Effects of Acute Thermal Stress on Texas Hornshell Mussel (*Popenaias popeii*)

by

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Abstract

Extreme weather conditions are placing increasing stress on aquatic ectotherms in the southern U.S. We investigated effects of thermal stress on the federally endangered Texas Hornshell (*Popenaias popeii*), the last freshwater mussel species in New Mexico. Using a combination of physiological assays, we examined effects of rapid temperature changes on scope for growth (SFG: energy surplus for growth and reproduction) and absolute aerobic scope (AAS: respiratory capacity in excess of that needed for basic maintenance). At the enzymatic level, respiratory capacity patterns differed from those of a previously examined ectotherm (marine shrimp), rendering estimates of AAS of minimal use in assessing thermal stress of Texas Hornshell. At the organismal level, diurnal changes in temperature typical of the summer months had a significant, negative impact on scope for growth. Maintaining sufficient daily flows during the hot summer months may be critical for the long-term survival of this species in New Mexico.

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List of Abbreviations

AAS	Absolute aerobic scope (mL O2/gWW/h)
AAS_{min}	Minimum observed AAS value (mL O2/gWW/h)
BLM	Bureau of Land Management
CEHMM	Center of Excellence; environmental organization in Carlsbad, New Mexico
CI	Confidence interval
CT_{max}	Critical thermal maximum; temperature at which an organism experiences loss of equilibrium (°C)
DO	Dissolved oxygen (mg/L)
DTF	Daily temperature fluctuations
ETS	Electron transport system
ETS_{max}	Maximum ETS or maximum MMR_{ets} activity (mL O2/gWW/h)
EWSFC	E.W. Shell Fisheries Center at Auburn University, AL
HAFW	Hard artificial freshwater
IPCC	Intergovernmental Panel on Climate Change
LOE	Loss of equilibrium
LPB	Larval-post larval-broodstock shellfish algae diet from Reed Mariculture Inc.
MMR_{ets}	Maximum metabolic rate as estimated by the ETS assay
MMR_{resp}	Maximum metabolic rate as estimated by respirometry; standard method
PMA	Potential metabolic activity
REML	Restricted maximum likelihood; statistical analysis method
RMR	Resting metabolic rate
RMR_{peak}	Peak resting metabolic rate

SD	Standard deviation
SE	Standard error
SFG	Scope for growth; energy available for growth and reproduction (J/gWWW/h)
SMR	Standard metabolic rate
SS	Smoothing spline; statistical analysis method
TAN	Total ammonia-nitrogen
THS	Texas hornshell mussel (*Popenaias popeii*)
TPC	Thermal performance curve
TSS	Total suspended solids
USEPA	U.S. Environmental Protection Agency
USFWS	U.S. Fish and Wildlife Service
USGS	U.S. Geological Survey

Background

Texas hornshell [*Popenaias popeii* (Lea 1857)] is a freshwater mussel in the family Unionidae. On February 9, 2018, the U.S. Fish and Wildlife Service (USFWS) ruled to list Texas hornshell (THS) as an endangered species under the Endangered Species Act of 1973 (83 FR 5720 5735; USFWS 2018a). Texas hornshell are native to the Rio Grande drainage in New Mexico and Texas, occurring in medium to large rivers and typically inhabit crevices, undercut riverbanks, travertine shelves, and under large boulders adjacent to runs (USFWS, 2018b). These habitats are thought to provide refuge from high water events, such as flooding which commonly occur in the species' historical range.

Most unionids, including THS, have a complex life history that requires a parasitic life stage of their larvae through utilization of host fishes. Texas hornshell larvae attach to the gills and fins of River Carpsucker (*Carpiodes carpio*), Red Shiner (*Cyprinella lutrensis*), or Gray Redhorse (*Moxostoma congestum*). The presence of these fish species is critical to the survival of THS mussels given that juveniles will not metamorphose into the juvenile life stage without a suitable host. Upon dropping off their host, juvenile mussels pedal feed and eventually use their foot to anchor into the substrate and filter feed. Texas hornshell life span is assumed to be 15 years or more (USFWS 2018a).

Significant declines in THS distribution are attributed to population isolation caused by reservoirs and unsuitable habitat due to unfavorable water conditions such as saline waters (USFWS, 2018b). These same barriers that isolate THS also act as barriers to their host fish passage furthering the isolation between previously connected populations. Texas Hornshell currently occupy approximately 15% of its historical range in the U.S. (USFWS, 2022). At present, there are six known populations remaining within the U.S. historical range: Black River,

Lower Pecos River, Rio Grande – Lower Canyons, Rio Grande – Laredo, Devils River (USFWS 2018b), and a small population in the confluence of Rio San Diego, Mexico (Hein et al. 2018).

