Effects of Hypoxia and Thermal Stress on a Rare Freshwater Mussel (Order: Unionida) in Relation to Sympatric and Allopatric Mussel Species

by

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Abstract

Pleurobema riddellii is proposed for federal listing as threatened. Remaining populations are largest in Texas and Louisiana. Respirometry was used to compare effects of temperature on hypoxia sensitivity of *P. riddellii* with sympatric and allopatric species. No detectable effect of temperature on hypoxia sensitivity was observed in *P. riddellii* under the conditions tested. Interspecific variation in ability to regulate respiration rate as dissolved oxygen declined was greater at 25°C than at 32°C. *Pleurobema riddellii* exhibited a lower critical thermal maximum and a steeper metabolic response to temperature than a widespread, sympatric species, *Cyclonaias pustulosa*. Behavioral responses to thermal stress varied among species and generally occurred near upper tolerance limits. Evaluating species-specific physiological responses to stressors is critical for assessing whether protective thresholds for mussels are suitable across species within a local community or region, or whether thresholds should be developed for individual species. This is particularly important considering climate change.

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In the preparation of this thesis, no Artificial Intelligence (AI) tools were used.

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AICc	Bias-corrected Akaike information criterion
AUC	Area under curve
CTM	Critical thermal maximum
DO	Dissolved oxygen
DO_{crit}	Critical dissolved oxygen
EWSFC	E. W. Shell Fisheries Center
gWWW	Grams whole wet weight
HAFW	Hard artificial freshwater
IQR	Interquartile range
LNVA	Lower Neches Valley Authority
Q_{10}	Temperature coefficient
RI	Regulation index
RO/DI	Reverse osmosis/deionized
SE	Standard error

Chapter 1: Effects of temperature and species on hypoxia tolerance in sympatric and allopatric, threatened and common species of freshwater mussels

INTRODUCTION

Freshwater mussels (Order Unionida) are among the most imperiled faunal groups in North America, with an estimated 65–70% of species considered endangered, threatened, or of conservation concern (Strayer et al., 2004; Haag, 2012). Long-term declines have been driven by multiple anthropogenic stressors, including sedimentation, invasive species, hydrological alteration, water quality deterioration, and other forms of habitat degradation (Vaughn & Taylor, 1999; Haag, 2012). More recently, global climate change has emerged as an intensifying threat to mussel persistence by altering temperature regimes, flow variability, and dissolved oxygen (DO) dynamics within aquatic systems (Embke et al., 2026).

Of particular concern is the increasing frequency of combined thermal and hypoxic stress, conditions expected to intensify under climate change and eutrophication. Elevated temperatures directly affect both environmental oxygen availability and organismal oxygen demand because, as water warms, DO solubility decreases, while metabolic rate and oxygen consumption increase (Pörtner & Knust, 2007). This creates a dual constraint on aerobic performance, narrowing the aerobic window available for essential functions such as growth and reproduction (Pörtner, 2010). Hypoxic events are common in shallow rivers, engineered channels, and eutrophic systems (Sparks & Strayer, 1998; Galbraith et al., 2012), suggesting that future climatic and land use trends may exacerbate oxygen limitation across mussel habitats. Unionids, which are often sedentary and rely on aerobic metabolism, may be particularly vulnerable to these interactions. While some species have evolved adaptations to low-oxygen

environments (Sparks & Strayer, 1998), others, especially those with restricted ranges or limited plasticity, may lack these capabilities.

Unionid mussels exhibit a range of behavioral and physiological responses to thermal and hypoxic stress, including reduced activity, valve closure, metabolic depression, and anaerobic metabolism (Haney et al., 2020; Sparks & Strayer, 1998). These responses can prolong survival during short-term hypoxia but may be unsustainable if oxygen limitation persists or is combined with thermal stress. Although several studies have examined single-stressor effects of temperature or oxygen on mussels, relatively few have quantified their interactive effects on metabolism, despite their likely ecological relevance. Therefore, integrative physiological studies are critical for understanding how mussels respond to multiple stressors and for predicting which species are most at risk in a warming, increasingly hypoxic world.

A useful framework for examining these stress responses lies in conservation physiology, which seeks to link physiological performance to environmental constraints and population persistence (Cooke et al., 2013). Within this context, two metrics have become especially valuable for quantifying hypoxia sensitivity in aquatic animals: the critical dissolved oxygen concentration (DO_{crit}) and the regulation index (RI; Haney et al., 2020). The DO_{crit} represents the oxygen level below which aerobic metabolism can no longer be sustained, forcing a transition to anaerobic respiration (Rogers et al., 2016). The DO_{crit} is typically estimated from respirometry by identifying the breakpoint where oxygen consumption begins to decline proportionally with ambient DO. Individuals with higher DO_{crit} values are thought to be more sensitive to oxygen limitation.

The DO_{crit} alone, however, cannot always capture the full complexity of metabolic response. The regulation index (RI) provides complementary information, describing the extent

to which an organism regulates its metabolic rate as oxygen declines. The RI is calculated using an area under the curve comparison between observed metabolism and theoretical regulator and conformer curves (Mueller & Seymour, 2011). Values near 1 indicate strong metabolic regulation (aerobic independence), whereas values near 0 indicate metabolic conformity (metabolism declines proportionally with DO). Because RI integrates the shape of metabolic response over the entire hypoxia trial, it is particularly useful when combined with DO_{crit}. Together, these indices provide a quantitative means of comparing hypoxia tolerance among species, life stages, or environmental conditions.

The interaction between temperature and oxygen availability can be understood through the oxygen and capacity-limited thermal tolerance (OCLTT) framework, which proposes that thermal limits arise when oxygen supply cannot meet rising metabolic demand (Pörtner, 2010). Though this framework has limitations, testing its concepts with freshwater animals can help explain how temperature and oxygen may or may not interact to define physiological limits across taxa (McBryan et al., 2013). Recent work has revealed that some freshwater mussels, like *Cyclonaias petrina*, exhibit increased sensitivity to hypoxia at higher temperatures, while others, such as *Cyclonaias pustulosa* (syn. *Pustulosa pustulosa*), show more thermal resilience (Haney et al., 2020).

Despite recent advances, many mussel species of conservation concern lack basic physiological data. *Pleurobema riddellii*, the Louisiana pigtoe, is a narrow-range endemic proposed for listing under the US Endangered Species Act. The species persists primarily in the Neches and Sabine River drainages of Texas and Louisiana (USFWS, 2023). One of the strongest remaining populations occurs within the Lower Neches Valley Authority (LNVA) canal system in southeastern Texas. Survey records and environmental assessment materials

document that the LNVA canal supports a substantial *P. riddellii* population and that mussels in this system have required relocation events during canal maintenance activities (USFWS, 2022). In 2019, BIO-WEST, Inc. conducted the primary mussel surveys and relocations within the LNVA canal, identifying *P. riddellii* as the most abundant state-threatened species (BIO-WEST, 2019).

Additional evidence from regional monitoring reveals that *P. riddellii* populations in the Neches River have been tracked for abundance and survival using multi-year mark-recapture approaches (Ford et al., 2022), verifying that the Neches River basin is likely a critical stronghold in the species' current range. However, the LNVA canal system is an engineered waterway subject to hydrologic manipulation and periodic dewatering, which are conditions that may expose mussels to elevated thermal and hypoxic stress (USFWS, 2022). No studies have yet characterized the thermal-hypoxic physiology of *P. riddellii*, and its sensitivity to hypoxia relative to sympatric and allopatric species remains unknown.

To address this knowledge gap, we quantified hypoxia responses of adult *P. riddellii* collected from the LNVA canal at two ecologically relevant temperatures (25 and 32°C). We compared these responses with those of sympatric *Cyclonaias pustulosa*, a widely distributed species collected from the same LNVA canal site. Additional comparisons were made with allopatric species, including *Fusconaia iheringi* (federally endangered) and *Lampsilis straminea* (common Gulf endemic), as well as species from Haney et al. (2020) and Pieper et al. (2025), to see where *P. riddellii* falls among other taxa that our lab has analyzed using the same methodology.

We hypothesized that sensitivity to hypoxia increases with temperature in all species, reflected by higher DO_{crit} and lower RI at 32°C. Additionally, we hypothesized that threatened or

geographically restricted species would exhibit lower thermal-hypoxic tolerance than widespread congeners. By integrating physiological and comparative data, this study advances conservation physiology for freshwater mussels and provides insight into environmental constraints affecting *P. riddellii* persistence under climate driven change.

METHODS

Mussel collection and laboratory acclimation

Pleurobema riddellii were collected from the LNVA canal in the lower Neches River drainage, Texas, on September 19, 2022, when canal temperatures were ~28.5°C. The mussels were promptly shipped to the EW Shell Fisheries Center (EWSFC) at Auburn University using protocols adapted from the guidelines in the *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels* (ASTM, 2013). Mussels were sandwiched between wet cotton towels with enough evenly spaced ice packs to maintain an intermediate temperature between the collection site (~28.5℃) and the initial holding system (18°C) in the lab. Fifty-three adult mussels were shipped overnight to EWSFC, but shipping was delayed one day, and they arrived on September 21, 2022. The internal temperature of the cooler was 25°C upon arrival and two mussel mortalities were noted. Surviving mussels appeared to be in good health and no additional mortality was observed in the subsequent two weeks after arrival.

Twenty-five *Cyclonaias pustulosa* were collected from the same canal site in the lower Neches River drainage on November 6, 2023, when canal temperatures were ~20°C, and shipped overnight to EWSFC. The mussels arrived the next morning with a cooler temperature of 15°C and no mortalities. No mortalities were recorded in the two weeks post arrival. The sexes of *P.*

riddellii and *C. pustulosa* individuals were not known because neither species is sexually dimorphic.

Data for the additional species included in comparisons were available for *Lampsilis straminea* (pond population; Auburn, AL), *Fusconaia iheringi* (Brazos River, TX), *Popenaias popeii* (Black River, NM), *Cyclonaias petrina* (Colorado River, TX), and *Cyclonaias necki* (Guadalupe River, TX). See Pieper et al. (2025) for information on the collection and holding of *P. popeii* and Haney et al. (2020) for methodology regarding the collection and holding of *C. petrina* and *C. necki*.

Twenty-one *Lampsilis straminea* were collected from three ~0.1-hectacre research ponds at EWSFC in Auburn, AL, from May 5 to May 12, 2023, when pond temperatures were between 21 and 26°C. *L. straminea* is sexually dimorphic, and only male individuals were collected and used in analyses to avoid potential effects of brooding on physiological data.

Six federally endangered *Fusconaia iheringi* individuals were collected from the Brazos River, Texas, on May 8, 2024, and shipped overnight under identical procedures to those described earlier, arriving without delay or mortality. The temperature of the cooler was 22.7°C upon arrival. Clear sexual dimorphism of *F. iheringi* has not yet been described, but one of the six individuals was noted as gravid in the collection data provided with shipping.

Mussels were weighed (gWWW), tagged with 8 x 4 mm external Hallprint shellfish tags (Hallprint Hindmarsh Valley, South Australia), and housed in upwellers containing ~95 liters of hard artificial freshwater (Smith et al., 1997; 151 L RO/DI water, 29.07 g sodium bicarbonate, 15.14 g calcium sulfate, 15.14 g calcium chloride, 9.08 g magnesium sulfate, 1.21 g potassium chloride; pH = 8.37, total hardness = 210 mg/L $CaCO_3$, and total alkalinity = 115 mg/L $CaCO_3$).

Upweller design was similar to Pieper et al. (2025). Bacterial biofilters were established in the upwellers using local *L. straminea* collected from ponds at EWSFC, which were held and fed in upwellers for a minimum of two weeks prior to receiving study animals.

Upwellers were dose-fed LPB™ Frozen Shellfish Diet every hour for 24 hours at a rate that maintained 30,000 cells/mL (± 5,000 cells/mL) using automated feeders (GHL Doser 2.2, GHL USA LLC, Wilmington, North Carolina). This algal concentration is based on values previously established in our laboratory for unionid care, which was selected to avoid pseudofeces production and was confirmed by the absence of pseudofeces. Water quality parameters in the upwellers (ammonia, nitrite, nitrate, hardness, alkalinity, and pH) were monitored two days per workweek using commercial ammonia test strips and 6-in-1 test strips, and once per week using a more precise YSI 9500 photometer.

Water changes were triggered when nitrates exceeded ~40 mg NO_3-N/L, which was chosen as a conservative threshold to remain below concentrations associated with sublethal effects on freshwater mussel early life stages (~56 mg NO_3-N/L; Moore & Bringolf, 2018). Ammonia was kept below 0.3 mg TAN/L, following chronic protective guidance based on freshwater mussel toxicity studies done by the U.S. Environmental Protection Agency (EPA; US EPA, 2013). Nitrite concentrations were maintained at undetectable levels. Hardness was maintained at or above 100 mg/L $CaCO_3$, and alkalinity was maintained at or above 80 mg/L $CaCO_3$, consistent with water quality at their collection site and U.S. EPA guidance for protection of freshwater aquatic life (US EPA, 1986). The pH was maintained between 7.0 and 8.5 to comply with general freshwater criteria (US EPA, 2013).

Animals were held at 18°C for a minimum of two weeks before acclimation to experiment temperatures. Then, mussels were randomly assigned to one of two temperature

groups (25 and 32°C). Temperatures were adjusted by ~1°C each day until the desired temperature was reached, consistent with standard temperature adjustment protocols commonly used in freshwater mussel studies (Galbraith et al., 2012). Mussels were then acclimated at that temperature for at least two weeks before initiating experiments. TECO chillers and temperature controllers with an associated 300-watt titanium heater bar were used to maintain desired holding or acclimation temperatures.

Hypoxia Tolerance

Respirometry experiments were conducted with 8-chamber fiber-optic respirometry systems using Auto-Resp software (Loligo Systems, Viborg, Denmark). Chambers were placed in two rectangular, fiberglass tubs, each approximately 122 x 76 x 33 cm in size, filled with ~151 L of hard artificial freshwater (HAFW). The two tubs were connected via two PVC pipes such that water circulated between the tubs when both tubs were held at the same temperature, but valves were closed to prevent circulation if each tub was run at a different temperature. Water temperature was controlled by titanium heater bars in each tub. Dissolved oxygen in each tub was maintained at ~100% saturation using two air stones that were 2.5–5-cm in length.

Each tub contained 4 acrylic respirometry chambers. Connected to each chamber were two Eheim submersible 300 L/h pumps (Eheim GmbH & Co., Deizisau, Germany). One pump was a flush pump, used during initial chamber acclimation to circulate oxygenated water through the chamber, while the other pump was a recirculating pump, used to circulate water in a closed loop through the chamber and past an optical dissolved oxygen (DO) sensor placed in the recirculation tubing that carried water from the chamber to the recirculating pump and back again (Haney et al., 2020). Prior to each respirometry run, all chambers and associated tubes and pumps were submerged in a 1% bleach solution for six hours to kill bacteria and minimize

background oxygen demand. Components were then rinsed thoroughly with tap water. Optical DO sensors were also disinfected but were submerged in the bleach solution for only 10 minutes as per user manual recommendations.

The appropriate respirometry chamber size was determined for each mussel by analyzing the chamber volume to animal mass ratio. The chamber must be small enough that the experiment will not take an excessive amount of time but big enough that respiration measurements are reliable. Chamber size was chosen such that the chamber volume to animal mass ratios were similar to the ratios used in Haney et al. (2020) and Pieper et al. (2025). For *P. riddellii*, medium sized chambers (347 mL vol. including tubes and pump) were used with an average chamber volume to animal mass ratio of 8:1. Small sized chambers (225 mL vol. including tubes and pump) were used for *C. pustulosa* with an average ratio of 11:1. Each chamber was considered an experimental unit with one mussel per chamber.

