2 runs per temperature. Temperature-acclimated individuals were removed from upwellers, scrubbed lightly with a soft-bristled brush to remove any algae, and weighed

(gWW) to the nearest 0.1 g. Mussels were then placed in PVC cups filled with pea gravel within the respirometry tub and held without food for ~24 hours to prevent feeding and digestion from affecting estimates of RMR.

Following the 24-hr fasting period, mussels were assigned to an appropriately sized respirometry chamber (accommodated a mussel's shell without touching sides or lid). A new PVC cup (~4 cm height) half-full of clean pea gravel was placed in each chamber to provide a substrate for the mussels to burrow into. Cups had 4 mm mesh screening on the bottom to allow for water recirculation and to reduce the chance of 'dead zones'. Flush pumps associated with each chamber were turned on and mussels allowed to acclimate to respirometry chambers for ~5 hours (see *Respirometry* results below). During this time, DO levels remained near 100% saturation. Respirometry rooms were held at a 12h light: 12h dark cycle.

Chamber acclimation was typically initiated between 3 and 6 pm. Following chamber acclimation, the flush pumps were turned off and closed pumps simultaneously turned on – creating a closed system where the volume of water recirculating within each chamber and associated tubing was constant, and no new, oxygenated water entered the system. Pumps were controlled remotely using TeamViewer 14 software (TeamViewer GmbH, Göppingen, Germany) to minimize any disturbance to the mussels within the respirometry rooms. AutoResp software calculated respiration rates every ~1 - 8 minutes (depending on temperature). Dissolved oxygen concentrations and respiration rates were displayed in real time. Mussels were allowed to respire until dissolved oxygen levels fell below ~0.2 mg O_2/L in the chambers if there were no signs of valve closure (i.e. rapid reduction of RMR to roughly 0 mg O_2/gWW/hr followed by a recovery of RMR to previous rate). Valve closure typically rendered respiration data unusable for regulation index (RI) or DO_{crit} analysis whereas RMR calculations were still possible on

occasion. Each run was ended when DO concentrations fell below ~0.2 mg O_2/L for all non-closure mussels within that run. Mussels were then removed from respiration chambers and returned to their temperature acclimation upwellers to recover. Following the completion of respiration experiments for *C. pustulosa* (Colorado) and *C. petrina*, we ran closed respirometry using the above protocol on similar numbers of *C. pustulosa* (Navasota) and *C. necki*.

To account for any background oxygen demand associated with bacteria, I measured the background respiration rate of each chamber (with gravel cup) before and after each run, under normoxic conditions (DO $\geq$ 5 mg O_2/L), for ~1.5 hrs without mussels present. The mean background oxygen demand (mussels absent) was then divided by the mean observed respiration rate (mussels present) under normoxic conditions to determine the proportion of total chamber respiration approximated as background respiration. This proportion was referred to as the correction factor. I assumed that this proportion remained constant as DO declined below normoxia and corrected our respirometry data in each chamber by multiplying the observed respiration rate by (1-correction factor).

Metabolic patterns

RIs were calculated using the methodology of Mueller and Seymour (2011). Corrected RMR (mg O_2/gWW/hr) values were plotted against DO (mg O_2/L) for each mussel tested. The upper limit of the DO range, for which RI was calculated, was held constant at 6 mg O_2/L to avoid bias in colder temperature runs where initial DO was much higher than warmer runs. Data were fitted with the curve (3-parameter exponential rise to maximum, 2-parameter hyperbola, or 2-segment piecewise regression) that showed the smallest bias-corrected Akaike information criterion (AICc: SigmaPlot 13.0; Systat Software, Inc., San Jose, CA, USA). We then 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 (Fig. 1.1a). RI was calculated as (Observed AUC-Conformation AUC)/(Regulation AUC-Conformation AUC). The RI thus provided a quantitative measure of the degree to which mussels were able to regulate oxygen consumption as ambient DO declined from 6 to < 0.2 mg O_2/L. DO_{crit} was calculated as the dissolved oxygen concentration showing the greatest distance between the observed RMR and the perfect conformation line (Mueller and Seymour 2011). From the curve with the lowest AICc, we also estimated RMR for each individual mussel at 6 mg O_2/L (RMR_6).

Food Requirements Calculation

Using temperature data from the San Saba gage on the Colorado River from 2009 (retrieved from Lower Colorado River Authority), I converted respiration rates from the RMR_6 predicted regressions (see *Statistical Analyses* and Fig. 1.4) to maintenance energy requirements that mussels would need to obtain from feeding and/or stored energy. The 2009 dataset was an example of a "warm" temperature regime where maximum temperatures reached or exceeded 36 °C. RMR_6 (mg O_2/gWW/hr) was converted to ml O_2/gWW/hr by dividing by 0.7 and then to μmol O_2/gWW/hr by dividing by 0.022391 (ICES Oceanography: https://ocean.ices.dk/tools/unitconversion.aspx). Lastly, the resulting RMR_6 were converted to energy requirements (Joules/gWW/hr) by multiplying by a conversion factor of 0.456 (Gnaiger 1983).

Statistical Analyses

Repeated measures one-way ANOVA was used to analyze intermittent respirometry data to determine the time needed to acclimate to respiration chambers. Each mussel tested (5-6 of each species) was represented at each time point. The time point after which the metabolic rate did not significantly change for the remainder of the run approximated the time required to acclimate to respiration chambers. The post-hoc Tukey's honestly significant difference (HSD) test was applied to determine statistically significant differences ($P < 0.05$) following ANOVA.

We used mixed-model analysis of covariance (ANCOVA) to compare the metabolic patterns (RMR_6, RI, and DO_{crit}) between species-locations of mussels. The ANCOVA accounted for variations in metabolic patterns that could be attributed to mussel's body weight and water temperature within respirometry runs. RMR_6 (mg O_2/gWW/hr), RI, and DO_{crit} (mg O_2/L) were the dependent variables in the three respective models while water temperature (°C) during respirometry run (T) and mussel wet mass (gWW) were covariates for each model. The species-location was the grouping variable. The least-squares adjusted means were used to compare the true effects of the treatments (species-location), adjusted for the effect of temperature. The Shapiro–Wilk test was utilized for normality analysis of the variables. For non-normally distributed data, the ANCOVA was performed on rank-transformed data. Statistical significance was set at $P < 0.05$. Statistical analyses were performed with SAS® version 9.4 (SAS 2013, Cary, NC, USA).

In order to describe the relationship between temperature and metabolic patterns within individual species and subpopulations, different regression curves (linear, quadratic, or 2-parameter hyperbola regression) were fitted through raw data of RMR_6, RI, and DO_{crit}. The model with the smallest AICc was selected. Residual analysis and outlier detection were

performed. Outlier observations (studentized residuals > 3) were removed from the model with the smallest AICc and refitted.

Valve closure events (> 0.5 mg O_2/L) were quantified for each individual in our trials (i.e. 0 for no closure, 1 for one closure, etc.). The mean number of closures per mussel was then regressed with a linear regression. Only significant regressions are shown ($P < 0.05$).

Results

Mussel Collection and Acclimation

Shipping techniques were successful with > 90% of mussels surviving from initial collection to 72 hrs after arrival in the receiving lab. Ammonia and nitrite remained at undetectable levels (< 0.5 mg/L) in the laboratory and temperature acclimation upwellers throughout the study. Nitrates were consistently detected, and water changes were triggered when nitrate concentrations reached or exceeded 20 - 40 mg/L. Survival at all experimental acclimation temperatures was 100% with no mortalities occurring in all four groups prior to respiration experiments. All species-location pairwise comparisons of gWW (wet mass: soft tissue plus shell) were significantly different (Kruskal–Wallis: $F_{4, 158} = 318.99$, $P < 0.0001$; Table 1.1) except for *C. pustulosa* (Navasota) and *C. necki*.

Respirometry

There were no significant differences in respiration rates after 4 hours in respirometry chambers for *C. pustulosa* (Kruskal–Wallis, Tukey HSD: $P > 0.05$ for all pairwise comparisons among ≥ 4.1 hour RMR means), or after 3 hours for *C. petrina* (Kruskal–

Wallis, Tukey HSD: $P > 0.05$ for all pairwise comparisons among ≥ 3.0 hour RMR means). Therefore, we used a conservative chamber acclimation time of ≥ 5 hours prior to initiating each respirometry run. Duration of each respirometry run, following chamber acclimation, was temperature dependent. At the coldest temperature (15 °C), it generally took > 8 hrs for DO to fall below 0.2 mg O_2/L whereas at the warmest temperature (36 °C), DO declined to < 0.2 mg O_2/L in less than 8 hours.

Resting metabolic rates estimated at 6 mg O_2/L (RMR_6) for all taxa across all temperatures were not normally distributed (Shapiro–Wilk: $W = 0.96$, $P = 0.0002$). Results of the mixed-model ANCOVA on rank-transformed data revealed a significant effect of species-location on RMR_6 (Table 1.2). RMR_6 of *C. pustulosa* (Colorado) was significantly higher than *C. pustulosa* (Navasota; $t_{(153)} = 9.71$, $P < 0.0001$), *C. petrina* ($t_{(153)} = 5.81$, $P < 0.0001$), and *C. necki* ($t_{(153)} = 7.99$, $P < 0.0001$). RMR_6 of *C. petrina* was significantly higher than *C. necki* ($t_{(153)} = 2.87$, $P = 0.0243$) and *C. pustulosa* (Navasota; $t_{(153)} = 3.97$, $P = 0.0006$; Fig. 1.3).

Water temperature showed a significant positive effect on RMR_6 as a covariate, but wet mass did not (Table 1.2). The interaction between temperature and species-locations was not significant (Table 1.2). The relationship between RMR_6 and temperature was best described by a linear regression for *C. pustulosa* (Colorado), *C. petrina*, and *C. necki* while *C. pustulosa* (Navasota) was best described by a quadratic regression (Fig. 1.4).

Regulation indices for all taxa were normally distributed (Shapiro–Wilk: $W = 0.99$, $P = 0.3430$). Results of the mixed-model ANCOVA revealed a significant effect of species-location on RI (Table 1.2). *C. pustulosa* (Navasota) had a significantly lower RI, regardless of temperature, than *C. pustulosa* (Colorado; $t_{(131)} = 3.28$, $P = 0.0072$), *C. petrina* ($t_{(131)} = 2.80$, $P = 0.0294$) or *C. necki* ($t_{(131)} = 2.71$, $P = 0.0379$; Fig. 1.5).

Water temperature had a significant negative effect on RI, but wet mass did not (Table 1.2). The interaction between temperature and species-locations was not significant (Table 1.2). The relationship between RI and temperature was best described by a hyperbola regression for *C. pustulosa* (Colorado) and *C. petrina*. There was no significant relationship between RI and temperature for *C. pustulosa* (Navasota) or *C. necki* (Fig. 1.6).

Critical dissolved oxygen estimates were not normally distributed among taxa tested (Shapiro–Wilk: $W = 0.94$, $P < 0.0001$). Results of the mixed-model ANCOVA on rank-transformed data revealed a significant effect of species-location on DO_{crit} (Table 1.2). *C. pustulosa* (Colorado) had a significantly higher DO_{crit} than *C. pustulosa* (Navasota; $t_{(131)} = 5.56$, $P < 0.0001$) and *C. necki* ($t_{(131)} = 3.42$, $P = 0.0046$). *C. pustulosa* (Navasota) also had a significantly lower DO_{crit} than *C. petrina* ($t_{(131)} = 3.57$, $P = 0.0028$; Fig. 1.7).

Water temperature had a significant positive effect on DO_{crit}, but wet mass did not (Table 1.2). The interaction between temperature and species-locations was not significant (Table 1.2). The relationship between DO_{crit} and temperature was best described by a hyperbola regression for *C. petrina* while there was no significant relationship for either *C. pustulosa* subpopulation or *C. necki* (Fig. 1.8).

Valve Closures

The mean number of closure events per mussel increased linearly with temperature for *C. petrina* and *C. necki* (Fig. 1.9). *C. pustulosa* (Colorado) only exhibited closure events at 36 °C, showing no significant pattern of valve closure events with temperature. *C. pustulosa* (Navasota) had no significant relationship of closures per mussel with temperature, showing little to no evidence of increased closures with rising temperature.

Energy Requirements

Results show that under a "warm" temperature regime, maximum energy requirements of *C. pustulosa* (Colorado) increased by 2-3x between March and July and were approximately twice as high as *C. pustulosa* (Navasota), *C. petrina*, or *C. necki* (Fig. 1.10).

Discussion

Rising stream temperatures have potential to lead to thermal stress in aquatic organisms as demand for oxygen rises while oxygen availability in warming waters declines. Mussels are at particular risk of thermal and hypoxia stress due to limited mobility – they cannot quickly move to cooler, normoxic refuges. Because they are one of the most threatened aquatic taxa (Master et al. 2000), susceptibility to thermal and hypoxia stress is of strong concern to a wide array of stakeholders including natural resource managers, conservationists, and river regulation authorities. Of particular importance is the question as to whether species and subpopulations can be managed as a common entity (i.e. one size fits all) or whether they exhibit differing environmental tolerances and present a need to be managed separately.