Historically, New Mexico had eight native freshwater mussel species; however, the only remaining species is the Texas hornshell. Global temperature increases and subsequent extreme weather events are straining the resiliency of this solely remaining species. Climate warming in the Southwest is expected to be greatest in the summer, with annual mean precipitation very likely to decrease in the Southwest (IPCC, 2023). Very low water levels are detrimental to THS populations. Droughts have occurred in recent years and have led to extremely low flows in rivers across the desert Southwest. The Black River THS population has some resiliency to drought because the river is spring fed. However, drought in conjunction with increased groundwater pumping and regulated reservoir releases may lead to lower river flows with longer duration than have been previously recorded (USFWS, 2018a). Recent pumping of water from the Black River, extreme drought events, along with other increased water withdrawals for commercial and other purposes have caused the amount of surface flow in the watershed to decline significantly (CEHMM, 2017). Additional reduction in flow could raise the water temperature, cause pools to stagnate, and cease surface flow altogether.

The Center of Excellence (CEHMM) partnered with the U.S. Fish and Wildlife Service (the Service), the Bureau of Land Management (BLM), New Mexico State Land Office, and several other state and federal agencies to develop a Candidate Conservation Agreement and a Candidate Conservation Agreement with Assurances for Texas hornshell and other impaired aquatic species in southeastern New Mexico and west Texas. This allowed voluntary participation of entities or individuals holding permits, leases, grants, and other authorizations

issued by the BLM to conduct activities on Federal lands to address the conservation of these imperiled species (CEHMM, 2017).

CEHMM awarded researchers a River Flow Regime Requirements Study which was approved and funded in October of 2020 for $168,772. The collaborative team of researchers from Miami, Texas A&M, and Auburn Universities were funded to conduct a series of laboratory experiments and field monitoring studies to examine lethal and sublethal effects of thermal and hypoxia stress on various life history stages of the THS. Relationships between flow, temperature, and dissolved oxygen in the Black River were also studied. Results will be used to help identify flow regimes most likely to induce mortality and/or thermal stress in THS. Combined with historical datasets, results will be used by both CEHMM and the Service. CEHMM will determine whether frequency of stressful periods has been increasing over time, and the Service will make specific flow recommendations for THS populations in the Black River. (Candidate Conservation Agreements THS, CHEMM 2021 Q2 Report).

Chapter I: Effects of Diurnal Temperature Increases on Scope for Growth of *Popenaias popeii*

Introduction

Extreme weather conditions and increasing demands for water by human populations are placing increasing stress on many aquatic ectotherms (Spooner and Vaughn, 2008; Haag and Williams, 2014). Unionids play crucial roles as ecosystem engineers through water clarification, biodeposition of organic matter and nutrient cycling, large biomass, and trophic interactions (Spooner and Vaughn, 2008; Haag, 2012). Approximately 65% of freshwater mussels in North America are endangered, threatened, or vulnerable (Haag and Williams 2014). Land-use changes, habitat destruction, pollution, commercial harvesting, and invasive species have all contributed to their imperilment (Luck and Ackerman, 2022).

Unionids live sedentary lifestyles, leaving them highly susceptible to environmental changes such as flow and temperature (Archambault et al. 2014). Understanding habitat needs and environmental tolerances of native species is crucial to their conservation. Bioenergetic modeling can provide critical information regarding sublethal and lethal effects of environmental stressors on at-risk populations. This information can then be used to inform adaptive management practices related to maintaining appropriate flow regimes and environmental conditions for continued survival of aquatic species of interest.

Growth of an organism depends primarily on food availability and is directly influenced by temperature. When sufficient food to support growth is available, growth rates peak at optimal temperatures and decline at temperatures above or below the optimal range. Growth incorporates the energy acquired by an organism through feeding and the energy it expends through metabolic processes and excretion (Widdows & Staff, 2006). Each of these processes is

affected by temperature (Woodward, Perkins, & Brown, 2010) and can be quantified using the same energetic units (J/h). Energy gains and energy costs can then be used to calculate net energy available for growth and reproduction, otherwise referred to as scope for growth (SFG) (Widdows and Johnson, 1988; Widdows, 1995; Widdows and Staff, 1997). It provides a method of determining an animal's physiological response to stress through a non-lethal approach. Although SFG has been used as a valuable method for evaluating how organisms are energetically impacted by environmental stressors and subsequently allow for the development of informed management strategies to aid in these species' recovery, the SFG model has rarely been applied to unionid mussels (Luck and Ackerman, 2022).