To initiate an experiment, six mussels were removed from upwellers at 8 AM, gently cleaned with a toothbrush to remove organismal growth, weighed to the nearest 0.1 g (gWWW), and placed in in PVC cups (diameter and height) – 1 mussel per cup, 3 cups per trough. Mussels were not fed from this point forward and fasted for ~24 hours prior to collection of data used for analysis. Each cup was filled halfway with clean pea gravel to allow mussels to orient naturally in substrate. A 4-mm mesh bottom on each cup retained the gravel while allowing for water circulation through the cup and gravel. At ~4 PM the same day, each mussel in its cup was transferred to a respirometry chamber and allowed to acclimate overnight while the flush pumps cycled on and off. The recirculation pumps always remained on as they circulate water past the optical DO sensor. Photoperiod was set at a 12:12 light:dark cycle with the light period from 7 AM to 7 PM.

At 9 AM the following day, two hours after the lights came on, the flush pumps were turned off for each chamber while the recirculation pumps remained on. Dissolved oxygen of each chamber was measured every 1 second and respiration rates were calculated every six minutes (360 seconds) by AutoResp software. The run was concluded once the dissolved oxygen level in each of the chambers containing a mussel fell below 0.2 mg O_2/L. At that point the flush pumps were turned back on, and mussels were removed from their chambers.

Background oxygen demand associated with bacteria was accounted for by running one control chamber (no mussel) in each of the two tubs. A correction factor was calculated by dividing the mean respiration rate of the two control chambers by the mean respiration rate of the mussel chambers during the range in DO values that overlapped between control and mussel chambers. For example, if the DO declined from 7.0 to 6.5 mg/L in the control, we used the mean mussel respiration rate estimated between 7.0 and 6.5 mg/L. This proportion was assumed to remain constant as DO in mussel chambers declined below the lowest DO value observed in the control, and mussel respiration rates were multiplied by (1-correction factor) to correct raw mussel chamber respiration rates for background (i.e. bacteria) respiration.

Mussels would occasionally close their valves during a respirometry run. This caused respiration rates to drop to zero or near zero during closure and then spike to high values upon reopening. This was sometimes followed by a decline to a lower, more stable pattern (Figure 1.1A), but sometimes closures were followed by a spike in respiration then a decline into another closure instead, with multiple back-to-back closures occurring (Figure 1.1B). In most cases, effects of valve closure on respiration patterns prevented accurate calculations of DO_{crit} and RI for that individual. *Pleurobema riddellii* was highly sensitive to disturbance and often exhibited valve closures for ~4 hours before opening and then closing again if disturbed once more.

Because of this we conducted runs at night during weekends for *P. riddelli* to minimize disturbance events. In this case, *P. riddelli* were acclimated to respiration chambers during the daytime and respiration runs were initiated at 9:00 pm rather than 9:00 am. Several individuals had to be run more than once to obtain a data set without valve closures. If an individual was used more than once, they were allowed to recover from the previous run for at least two weeks before repeating the run. During the recovery period they were placed back in their original upweller at experimental temperature and fed as described previously.

Calculation of RI and DO_{crit}

Raw data from each chamber were trimmed for analysis to have all starting or maximum observed DO values be ~6 mg/L O_2. Respiration rates were included only from intervals where oxygen declined in a steady, linear manner consistent with active ventilation. For each trial, DO concentration was plotted against time, and slopes were generated by AutoResp software. Segments that produced an R^2 value below 0.9 were removed prior to analysis because nonlinearity in respirometry trials typically reflects behavioral interruptions (e.g., valve closure) or unstable oxygen consumption and therefore does not provide reliable estimates of metabolic rate (Chabot et al., 2021). While overly restrictive criteria can bias metabolic estimates by excluding legitimate low-rate measurements (Chabot et al., 2021), excluded intervals in this study were characterized by clear deviations from linear oxygen decline rather than shallow but consistent slopes. Approximately 2.3% of measurement intervals were removed due to R^2 prior to analysis.

Data for each animal were graphed in SigmaPlot (version 15.0) and the best fit regression was found by assessing AICc score and visual fit. Three types of regression that have been shown to fit similar data in previous studies by our lab (Haney et al., 2020) were graphed and

their fit compared – hyperbola, exponential rise to maximum, and 2-segment piecewise regressions. Typically, the regression with the lowest AICc score was considered the best fit. However, if the two lowest AICc scores were very similar (i.e. no more than 5% difference) we picked the regression with the best visual fit.

To quantify the degree of metabolic regulation, we followed the approach of Mueller and Seymour (2011) and calculated the area under the curve (AUC) for three reference points using SigmaPlot. These consisted of a horizontal line representing perfect regulation, a linearly decreasing (diagonal) line representing perfect conformity, and the measured oxygen consumption data. The regulation index (RI) was then calculated using the following formula:

$$RI = (AUC_{observed} - AUC_{conformation}) / (AUC_{regulation} - AUC_{conformation})$$

DO_{crit} was determined as the point at which the observed metabolic rate differed most from the perfect conformity line (Mueller & Seymour 2011).

Statistical Analyses

The following statistical analyses were conducted in SigmaPlot (version 15.0). Two-way analysis of variance (ANOVA) was used to analyze the main effects of species (*P. riddellii* and *C. pustulosa*) and temperature (25°C and 32°C) on each endpoint (DO_{crit} and RI), as well as to detect interactions between temperature and species on endpoints. A Tukey's post-hoc test was used to confirm which treatments differed. Additionally, a one-way ANOVA with Tukey's post-hoc test was used to test for differences between the focus species of this study and several other southern species that our lab has tested using the same methodology at the same two temperatures. During these analyses, if the test for equal variance failed, we used a Kruskal-Wallis ANOVA on ranks followed by a Dunn's post-hoc test. For the comparison of DO_{crit}

among several species at 32°C, the Kruskal-Wallis analysis detected a significant difference, but Dunn's Method post-hoc test did not find significant differences between any groups. Thus, a Welch's ANOVA with a Games Howell post-hoc test was executed. One-way, two-way, and Welch's ANOVAs tested for differences among mean values, whereas Kruskal-Wallis ANOVA on ranks tested for differences among median rank-transformed values. In all tests, statistical significance was determined as $p < 0.05$.

RESULTS

Hypoxia Tolerance

There was no main effect of species on DO_{crit} for *P. riddelli* and the sympatric *C. pustulosa* ($F_{1,19} = 0.159$; $p = 0.694$; Figure 1.2A). There was also no main effect of temperature on DO_{crit} ($F_{1,19} = 0.598$; $p = 0.449$; Figure 1.2B). No interaction between species and temperature on DO_{crit} were found ($F_{1,19} = 0.376$; $p = 0.547$; Figure 1.2C).

There was a significant main effect of species on regulation index (RI), with *P. riddellii* having lower RI than *C. pustulosa* ($F_{1,19} = 6.792$; $p = 0.017$; Figure 1.3A). However, there was no main effect of temperature on RI ($F_{1,19} = 0.0150$; $p = 0.904$; Figure 1.3B). There was a significant interaction between species and temperature on RI ($F_{1,19} = 6.815$; $p = 0.017$). Post-hoc analysis revealed that *P. riddellii* had a lower RI than *C. pustulosa* at 25°C (Tukey Test: $p < 0.001$) but not at 32°C (Figure 1.3C).

With the inclusion of additional southern taxa, the comparison of DO_{crit} at 25°C showed no differences between species ($F_{4,27} = 1.678$; $p = 0.184$; Figure 1.4A). At 32°C, DO_{crit} differed

among species ($F_5 = 8.57$; $p = 0.002$), with *C. petrina* having a significantly higher DO_{crit} than *C. pustulosa* (Games Howell: $p = 0.036$) and *L. straminea* ($p = 0.001$; Figure 1.4B).

At 25°C, differences in RI were found across this broader group of species ($F_{4,27} = 46.882$; $p < 0.001$). Most comparison groups differed significantly from each other, except for *L. straminea* vs. *C. pustulosa* and *F. iheringi* vs. *P. riddellii* (Table 1), and all significant pairwise comparisons had $p < 0.001$ (Tukey Test). Species also showed differences in RI at 32°C ($H_5 = 17.516$; $p = 0.004$), but only *P. popeii* and *C. petrina* were found to be significantly different from each other during pairwise comparisons, with *P. popeii* having a higher RI (Dunn's Method: $p = 0.035$; Table 1).

DISCUSSION

The results of this study contribute important new insights into how freshwater mussels respond physiologically to interacting thermal and hypoxic stressors, particularly for *Pleurobema riddellii*, a narrow-range species proposed for federal protection. Previous work has documented considerable interspecific variation in metabolic responses to environmental stress among unionids (Haney et al., 2020; Chen et al., 2001), and the present study expands this knowledge by examining the hypoxia tolerance of *P. riddellii* relative to a sympatric, widely distributed congener, *Cyclonaias pustulosa*, under two ecologically relevant temperatures.

Notably, our findings reveal that both *P. riddellii* and *C. pustulosa* maintained low critical dissolved oxygen concentrations (DO_{crit}) at both 25 and 32°C and did not exhibit the expected increase in hypoxia sensitivity at higher temperatures. In many ectotherms, warming is expected to increase metabolic oxygen demand and potentially reduce the margin between

oxygen supply and demand, as proposed by broader conceptual frameworks such as the oxygen and capacity limited tolerance hypothesis (Pörtner, 2010). However, empirical evidence for this pattern in freshwater mussels is mixed. For example, Haney et al. (2020) found that increasing temperature significantly increased metabolic rate across three *Cyclonaias* species, indicating elevated energetic demand, but had weak and inconsistent effects on DO_{crit}. Only *C. petrina* exhibited a significant increase in DO_{crit} with temperature, whereas *C. necki* and two populations of *C. pustulosa* showed no significant relationship. These results suggest that although warming increases metabolic demand in unionid mussels, this does not necessarily translate into increased hypoxia sensitivity. Similarly, the lack of temperature related increases in DO_{crit} observed in *P. riddellii* and *C. pustulosa* from the Neches River indicates that these species may maintain effective oxygen regulation across this thermal range despite elevated metabolic demand.

Importantly, growing evidence indicates that the interpretation of critical oxygen thresholds is inherently dependent on the metabolic state used to define them because oxygen demand varies with metabolic activity (Seibel et al., 2021). Critical oxygen levels derived from standard metabolic rate (SMR) typically occur at lower oxygen concentrations than those derived from routine metabolic rate (RMR), and especially maximum metabolic rate (MMR), which reflects the highest oxygen demand and the highest critical oxygen threshold (Reemeyer & Rees, 2019; Seibel et al., 2021). As a result, comparisons of hypoxia tolerance across studies require careful consideration of the metabolic state used to determine these values.

In this study, mussels were fasted for 24 hours and constrained in PVC cups to minimize movement. However, defining a true SMR in bivalves remains inherently difficult because mussel oxygen consumption varies along a continuum of valve states, from fully open ventilation to partial closure and full closure. SMR is typically estimated from RMR by isolating the low

end of aerobic metabolic variation in taxa such as fish or crayfish (Seibel et al., 2021), but that approach does not translate cleanly to mussels because low oxygen consumption values may reflect different degrees of valve closure and periodic shifts toward anaerobic respiration rather than a stable basal aerobic state. Thus, respiration is behaviorally controlled by valve position in mussels, as demonstrated by Figure 1.1, and it is challenging to assess what range in respiration rates count as baseline in SMR estimation. As a result, our measurements likely represent a low routine metabolic rate, often referred to as a resting metabolic rate, that is probably somewhat higher than true SMR. Because DO_{crit} depends on the metabolic state being quantified and usually increases with increasing metabolic rate, our estimates should be interpreted as reflecting oxygen thresholds associated with a low-maintenance resting state. In this context, the apparent thermal invariance in DO_{crit} for *P. riddellii* may be partly due to the metabolic state at which DO_{crit} was quantified, in addition to genuine physiological resilience to hypoxia across the thermal range tested.

Interpretation of these patterns requires consideration of respiratory strategy, metabolic regulation, behavioral responses, and possible local adaptation. The regulation index (RI) provides corresponding insight alongside DO_{crit} by characterizing the degree to which an organism maintains metabolic rate as oxygen declines (Mueller & Seymour, 2011). In this study, *P. riddellii* consistently showed intermediate RI values (on a scale of zero to one) at both temperatures, indicating that it neither strictly conforms to ambient oxygen nor highly regulates metabolic rate as dissolved oxygen declines.

An intermediate regulation strategy indicates that oxygen consumption was partially maintained as ambient oxygen declined, but not to the extent characteristic of strong regulation nor as dramatically dependent on environmental oxygen as pure conformity (Mueller &

Seymour, 2011). This intermediate position is consistent with physiological trade-offs often observed in aquatic ectotherms, where maintaining high levels of aerobic regulation can be energetically costly and may not always present a clear advantage in environments where prolonged hypoxic conditions are less common (Chen et al., 2001). Studies of unionid mussels have shown that those from habitats with more frequent or prolonged hypoxia tend to exhibit stronger regulation, likely because sustained aerobic metabolism under low oxygen improves performance during extended declines (Chen et al., 2001). In the case of *P. riddellii*, the intermediate RI may reflect a balance between metabolic costs and regulatory capacity rather than a specialized hypoxia strategy.

Behavioral responses further highlight these results. *P. riddellii* exhibited prolonged valve closures during experimental runs, sometimes lasting several hours, which required repeated respirometry attempts to obtain sets of metabolic rates without prolonged closures. Valve closure is a well-documented stress response among freshwater mussels exposed to thermal or hypoxic stress (Haney et al., 2020). By closing valves, mussels temporarily slow metabolic processes and isolate themselves from external stressors, effectively reducing oxygen consumption. Although valve closures complicate respirometry measurements, they represent an ecologically relevant mechanism of coping with stressful conditions. The frequency and duration of closures observed in *P. riddellii* may indicate a heightened sensitivity to disturbance rather than to hypoxia per se, as individuals frequently reopened and reclosed in response to minor environmental fluctuations such as vibrations, shadows, and sounds. This behavioral sensitivity suggests that *P. riddellii* may prioritize a shut-down response when confronted with multiple stressors.

Potential local adaptation may also contribute to the patterns observed. The Lower Neches Valley Authority (LNVA) canal system, where the study population of *P. riddellii* originated, is an engineered waterway subject to hydrologic manipulation and routine water quality monitoring that includes dissolved oxygen saturation and temperature among its parameters. These sensors are located in the main canal segment at 30.1411111, -94.1744444, approximately 6.26 km upstream of our sample populations of *P. riddellii* and *C. pustulosa*. Available data indicate DO concentrations that vary seasonally, typically ranging from ~4–7 mg/L during the warmest months (~25–34°C, Jun.-Sep.) and ~7–12+ mg/L in cooler months (<25°C, Nov.-Feb.), although some years show DO declines that reach the DO_{crit} of *P. riddellii* (~1 mg/L; Figure 1.6).

While hypoxic events appear to be rare in this system, these lower end values (~4 mg/L DO) are below the typical DO ranges considered optimal for supporting healthy aquatic life in freshwater systems (USEPA, 1986), and these values are closer to ranges where sublethal effects have been documented in the broader aquatic invertebrate literature. Meta-analyses of aquatic invertebrate responses to declining oxygen availability show that respiration, growth, and reproduction begin to decline at DO concentrations above standard hypoxia definitions (<2–3 mg/L), with sublethal impairments observed at ~0.77–6.4 mg/L in diverse invertebrate taxa under experimental conditions (Galic et al., 2019). Research on unionid communities has shown increased mortality and assemblage changes associated with DO concentrations below ~5 mg/L during drought and low-flow events (Gagnon et al., 2004).

An additional ecological consideration is that low-end dissolved oxygen concentrations in the range reported for the LNVA canal system may present greater risk for the fish hosts required for unionid reproduction. Most freshwater mussels rely on an obligate parasitic larval stage that

must attach to suitable host fish, linking mussel population persistence directly to the physiological tolerances and survival of fish hosts. Many freshwater fish species experience impaired growth, reduced activity, and increased stress as DO declines toward ~4 mg/L, particularly in warm water where metabolic demand is elevated (USEPA, 1986). Consequently, while adult mussels may tolerate short-term exposure to DO concentrations near or below ~4 mg/L, these conditions could still affect mussel populations indirectly by reducing survival, performance, or availability of host fishes necessary for reproduction. Host fish availability may also be impacted by the numerous dams on these canals, hindering the ability of mussels to spread upstream and isolating these populations.