Across all species and subpopulations tested, thermal stress up to 36 °C was associated with only sublethal effects. There was no mortality observed during ≥ 7-day acclimations to even the highest (36 °C) temperature. Similarly, no mussels died during respirometry runs even though some individuals spent several hours at < 0.5 mg O_2/L at even the highest temperatures. Thus, effects of extreme temperature events of up to 7 days duration, and short-term hypoxia, in natural populations would not be expected to result in significant adult mortality for any species

or subpopulation tested. These results were similar to Ganser et al. (2015) who saw no mortality of four mussel species after exposure to temperatures up to 35 °C during 21-day trials.

However, also similar to Ganser et al. (2015), we found evidence of sublethal thermal stress with important implications for growth and reproduction. When mussels were open and actively respiring, all three species exhibited increasing energy demands with increasing temperature, but characteristics of this response differed among species and subpopulations. Whereas *C. petrina*, *C. necki*, and *C. pustulosa* (Colorado) exhibited a positive, linear relationship between RMR_6 and temperature from 15 – 36 °C, *C. pustulosa* (Navasota) exhibited thermal compensation, often termed metabolic depression or compensation, at around 28 °C. Implications of thermal compensation zones are poorly understood. Brown (1989) found that sculpin species exhibited thermal compensation between 20 and 25 °C and died at 27.5 °C, suggesting that metabolic depression is a sign of thermal stress as metabolic processes are unable to function efficiently. In contrast, Crocker and Cech (1997) linked this depression in white sturgeon to hypoxia and suggests it is a mechanism for fish to survive long periods of time in hypoxic environments. While the implications of metabolic depression are unclear for mussels, it is of interest to note that the major difference in RMR_6 trajectory with increasing temperature occurred between two subpopulations of a single, widely distributed species (*C. pustulosa*) rather than between two narrowly distributed species (*C. petrina* and *C. necki*).

Differences in mean RMR_6, adjusted for the effect of temperature, also occurred between subpopulations and among species with *C. pustulosa* (Colorado) having a higher RMR_6 than *C. pustulosa* (Navasota), and *C. petrina* having a higher RMR_6 than *C. necki*. Increases in RMR_6 with temperature translate to higher energy requirements for basal metabolic processes and maintenance. Because mussels have limited energy to distribute among maintenance, growth,

and reproduction (Burton et al. 2011), increases in RMR can be detrimental to fitness as energy reserves are shunted away from growth and reproduction (Ganser et al. 2015). Thus, the *C. pustulosa* subpopulation from the Colorado River appears to be most at risk for food limitation, reduced growth rate, and reduced reproductive capacity at high temperatures due to it having a greater rate of increase in RMR_6 with temperature, and the highest mean RMR_6 (regardless of temperature) of any species or subpopulation tested.

Although energy requirements generally increase with temperature, many bivalves exhibit an increased frequency of valve closure as temperatures warm. These closure periods have been interpreted as a mechanism to offset thermally induced increases in energy demand (Anestis et al. 2007) since aerobic respiration essentially ceases during valve closure. On the other hand, feeding ability is mostly compromised during valve closure, which means internal energy stores must be accessed to meet remaining energetic needs. The mussels in our study appeared to use different strategies to deal with these trade-offs between reductions in energy demand and increased reliance on internal energy stores. The subpopulation with the highest energy demand (*C. pustulosa*, Colorado River) refrained from valve closure until the highest temperature. This strategy would reduce depletion of energy stores when sufficient food was available to meet increasing energy demands but would be very risky under low food conditions. The other subpopulation (*C. pustulosa*, Navasota River) exhibited metabolic depression at warm temperatures with little evidence of increased closure at high temperatures. This would reduce their risk of food limitation while minimizing depletion of internal energy stores but indicates a reduction in metabolic investment in basic physiological processes (Burton et al. 2011). The remaining two species showed a linear increase in closure frequency with warming temperatures, indicating an increased reliance on internal energy stores, but also exhibited lower RMR_6 than *C.*

pustulosa (Colorado River) which would reduce the rate of depletion. These results are intriguing and suggest additional research as to trade-offs between metabolic rates, energy-reserve depletion, and valve closure. Results also support the importance of understanding food sources, quantity, and quality in natural systems – an area of mussel ecology that is still poorly understood.

In addition to increasing metabolic demands, mussels face increasing risk of hypoxia as temperatures increase. Traditionally, the critical oxygen level (DO_{crit}) has been described as the dissolved oxygen threshold below which the organism switches from aerobic to anaerobic respiration (Mueller and Seymour 2011) and has been considered a useful predictor of hypoxia tolerance. Under this paradigm, tolerance of hypoxia increases as DO_{crit} decreases. However, the use of DO_{crit} as a predictor of hypoxia tolerance has been recently challenged (Wood 2018). Loss of equilibrium was suggested as a preferred alternative to critical oxygen levels, but this isn't feasible with unionids. We retained the use of DO_{crit} for ease of comparison with previous studies, but as Wood (2018) suggests, we accompanied DO_{crit} measures with the regulation index. The RI is an improved quantitative indicator of the ability of organisms to maintain a consistent respiration rate in the face of declining DO. A decline in RI is typically interpreted as indicating a decline in hypoxia tolerance (Mueller and Seymour 2011).

An important consideration for managers is whether sensitivity to hypoxia increases as water temperatures rise and dissolved oxygen concentrations decline. In our study, sensitivity to hypoxia, as indicated by changes in DO_{crit} and RI, did not always increase with temperature but, rather, varied among species and subpopulations. *C. petrina* exhibited declining RI and increasing DO_{crit} with warming temperatures, suggesting the greatest reduction in hypoxia tolerance with increasing temperature. *C. pustulosa* (Colorado) was intermediate with a declining

RI but no change in DO_{crit}. *C. pustulosa* (Navasota) and *C. necki* showed no evidence of decreasing hypoxia tolerance as RI and DO_{crit} remained stable across a range of temperatures. Thus, species and subpopulations are likely to be differentially affected by rising temperatures in riverine systems, with *C. petrina* being the most at-risk of the mussel taxa tested.

When temperature was not considered a factor, differences in sensitivity to hypoxia were less clear. *C. pustulosa* (Navasota) had the lowest RI of any species or subpopulation indicating the greatest sensitivity to hypoxia. However, it also had one of the lowest DO_{crit} values, suggesting the least sensitivity to hypoxia. Amongst the other three mussel groups, there was no difference in RI, while DO_{crit} was lower for *C. necki* than *C pustulosa* (Colorado). These results suggest that temperature-dependent changes in RI and DO_{crit} may be better, more consistent, indicators of relative sensitivity to hypoxia than RI and DO_{crit} alone.

Because all mussel species examined were candidates for federal listing during the study period, permit limitations and logistics precluded us from collecting sufficient numbers of each species and subpopulation that were all of similar size and collected within the same time period. In general, small individuals have a higher mass-specific metabolic rate than larger individuals (Bayne et al. 1976). Because there were significant differences in wet mass among species and subpopulations, differences in metabolic patterns could simply be an artifact of mussel size. However, we found no significant effect of mussel wet mass on RMR_6, RI, or DO_{crit}. Furthermore, the subpopulation with the highest RMR_6 (*C. pustulosa*, Colorado River) had a larger mean size than the subpopulation (*C. pustulosa*, Navasota River) and species (*C. necki*) with the two lowest mean RMR_6 values. Thus, patterns were not simply a function of allometry.

Patterns could also have been biased by season of collection as *C. pustulosa* (Colorado) and *C. petrina* were collected in spring/summer while *C. pustulosa* (Navasota) and *C. necki* were

collected in the fall/winter. We tried to minimize this possibility by acclimating all animals to laboratory conditions and cool temperatures (18 °C) for a minimum of two weeks, and experimental temperatures for a minimum of one week (plus the time to reach experimental temperatures) prior to conducting respirometry. However, we acknowledge it is still possible that time of collection affected our results.

Results of this study found that metabolic demand increased in all species and subpopulations with temperature although the shape and slope of the patterns varied. Additionally, response of mussels to hypoxia with rising temperatures varied among species and subpopulations. Our results suggest that *C. pustulosa* (Colorado River) is at most risk for food limitations, reduced growth, and depletion of energy reserves as temperatures rise. With subsequent changes in DO saturation and availability from rising temperatures, *C. petrina* is likely to experience a reduction in the ability to meet metabolic demands and gather sufficient oxygen from the water in response to hypoxic events. With rapid declines in unionid populations in the United States and potential threats from rising stream temperatures, this study stresses the importance of applied research geared towards thermal and hypoxia tolerance of unionids as this is a topic with little research. Furthermore, this study provides evidence that not all unionids respond to stressors in the same way, thus revealing a greater need for species-specific management to protect populations that are at most risk of stressors, not just a one-size-fits-all strategy.

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Figures and Tables

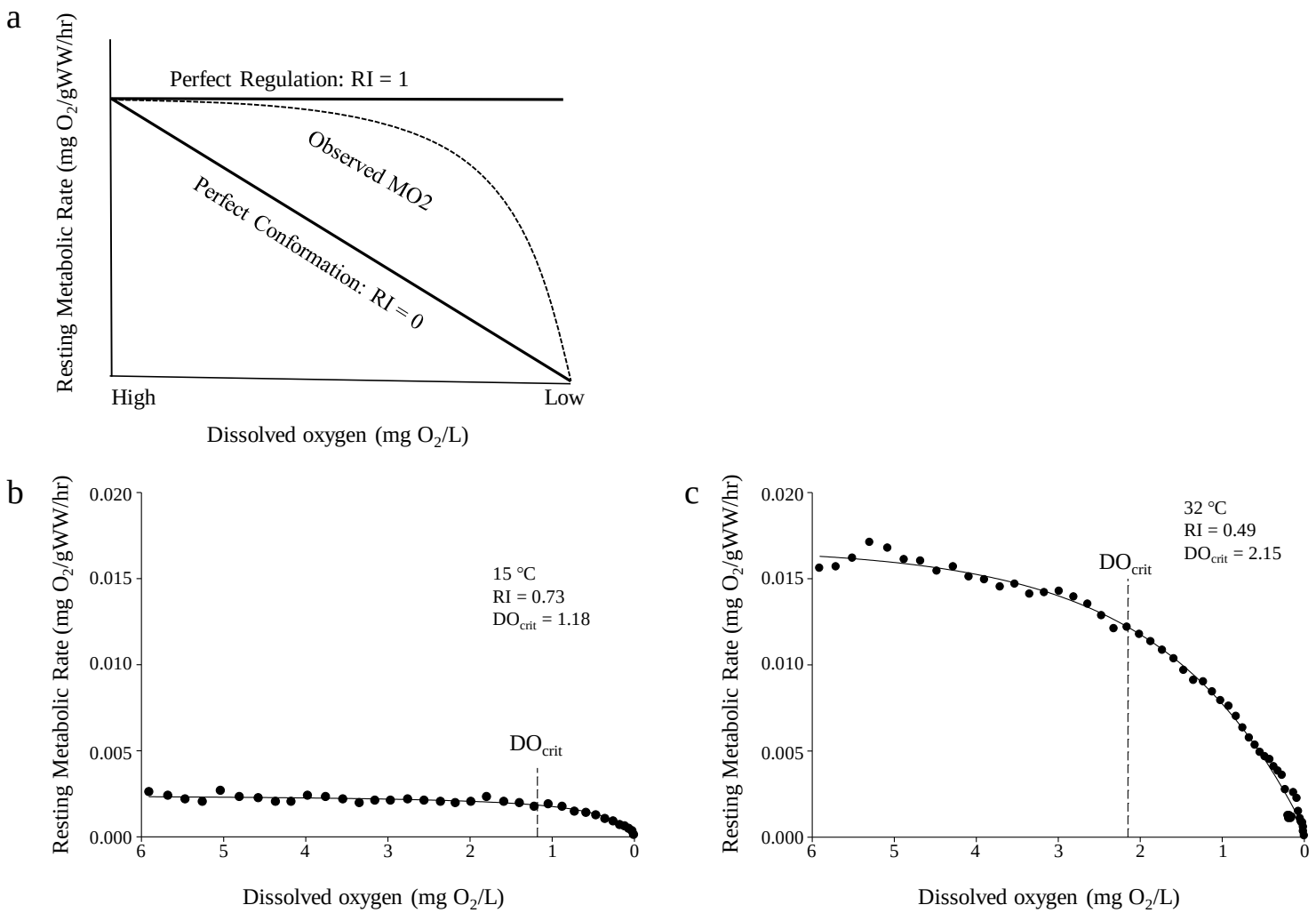

Figure 1.1. Resting metabolic rates (RMR) graphed as a function of declining dissolved oxygen showing a) perfect regulation, perfect conformation, regulation index (RI) values associated with each, and an intermediate pattern more common than "perfect" patterns (Adapted from Mueller and Seymour 2011). b) An example of "high" RI and a "low" critical oxygen level (DO_{crit}) and c) An example of a "low" RI and a "high" DO_{crit} observed in this study. Dotted lines indicate DO_{crit}.