Standard methodology for SFG estimation calls for repeated measurements of required parameters at a constant temperature on individuals acclimated to that temperature (Widdows and Staff, 1997). This process is then repeated for a different set of animals held at a different constant temperature. However, in the wild, temperatures are not constant and individual organisms experience daily, monthly, and annual temperature swings (Gunderson et al. 2016). These fluctuations are likely to be extremely important for dictating how organisms respond to prevailing conditions and to increasing environmental change (Vincenzi 2014). The effects of diurnal temperature swings and other dynamic environmental factors on physiological responses are becoming increasingly studied with the hopes of generating more realistic inferences about the effects of global change (Gunderson et al. 2016). The effects of diurnal temperature fluctuations in unionid bioenergetics are not yet well studied. It may be that daily changes in temperature are too small to affect SFG. On the other hand, daily changes in Spring or Fall may be beneficial by increasing SFG from morning to afternoon as temperatures approach an

optimum. Meanwhile, daily shifts in mid-summer may be detrimental as temperatures rise above the optimum and become stressful.

The Texas Hornshell mussel (*Popenaias popeii*) is a unionid found in the Rio-Grande basin in New Mexico, Texas, and Mexico. It is federally listed as "Endangered" and is the only remaining freshwater mussel in New Mexico where it experiences daily temperature fluctuations of 4°C or even higher (USFWS, 2018a). Key threats to this species include the effects of extreme weather events. Extreme weather events such as high temperatures, storms, droughts, floods, cold spells and heat waves are increasing in magnitude and frequency and have serious implications for ecosystems and societies (IPCC 2007; Jentsch et al. 2007). These effects coupled with the implications of anthropogenic stressors such as damming, water extraction, and run-off have driven this species to be at-risk of extinction (USFWS 2018b; CEHMM 2017; Aldridge et al. 2022). With the increasing threats to this species and its habitat, understanding their needs and stressors has become crucial to their conservation. To investigate THS response to thermal increases, we conducted experiments evaluating their scope for growth (SFG) under ecologically relevant diurnal changes in temperature.

Recent studies following traditional methodology and measuring SFG of acclimated THS held at constant temperatures revealed a relatively constant SFG from 16-24°C, increasing SFG from 24-28°C, followed by a decreasing SFG from 28-32°C (Pieper, 2023). However, reaches of the Black River, NM, that host one of the few remaining stable populations of THS (Inoue et al., 2014), frequently experience a 4°C diurnal temperature increase from morning to late afternoon, particularly from late Spring through early Fall (Figure 1.1). In this study, we investigate whether short term shifts in temperature, representative of THS's natural environment, cause changes in SFG similar to those observed in traditional, constant temperature experiments. We conducted a

series of dynamic SFG assays on *P. popeii* exposed to diurnal temperature swings mimicking those typically experienced in the Black River, New Mexico in the Spring, Summer, and Fall. We hypothesized:

1) Diurnal swings of 16-20℃ and 20-24℃ will have little effect on mussel SFG
2) A diurnal swing of 24-28℃ will result in a diurnal increase in SFG
3) A diurnal swing of 28-32℃ will result in a diurnal decrease in SFG

Methods

Experimental Animals

Fifty adult THS were collected from the Black River near Carlsbad, NM in October 2021 under federal collection permit #TE78507C-0. The total population of the Black River has been estimated at ~48,000 mussels (Inoue et al. 2014). Mussels were primarily found in submerged portions of undercut banks, burrowed in muddy substrate. Adult mussels over 70mm were collected for experiments whereas subadults and juveniles were noted and returned to the substrate. The first day of the collection trip was spent locating and marking areas containing mussels so they could be quickly collected by hand on the second day. Less than 50% of detected mussels were collected from any given site and collections were spread out across three sites. After collection, each mussel was cleaned, photographed, wrapped in moist towels, then placed in coolers with a small ice pack for overnight shipping to the E.W. Shell Fisheries Center (EWSFC) at Auburn University, AL.

Upon arrival at EWSFC, mussels were cleaned, weighed, and marked with FPN glue-on shellfish tags (Hall Print Fish Tags, South Australia) for individual identification. Mussels were held in upweller systems filled with Hard Artificial Freshwater (HAFW) (Smith et al. 1997) and gravel substrate. Automatic feeders (GHL Doser 2.2; GHL International, Germany) connected to

each upweller provided a constant algae supply maintained 25,000-35,000 cells/mL of LPB Shellfish Diet (Reed Mariculture Inc., Campbell, CA) stock solution. The algal source for each automatic feeder was a stock solution in 2L beakers, kept in suspension by a stir plate, and placed in a mini fridge to maintain temperature. Stock solutions were replenished weekly and feeding dosages adjusted to maintain proper algae concentrations. Total ammonia-nitrogen (TAN), nitrate, and nitrite were analyzed twice/week using a YSI 9300 Photometer (YSI Inc., Yellow Springs, OH). A partial water exchange was triggered when TAN increased above 0.1 mg/L.