Although the LNVA canal does not appear to be chronically hypoxic, mussels in the system experience variable environmental conditions, including high temperatures (up to ~34°C), that could impose selection on metabolic regulation traits. Evidence for population specific physiological variation has emerged in other unionids. For example, Haney et al. (2020) reported substantial differences in thermal-hypoxic sensitivity among geographically distinct subpopulations of *C. pustulosa*. The consistency of low DO_{crit} and intermediate RI in LNVA *P. riddellii* across temperatures could reflect long term exposure to environmental variation that shapes metabolic response patterns, favoring individuals with resilience to variable temperatures and an intermediate regulation response rather than strong regulation or pure conformity.

Species comparisons add further context. *C. pustulosa* displayed higher RI values than *P. riddellii* at 25°C, indicating a greater ability to maintain a consistent level of aerobic metabolism as DO declines under cooler conditions. However, this difference disappeared at 32°C, where RI values for the two species largely overlapped. This convergence was not driven by significant shifts in mean RI within either species, but instead by increased intraspecific variability and

opposing changes in the distribution of individual responses. In *P. riddellii*, RI values overlapped across temperatures but extended toward higher values at 32°C, whereas *C. pustulosa* showed a downward spread with more low RI values at 32°. These contrasting shifts increased overlap between species at the higher temperature, effectively reducing interspecific differences in apparent regulation ability.

Comparisons with broader datasets, including southern species from Texas, Alabama, and New Mexico, revealed that DO_{crit} values remained considerably low for all species at both temperatures, meaning that their threshold for transitioning to anaerobic respiration is at a very low DO level, regardless of temperature (~1–2 mg/L O_2). This is consistent with the general hypoxia tolerance characteristics of unionids, which often inhabit sedimentary or low-flow habitats where oxygen limitation is frequent (Strayer et al., 2004). However, RI patterns showed greater variability across species, and species differences were stronger at 25°C than at 32°C. *P. riddellii* had the lowest RI score compared to both the common and rare species tested at the lower temperature, and *P. popeii* exhibited the highest RI, yet both species are rare. This suggests that rarity alone does not predict hypoxia regulation strategy, supporting conclusions that physiological sensitivity does not universally correlate with conservation status. Instead, metabolic strategies likely represent a combination of evolutionary history, habitat conditions, and species-specific life histories.

The ecological and conservation implications of these findings are multifaceted. The low DO_{crit} values exhibited by *P. riddellii* and *C. pustulosa* suggest that adult individuals of both species are capable of withstanding acute hypoxia across the temperatures tested. This may be advantageous in variable systems like the LNVA canal, where DO and temperature fluctuate seasonally or episodically, even if conditions do not reach chronic hypoxia. At the same time, *P.*

riddellii had relatively low RI values, although RI values remained intermediate rather than indicative of strict oxygen conformity. Partial regulation may reduce the energetic demands associated with sustaining high ventilation under declining oxygen, which can increase energetic expenditure and oxidative stress in fully regulating species (Pörtner, 2010). However, limited regulatory capacity may constrain performance during prolonged hypoxia (Wu, 2002). Thus, rather than being inherently detrimental, an intermediate metabolic regulation response is best interpreted as a flexible respiratory strategy that balances oxygen acquisition with energetic costs, consistent with the wide interspecific variation in hypoxia responses observed among bivalves and other aquatic animals (Rogers et al., 2016).

The fact that *P. riddellii* did not show increased hypoxia sensitivity at higher temperatures can be interpreted as an indication of climate resilience regarding short-term hypoxic events. However, acute responses may not predict long-term performance, and chronic exposure to sublethal stress often leads to cumulative energetic deficits. Pieper et al. (2025), for example, found that scope for growth sharply declined under increasing thermal conditions in *P. popeii*, and *P. popeii* had the highest RI at both temperatures compared to all species in our broader taxa analysis. Thus, the absence of increased DO_{crit} and decreased RI at 32°C in *P. riddellii* does not rule out substantial energetic strain or reduced performance under prolonged warming.

Future research should build on the physiological framework established here by examining how DO_{crit} and RI in *P. riddellii* vary across different temperatures, populations, and life stages. Juveniles and glochidia are often more sensitive to environmental stressors than adults (Moore & Bringolf, 2018), meaning that thermal-hypoxic tolerance in early life stages may represent a critical bottleneck for *P. riddellii* persistence. Studies of aerobic scope, feeding

efficiency, and recovery after stress exposures would also provide valuable context for interpreting the ecological relevance of metabolic strategies. Population comparisons across the Neches and Sabine River drainages would help differentiate local adaptation from species-level traits, while genomic approaches could explain whether physiological responses have an underlying genetic basis relevant for conservation planning.

In conclusion, this study provides the first detailed assessment of thermal-hypoxic physiology in *Pleurobema riddellii*, revealing a metabolic strategy characterized by low DO_{crit}, intermediate RI, and pronounced behavioral sensitivity. These traits suggest that *P. riddellii* adults possess substantial acute hypoxia tolerance, but their intermediate regulation ability presents potential consequences for long-term performance under chronic stress. Understanding these dynamics is essential for effective conservation and management of this vulnerable species, particularly when thermal and hypoxic events are likely to intensify under future climatic scenarios.

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Species	25°C		32°C	
	RI Mean (*SE*)	*n*	RI Median (*Q1–Q3*)	*n*
P. riddellii	0.492 (0.0128)[c]	6	0.593 (0.473–0.662)[ab]	4
C. pustulosa	0.668 (0.0186)[b]	9	0.589 (0.448–0.691)[ab]	4
L. straminea	0.707 (0.0081)[b]	8	0.755 (0.646–0.797)[ab]	9
P. popeii	0.858 (0.0164)[a]	4	0.777 (0.707–0.827)[a]	6
F. iheringi	0.531 (0.0347)[c]	5	-	-
C. petrina	-	-	0.56 (0.447–0.581)[b]	4
C. necki	-	-	0.575 (0.332–0.693)[ab]	5

Table 1.1: Results of one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test comparing regulation index (RI) at 25°C across several Southern species (TX, AL, NM); results of a Kruskal-Wallis one-way ANOVA on ranks followed by a Dunn's Method post-hoc test comparing RI at 32°C across species. Significant differences ($p < 0.05$) in RI between species at a given temperature are denoted by lowercase letters. Dash (-) indicates no data available for given temperature.

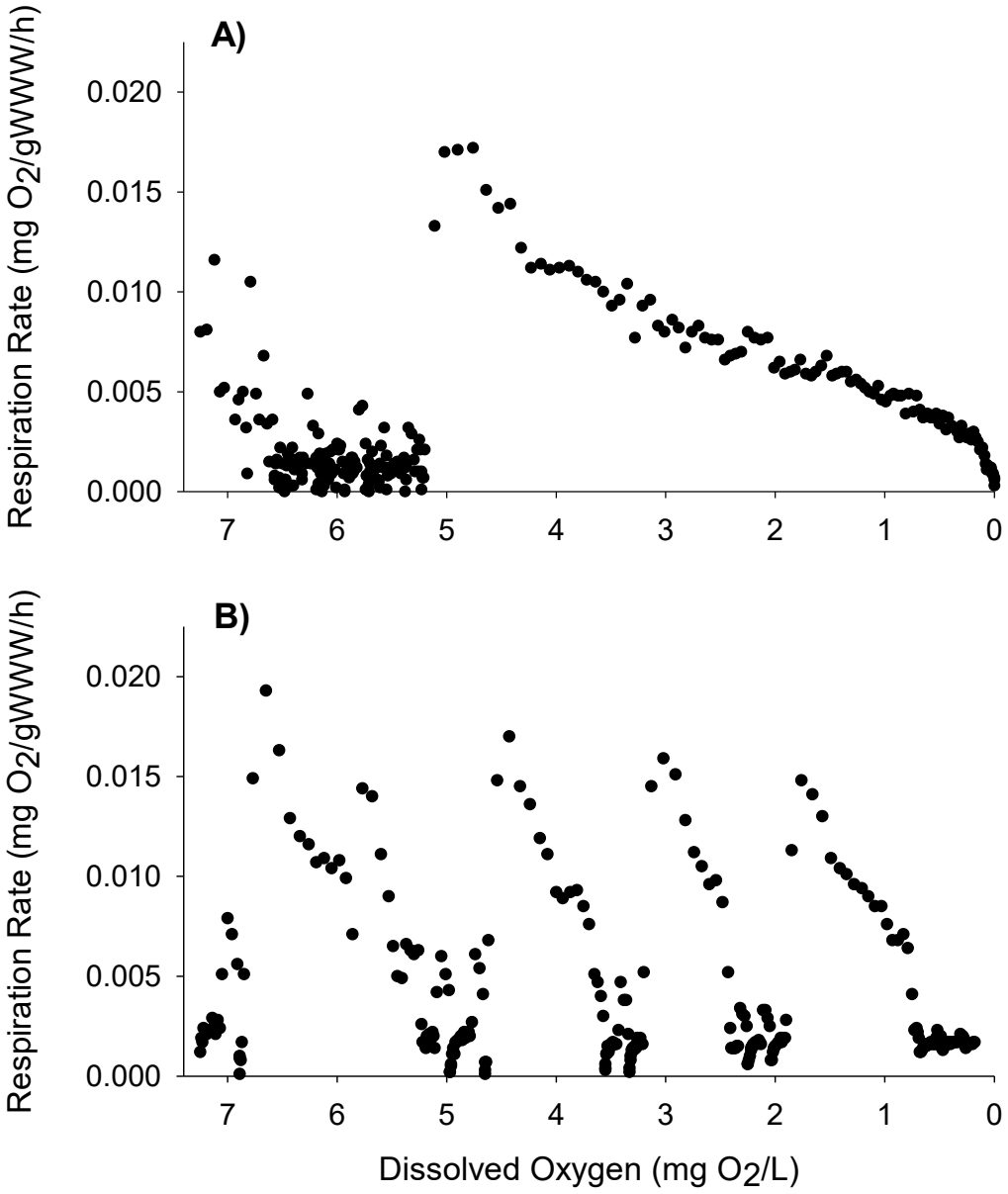

Figure 1.1: A) Example of a valve closure event with *P. riddellii* where respiration was near zero and spiked after the mussel reopened, then declined to a lower, more stable pattern. B) Example of an instance with *P. riddellii* where a repeated closure-spike-closure pattern was observed as dissolved oxygen (DO) declined.

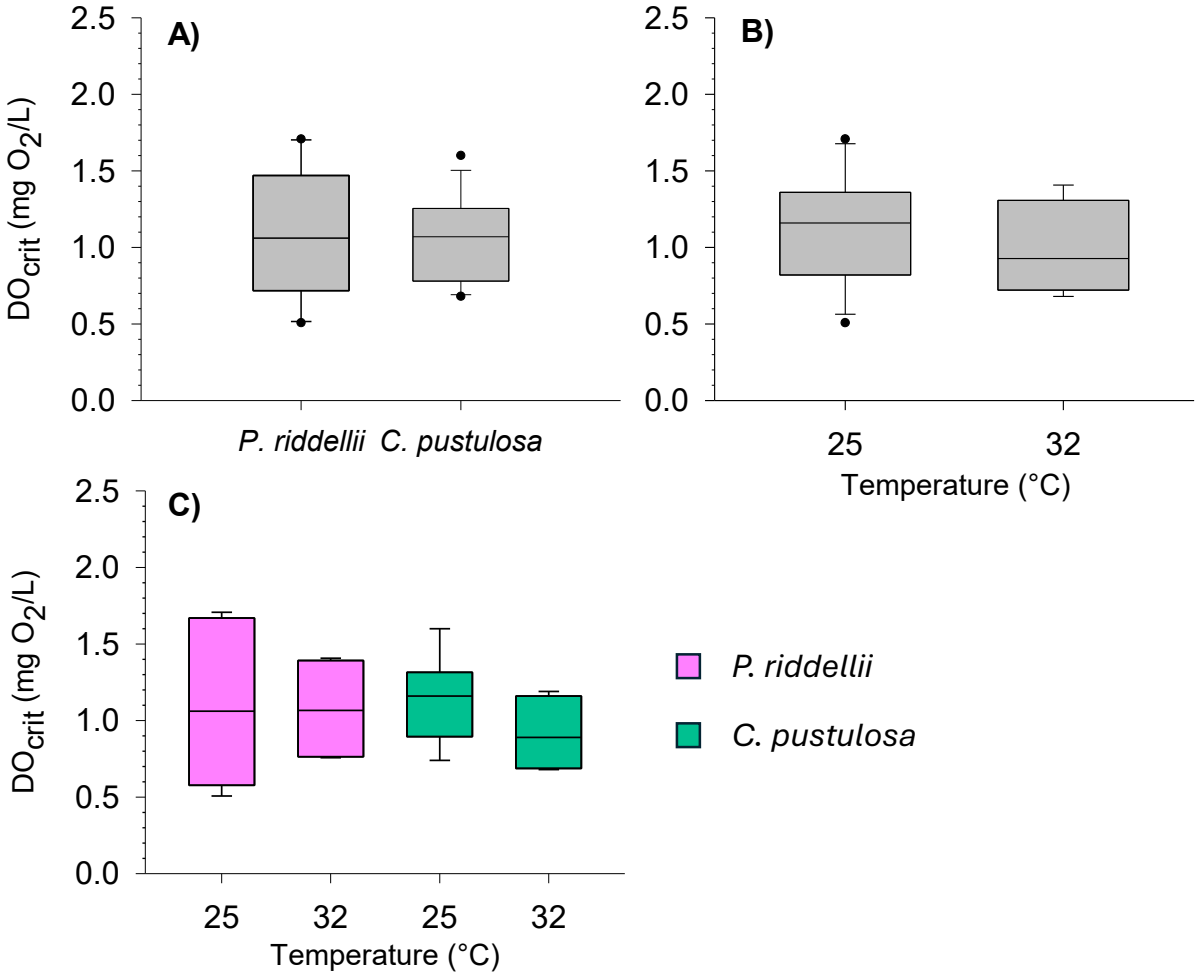

Figure 1.2: A) Critical dissolved oxygen (DO_{crit}) of each species regardless of temperature. B) DO_{crit} of *P. riddellii* and *C. pustulosa* at each temperature, regardless of species. C) Interaction between species and temperature on DO_{crit}. Boxes show interquartile range (IQR; 25^{th}-75^{th} percentile), the horizontal line inside of a box indicates the median, and whiskers show data extending to the smallest and largest values within 1.5 × IQR. Individual points indicate outliers.