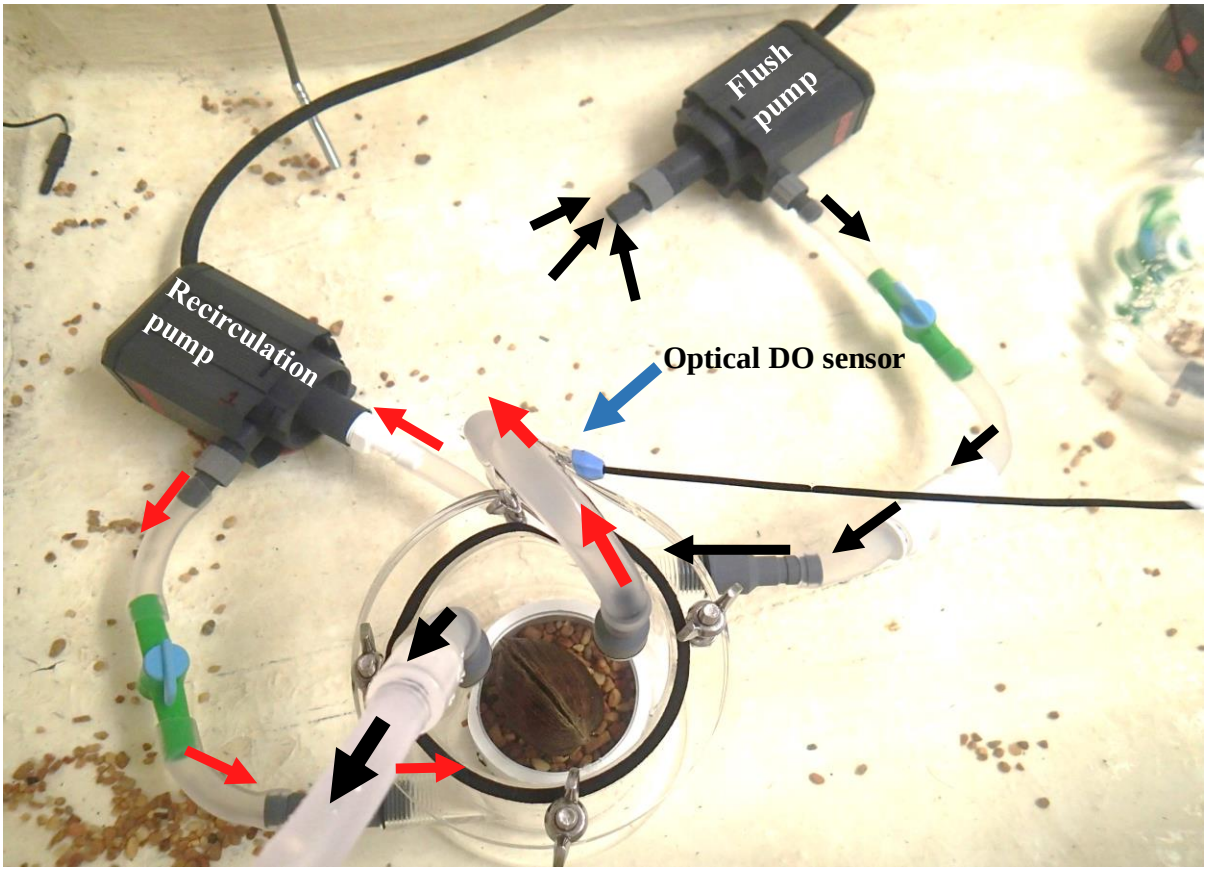

Figure 1.2. General closed respirometry configuration of chambers, tubing, and oxygen sensor. The oxygen sensor was inserted into the recirculation tubing.

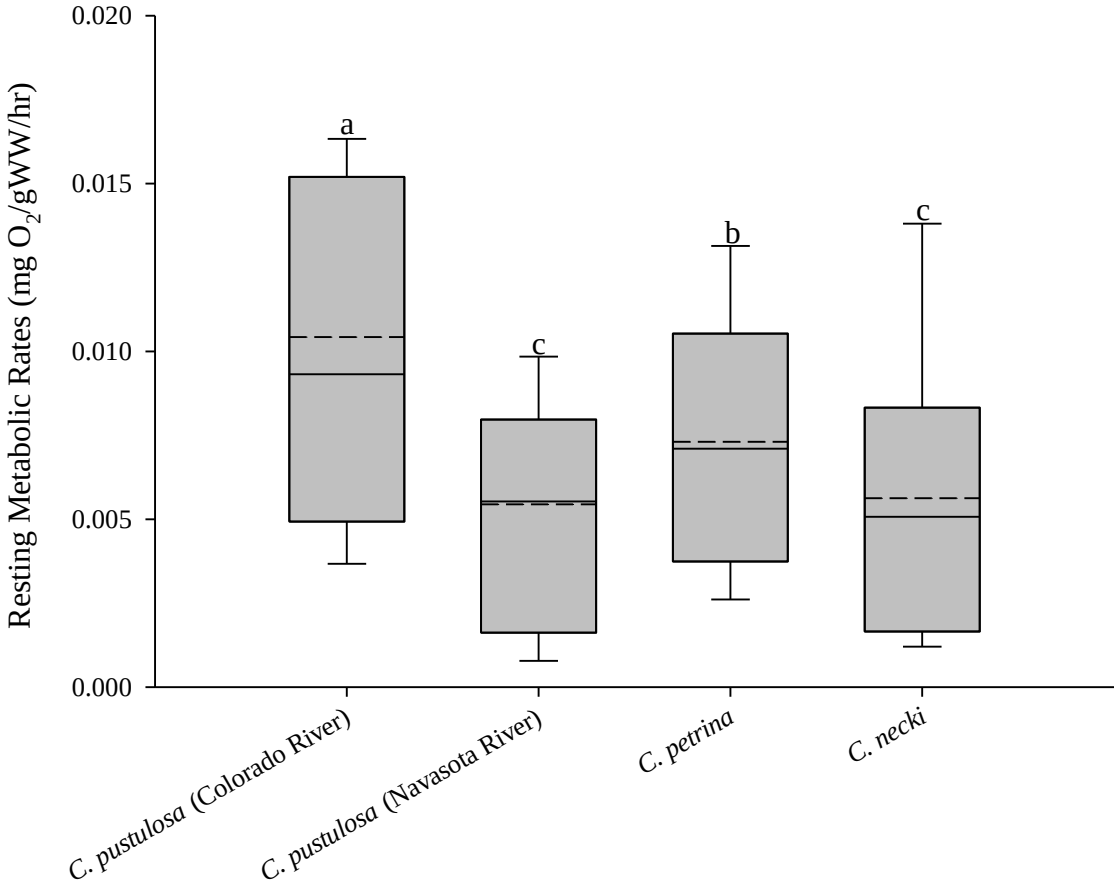

Figure 1.3. Box plot of resting metabolic rates (RMR_6) of each species × location combination. Solid and dashed horizontal lines within each box represent median and mean values, respectively. Different letters represent significant differences between species-location using post-hoc Tukey HSD test with temperature accounted for as a covariate in ANCOVA model.

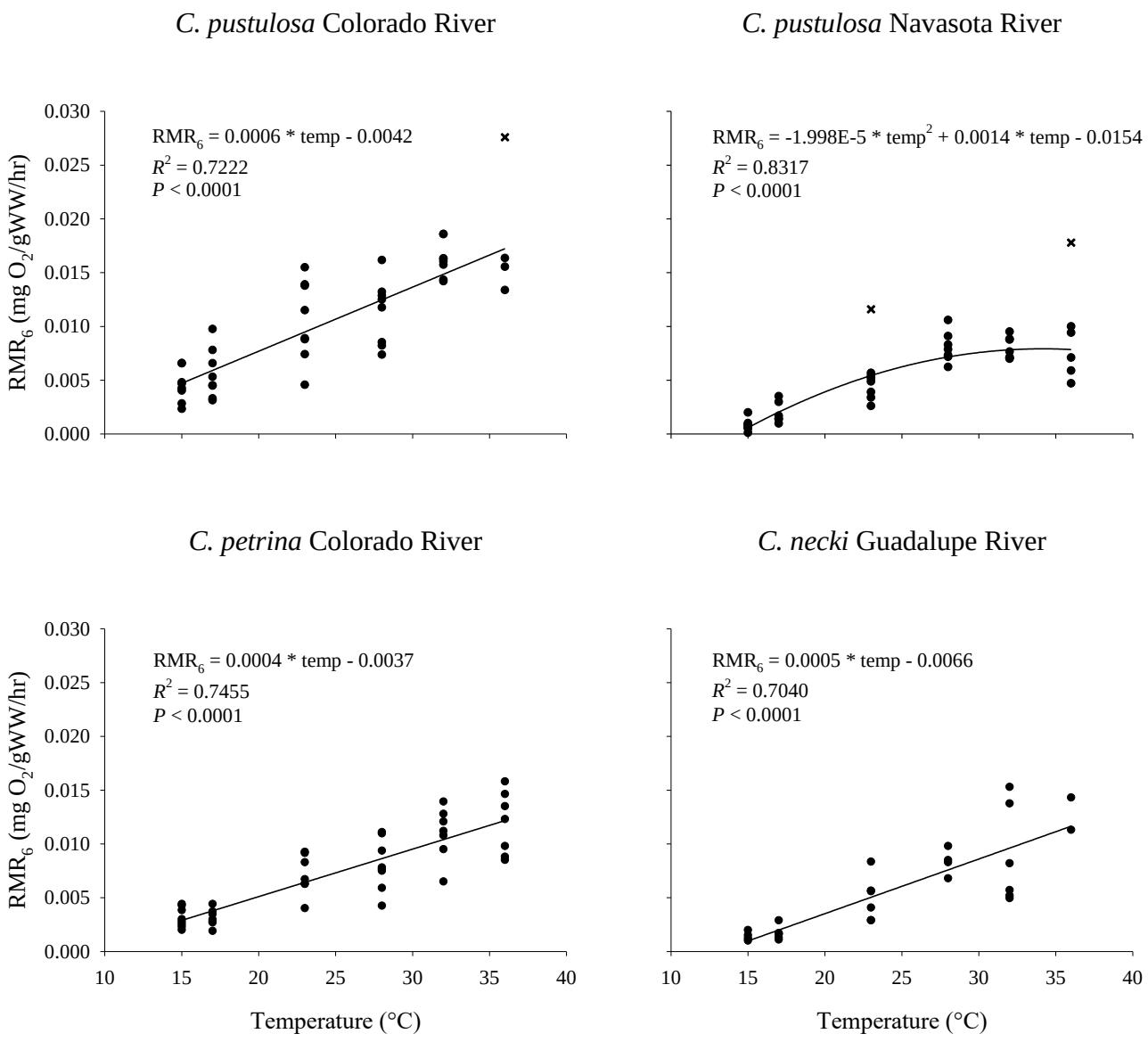

Figure 1.4. Relationship between RMR_6 and temperature for the four mussel populations tested. Black circles represent respiration rates of individual mussels. "X" symbols represent outliers (studentized residuals >3) that were removed. Lines represent the best fit regression through each dataset excluding outliers.