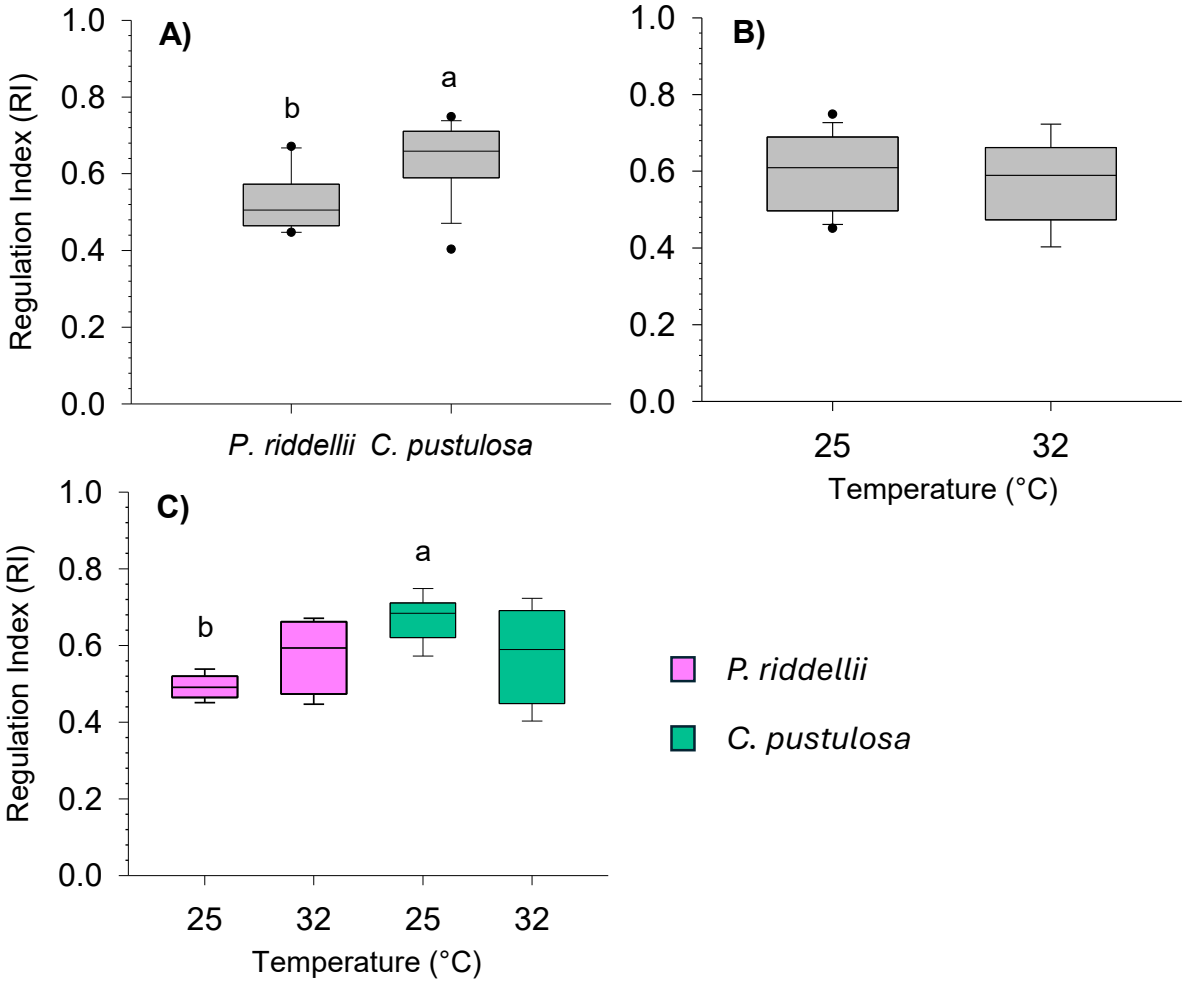

Figure 1.3: A) Regulation index (RI) of each species regardless of temperature. B) RI of *P. riddellii* and *C. pustulosa* at each temperature, regardless of species. C) Interaction between species and temperature on RI. Lower case letters indicate significant differences in mean RI among categories. Boxes show interquartile range (IQR; 25th-75th percentile), the horizontal line inside of a box indicates the median, and whiskers show data extending to the smallest and largest values within 1.5 × IQR. Individual points indicate outliers.

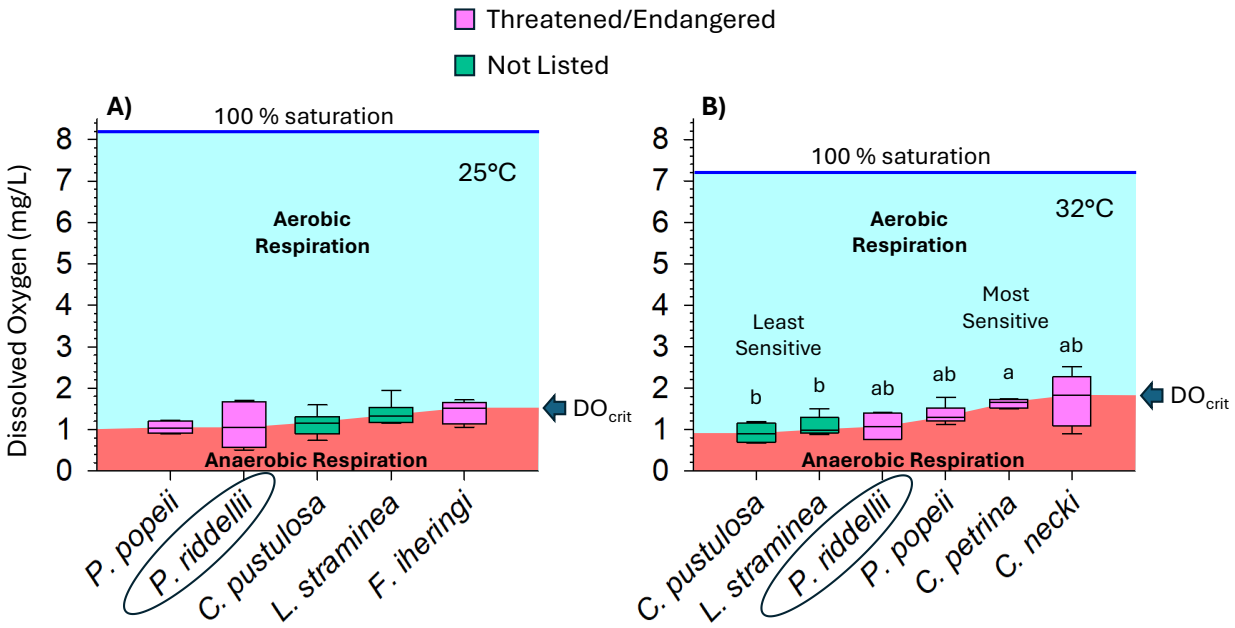

Figure 1.4: Comparisons of critical dissolved oxygen (DO_{crit}) at A) 25°C and B) 32°C among species tested by our lab in this and previous studies using the same methodology. Blue shading shows the range in DO where each species primarily uses aerobic respiration whereas red shading shows the DO range where each species primarily uses anaerobic respiration. The breakpoint between aerobic and anaerobic respiration is defined as DO_{crit}. Lower case letters indicate significant differences in mean DO_{crit} among species. Boxes show interquartile range (IQR; 25th-75th percentile), the horizontal line inside of a box indicates the median, and whiskers show data extending to the smallest and largest values within 1.5 × IQR.

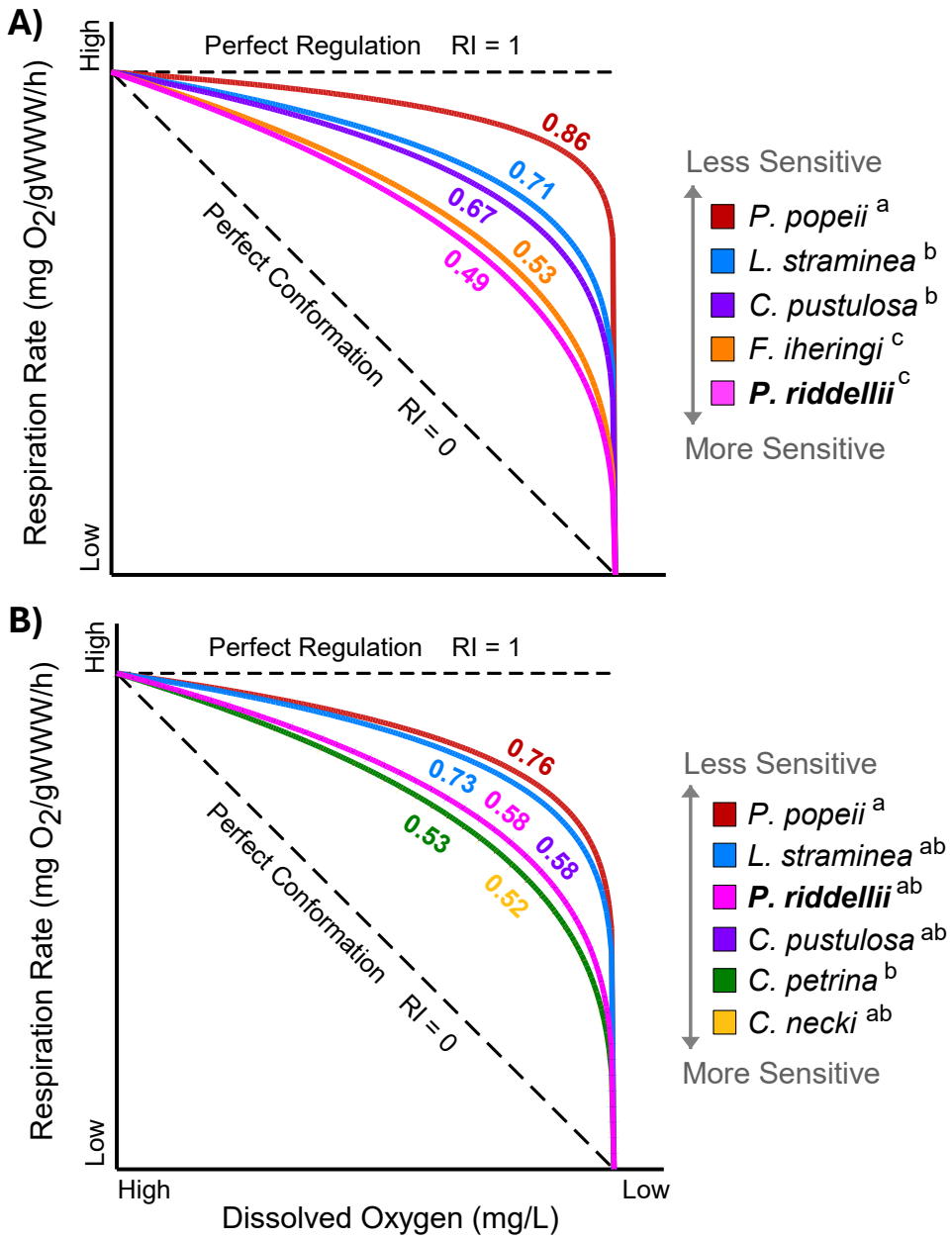

Figure 1.5: Comparisons of mean regulation index (RI) at A) 25°C and B) 32°C among species tested by our lab in this and previous studies using the same methodology. Lower case letters indicate significant differences in mean (25°C) and median rank-transformed (32°C) RI among species. Lower RI values suggest higher sensitivity to low DO, indicated by gray arrow beside legend.

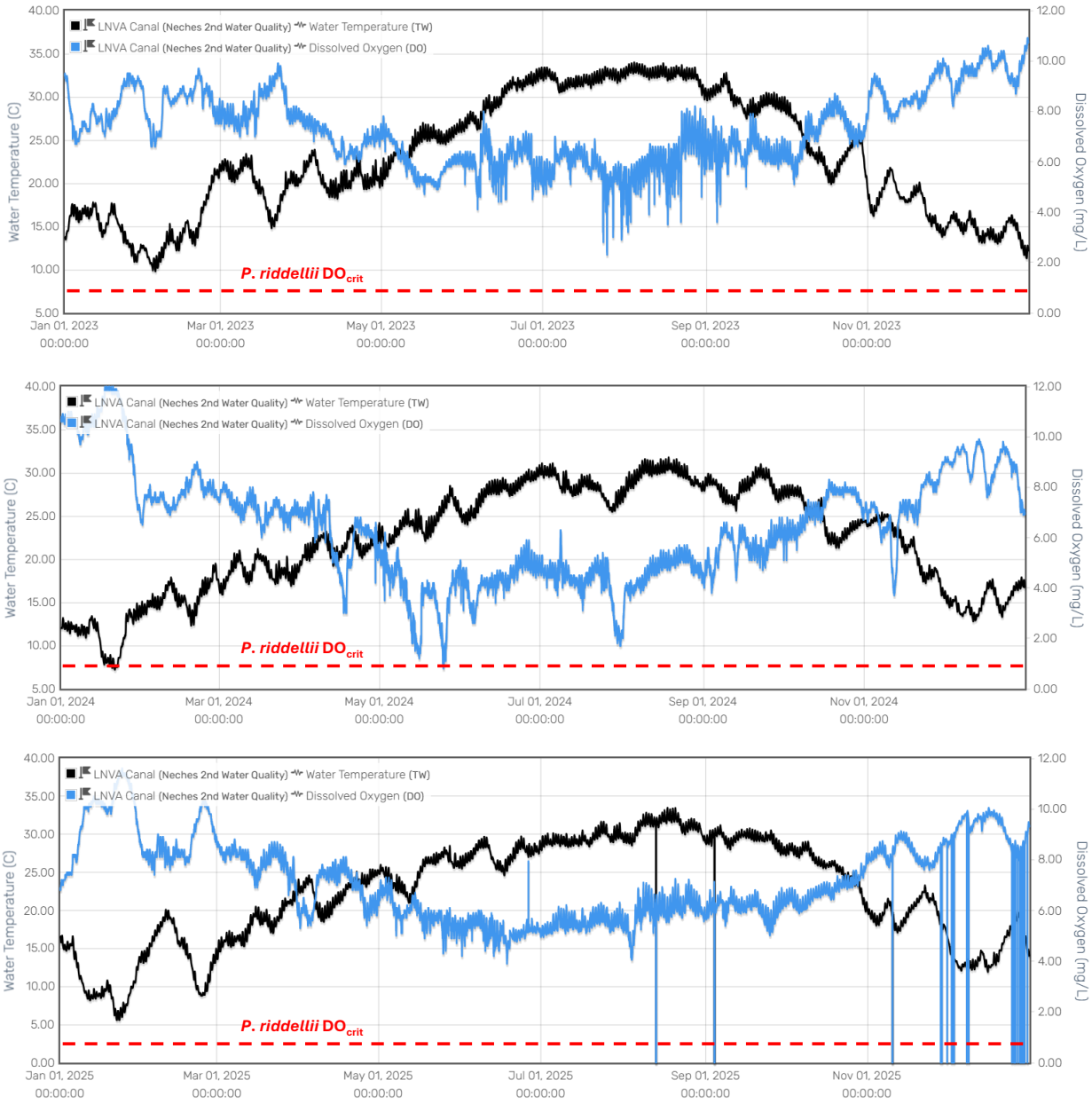

Figure 1.6: Temperature and dissolved oxygen data in 2023, 2024, and 2025, as recorded by the Lower Neches Valley Authority (LNVA) sensors in the LNVA Canal (30.1411111, -94.1744444). The critical dissolved oxygen (DO_{crit}) of *P. riddellii* is marked by a red dotted line. Sudden, straight vertical drops in data are presumed to be sensor errors.

Chapter 2: Physiological and behavioral responses to acute thermal stress in allopatric and sympatric, threatened and common freshwater mussels.

INTRODUCTION

Freshwater mussels (order: Unionida) are a keystone taxa in aquatic ecosystems, providing ecological functions such as water filtration, nutrient cycling, and substrate stabilization (Vaughn & Hakenkamp, 2001). However, unionids are also among the most endangered taxa in North America, with ~70% of species considered imperiled or extinct (Haag, 2012). Threats to mussel survival include habitat modification, invasive species, pollution, and increasingly, climate change (Newton et al., 2023). Acute temperature fluctuations, particularly extreme heat events, are of growing concern, yet quantitative data on how unionid species respond physiologically and behaviorally to thermal stress remains limited and are available for only a limited number of taxa (Fogelman et al., 2023).

Climate change is expected to increase both frequency and intensity of thermal stress in freshwater systems, including greater thermal variability and more frequent extreme heat and drought events (IPCC, 2021; USGCRP, 2018). As ectotherms, unionid mussels rely on ambient water temperature to regulate physiological processes, making them particularly sensitive to thermal stress. Chronic exposure to elevated temperatures can impair growth, reproduction, and long-term survival by increasing metabolic demand and reducing energy available for maintenance and fitness (Embke et al., 2026). In contrast, acute thermal stress associated with short-term heat spikes may exceed physiological tolerance limits, resulting in rapid loss of function or mortality if temperatures approach or surpass species-specific thermal maximums (Fogelman et al., 2023; Archambault et al., 2014).

The biological consequences of thermal stress therefore depend on both the magnitude and duration of exposure, as well as the organism's capacity for recovery. Acute heat events are of specific concern in shallow, isolated, or flow-altered habitats where water temperatures can rise rapidly and thermal refugia may be limited (Poole & Berman, 2001). For example, Galbraith et al. (2010) observed that reduced river flow, associated with drought and changes in local water management, fragmented previously flowing segments of an Oklahoma river into shallow, isolated habitats that experienced temperatures up to more than 40°C, resulting in disproportionately high mortality in thermally sensitive mussel species relative to more tolerant species. Therefore, understanding species-specific thermal tolerance and stress responses across both chronic and acute timescales is critical for predicting freshwater mussel persistence under future climate scenarios.

The Louisiana pigtoe (*Pleurobema riddellii*), federally proposed as threatened, represents a case of particular concern. Historically widespread in Texas and Louisiana rivers, it is now restricted to a handful of populations (USFWS, 2023). Recent surveys indicate that the Neches River system in Texas contains some of the largest documented present-day populations of *P. riddellii,* and there is evidence of active reproduction (Ford et al., 2022). These populations are regarded as some of the last relatively stable strongholds for the species. Notably, the population in the Lower Neches Valley Authority (LNVA) canals is in an engineered system subject to occasional maintenance requiring dewatering and mussel relocation (BIO-WEST, 2019), raising concerns about the long-term viability of this anthropogenic ecosystem as suitable habitat for a mussel species in decline. However, the relatively stable conditions of some artificial and modified water bodies have been found to act as refuges for freshwater biodiversity, with transport canals being reported as among the most diverse across aquatic taxa (Chester &

Robson, 2013). Thus, information regarding the stress tolerances of rare, threatened, or endangered animals found within the LNVA canal system would help inform the management and enhancement of this system such that it may conserve biodiversity and facilitate adaptation to climate change.

Physiological responses of mussels to thermal stress can be analyzed using metrics like respiration rate (MO_2) and temperature coefficient (Q_{10}), which quantify oxygen consumption across temperature gradients and offer insight into metabolic sensitivity (Schulte et al., 2011). Acute upper thermal tolerance is frequently assessed using the critical thermal maximum (CTM) dynamic ramping assay, defined as the temperature at which an organism experiences a loss of critical physiological function during a progressive temperature increase (Lutterschmidt & Hutchison, 1997). In freshwater mussels, CTM is commonly identified using behavioral loss-of-function endpoints such as extreme gaping, foot extension, and loss of responsiveness to stimuli (Galbraith et al., 2012). Importantly, CTM represents a threshold of functional failure rather than immediate mortality, and individuals are expected to recover if thermal stress is relieved (Lutterschmidt & Hutchison, 1997).

Behavioral responses including gaping, foot extension, and reduced responsiveness have been widely documented in unionid mussels experiencing physiological stress (Sparks & Strayer, 1998; Byrne & McMahon, 1994). In some studies, extreme gaping, foot extension, mantle tissue retraction, and loss of responsiveness are used explicitly as endpoints to define CTM (Galbraith et al., 2012), whereas in others, similar behaviors are used as indicators of mortality in lethal tolerance (LT) experiments (e.g., Khan et al., 2020). As a result, distinguishing between early stress behaviors, functional thermal limits (CTM), and irreversible mortality is not always straightforward, and interpretations of these behaviors can differ among studies. Clarifying how

behavioral responses progress relative to physiological collapses and lethal thresholds is therefore critical for understanding species-specific vulnerability to acute thermal stress.

Mildenberger et al. (2025) analyzed the acute upper thermal limits (LT_{05} and LT_{50}) of *P. riddellii* from the LNVA Canal and the widespread *Cyclonaias pustulosa* from the upper Sabine River. They found that *P. riddellii* was more sensitive to thermal stress, but individuals of each species were collected from different river basins. Although CTM values have been reported for other unionid species (e.g., *Alasmidonta varicosa*, *Elliptio complanata*, *Strophitus undulatus*; Galbraith et al., 2012), no published studies have quantified CTM for adult *P. riddellii*. Previous work has demonstrated that CTM varies among unionid species and is influenced by recent thermal history (Fogelman et al., 2023; Galbraith et al., 2012). Other studies have examined physiological responses or changes in behavior in mussels under elevated or acute thermal stress (Ganser et al., 2015; Archambault et al., 2014). However, few studies have explicitly integrated quantitative physiological measurements with discrete behavioral responses, analyzed across a continuous temperature ramp extending to near-lethal limits, particularly in a comparative framework that includes both sympatric and allopatric species.

These distinctions are ecologically important because species that co-occur may experience similar environmental conditions yet differ substantially in physiological sensitivity and metabolic capacity, potentially leading to differing vulnerability during extreme heat events (Payton et al., 2016; Pandolfo et al., 2010; Fogelman et al., 2023). Comparisons with allopatric species further provide insight into how evolutionary and environmental history may shape thermal tolerance. Understanding these differences is especially important for rare, threatened or endangered species such as *P. riddellii*, as it can inform conservation and management strategies

by identifying taxa or populations that may be disproportionately vulnerable to acute thermal stress.

To address these knowledge gaps, this study focused on the proposed threatened species *P. riddellii* from the LNVA canal system, a common, sympatric species (*Cyclonaias pustulosa*; syn. *Pustulosa pustulosa*) collected from the same site, and an allopatric, federally endangered species (*Fuscanaia iheringi*) from the Brazos River, TX. These species vary in conservation status, habitat distribution, and potentially, thermal sensitivity. The objectives of this study were to link behavioral and physiological responses to increasing thermal stress, determine the acute critical thermal maximum (CTM), and compare these thermal endpoints across sympatric and allopatric, common and threatened species.

Specifically, we tested the hypothesis that *C. pustulosa* would exhibit a higher CTM than the rarer *P. riddellii*, reflecting the possibility that differences in breadth of geographic distribution and evolutionary history among species may be associated with variation in upper thermal tolerance (Mildenberger et al., 2025; Payton et al., 2016; Galbraith et al., 2012). Similarly, we hypothesized that rare species (*P. riddellii* and *F. iheringi*) would exhibit earlier behavioral indicators of thermal stress at lower temperatures than the widespread species *C. pustulosa*. Finally, we tested the hypothesis that metabolic rate would increase more steeply with rising temperature in the rare species, considering the prospect that higher metabolic sensitivity may be limiting adaptation to environmental variation in narrowly distributed species.

METHODS

Mussel collection and holding

Pleurobema riddellii were collected from the LNVA canal within the lower Neches River drainage, Texas, on September 19, 2022. Canal temperatures were ~28.5°C during collection. Specimens were shipped to Auburn University following protocols adapted from the *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels* (ASTM, 2013). Mussels were wrapped in wet cotton towels with evenly spaced ice packs such that an intermediate temperature was maintained between the collection site (~28.5°C) and the initial holding system (18°C) in the lab. A total of 53 adult mussels were shipped overnight; however, due to a one-day shipping delay, they arrived on September 21st with two mortalities. Survivors appeared healthy upon arrival, and no additional mortality was observed within the following two weeks after arrival.

Using the same shipping procedures, 25 *Cyclonaias pustulosa* were collected from the same site on August 25th, 2023, when canal temperatures were ~33°C, and shipped to Auburn University. A two-day shipping delay occurred, and four mussels were dead on arrival. Temperature of the cooler was not measured on arrival, but all ice packs were melted and warm. Survivors of this initial shipment exhibited high post-arrival mortality, with only 16 mussels surviving two weeks after delivery, prompting a second shipment request. Twenty-five additional *C. pustulosa* were collected and shipped overnight on November 6, 2023, when canal temperatures were ~20°C. Mussels arrived without delay or mortality, and the temperature of the cooler was 15°C. No mortalities were recorded in the subsequent two weeks.

For non-sympatric, rare species comparison, six federally endangered *Fusconaia iheringi* were collected from the Brazos River, Texas, on May 8, 2024, and shipped overnight under identical procedures, arriving without delay or mortality. The temperature of the cooler was 22.7°C upon arrival.

All mussels were housed in ~95 liter upweller systems, which were similar in design to Pieper et al. (2025), containing hard artificial freshwater (Smith et al., 1997; 151 L RO/DI water, 29.07 g sodium bicarbonate, 15.14 g calcium sulfate, 15.14 g calcium chloride, 9.08 g magnesium sulfate, 1.21 g potassium chloride; pH = 8.37, total hardness = 210 mg/L $CaCO_3$, and total alkalinity = 115 mg/L $CaCO_3$). Automated feeders (GHL Doser 2.2, GHL USA LLC, Wilmington, North Carolina) were used to feed mussels an hourly dose of LPB™ Frozen Shellfish Diet calibrated to maintain an ambient concentration of 30,000 ± 5,000 cells/mL.

Upweller water temperature was controlled by TECO TK-2000 heater/chiller units (TECO US) or temperature controllers with an associated 300-watt titanium heater bar. Mussels were held at 18°C for a minimum of two weeks to reduce stress associated with collection and shipping, and to acclimate them to laboratory holding conditions (AFW, artificial feed). After acclimation to laboratory conditions, water temperatures were increased at a rate of approximately 1°C/day to an experimental baseline temperature of 25°C, consistent with temperature adjustment protocols commonly used in freshwater mussel thermal physiology studies (Galbraith et al., 2012; Ganser et al., 2015).

A minimum of 17 adult individuals of each species (except *F. iheringi*, for which we only had six individuals) were acclimated to 25°C for at least two weeks prior to subjecting them to acute thermal tolerance experiments. Some individuals were used in previous sublethal hypoxia studies detailed in Chapter 1, but these individuals were allowed to recover for a minimum of two weeks before being used in subsequent experiments.

Water quality monitoring followed the same procedures described in Chapter 1. Routine water quality checks were performed using commercial multi-parameter test strips twice per week (used as a rapid check of hardness/alkalinity, nitrogen, and ammonia), along with an

electronic handheld pH reader. A YSI 9500 photometer was used once per week to record more precise measurements.

Behavioral responses to thermal stress

The afternoon prior to initiating a trial, ten mussels of a given species (or for *C. pustulosa*, ten individuals from each shipment) were transferred from upwellers to a rectangular observation tank approximately 122 x 76 x 33 cm in size, filled with ~151 L HAFW maintained at 25°C and allowed to acclimate overnight under experimental conditions. Dissolved oxygen in each tub was maintained at ~100% saturation using two air stones that were 2.5-5-cm in length, and water temperature was controlled by temperature controllers and 800-watt titanium heater bars in each tub. Only one heater bar per tub was required to maintain 25°C during acclimation, two in each tub were used during the heat ramp, and a third was added to each, if necessary, near peak temperatures. Mussels were placed individually into open plastic beakers with a ~5 cm deep gravel cup, which allowed unrestricted valve movement and foot extension. Plastic beakers had a ~10x10 cm section cut out on each side and replaced with mesh to allow for water flow through the beaker. Experiment photoperiod matched that of acclimation: a 12 h light cycle from 09:00 to 19:00. Collection of experimental data for analysis was initiated at 09:00, two hours after lights came on.

Starting at 09:00, water temperature was increased at a rate of 2°C / hour, and mussels were observed at 15-minute intervals for behavioral responses. Behaviors were defined and recorded according to established unionid research (Matteson, 1948). Foot extension was defined as limp protrusion of the foot beyond the shell margin. Gaping-responsive was defined as an extreme gape with relaxation of the adductor muscles and parting of the mantle edges, but the mussel still reacts to tactile probing using dull metal probes. Gaping-unresponsive was defined as

extreme gaping without a response to probing and was used as the critical thermal maximum (CTM) endpoint. A team of 3-4 trained observers monitored behaviors, with mussels being divided into groups that would alternate between people every 15 minutes to reduce observer bias. Temperature, time, and notes were recorded every time a behavior was observed.

This approach differs from previous unionid CTM assays in heating rate and utilizes slightly modified behavioral criteria. Galbraith et al. (2012) increased temperature at approximately 0.35°C per minute (~21°C/h) with continuous behavioral monitoring and defined CTM using a composite loss-of-function endpoint that included extreme gaping, mantle tissue retraction, foot extension, and loss of responsiveness. The slower ramp rate used here falls within the lower bounds discussed by Lutterschmidt and Hutchison (1997) and was selected to minimize thermal lag, allow determination of intermediate behavioral transitions prior to loss of responsiveness, and allow for measurements of respiration at each temperature interval. While not directly measured, we assumed that this rate would ensure mussel body temperature closely matched ambient water temperature, while still being fast enough to generally prevent thermal acclimation (Lutterschmidt & Hutchison, 1997).

In contrast to Galbraith et al. (2012), who defined CTM as the temperature at which extreme gaping, foot extension, mantle retraction, and loss of responsiveness occurred simultaneously, CTM in this study was defined as the temperature at which an individual gaped and became unresponsive to probing, regardless of foot position or mantle tissue retraction because several individuals did not display these behaviors prior to gaping with no response. Observations continued until all individuals reached the CTM endpoint. *P. riddellii* were then placed in a bucket of aerated experiment water that gradually cooled to room temperature and observed for recovery (i.e. ability to close valves in response to probing) the following morning.

For *C. pustulosa*, the behavioral trial was extended past CTM to observe possible mantle tissue retraction and siphon disjunction, as these were not observed in *P. riddellii* prior to experiment termination.

Physiological effects of thermal stress

In a separate system, we used intermittent-flow respirometry to record the respiration rates of seven individuals per species as temperatures were raised 2°C per hour, starting from 25°C. For *P. riddellii*, the respiration trial was conducted at the same time on the same day as the behavioral observation trial. However, the respirometry trial for *C. pustulosa* was conducted the week after the behavioral trial due to the larger number of individuals requiring observations during the behavioral trial and limitations in personnel. Intermittent-flow respirometry is a widely used technique that alternates between closed measurement periods, during which oxygen decline in the chamber is recorded, and flush periods, during which metabolic rates are not recorded and the chamber is replenished with fully oxygenated water that also flushes metabolic wastes.

The respirometry system was the same as described in Chapter 1, but the process differed in that intermittent-flow respirometry was used instead of closed-chamber respirometry, and only a single control chamber was run in parallel with mussel chambers. Intermittent respirometry cycles consisted of flush, wait, and measurement periods. Measurement period lengths were set to ensure that the DO in chambers did not fall below 80% oxygen saturation (Clark et al., 2013). Flush periods were set such that the DO in chambers returned to ~100% saturation for several minutes before the start of the next measurement period. Period lengths were adjusted as needed to meet these parameters while temperatures were increasing.

Seven mussels were placed individually into one of seven acrylic, vertical respirometry chambers (Loligo Systems) submerged within two connected experimental tanks, each with ~151 L aerated HAFW, maintained at the target water temperature. Each chamber was supplied with one recirculating pump and one flush pump (Eheim submersible 300 L/h pumps, Eheim GmbH & Co., Deizisau, Germany). A fiber-optic oxygen probe was placed via tubing between the chamber and the recirculating pump, such that the recirculating pump was always pumping water from the chamber past the oxygen sensor. Oxygen concentrations were logged continuously using AutoResp software (Loligo Systems). Background respiration was monitored in an eighth (control) chamber that did not contain a mussel and was subtracted from mussel MO_2 values to correct for microbial respiration. All chambers, including control chambers, ran on identical cycle times.

The appropriate respirometry chamber size was determined for each mussel by analyzing the chamber to animal volume ratio. For *P. riddellii*, medium sized chambers (347 mL vol. including tubes and pump) were used with the chamber volume to animal mass ratios averaging 8:1, similar to Haney et al. (2020) and Pieper et al. (2023). Small sized chambers (225 mL vol. including tubes and pump) were used for *C. pustulosa* with ratios averaging 11:1. Each chamber was considered an experimental unit with one mussel per chamber.