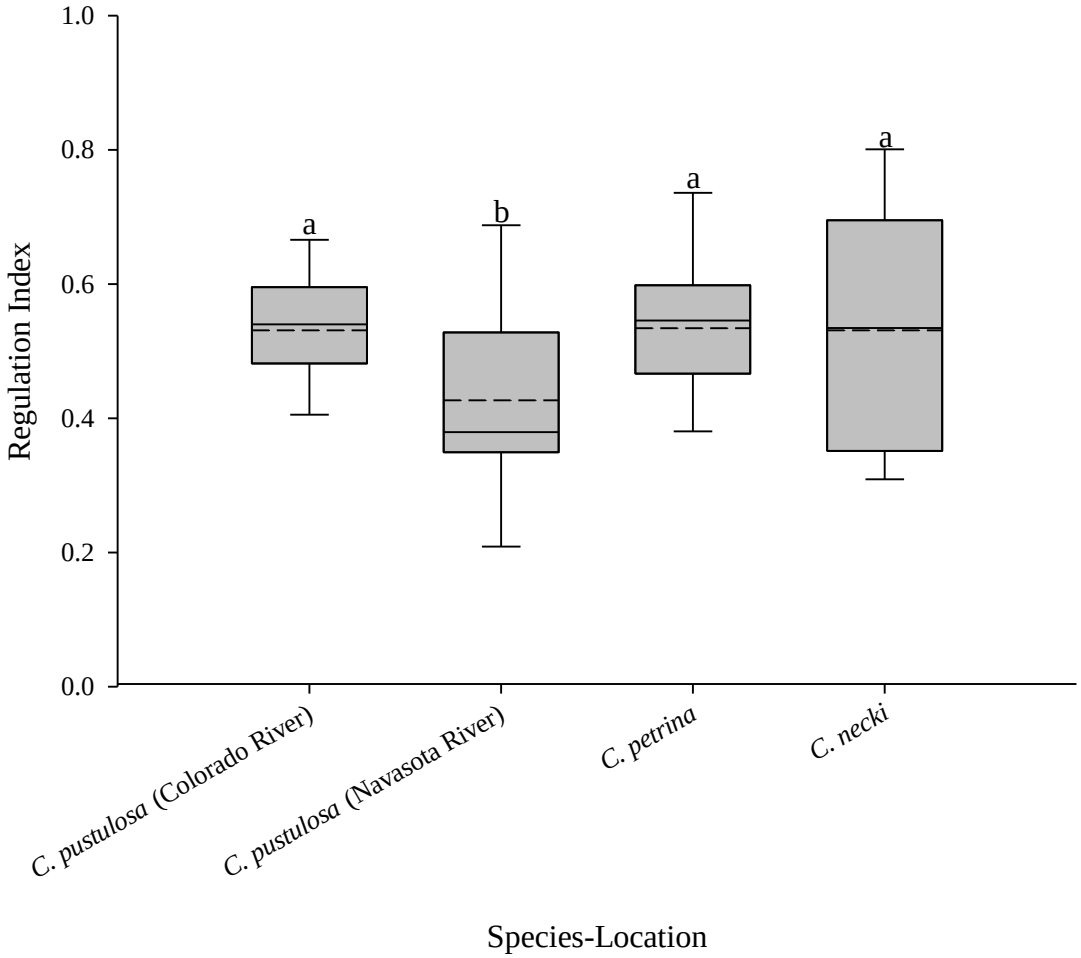

Figure 1.5. Box plot of regulation index (RI) values for each species × location combination. Solid and dashed horizontal lines within each box represent median and mean values, respectively. Different letters represent significant differences between species-location using post-hoc Tukey HSD test with temperature accounted for as a covariate in ANCOVA model.

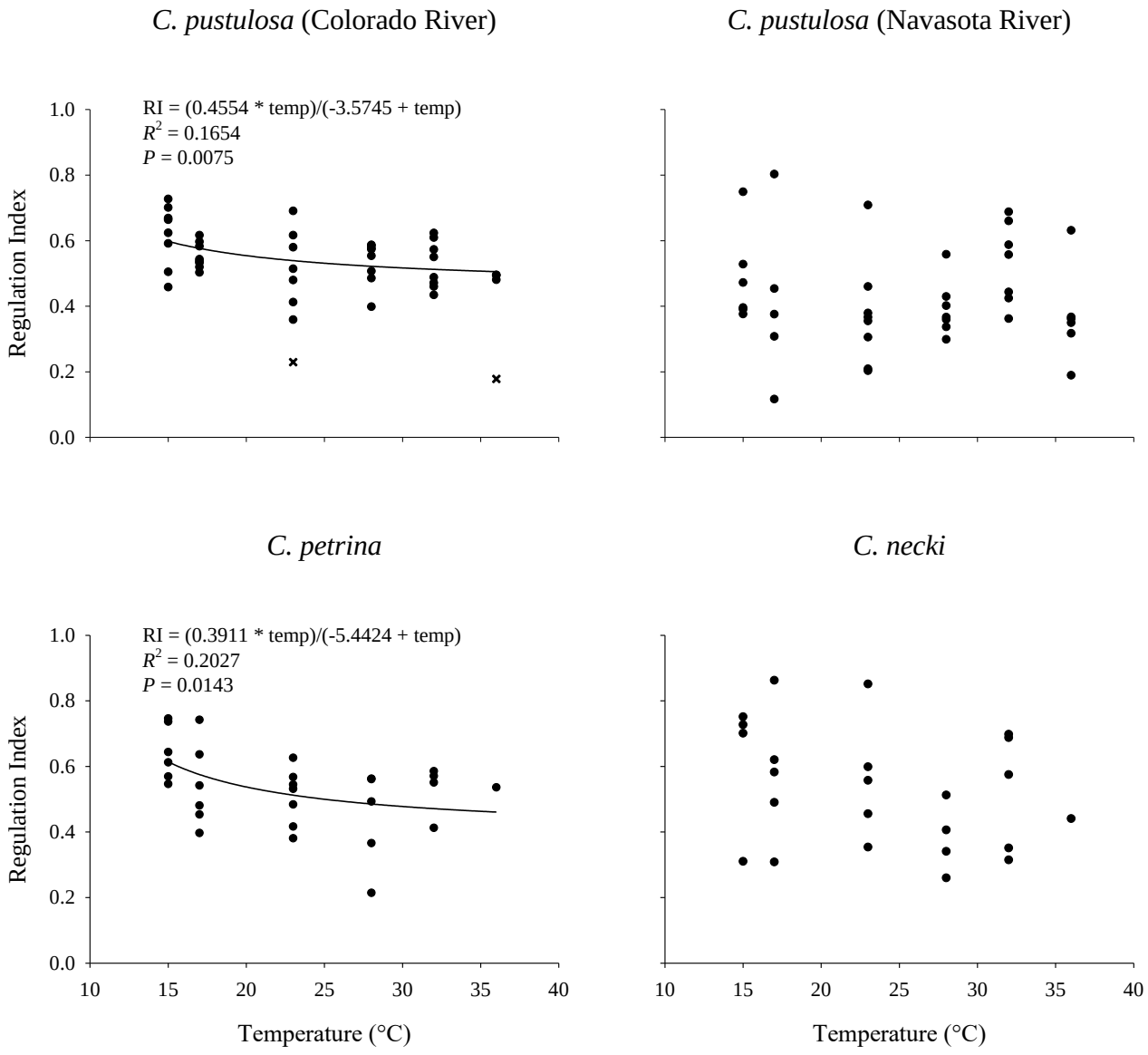

Figure 1.6. Relationship between regulation index and temperature for the four mussel populations tested. Black circles represent regulation indices of individual mussels. "X" symbols represent outliers (studentized residuals >3) that were removed. Lines represent the best fit regression through each dataset excluding outliers. Only significant ($P < 0.05$) regressions are shown.

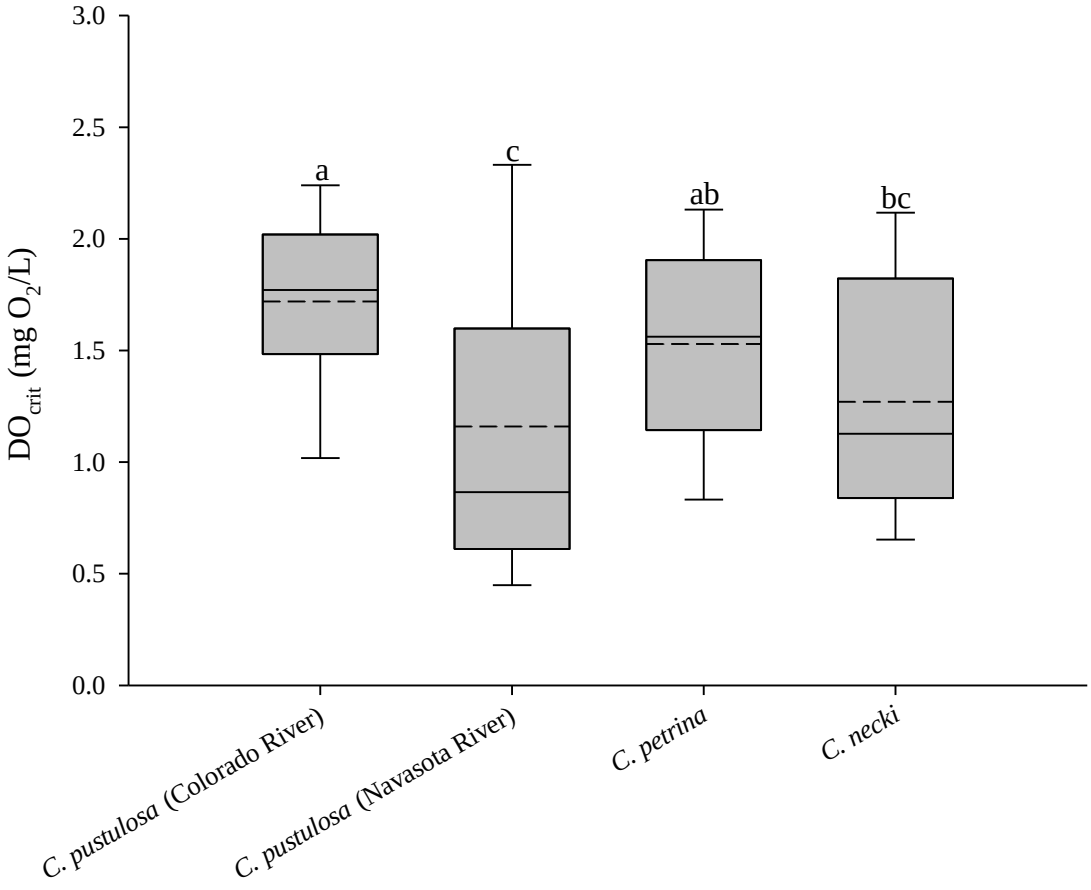

Figure 1.7. Box plot of critical oxygen levels (DO_{crit}) of each species × location combination. Solid and dashed horizontal lines within each box represent median and mean values, respectively. Different letters represent significant differences between species-location using post-hoc Tukey HSD test with temperature accounted for as a covariate in ANCOVA model.

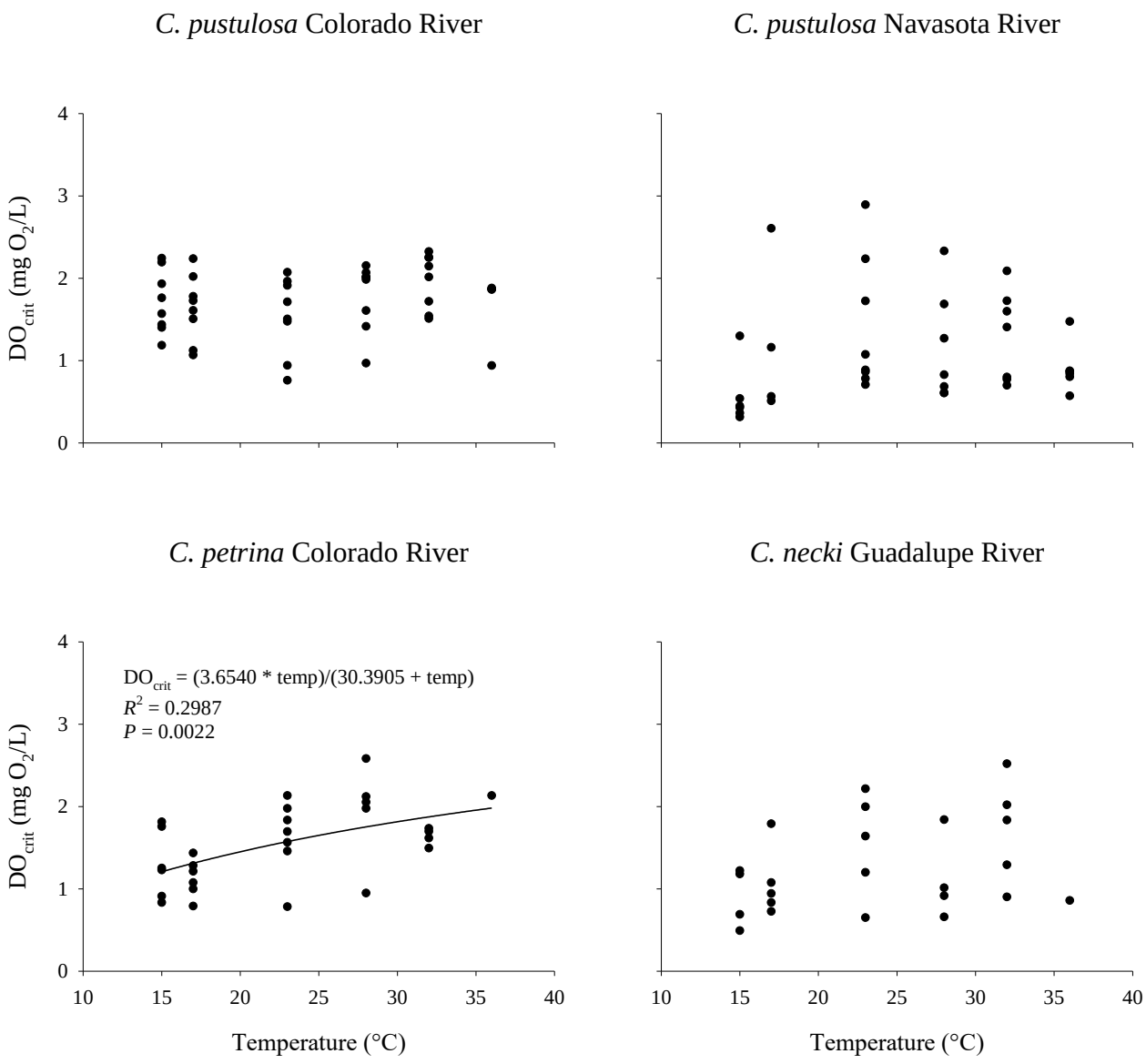

Figure 1.8. Relationship between critical dissolved oxygen concentration (DO_{crit}) and temperature for the four mussel populations tested. Black circles represent critical oxygen concentrations of individual mussels. No outliers were identified in these data sets. Lines represent the best fit regression through each dataset. Only significant ($P < 0.05$) regressions are shown.