Animals were fasted for 24 h prior to trials to minimize the effects of specific dynamic action (SDA), the post-prandial elevation in metabolic rate associated with digestion, and to ensure that measurements reflected resting metabolic rate (Secor, 2009; Chabot et al., 2016). Animals were then acclimated to intermittent flow respirometry conditions for approximately 12 hours overnight prior to the experiment. Haney et al. (2020) tested acclimation times in freshwater mussels and found that an acclimation period of ≥ 5 hours was sufficient to stabilize

respiration rates after disturbance stress associated with transfer of mussels to chambers. Oxygen probes were calibrated with a 0% solution (sodium sulfite) and 100% air-saturated water before use.

For *P. riddellii*, the respirometry trial was ended at the same temperature at which all animals in the behavioral assay room reached CTM to allow for possible recovery because this species is rare. All individuals were transferred into aerated buckets containing experimental water that was allowed to cool gradually to room temperature to facilitate recovery chances. However, ending the respirometry trial at CTM consequently prevented us from capturing a respiration rate decline in *P. riddellii* (Figure 2.1). For the common *C. pustulosa*, the respiration trial was extended until metabolic rate approached zero, allowing us to capture a full decline in respiration rate in response to extreme acute heat stress (Figure 2.1). *F. iheringi* were assessed only in respirometry chambers due to the limited number of animals available. Behaviors were observed through respiration chambers, but responsiveness to probing could not be recorded. Though *F. iheringi* are also rare, the respiration trial was run until metabolic rate approached zero so we could capture a decline in respiration rate (Figure 2.1). Because trials with *C. pustulosa* and *F. iheringi* were extended until thermal extremes past CTM (upwards of 45°C), they were presumed dead, and wet tissues were removed and weighed separately from shell weight immediately after experiment termination.

Statistical Analyses

For each species, the Q_{10} temperature coefficient was calculated to measure the factor by which the rate of change in respiration increases for every increase in temperature by 10°C (Lampert, 1984) using the following formula:

$Q_{10} = (R_2 / R_1) \wedge [10 / (T_2 - T_1)]$

Where:

$R_1 = MO_2$ (mg O_2/g/h) at temperature T_1 (where $T_1 < T_2$)

$R_2 = MO_2$ (mg O_2/g/h) at temperature T_2 (where $T_2 > T_1$)

T_1 = temperature (°C) at measurement of R_1 (where $T_1 < T_2$)

T_2 = temperature (°C) at measurement of R_2 (where $T_2 > T_1$)

To test for differences in critical thermal maximum (CTM) between *P. riddellii* and *C. pustulosa*, we ran a one-way ANOVA. Data failed the normality test (Shapiro-Wilk), and a Kruskal-Wallis one-way ANOVA on ranks was executed. An all pairwise multiple comparison procedure (Dunn's Method) was used to isolate groups that differed from the others. To test for differences in the temperature that gaping-responsive behavior occurred among species, we also used a Kruskal-Wallis one-way ANOVA on ranks due to data failing the test for normality. A two-way ANOVA with a Tukey's post-hoc analysis was used to analyze the main effects of species, temperature, and the interaction between species and temperature on MO_2. The two temperatures tested were the initial acclimation temperature (26 ± 1°C) and the temperature near peak MO_2 (40 ± 1°C; Figure 2.1). One and two-way ANOVAs tested for differences among mean values, whereas Kruskal-Wallis ANOVA on ranks tested for differences among median rank-transformed values.

RESULTS

Foot extension was almost entirely lacking in *C. pustulosa*, as only one of the nine individuals from the stressed shipment exhibited this behavior at 43°C, and zero of ten mussels from the non-stressed shipment showed foot extension at any temperature (Figure 2.2). Foot extension was more common in the other species as six of ten *P. riddellii* showed foot extension at a mean temperature of 39.3°C ± 0.174 (*SE*), and three of the six *F. iheringi* showed foot extension at a mean temperature of 36.9°C ± 1.93 (*SE*; Figure 2.2).

Nine of ten *P. riddellii* were observed gaping but responsive at a mean temperature of 39.9°C ± 0.319 (*SE*), and all six *F. iheringi* were gaping (responsiveness unknown) at a mean temperature of 41°C ± 0.507 (*SE*). Six of ten *C. pustulosa* from the non-stressed shipment were gaping but responsive at a mean temperature of 40.5°C ± 0.5 (*SE*), and seven of nine *C. pustulosa* from the stressed shipment were gaping but responsive at a mean temperature of 41.1°C ± 0.634 (*SE*). Individuals that were not recorded gaping and responsive were non-responsive as soon as they gaped. No significant difference was found between species and/or shipments of *C. pustulosa* regarding the temperature at which gaping (responsive) was observed ($H_2 = 5.16$; $p = 0.076$; Figure 2.3). For *C. pustulosa*, mantle retraction was not observed until respiration rate approached zero, well past CTM, and full siphon disjunction was not clearly observed, possibly due to the shape and limited extreme gape width of this species.

The analysis of respiration rates (MO_2) at the beginning of the heat ramp (26±1°C) and close to peak (40±1°C) revealed a significant effect of species on respiration rate ($F_{2,78} = 35.77$; $p < 0.001$), with *C. pustulosa* having a higher respiration rate than *P. riddellii* (Tukey Test: $p < 0.001$) and *F. iheringi* having a higher respiration rate than *P. riddellii* ($p < 0.001$) and *C. pustulosa* ($p = 0.001$; Figure 2.4A). Additionally, there was a significant effect of temperature on respiration rate ($F_{1,78} = 297.16$; $p < 0.001$), with respiration rate being significantly higher at

40±1°C than at 26±1°C (Tukey Test: $p < 0.001$; Figure 2.4B). There was not a significant interaction between species and temperature on respiration rate ($F_{2,78} = 1.22$; $p = 0.300$; Figure 2.4C). We found that *P. riddellii* had the highest Q_{10}, *C. pustulosa* fell in the middle, and *F. iheringi* had the lowest Q_{10} (Figure 2.5).

Pleurobema riddellii reached CTM at a mean temperature of 41.7°C ± 0.178 (*SE*). *Cyclonaias pustulosa* from the non-stressed shipment had a mean CTM of 42.9°C ± 0.218 (*SE*), and *C. pustulosa* from the stressed shipment had a mean CTM of 42.8°C ± 0.396 (*SE*). Significant differences in CTM were found ($H_2 = 10.9$; $p = 0.004$). *P. riddellii* had a significantly lower critical thermal maximum (CTM) than both the stressed shipment of *C. pustulosa* ($p = 0.022$) and the non-stressed shipment of *C. pustulosa* ($p = 0.010$), but CTM did not significantly differ between *C. pustulosa* from the stressed and non-stressed shipments ($p = 1.00$; Figure 2.6). Following the 24-hour recovery period after the behavioral and respirometry thermal ramps, none of the 17 *P. riddellii* (10 from behavior trial, 7 from respirometry trial) recovered.

DISCUSSION

This study provides comparative data on acute thermal tolerance in three freshwater mussel species differing in conservation status and geographic distribution. Our findings reveal that *Pleurobema riddellii,* federally proposed as threatened, has a significantly lower critical thermal maximum (CTM) than common, sympatric *Cyclonaias pustulosa.* This supports our hypothesis that the rarer, range-limited species would have a lower upper thermal maximum than the common, widespread species, despite sympatry. Additionally, we found that *P. riddellii* has a steeper metabolic response to temperature (higher Q_{10}) than both *C. pustulosa* and federally

endangered, allopatric *Fuscanaia iheringi,* with *F. iheringi* having a slightly lower Q_{10} than *C. pustulosa*. This does not fully support our hypothesis that rarer species would show greater metabolic sensitivity to temperature, as *F. iheringi* is the most threatened of all three species, yet possesses the lowest Q_{10}.

Differences in Q_{10} among species indicate variation in thermal sensitivity of metabolic processes, with higher Q_{10} values reflecting greater increases in metabolic demand with temperature. Such differences may influence how rapidly species approach physiological limits under warming, but the mechanisms of physiological collapse vary and are not fully described by single-mechanism explanations (Schulte, 2015). Nevertheless, the species with the lowest CTM in this study simultaneously exhibited the highest Q_{10} value. These findings suggest that *P. riddellii* has a higher vulnerability to acute heat stress than *C. pustulosa,* which may be partially explained by differences in metabolic sensitivity.

Notably, none of the 17 *P. riddellii* individuals recovered following exposure to CTM conditions, indicating that the endpoint used in this study may have exceeded the reversible loss of function that CTM protocols are intended to measure, and/or our post-CTM cooling procedure did not adequately promote recovery. The CTM framework was originally defined as a non-lethal approach where organisms are progressively warmed until they reach loss of equilibrium or responsiveness, and recovery is expected if individuals are returned to harmless conditions (Lutterschmidt & Hutchison, 1997). With freshwater mussels, Galbraith et al. (2012) used essentially the same CTM endpoint that was used in our study (extreme gaping and unresponsive to probing) and reported minimal immediate mortality. However, delayed mortality in one Galbraith et al. (2012) species-treatment group (*E. complanata* with lower temperature acclimation and lower oxygen treatment) was substantial, with approximately 66% of mussels

dying within one week of the experiment. This, along with the observations from the current study, potentially suggest that CTM studies using extreme gaping coupled with unresponsiveness as endpoints may fall closer to representing physiological death than merely loss of function in some circumstances.

Post-trial cooling procedures were similar between the present study and Galbraith et al. (2012), with animals in both studies being cooled gradually rather than being rapidly transferred to lower temperatures. A key methodological difference is that Galbraith et al. (2012) used a more rapid warming protocol (~0.35°C/min), whereas the slower rate used here (~2°C/h) was favored to record oxygen consumption during intermittent respirometry. Slower ramping rates are commonly used in physiology-focused CTM studies for similar reasons, but they prolong the duration of exposure to temperatures near the upper thermal limit (Sandrelli & Gamperl, 2023). Many CTM studies that use faster ramping rates and immediate post-CTM cooling report high rates of recovery in specimens, such as Enders and Durhack (2022), which examined the CTM of salmonids. They transferred animals immediately from CTM temperatures to a ~10°C recovery bath, rapidly terminating thermal exposure, and reported only one mortality out of twenty fish.

In the present study, the combination of the slower ramping rate with passive cooling following CTM exposure likely further extended total exposure duration at stressful temperatures. Future studies may benefit from a more rapid post-CTM cooling methodology and/or adopting an earlier behavioral endpoint, such as extreme gaping with responsiveness, which was more consistently observed across species than foot extension and usually occurs simultaneously with foot extension or between foot extension and unresponsive extreme gaping. Khan et al. (2020) used unresponsive extreme gaping as their endpoint for mortality in a lethal tolerance (LT) analysis of freshwater mussels, further highlighting the confusion surrounding

where this endpoint falls between loss of equilibrium and mortality. This uncertainty suggests that it would be beneficial to re-evaluate which behavioral endpoint for freshwater mussels best represents the physiological state that CTM is intended to measure (Lutterschmidt & Hutchison, 1997), with careful consideration of how rates of temperature increase and post-CTM cooling methodology impact survival outcomes when using different endpoints. Since threatened or rare species are difficult to acquire, a more cautious methodology may better facilitate chances of survival and allow for re-use of animals in additional experiments.

Current published data on thermal tolerance for *Pleurobema riddellii* are limited to acute static lethal exposure experiments rather than dynamic heat-ramp assays. Mildenberger et al. (2025) examined the lethal thermal tolerances of three species of East Texas freshwater mussels (*P. riddellii*, *C. pustulosa*, and *Fusconaia askewi*). The *P. riddellii* mussels used in that study were from the LNVA Canal population, but the *C. pustulosa* and *F. askewi* mussels were from the upper Sabine River. They acclimated adult mussels to 27°C and then exposed mussels to constant temperatures of 30°C, 33°C, 36°C, or 39°C for 240 hours. For *P. riddellii*, the temperature associated with 5% mortality (LT_{05}) was 34.17°C over 240 hours, and the LT_{50} was 34.29°C over 240 hours. In contrast, at the same timepoint, *C. pustulosa* exhibited a higher LT_{50} (35.62°C), whereas *F. askewi* had the same LT_{50} as *P. riddellii* (34.29°C). This suggests species-specific differences in lethal thermal thresholds during prolonged exposure, and as the authors of that study note, *F. askewi* is a close relative of *P. riddellii*. Importantly, our CTM analysis also found that *P. riddellii* was more sensitive to acute thermal stress than *C. pustulosa*.

Although *P. riddellii* exhibited the lowest CTM among the two species tested here, its value (mean 41.7°C) falls in the middle of the observed range for unionids acclimated to 25°C when compared with published data. Across species, adult CTM values range from 32.1-42.7°C

under dynamic ramping protocols, depending on acclimation history and methodology (Fogelman et al., 2023), with CTM values ranging from 41.1-42.3°C when mussels are acclimated to 25°C (Galbraith et al., 2012). We found that *C. pustulosa* has a mean CTM of 42.9°C, higher than any other mussel species reported in published data. Importantly, Galbraith et al. (2012) used a rate of temperature increase that was 10.5 times faster than the rate used in the present study, and it is necessary to acknowledge the potential impacts of temperature rate on CTM values when making direct comparisons between studies (Lutterschmidt & Hutchison, 1997). The possibility must be considered that the mussels analyzed by Galbraith et al. (2012) might exhibit a lower CTM if re-analyzed at a slower ramp rate (i.e. 2°C/h).

Thus, the conservation concern for *P. riddellii* is not that its CTM is low across taxa, as it is among the highest recorded across published data for freshwater mussels, even though its analysis consisted of a much slower rate of temperature increase than comparable published studies. Rather, the potential concern is that its CTM is lower relative to its sympatric cohabitor and is coupled with a steep metabolic response to temperature. The findings in this study along with the findings of Mildenberger et al. (2025) indicate that regulations protective of common species such as *C. pustulosa* may not be protective of rarer species such as *P. riddellii*.

These results must be viewed in the context of the conservation and management of this species. The Neches River currently holds some of the largest documented populations of *P. riddellii*, and these populations have been viewed as a rare refuge for a species in steep decline (Ford et al., 2022). However, the engineered nature of the LNVA canals may present long-term complications for the aquatic communities they support, as scheduled dewatering events for maintenance have previously required mussel relocation (BIO-WEST, 2019). Available water temperature data for the LNVA canal system is recorded by the LNVA sensor (30.1411111, -

94.1744444), approximately 6.26 km upstream from the sample populations used in this study. From 2023 through 2025, data shows that temperatures did not approach the CTM of *P. riddellii* (max canal temp.: ~34°C; Figure 2.7). As discussed earlier, our recorded CTM value for *P. riddellii* might be more indicative of physiological death than recoverable loss of function, with the latter possibly being more accurately described by responsive extreme gaping (mean 39.9°C). Because recorded canal temperatures from the examined years do not approach the temperature of either endpoint, this aligns with the idea that anthropogenic habitats can, under certain conditions, provide stable environmental conditions and function as refuges for aquatic taxa (Chester & Robson, 2013).

Importantly, Chester and Robson (2013) demonstrated that human-made systems may offer refuge through maintained flow, depth, and water quality, but their suitability depends on site-specific management and environmental context. In this study, the LNVA canal system appears to provide such conditions under current regimes. Mildenberger et al. (2025) used in situ temperature recording at their LNVA canal collection site for *P. riddellii* alongside their lethal thermal tolerance analysis and found that temperature rarely exceeded thermal tolerance estimates, but exceedances increased during drought. Thus, these findings suggest that future increases in extreme heat and drought (USGCRP, 2018; IPCC, 2021) could threaten even this robust population if water levels drop or thermal refuge is unavailable. Consequently, the recognition, management, and enhancement of the LNVA canal system as potential refuge habitat may help mitigate these risks in a manner consistent with the conceptual perspective outlined by Chester and Robson (2013).