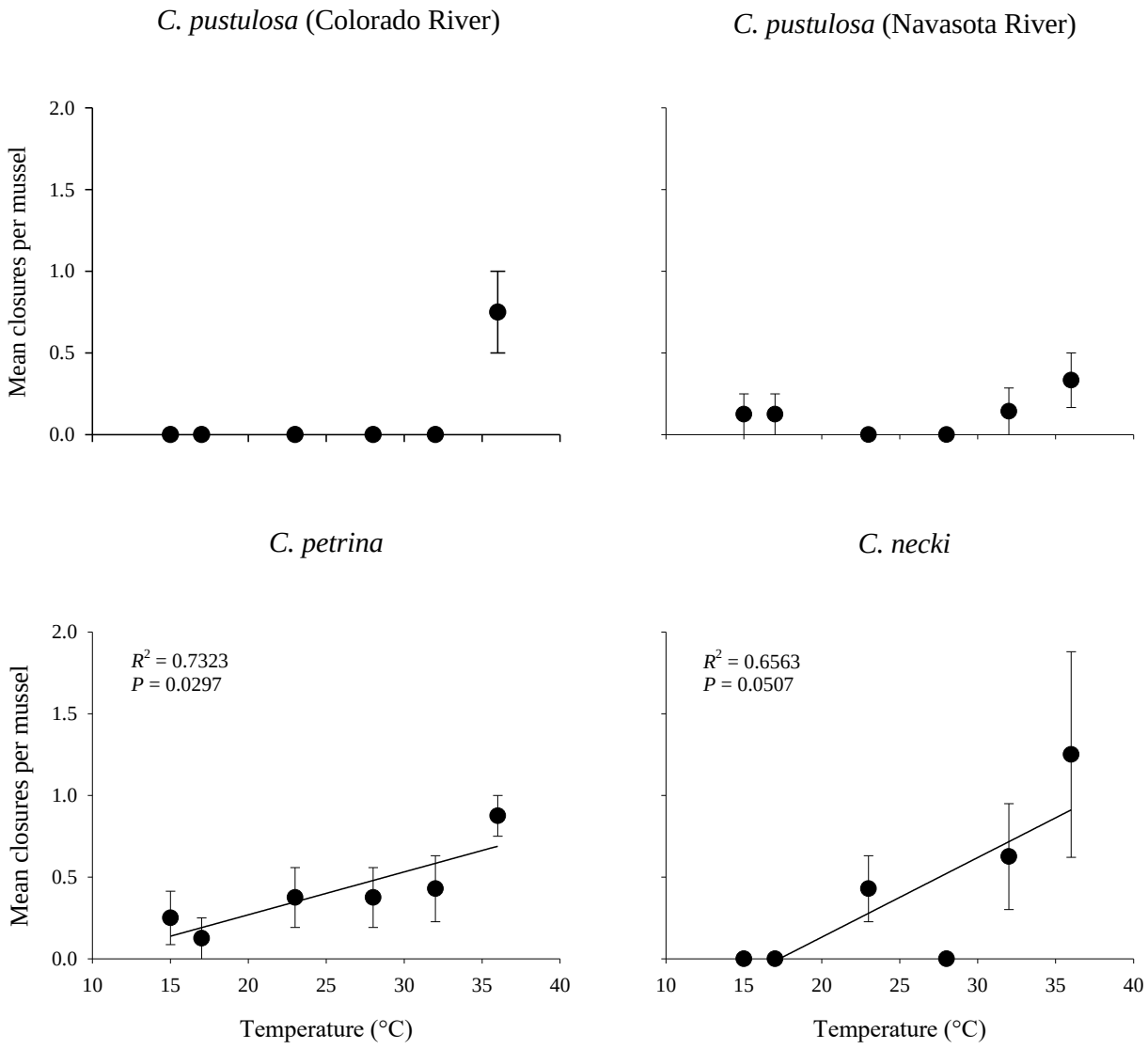

Figure 1.9. Mean number of closures per mussel at each temperature for *C.* pustulosa, C. *petrina*, and *C. necki*. Error bars represent standard errors. Only significant regressions ($P < 0.05$) are shown.

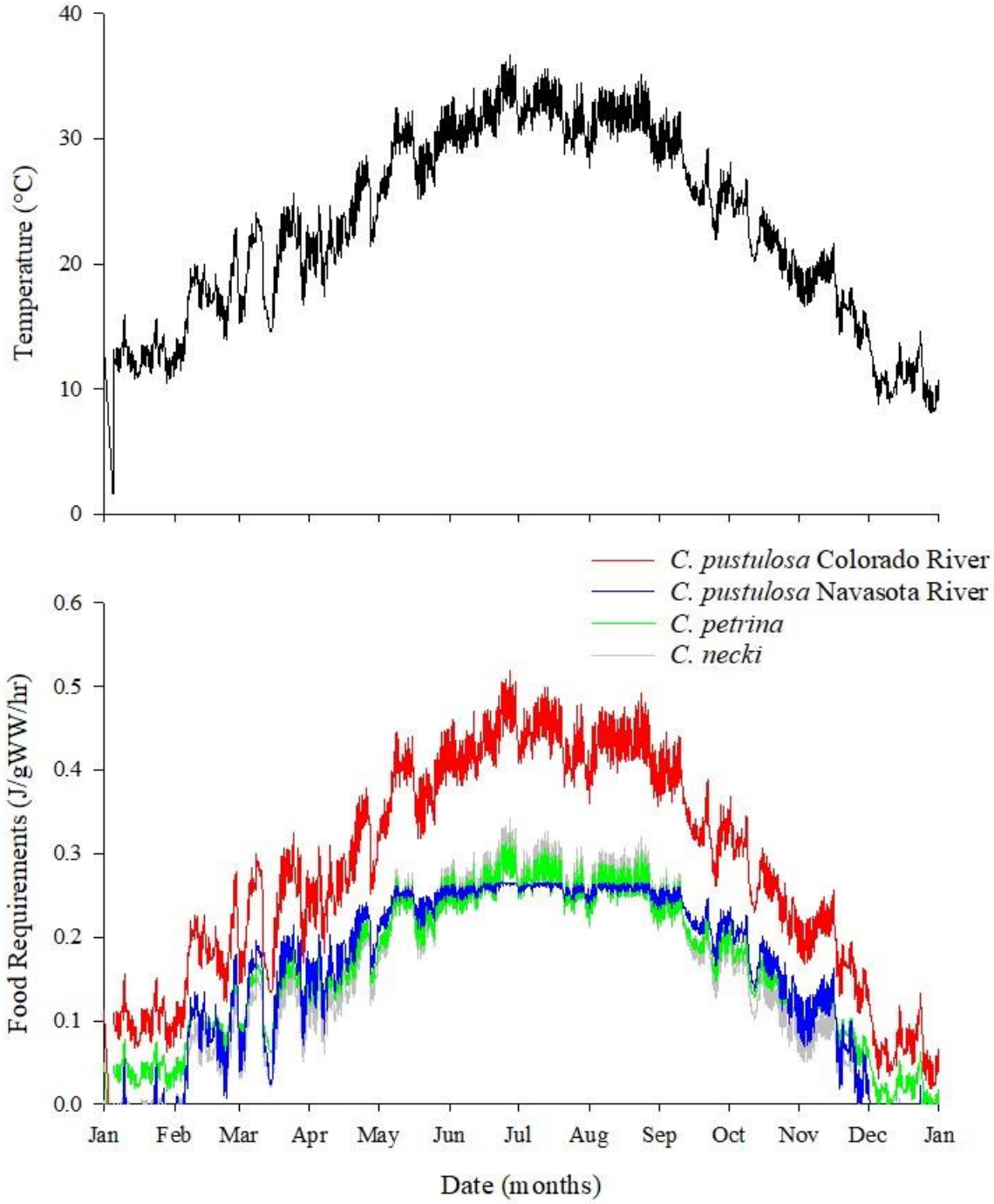

Figure 1.10. Top panel: Example of seasonal thermal regime in the Colorado River during a "warm" year when temperatures reached or exceeded 36 °C. Data are from the San Saba gage site (Lower Colorado River Authority). Bottom panel: Seasonal changes in energy requirements to meet basic metabolic needs if each population was subjected to the San Saba thermal regime. Energy requirements were calculated based on the relationship between resting metabolic rate and temperature for each mussel population tested.

Table 1.1. Taxonomy (initial and revised), sampling locations, and mean wet mass of mussels used in this study. Different letters indicate significant differences in mean wet mass at $P < 0.05$ (Tukey HSD test).

Initial genus + species	River basin	Latitude Longitude (DD)	Revised species (Burlakova et al. 2018; Johnson et al. 2018)	Mean wet mass (shell + soft tissue) grams (*SE*)
Cyclonaias houstonensis	Colorado River	29.555553 -96.40158	*C. pustulosa*	51.94 (2.80) B
	Navasota River	31.254239 -96.33057	*C. pustulosa*	35.39 (1.65) C
Cyclonaias petrina	Colorado River	29.555553 -96.40158	N/A	96.52 (5.83) A
		31.206155 -98.568903		
	Guadalupe River	29.434542 -97.37924	*C. necki*	35.84 (1.85) C

Table 1.2. Results of analysis of covariance (ANCOVA) for main effects and interactions of species location combination, wet mass (gWW), and temperature on resting metabolic rate (RMR_6), regulation index (RI) and critical dissolved oxygen concentration (DO_{crit}).

Effect	**RMR_6**			**RI**			**DO_{crit}**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Spp-Location	4	13.84	< **0.0001**	4	61.48	< **0.0001**	4	4.93	**0.001**
gWW	1	1.24	0.2674	1	0.05	0.8308	1	0.53	0.4697
gWW*Spp-Location	4	0.54	0.7084	4	2.36	0.0569	4	0.81	0.5212
Temp	1	405.4	< **0.0001**	1	7.85	**0.0059**	1	8.81	**0.0036**
Temp*Spp-Location	3	0.52	0.6661	3	0.92	0.4308	3	1.34	0.2657

Chapter 2: Impacts of acute and chronic heat stress on potential metabolic activity (PMA) of freshwater mussels from central Texas

Introduction

Aquatic organisms face chronic and acute thermal stressors in their natural habitat. Factors such as seasonal cycles result in gradual changes that allow organisms to slowly acclimate and adjust to rising temperatures. These changes include enzyme structure, enzyme quantity, and other critical changes (Randall et al. 1997; Simcic et al. 2014). Other factors such as altered flows (i.e. water withdrawals, retention of water by dams), heated discharges, and rapid dewatering regimes (i.e. stranding in isolated pools) may expose organisms to rapidly rising temperatures and acute heat shock. In contrast to chronic thermal stress, acute thermal stress exposes the existing enzymatic suite to rapidly rising temperatures with little to no time for acclimation.

Metabolic rates typically rise with increasing temperature and organisms must obtain ever-increasing quantities of oxygen to meet increased metabolic demand. Oxidation of the coenzyme Q-cytochrome b complex is thought to be the rate-limiting step that ultimately determines the maximum rate of oxygen consumption an organism can maintain (Fanslow et al. 2001). This potential metabolic activity (PMA) can be quantified using the electron transport system (ETS) assay (Packard et al. 1971, Fanslow et al. 2001) and has been examined in a range of aquatic invertebrates and fish (Zagar et al. 2018). PMA typically increases with increasing temperature, peaks, and then declines. Increasing enzymatic activity is primarily due to increased kinetic energy of substrate molecules. However, denaturation of enzymes also increases with increasing temperature. Peak enzymatic activity occurs at the temperature where increased substrate energy is counterbalanced by increased enzyme denaturation. Beyond this temperature, enzyme denaturation exceeds any further increases in substrate energy and enzyme activity begins to decline (Randall et al. 1997). Thus “optimal enzymatic temperatures” are optimal for

overall enzyme activity, but not necessarily for the enzymes themselves as denaturation may already be occurring at "optimal" temperatures. As the increase in PMA slows, stops, and then declines beyond the optimum, metabolic demand (as measured by respiration rate) often continues to increase (see Chapter 1). Thus, organisms are likely to experience a reduced ability to support increasing metabolic requirements as the enzymatic activity optimum is approached and any PMA increases are outpaced by the rate of increase in energy demand. In support of this scenario, previous studies have shown that the temperature which supports maximum potential scope for growth can be several degrees lower than that which supports maximum PMA (Simcic et al. 2014).

Unionid mussels make up a large portion of freshwater biodiversity but are in decline throughout the United States (Master et al. 2000). Unionids may be particularly impacted by acute and chronic thermal stress due to their limited mobility. Many species may already be living near their upper thermal tolerance limits (Pandolfo et al. 2010). In light of natural and anthropogenic drivers of temperature, management, and conservation of our remaining unionid fauna will require an understanding of how PMA responds to rapid as well as gradual changes in temperature.

In this study, we examined the effect of gradual and rapid temperature changes on the PMA of three mussel species native to central Texas. Our objectives were to:

1) Determine whether thermal performance curves of mussel respiratory enzyme systems differ among species and subpopulations with and without temperature acclimation.
2) Determine if gradual acclimation allows mussels to adapt to warming temperatures as evidenced by an increase in optimal enzymatic temperatures, an increase in optimal temperature breadth, and an increase in PMA.

Methods

Mussel Collection and Laboratory Acclimation

In 2017, *Cyclonaias pustulosa* were collected from the lower Colorado River near Altair, TX on April 28 and May 17, and from the Navasota River near Easterly, TX on July 17. *Cyclonaias petrina* were also collected from the lower Colorado River near Altair on April 28 and May 17, as well as from an additional site near Lometa, TX. *Cyclonaias necki* were collected from the Guadalupe River on August 17 and November 11 (Fig. 2.1).

Shipping methodology was adapted from the recommendations of the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (ASTM, 2013). Mussels were placed in coolers between layered moist cotton towels at field sites in preparation for shipments. Sufficient 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 of 18 °C at the receiving lab. All coolers were shipped overnight to the South Auburn Fisheries Research Station (SAFRS), Auburn University, Auburn, AL.

Upon arrival, mussels were tagged with 8 x 4 mm external Hallprint shellfish tags (Hallprint, Hindmarsh Valley, South Australia), measured (length), and placed in upwellers containing ~80 L of hard artificial freshwater (HAFW: 0.192 g $NaHCO_3$, 0.10 g $CaSO_4 \cdot 2H_2O$, 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); pH = 8.35, total hardness = 197.5 mg/L $CaCO_3$, and total alkalinity = 120 mg/L $CaCO_3$). Bacterial biofilters in upwellers were established for > 2 weeks prior to the arrival of experimental mussels using local mussels from ponds at the research station. Mussels in each upweller were fed 2 mL of Shellfish Diet 1800 (Reed Mariculture, Inc., Campbell, CA, USA) in the morning and 1 mL in the afternoon daily. Water quality (ammonia,

nitrites, and nitrates) was measured 3 times/week using either Tetra 6-in-1 and Ammonia Aquarium Test Strips (Spectrum Brands, Inc., Blacksburg, VA, USA) or API 5-in-1 and Ammonia Test Strips (MARS Fishcare, Inc., Chalfont, PA, USA). All newly arrived mussels were acclimated to the same initial holding conditions (18 °C and 12:12 L:D cycle) for > 2 weeks before being acclimated to experimental temperatures in order to minimize any differences associated with collection season.

Acclimated PMA

Following the laboratory acclimation, eight mussels per species or subpopulation were assigned with random number generation to each of six temperature treatments (15, 17, 23, 28, 32, and 36 °C). Assigned mussels were placed in insulated upwellers (~70 L) equipped with chillers (AquaEuroUSA, Gardena, CA, USA) and/or heaters (Finnex TH-300, Illinois, USA) with temperature control (4 mussels/species/cooler, 2 coolers per temperature treatment). Water temperatures were slowly adjusted up or down at a rate of 1 °C/day until the target temperature was reached, and mussels subsequently acclimated to the target temperature for > 1 week (Chen et al. 2001). During the temperature acclimation period, mussels were fed Shellfish Diet 1800 (Reed Mariculture, Inc., Campbell, CA, USA) twice daily (2 mL morning, 1 mL afternoon per ~70 L upweller) and held at a 12h light: 12h dark cycle. After the > 1 week acclimation period, respiration of each mussel was measured as described in Chapter 1.

Within 24 hours of each respirometry run, we randomly selected four mussels from each of the original six temperature treatments, gently pried their shells open, and collected two, ~10 mg tissue plugs from the foot of each mussel using a nasal biopsy tool (Karl Storz nasal biopsy tool #453733; Fritts et al. 2015). Tissue plugs were placed in cryovials and immediately frozen at

-80 °C. In order to increase the number of temperatures for the acclimated PMA study, we then randomly selected two mussels remaining in each temperature, transferred them to a temperature-controlled upweller, and assigned them to a new acclimation temperature of 20, 25, or 30 °C. Temperatures were adjusted at a rate of 1 °C/day until the target temperature was reached. Mussels were then acclimated for >1 week at the new temperatures. Tissue plugs were collected and stored in the same manner as described previously. In total, we collected tissue plugs from approximately four mussels for each species and subpopulation acclimated for >1 week to each of 9 temperatures (15, 17, 20, 23, 25, 28, 30, 32, 36 °C). Due to scarcity of animals in the field and collection permit limitations, numbers of *C. necki* at each temperature were slightly less than those of the other species and subpopulations.

PMA of mussels was measured using standard methodologies adapted from Packard et al. (1971) and Simcic et al. (2014). Frozen tissue plugs collected from a single mussel were weighed and placed in a self-standing, nominal 5 ml sample tube (Globe Scientific, Inc., Mahwah, NJ, USA, via VWR# 89497-730: note that each vial actually held up to 7 ml). Each tube was filled to the 4 mL mark with 1.0 mm diameter glass beads (BioSpec Products, Inc., Bartlesville, OK, USA, Cat. No. 11079110) and 4 mL of homogenization buffer (0.1 M sodium phosphate buffer pH = 8.4; 75 μM $MgSO_4$; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (v/v) Triton-X-100) was added. Tissue was then homogenized with a BeadBeater (MiniBeadBeater-24; BioSpec Products, Inc., Bartlesville, OK, USA) for 1 minute and chilled for 1-2 minutes in a freezer to compensate for heating during the bead-beating process. The bead-beating/chilling cycle was repeated for 3-4 cycles until the tissue was thoroughly homogenized. The vial was then centrifuged for 4 minutes, at 10000 rpm, at 0 °C in a refrigerated centrifuge (Allegra X-30R, Beckman Coulter, Brea, CA, USA). Homogenate generated from a given mussel was placed in a flask, diluted to 2.5 mg

tissue/mL using reagent grade DI water (RICCA, cat# 9150-1), mixed with a stir bar, distributed amongst 2 mL vials (Eppendorf® Safe-Lock microcentrifuge tubes) and frozen at -80 °C. PMA measurements were completed within 6 weeks of tissue homogenization date.

To measure PMA, two replicate, 0.5 mL subsamples of thawed homogenate were each incubated in 1.5 mL substrate solution (0.1M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (v/v) Triton-X-100) with 0.5 mL INT solution (2.5 mM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride) for 30 minutes, in the dark, at the temperature at which the mussel had been acclimated. The reaction was then stopped by adding 0.5 mL of stopping solution (Formalin: H_3PO_4 = 1:1). A blank for the replicate samples was made by combining 1.5 mL substrate solution with 0.5 mL INT solution, incubated, and then the reaction was stopped along with the samples. Following addition of stopping solution, 0.5 mL of the corresponding homogenate was added to the blank. Absorbance (490 nm) of the replicate samples was measured with a spectrophotometer (Genesys 10S UV-VIS, ThermoScientific, Waltham, MA, USA) and corrected for absorbance of the blank. PMA was calculated according to the following formula (Kenner and Ahmed 1975):

$$\text{PMA (ml } O_2 \text{ g}^{-1} \text{ WW h}^{-1}) = ((ABS^{490nm} \times V_h \times V_r \times 60)/(V_a \times S \times t \times 1.42)) / 1000,$$

where ABS^{490nm} is the absorbance of the sample corrected for blank; V_h is the volume of the homogenate (4 mL) prior to removal of subsamples; V_r is the volume of the reaction mixture (homogenate subsample + substrate solution + INT solution + stopping solution = 3mL); V_a is the volume of the homogenate subsample (0.5 mL); S is the mass of the tissue sample (g); t is the

incubation time (30 min); 60 is a correction factor to convert the rate to hours, and 1.42 is the factor for conversion to volume O_2.

Non-acclimated PMA

Following respiration experiments, two mussels were randomly selected from each of six experimental temperatures, placed in temperature-controlled upwellers, and brought to 21 °C at a rate of 1 °C/day. All mussels were held at 21 °C for at least 1 week. Following the > 1 week holding period, each mussel was sacrificed by severing the adductor muscles with a scalpel and opening the shell. Approximately 100 mg of tissue was immediately collected from the foot of each mussel using a nasal biopsy tool. Tissue plugs were placed in cryovials and immediately frozen at -80 °C. Tissue was subsequently homogenized as previously described. Enzymes extracted from two replicate samples from each mussel were exposed for 30 minutes to each of nine temperatures (15, 17, 20, 23, 25, 28, 30, 32, and 36 °C) to which they had not been acclimated. PMA at each temperature was estimated using the previously described methodology.

Data analyses

PMA data were plotted against temperature for each group and acclimation type to create a composite thermal performance curve (TPC; SigmaPlot 13.0). For the acclimated TPCs, each data point represented a unique individual only acclimated to one temperature. For the non-acclimated TPCs, each data point at a given temperature represented a unique individual, but the same individuals were represented at each temperature. Each composite curve was fitted with a

quadratic regression. If normality assumptions were violated, the quadratic regression was fitted to log-transformed PMA data.

Because a single, composite dataset was generated for each species-location × acclimation method combination, we used nonparametric bootstrapping (Efron and Tibshirani 1994; R 3.5.1) to compare between species-location groups and acclimation type similarly to Bender et al. 1996. Random subsamples with replacement (Bootstrapping) of size *n* – original sample size for each group and acclimation type – from each of the original composite curves were taken and fit with quadratic regressions to estimate PMA endpoints. Bootstrapping was repeated and produced 1000 iterations of the endpoints for each group and acclimation method. Endpoints were T_{opt} (the temperature which exhibited the highest PMA), T_{lower} (the lowest temperature where PMA was within 90% of its peak value), $T_{breadth}$ (T_{lower} subtracted from T_{opt}), and PMA_{max} (maximum PMA value; Fig. 2.2). Pairwise 95% confidence intervals (95% CI) of differences were estimated for each PMA endpoint by using bootstrap resampling procedures. Statistically significant differences in PMA endpoints within each acclimation method and differences between acclimated and non-acclimated methods within a single population were concluded when 95% CI did not include zero.

Results

Composite thermal performance curves for each acclimation type and group are shown in Figure 2.3. When not acclimated to each individual temperature, T_{opt} of *C. pustulosa* (Colorado) was significantly higher than *C. pustulosa* (Navasota) and *C. necki. C. petrina* was also significantly higher than *C. necki*. T_{lower} of *C. petrina* and *C. pustulosa* (Colorado) was significantly higher than *C. pustulosa* (Navasota) and *C. necki.* For $T_{breadth}$, *C. pustulosa* (Colorado), *C. pustulosa*

(Navasota), and *C. necki* were significantly higher than *C. petrina*. When acclimated to individual temperatures, T_{opt} of *C. pustulosa* (Colorado) and *C. petrina* were significantly higher than *C. pustulosa* (Navasota) but not *C. necki.* The same trend followed with T_{lower} where *C. petrina* and *C. pustulosa* (Colorado) were significantly higher than *C. pustulosa* (Navasota) but not *C. necki*. No significant differences were found between groups for $T_{breadth}$ for acclimated individuals (Table 2.1).

T_{opt} estimates were significantly higher for acclimated than non-acclimated *C. pustulosa* (Colorado) and *C. petrina*. T_{lower} and $T_{breadth}$ were not significantly different between acclimated and non-acclimated mussels for any group (Table 2.1; Fig. 2.4). PMA_{max} was significantly higher for acclimated than non-acclimated individuals for *C. pustulosa* (Colorado) and *C. petrina*, but lower for *C. pustulosa* (Navasota) and *C. necki* (Table 2.2; Fig. 2.5).

Discussion

Freshwater mussels are among the most threatened aquatic taxa in the United States (Master et al. 2000). Climate change and other thermal stressors present threats to mussel populations due to low mobility and reduced ability to move to thermal refuges. Since heat stress can be present as acute heat-shock (dewatering regimes, heated discharges, etc.) or a gradual increase in temperature (seasonal changes), it is important to understand whether mussel physiological responses differs to rapid, as opposed to gradual, changes in temperature. For natural resource managers, it is particularly important to determine whether response patterns are similar amongst species and subpopulations – allowing for one-size-fits-all management strategies – or differs amongst taxa and subpopulations – requiring management strategies tailored to specific groups.