The first shipment of *C. pustulosa* that we received was delayed for two days during a very hot period of the year (August) and suffered several mortalities in the two weeks after

arrival. Because there were enough individuals remaining to analyze them in the CTM experiment, we decided to include them in analyses alongside the second shipment we requested, which experienced no stressful shipping delays. We found no differences between shipments in CTM or any other behavioral endpoints, suggesting full recovery from the conditions that caused 36% mortality in the stressed shipment. This also indicates that if the stressful shipping exerted selection for survival of more thermally tolerant individuals, it was not significant enough to be detected by CTM analysis. However, a wider spread in data was observed in the stressed shipment across endpoints, indicating greater variability between individuals from that shipment. Both shipments of *C. pustulosa* showed a significantly higher CTM than *P. riddellii*, further supporting that stressful shipping did not significantly impact the upper thermal tolerance of *C. pustulosa* after they recovered.

Results from the behavioral trials showed considerable variability between species regarding the behavioral indicators observed in response to thermal stress. *P. riddellii* exhibited frequent foot extension at high-end temperatures, a behavior associated with stress response in mussels (Galbraith et al., 2012), whereas *C. pustulosa* hardly used this behavior. *F. iheringi* were observed displaying foot extension at lower temperatures than the other two species, suggesting species-specific thresholds of behavioral response. These observations support our hypothesis that rarer species would show signs of stress at lower temperatures than widespread species.

In a previous emersion and thermal tolerance study that included *C. pustulosa* (syn. *Quadrula pustulosa*) from Wisconsin, Bartsch et al. (2000) observed that foot extension did not occur in *C. pustulosa*, nor was it observed in *Lampsilis cardium*, but foot extension was observed in *Elliptio dilatata*. Bartsch et al. (2000) noted that there appeared to be a relationship between the occurrence of these behaviors and survival in the species examined, with *E. dilatata*

exhibiting foot extension and having the lowest survival during emersion at higher temperatures. Our findings in the present study support this idea, as the taxa that exhibited the most frequent foot extension also had a lower CTM. If frequent foot extension can be used as an indicator of lower thermal tolerance relative to species that exhibit less frequent foot extension, it could be a useful management tool, but additional research is required to determine whether this is a widely observed pattern. These results support the idea that foot extension behaviors act as valuable indicators of stress, but it remains uncertain for many species whether this behavior occurs early enough prior to physiological death to allow for timely management interventions if observed in the field, such as rescue or translocation to prevent mortality.

In *P. riddellii*, since foot extension occurred prior to CTM, it should be expected that they would recover if they were found still responsive and promptly moved to non-stressful conditions. However, *P. riddellii* exhibited foot extension at a mean temperature of 39.3°C, which is likely far too close to the mean CTM of 41.7°C to be considered an early indicator of sublethal stress. The same situation was observed with gaping-responsive behavior, as both *P. riddellii* and *C. pustulosa* were observed gaping yet responsive too close to CTM for it to be considered an early indicator of stress in these species. This is further highlighted by the finding that both *P. riddellii* and *C. pustulosa* had at least one individual that was non-responsive with the first sign of gaping, with this being more frequently observed in *C. pustulosa*. Field observations of foot extension and gaping in this population of *P. riddellii* should be taken very seriously as these behaviors occurred within 3°C of CTM.

These findings indicate that for some species or populations, behaviors such as foot extension and responsive gaping should be regarded as late indicators of stress, positioning them more as indicators of imminent mortality. Additionally, this supports the idea that foot extension

and/or responsive extreme gaping endpoints may be valid indicators of CTM (recoverable loss of function) because unresponsive gaping may occur too close to physiological death in some species to accurately describe CTM. However, it is worth noting that if this approach were taken in this study and responsive gaping was used as our CTM endpoint, no significant difference in CTM would have been observed between *P. riddellii* and *C. pustulosa*. Behavioral interpretation gets further complicated by the finding that only one of nineteen *C. pustulosa* individuals were observed using foot extension, raising questions about why some species do not seem to show this behavior. Thus, additional research is needed on the timing and predictive value of behavioral responses.

In conclusion, while *P. riddellii's* CTM is high compared to other unionids, its lower tolerance and higher metabolic sensitivity relative to sympatric *C. pustulosa* reinforces the idea that management strategies protective of widespread species may not be adequate for more rare species. The importance of this finding is highlighted by the fact that none of the *P. riddellii* individuals recovered after being exposed to CTM conditions. CTM methodological considerations, including ramping rate and behavioral endpoints, emphasize the need for careful interpretation of CTM-derived thresholds. While the LNVA canal system currently appears to provide thermal conditions that remain well below critical thresholds for *P. riddellii*, strong populations in natural rivers, such as those in the Neches River, may be at future risk of declining under acute heat events if drought occurrence increases alongside changes in local water management practices. Similar to the events recorded by Galbraith et al. (2010), this scenario could lead to shallow-water heating that would likely approach the CTM of *P. riddellii*. However, embracing the potential for the LNVA canal system to provide thermal and hydrological refuge for *P. riddellii* might protect this species from disturbances to natural

habitats expected to intensify with climate change. These findings highlight the importance of evaluating behavioral and metabolic responses across species and within their environmental context to better assess vulnerability to climate change. Future work should clarify the relationship between behavioral endpoints and methodological influences on post-CTM survivorship in freshwater mussels, examine the thermal tolerances of different life stages of *P. riddellii*, and analyze whether the LNVA canal can effectively serve as refuge habitat for host fish as well.

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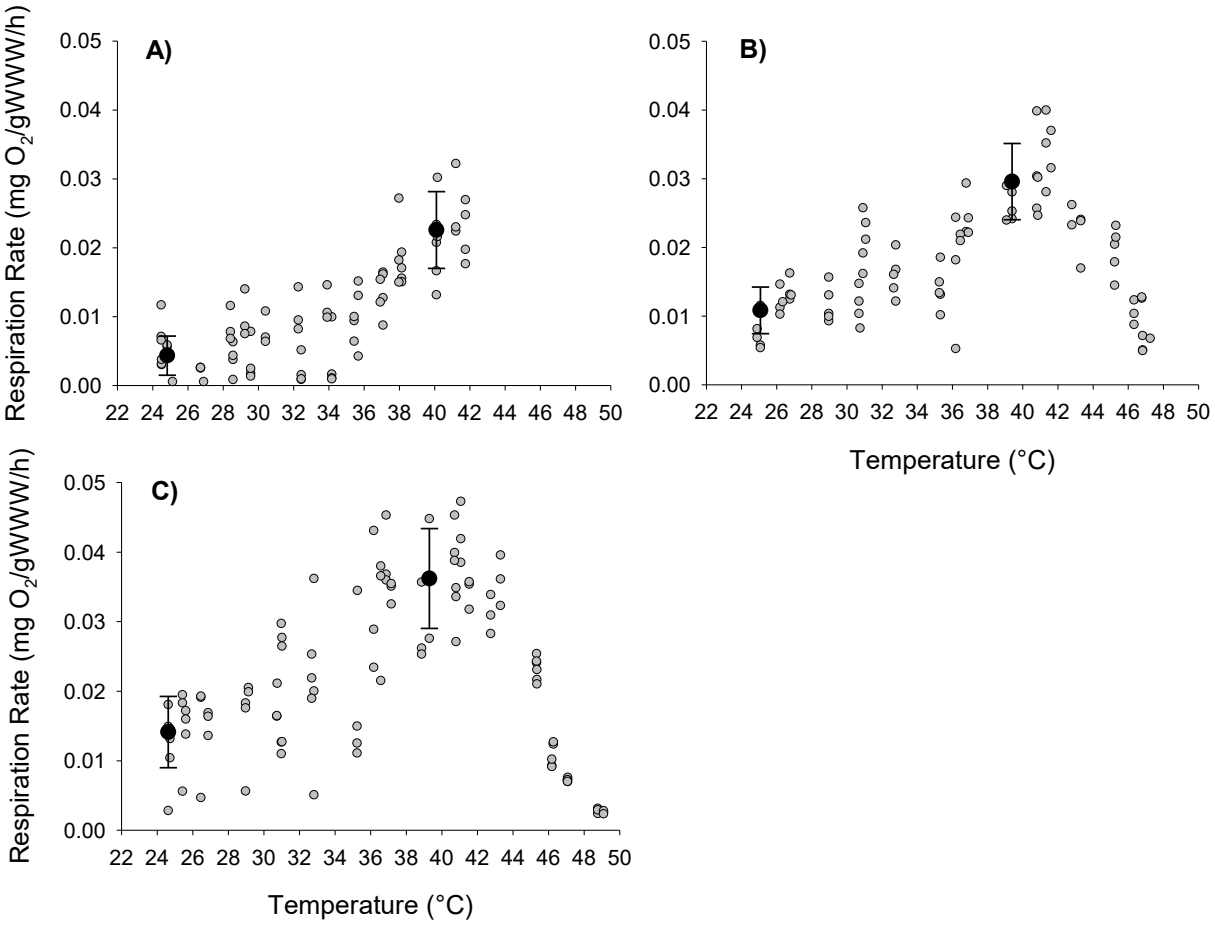

Figure 2.1: Respiration rate (MO_2) of A) *P. riddellii,* B) *C. pustulosa,* and C) *F. iheringi* as temperatures increased 2°C/h. Grey circles represent raw data, while black circles indicate mean MO_2 values at 26 ±1°C and 40 ±1°C. Error bars represent ± SD.

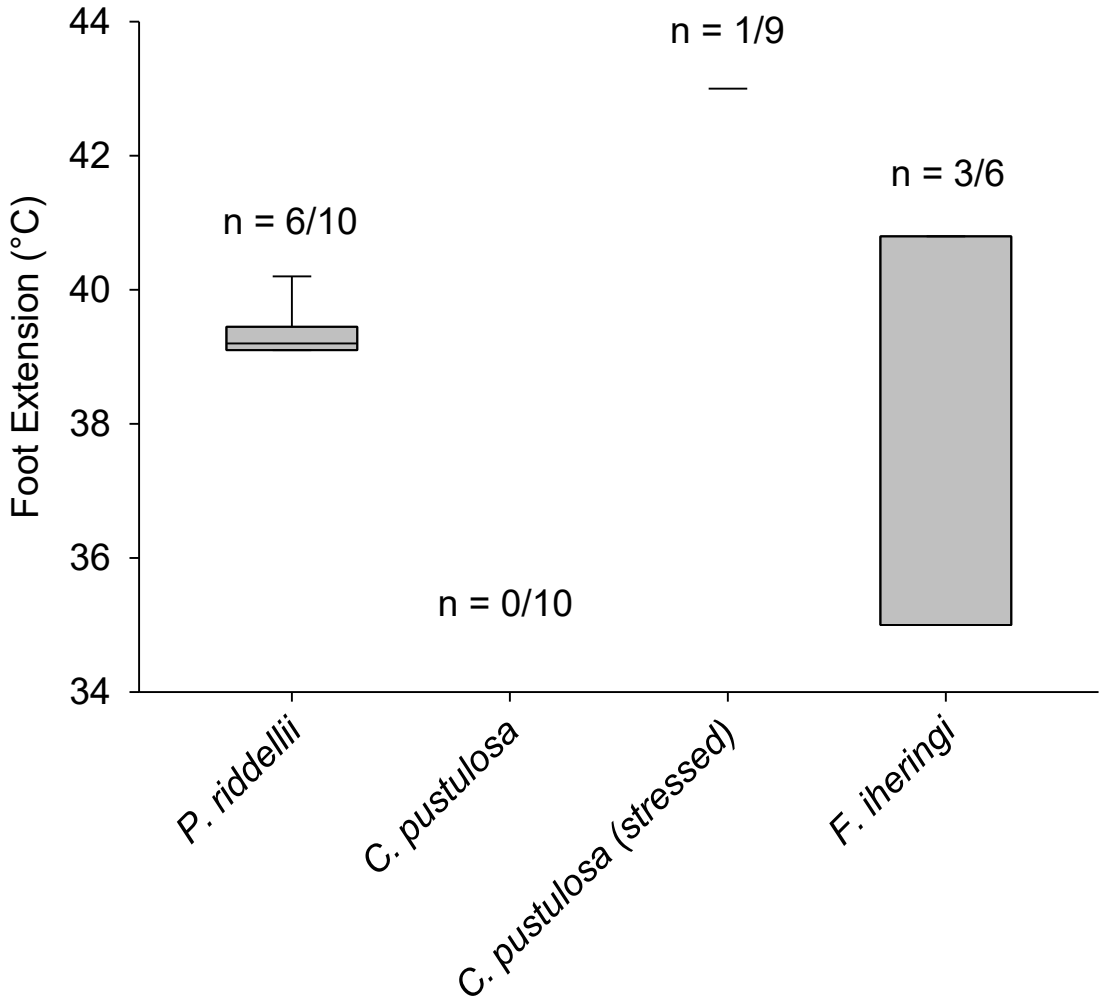

Figure 2.2: The temperature at which foot extension was observed for each species. The number of individuals that exhibited the behavior out of the total number of individuals of a given species are denoted by "n". Boxes show interquartile range (IQR; 25th-75th percentile), the horizontal line inside of a box indicates the median, and whiskers show data extending to the smallest and largest values within 1.5 × IQR.

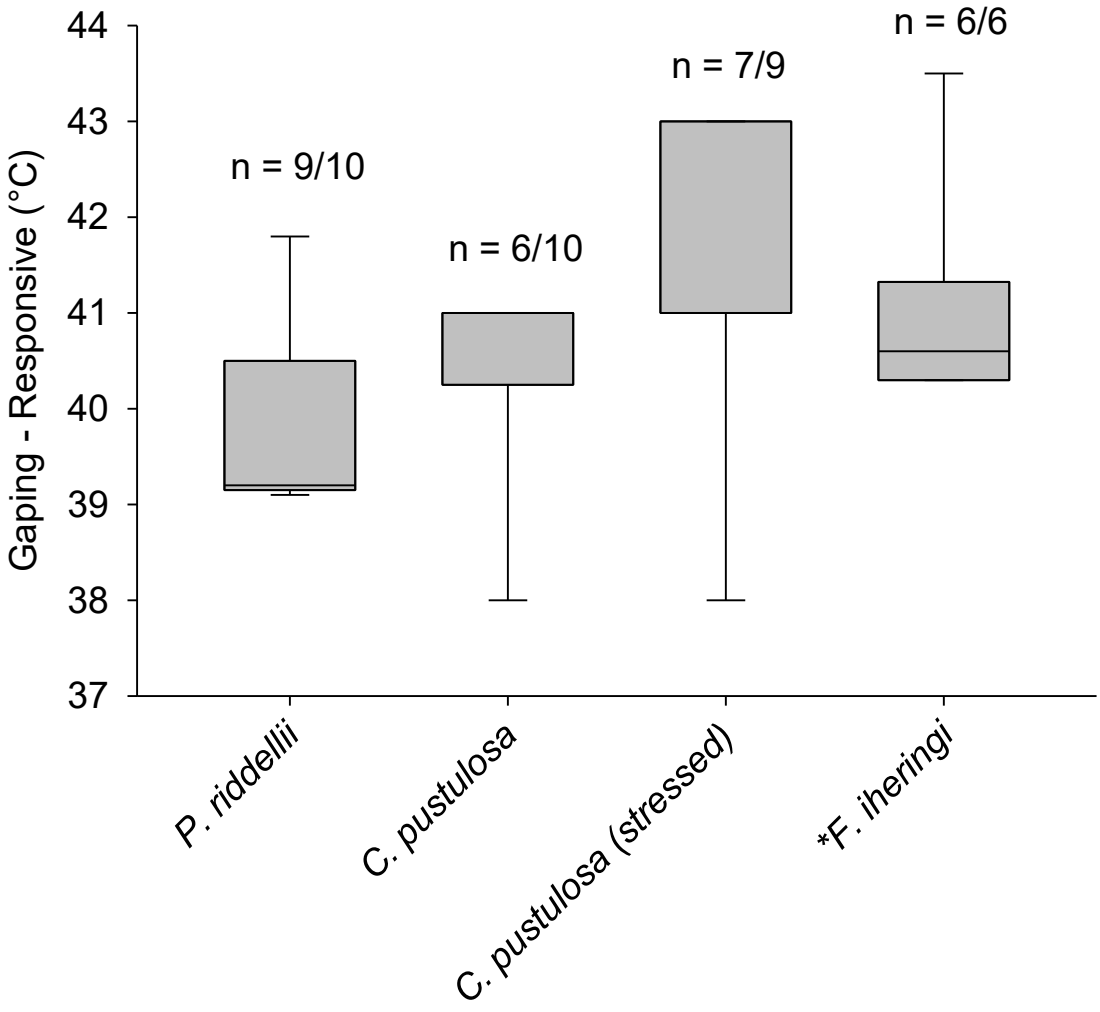

Figure 2.3: The temperature at which gaping (responsive) was observed for each species. The number of individuals that exhibited the behavior out of the total number of individuals of a given species are denoted by "n." Boxes show interquartile range (IQR; 25th-75th percentile), the horizontal line inside of a box indicates the median, and whiskers show data extending to the smallest and largest values within 1.5 × IQR. **F. iheringi* were observed in respirometry chambers and responsiveness could not be measured.