Results of this study show that the response of respiratory enzyme systems to acute increases in temperature can differ among mussel subpopulations as well as species. *C. petrina* appeared to be a high-temperature specialist relative to other taxa. The respiratory enzymes of *C. petrina* were more stenothermal than *C. necki* or the *C. pustulosa* (Navasota) subpopulation, as evidenced by a significantly smaller $T_{breadth}$. Conversely, T_{lower} (the lowest temperature at which PMA is within 90% of its maximum rate) was significantly higher for *C. petrina* than either *C. necki* or *C. pustulosa* (Navasota) and T_{opt} was significantly higher than *C. necki*. Enzymes of the *C. pustulosa* (Colorado) subpopulation also appeared to be more thermally tolerant than *C. necki* or the *C. pustulosa* (Navasota) subpopulation – as evidenced by significantly higher T_{lower} and T_{opt} – but were more eurythermal than *C. petrina* – as evidenced by a significantly greater $T_{breadth}$. Taken as a whole, evidence suggests that the respiratory enzymes of *C. petrina* are more tolerant of rapid temperature changes than *C. necki*. Similarly, respiratory enzymes of the *C pustulosa* (Colorado) subpopulation were more tolerant of rapid temperature changes than the *C. pustulosa* (Navasota) subpopulation.

We hypothesized that exposure to gradual increases in temperature would result in an increase in T_{opt}, $T_{breadth}$, and T_{lower} for all mussel populations – indicating the ability to adapt to, and better tolerate, thermal stress following acclimation. However, evidence of the ability of mussel enzymatic systems to adapt to gradual increases in temperature in terms of these parameters was absent or inconsistent. None of the four populations exhibited a significant increase in $T_{breadth}$ or T_{lower} following acclimation. Only *C. petrina* and the *C. pustulosa* (Colorado) subpopulation showed a significant increase in T_{opt}.

The most consistent effect of acclimation was a change in the PMA at T_{opt}. Because energetic demand increases with temperature and PMA represents the maximum metabolic (i.e.

respiration) rate of an organism, an increase in PMA would represent an increase in the ability of an organism to deal with an increased metabolic rate. The two populations (*C. petrina* and *C pustulosa* Colorado) that exhibited an increase in T_{opt} following acclimation also exhibited an increase in PMA. However, the two populations (*C. necki* and *C. pustulosa* Navasota) that showed no increase in T_{opt} exhibited a significant decrease in PMA following acclimation. Taken as a whole, these results indicate that while the enzyme systems of *C. petrina* and the *C. pustulosa* (Colorado) subpopulation were able to adapt to slowly increasing temperatures, the enzymes systems of *C. necki* and the *C. pustulosa* (Navasota) subpopulation were not only unable to adapt but may have been damaged by chronic exposure (i.e. acclimation) to high temperatures.

Using the concept of aerobic scope, we can hypothesize potential effects of these changes in enzymatic thermal optima and PMA with acclimation as it relates to mussel fitness and energy distribution, although aerobic scope must be viewed cautiously (Clark et al. 2016). Aerobic scope is generally considered the difference between the maximum respiration rate an organism is capable of and minimum rate required for basic metabolic functions. In our study, PMA represents the maximum rate and RMR represents the minimum rate. The oxygen- and capacity-limited hypothesis states that growth, reproduction, and movement are maximized at the temperature where an organism exhibits the greatest aerobic scope and decrease above and below this temperature (Sokolova et al. 2012; Clark et al. 2016). Assuming respiration rate rises linearly with temperature up to 36°C (demonstrated in chapter 1) for *C. pustulosa* (Colorado) and *C. petrina*, the observed increase in PMA and T_{opt} following acclimation would increase aerobic scope at warmer temperatures. In contrast, *C. necki* exhibited no change in T_{opt} and a decrease in PMA following acclimation, which would decrease aerobic scope. *C. pustulosa* (Navasota) also

had no change in T_{opt} and a decrease in PMA, but its RMR leveled off at ~30°C (see Fig. 1.4, Chapter 1) – potentially offsetting loss in aerobic scope due to a declining PMA. Thus, we would predict that negative impacts of chronic thermal stress on growth and reproduction would be greatest for *C. necki* as it was the only species that did not exhibit a mechanism to increase aerobic scope after prolonged exposure to thermal stress. It is important to note that these scenarios rely on the assumptions that RMR of unacclimated individuals followed the same pattern as observed for acclimated individuals in Chapter 1 or increased at a faster rate. Further research relating PMA and RMR of both acclimated and non-acclimated individuals to aerobic scope, growth, and reproduction is necessary to provide conclusive evidence confirming these hypotheses.

Similar to aerobic scope, increasing attention has been focused on development of adverse outcome pathways that connect effects of stressors at multiple organization levels from cellular to community. Future research is needed to link effects of thermal stress on mussel enzymatic systems with those at the individual level. For example, loss of equilibrium and muscle spasms are often used to define upper thermal limits at the organismal level (Vinagre et al. 2015; Westhoff and Rosenberger 2016) in many taxa, and loss of equilibrium has been recommended as an important endpoint to link with respiratory stress. Recent work in our lab has shown that loss of equilibrium in crayfish occurs at the upper optimal temperature for PMA in unacclimated animals (Abdelrahman et al. unpublished data). Loss of equilibrium is not a viable endpoint for unionid mussels, but gaping behavior and valve closure strength of bivalves has been linked with thermal stress (Galbraith et al. 2012) and overall health (Aoki et al. 2010). These endpoints may hold promise as ways to link thermal performance of unionid respiratory enzymes to indicators of thermal stress at the individual level.

In summary, the response of respiratory enzymes and PMA varied across species and subpopulations tested. *C. pustulosa* (Colorado) and *C. petrina* appear to favor and adapt best to slow, gradual temperature changes as evidenced by an increase in thermal optima and PMA with acclimation. Contrarily, these chronic temperature changes (i.e. acclimation) may have a negative effect on *C. pustulosa* (Navasota) and *C. necki*, demonstrated by no change in thermal optima and a decrease in PMA with acclimation. These results may have implications for changes in aerobic scope, growth, and reproduction for these mussels. Overall, a lack of a fixed enzymatic response to acute or chronic heat stress, as well as inconsistent effects of acclimation on mussels tested in this study, further demonstrate the importance of understanding and testing species-specific responses to thermal stress to better manage and protect unionids as a whole.

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Figures and Tables

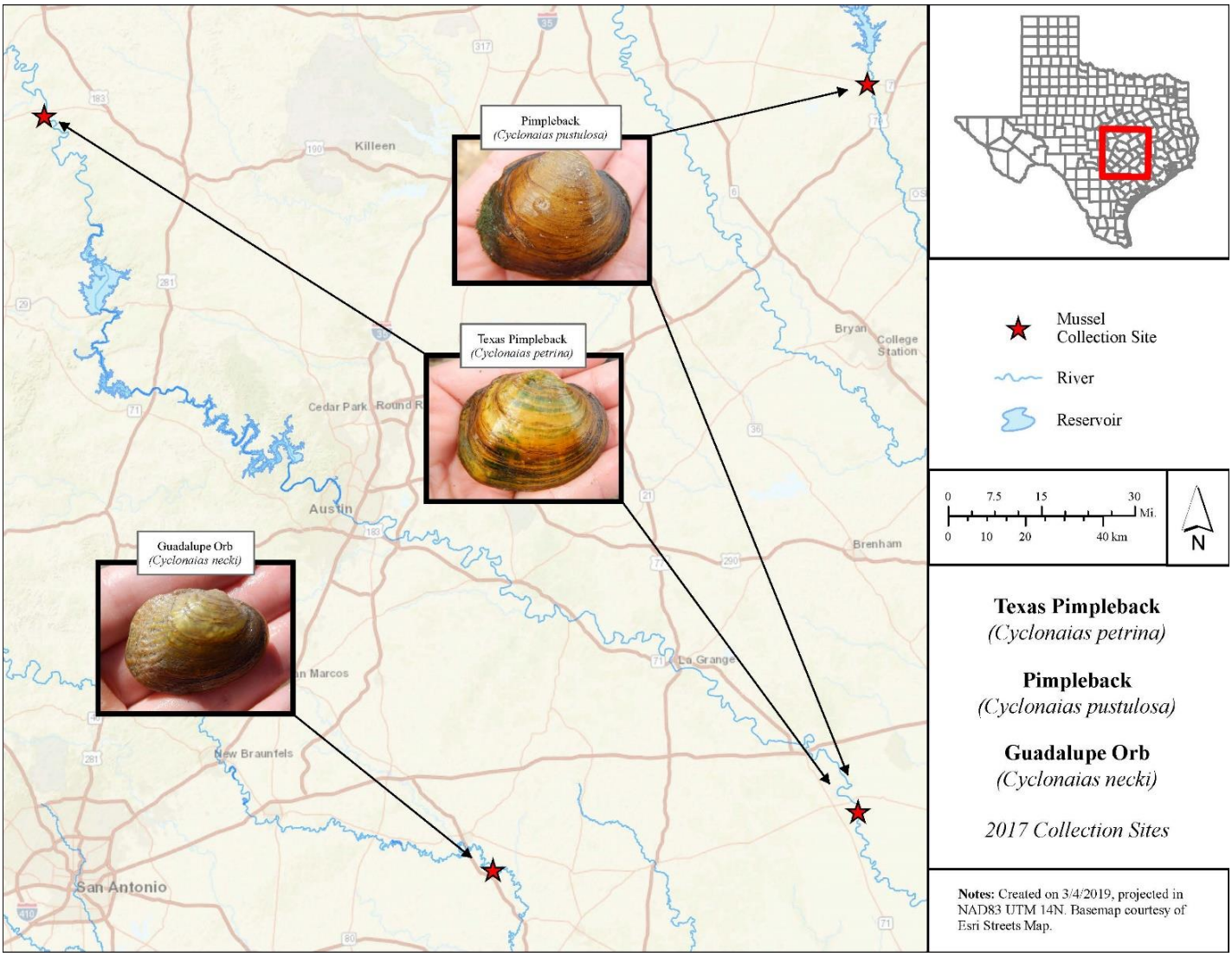

Figure 2.1. Collection sites for *Cyclonaias pustulosa*, *Cyclonaias petrina*, and *Cyclonaias necki* throughout central Texas.

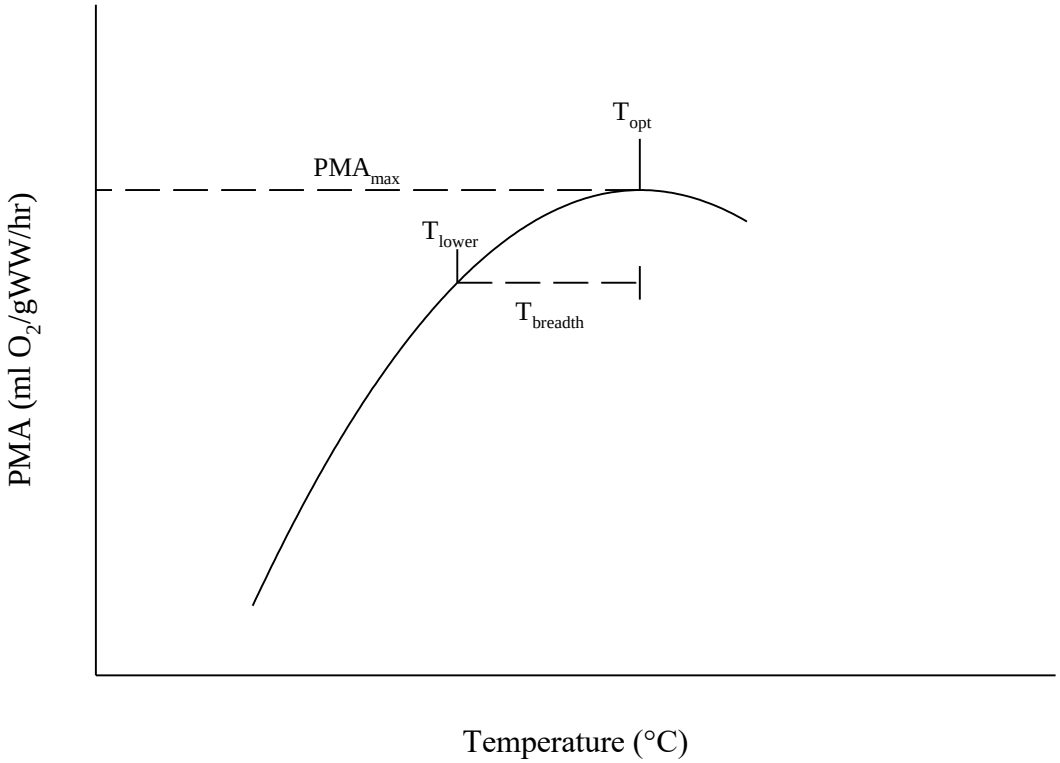

Figure 2.2. Theoretical thermal performance curve (TPC) showing hypothetical potential metabolic activity (PMA) endpoints of enzymatic thermal optimum (T_{opt}), lower enzymatic thermal optimum (T_{lower}), enzymatic thermal optimum range ($T_{breadth}$), and maximum PMA (PMA_{max}) on a quadratic regression.

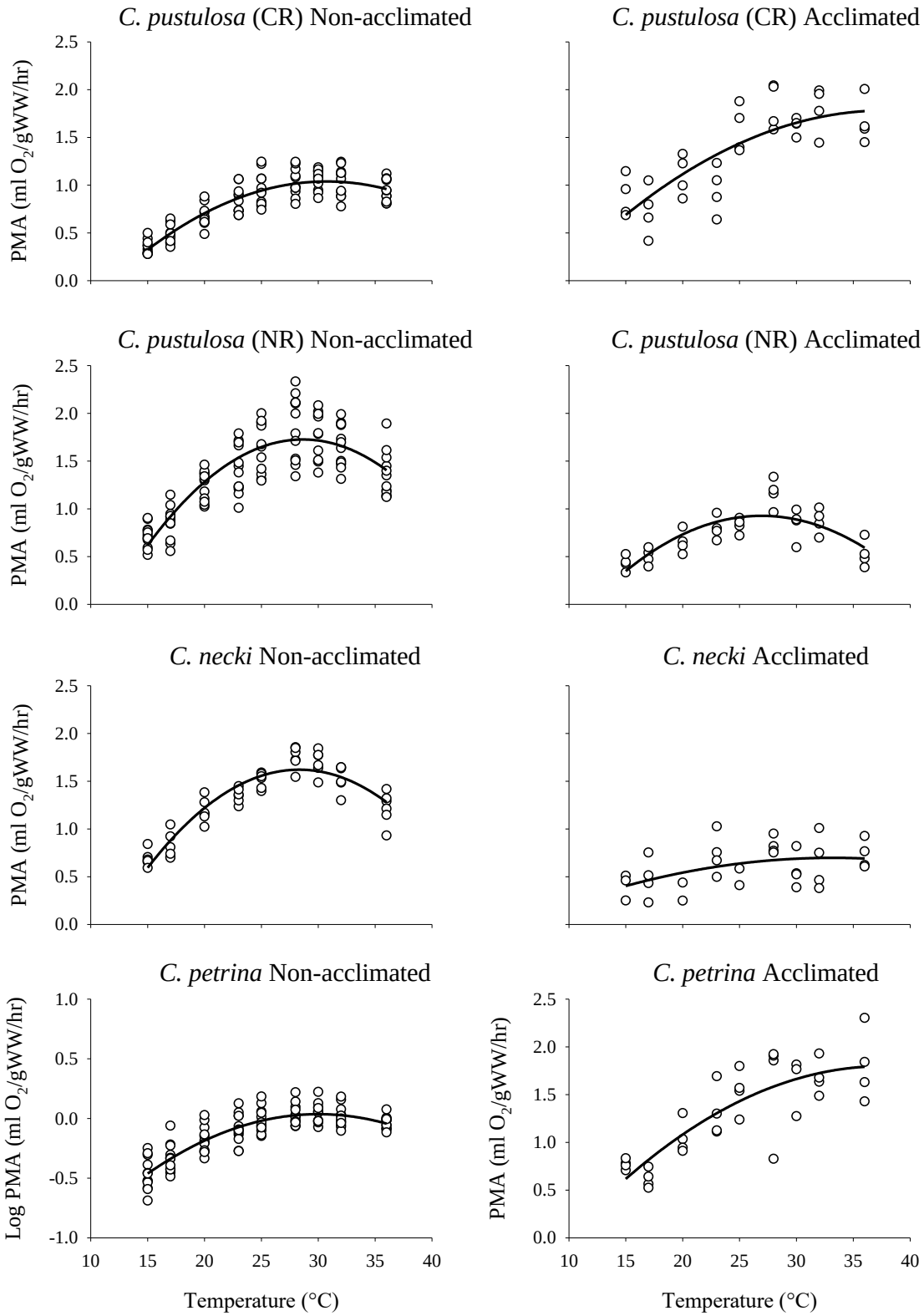

Figure 2.3. Composite thermal performance curves for each group and acclimation method fitted with quadratic regressions. Each dot represents the mean potential metabolic activity (PMA) for an individual at each temperature. For non-acclimated curves, each mussel is represented at every temperature whereas each dot is a unique individual for acclimated curves.

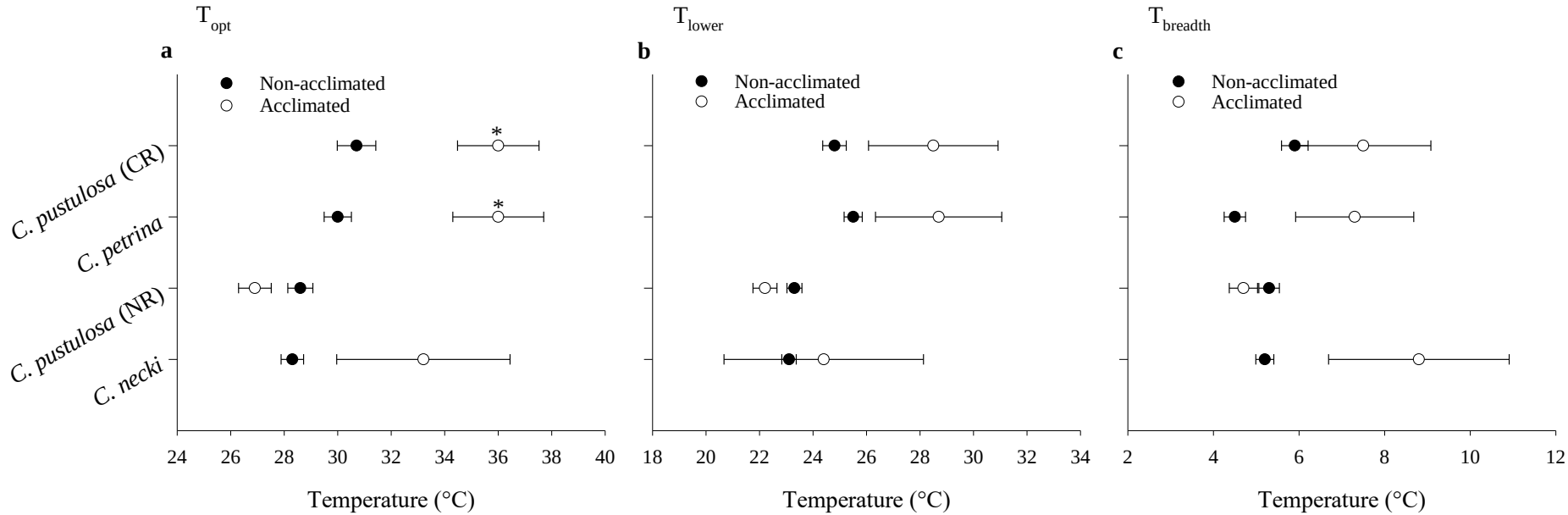

Figure 2.4. Shifts in a) enzymatic thermal optima (T_{opt}), b) lower enzymatic thermal optima (T_{lower}), and c) enzymatic thermal optima range ($T_{breadth}$) for non-acclimated and acclimated mussels. Asterisks indicate significant changes in endpoints between acclimated and non-acclimated mussels within each population tested.

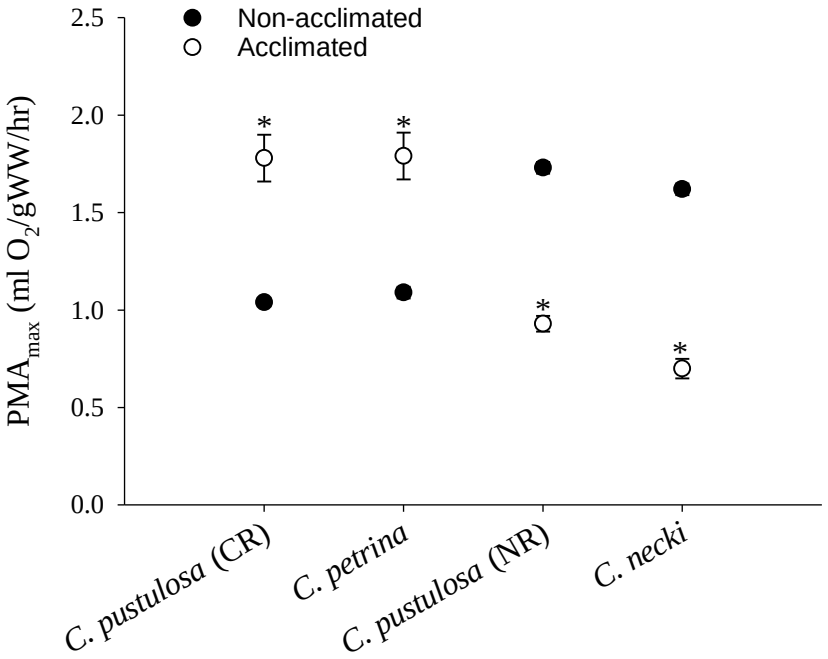

Figure 2.5. Maximum potential metabolic activity (PMA) of non-acclimated and acclimated mussels from each population tested. Asterisks indicate significant changes in PMA between non-acclimated and acclimated mussels.

Table 2.1. Estimates for non-acclimated and acclimated potential metabolic activity (PMA) endpoints from quadratic regressions of bootstrapped data. Standard errors around the estimates are bootstrapped. Comparative 95% confidence intervals were used to determine significant differences between populations within populations. Different letters represent statistically significant differences between populations in the same column at $P < 0.05$ (Tukey HSD test). Asterisks indicate significant differences in endpoints between non-acclimated and acclimated mussels within the same population. Difference estimates were determined from bootstrapping and only shown where there was a significant difference between non-acclimated and acclimated endpoints. CR= Colorado River. NR= Navasota River.

	Population	Non-acclimated (± *SE*)	Acclimated (± *SE*)	Difference	
				P	**Estimate (± *SE*)**
T_{opt} (°C)	*C. pustulosa* CR	30.7 (0.72)^{a}	36.0 (1.52)^{a}	0.03*	5.26 (1.64)
	C. petrina	30.0 (0.51)^{ab}	36.0 (1.70)^{a}	0.026*	5.99 (1.78)
	C. pustulosa NR	28.6 (0.47)^{bc}	26.90 (0.61)^{b}	0.962	–
	C. necki	28.3 (0.42)^{c}	33.2 (3.24)^{ab}	0.26	–
T_{lower} (°C)	*C. petrina*	25.5 (0.34)^{a}	28.7 (2.36)^{a}	0.108	–
	C. pustulosa CR	24.8 (0.44)^{a}	28.5 (2.42)^{a}	0.072	–
	C. pustulosa NR	23.3 (0.28)^{b}	22.20 (0.45)^{b}	0.968	–
	C. necki	23.1 (0.27)^{b}	24.4 (3.73)^{ab}	0.62	–
$T_{breadth}$ (°C)	*C. pustulosa* CR	5.9 (0.31)^{a}	7.5 (1.58)^{a}	0.65	–
	C. pustulosa NR	5.3 (0.24)^{a}	4.70 (0.33)^{a}	0.906	–
	C. necki	5.2 (0.21)^{a}	8.8 (2.11)^{a}	0.304	–
	C. petrina	4.5 (0.25)^{b}	7.3 (1.38)^{a}	0.31	–

Table 2.2. Estimates for non-acclimated and acclimated maximum potential metabolic activity (PMA_{max}) from quadratic regressions of bootstrapped data. Difference estimates are determined from bootstrapping. Standard errors around the estimates are bootstrapped. Comparative 95% confidence intervals were used to determine significant differences between acclimation type within a population ($P < 0.05$). Asterisks indicate significant differences in endpoints between non-acclimated and acclimated mussels within the same population. CR= Colorado River. NR= Navasota River.

	PMA_{max} (ml O_2/gWW/hr)			
Population	**Non-acclimated (± *SE*)**	**Acclimated (± *SE*)**	**Difference**	
			P	**Estimate (± *SE*)**
C. pustulosa NR	1.73 (0.03)	0.93 (0.04)	< 0.002*	-0.80 (0.05)
C. necki	1.62 (0.03)	0.70 (0.05)	< 0.002*	-0.92 (0.06)
C. petrina	1.09 (0.03)	1.79 (0.12)	< 0.002*	0.70 (0.13)
C. pustulosa CR	1.04 (0.02)	1.78 (0.12)	< 0.002*	0.74 (0.12)