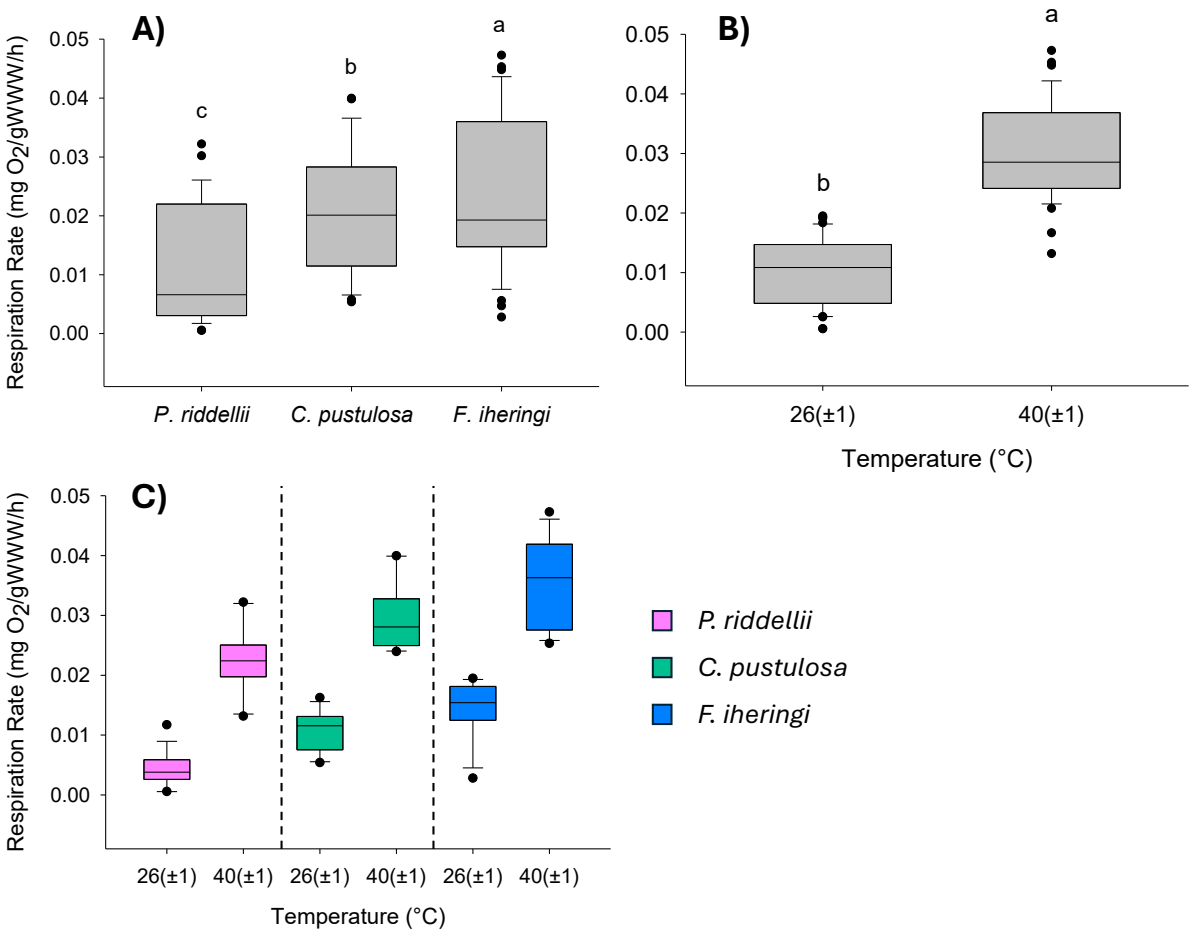

Figure 2.4: A) Effect of species on respiration rate. B) Effect of temperature on respiration rate. C) Interaction between species and temperature on respiration rate. *C. pustulosa* were from the non-stressed shipment only. Lower case letters indicate significant differences in mean respiration rate among categories. Boxes show interquartile range (IQR; 25th-75th percentile), the horizontal line inside of a box indicates the median, whiskers show data extending to the smallest and largest values within 1.5 × IQR. Individual points indicate outliers.

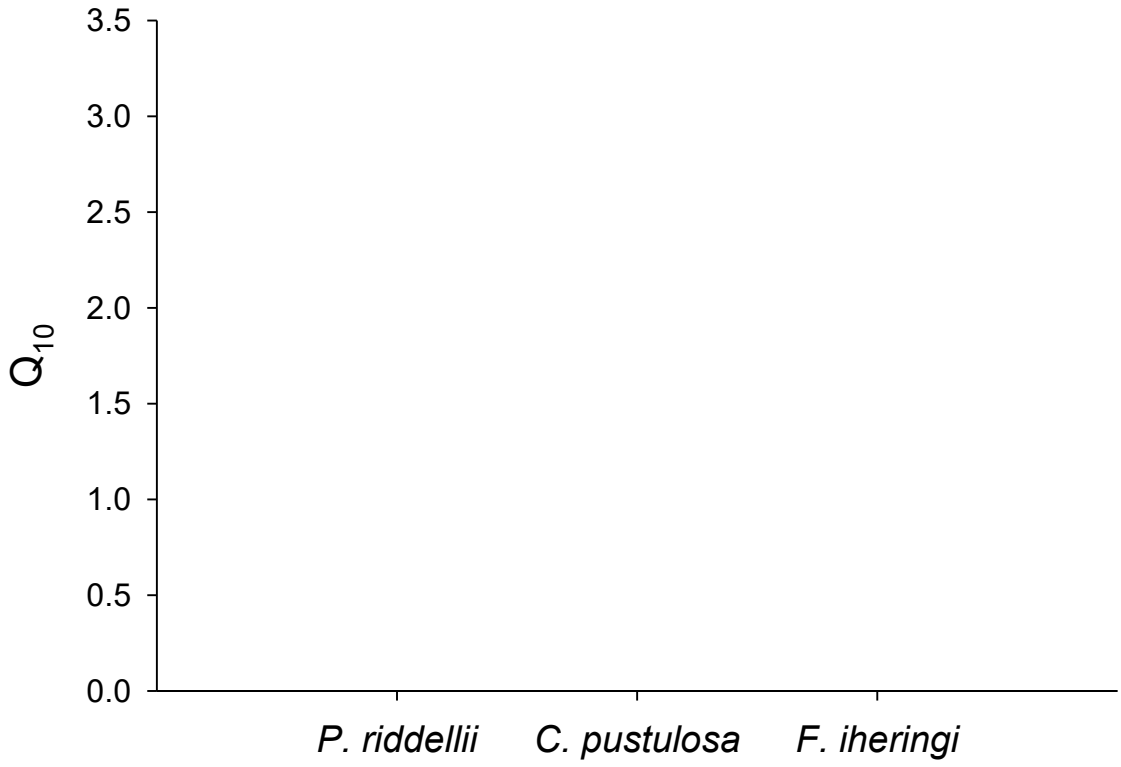

Figure 2.5: Temperature coefficient (Q_{10}) across species.

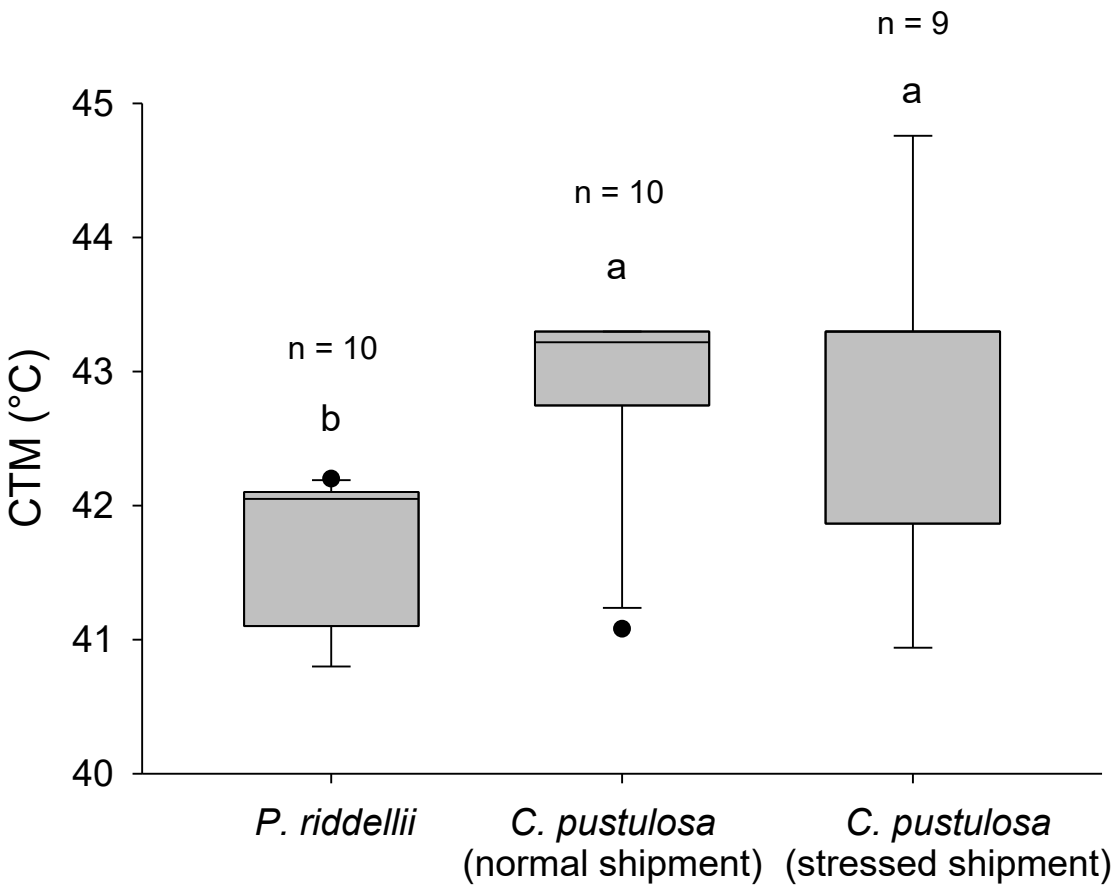

Figure 2.6: The temperature at which critical thermal maximum (CTM) was observed for each species. The numbers of experimental individuals are denoted by "n." Lowercase letters indicate significant differences in median rank-transformed CTM among species and shipments. Boxes show interquartile range (IQR; 25^{th}-75^{th} percentile), the horizontal line inside of a box indicates median temperature, and whiskers show data extending to the smallest and largest values within 1.5 × IQR. Individual points indicate outliers.

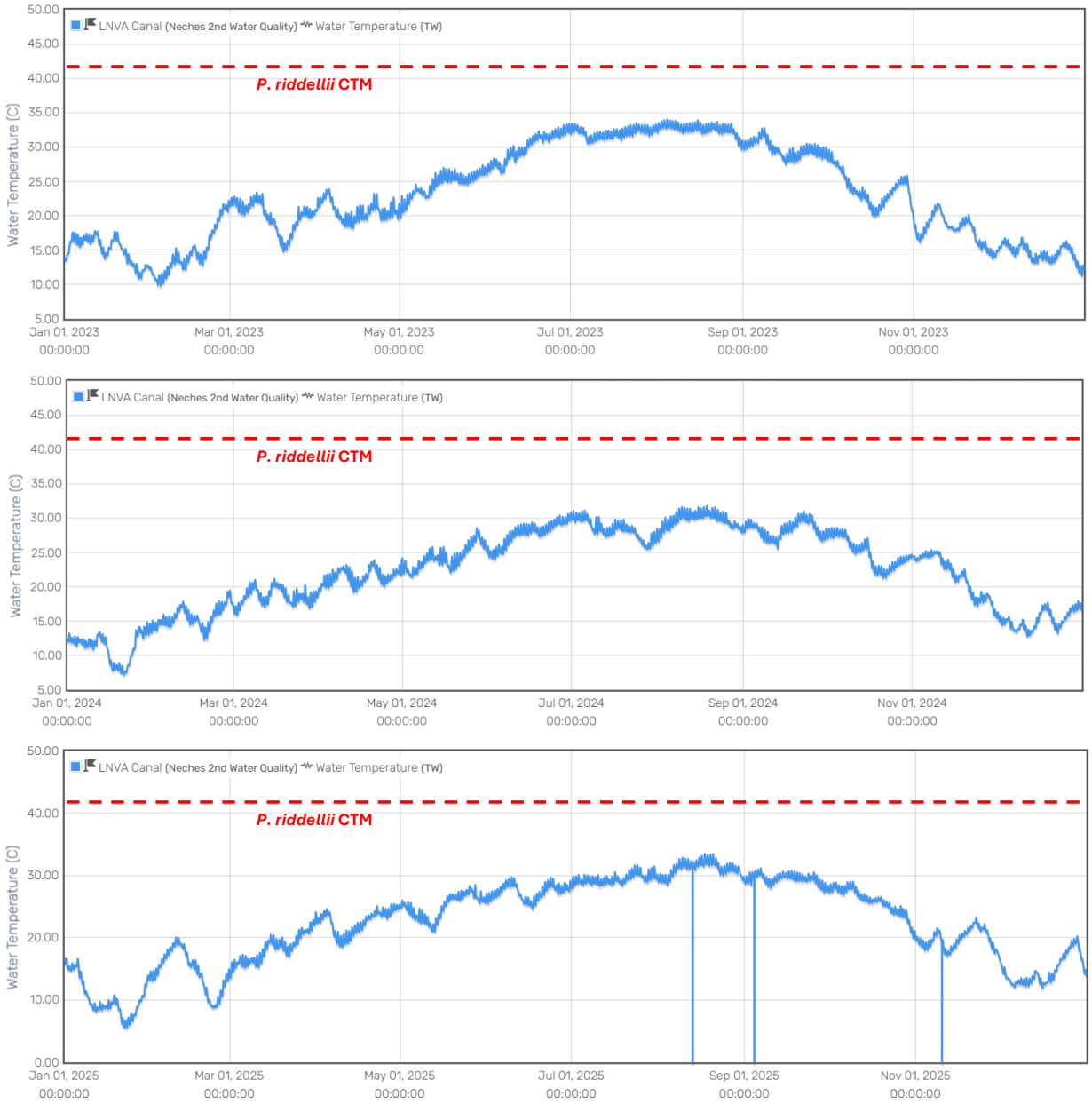

Figure 2.7: Temperature data in 2023, 2024, and 2025, as recorded by the Lower Neches Valley Authority (LNVA) sensors in the LNVA Canal (30.1411111, -94.1744444). The critical thermal maximum (CTM) of *P. riddellii* is marked by a red dotted line. Sudden, straight vertical drops in data are presumed to be sensor errors.