General SFG Assay design

After acclimation to laboratory conditions for >2 weeks, mussels were randomly assigned to one of four thermal ramp treatments (16-20, 20-24, 24-28, 28-32°C), with six mussels per ramp for a total of 24 mussels. All mussels were acclimated to their assigned starting temperature for ≥ 2 weeks prior to being exposed to the thermal ramp. For example, mussels assigned to the 20-24°C ramp treatment were first acclimated for ≥ 2 weeks at 20°C. Immediately before entering experimental assays, mussels were cleaned with a soft bristle brush, towel dried, and whole wet weight recorded. During a given thermal ramp treatment, temperature was increased at a rate of 0.5°C/h for 8h.

During acclimation to their assigned starting temperature, and all subsequent assays, mussels were fed ~30,000 cells/mL using automated feeders (GHL Doser 2.2) as described previously. Algal density within experimental systems was monitored using a spectrophotometer (GENESYS 10S UV-Vis, Thermo Fisher Scientific Inc., Waltham, MA) at 450nm wavelength and 50 mm Rectangular Long Pathlength Cell glass cuvettes. We first established a calibration curve by creating a range of LPB Diet standards in 1L HAFW ranging from 3,000 to 350,000

cells/ml. A hemocytometer was used to verify the cell density of each standard (2 replicate counts per standard). Three 15mL samples of each standard were then placed in a long pathlength cuvette and absorbance measured. The mean hemocytometer count of each standard was then plotted against mean absorbance and a linear regression fitted through the data (SigmaPlot, 2015 Systat Software Inc. version 13.1). The resulting LBP Diet calibration curve ($R^2 = 0.997$) was used in all experimental runs to convert absorbance measurements to algal density (cells/mL).

To test for changes in SFG during a given thermal ramp, we first measured respiration rate of all mussels throughout their assigned ramp. Mussels were then allowed to cool back down to the ramp base-temperature overnight. The next day, clearance rates of the same mussels were measured as the thermal ramp was repeated, and the feces of each individual were collected at the end of the run. SFG was calculated using the following simplified energy equation given by Widdows & Staff 2006:

$$SFG = A - R$$

Where,

A = absorbed food energy;

R = respiratory energy expenditure;

SFG = energy available for growth and reproduction.

Specific assay methodologies and equations used in quantifying each variable incorporated into SFG measurements are described below.

Respiration Rates

Resting metabolic rates (RMR) for each individual were measured using intermittent respirometry via an 8-chamber optical respirometry system coupled with Auto-Resp software

(Loligo Systems, Viborg, Denmark). Chambers were made of acrylic and ranged in volume from 200 to 700 mL. Mussels were paired with an appropriately sized chamber such that the ratio of chamber volume (mL) to mussel whole wet weight (g) was approximately 6.7 mL:g (± 0.48 SE), similar to that of Haney et al. (2020). Each chamber was connected to two Eheim submersible 300 L/h pumps (Eheim GmbhH & Co., Deizisau, Germany). The flush pump circulated fresh, oxygenated water from the trough through the chamber during initial chamber acclimation and during flush cycles. The closed pump circulated water within a closed loop, including the chamber, during measurement periods (Figure 1.2). A flow-through oxygen cell with an optical dissolved oxygen (DO) sensor was inserted in the closed pump tubing to measure the DO of recirculating water. Two temperature sensors placed one on either end of the experimental tank continually measured the temperature of each run.

Respirometry chambers, pumps, and sensors were submerged in a 400 L rectangular fiberglass tank filled with ~250 L hard artificial freshwater (HAFW). The tank water was aerated to maintain ~100% oxygen saturation. Water temperatures were controlled with a TECO TK-2000 chiller/heater unit (TECO US). To reduce ambient oxygen demand from bacteria, all chambers, tubing, and pumps were submerged in a 3% chlorinated water solution (30 mL bleach/L of tap water) before each trial and rinsed thoroughly. To correct for the background respiration (i.e. bacteria), two respirometry chambers without mussels were included in every experiment, while the other six chambers contained mussels.

On day 0 of each respirometry trial, each mussel was placed in a 2-inch PVC cup for maintaining proper mussel orientation, and each cup placed in an appropriately sized respirometry chamber. Mussels were then held overnight to allow sufficient time to acclimate to the cup and chamber (Haney et al. 2020). Starting at 08:00 on Day 1, we began collecting

respiration data at the base temperature for inclusion in analysis. The temperature was then increased, starting at 09:00 by 1°C every 2h, until the 4°C diurnal ramp was complete at 17:00. We used intermittent respirometry with 20 minute measurement periods separated by 20 minute flushing plus 6 minute waiting periods. During the measurement periods, only the closed pump was turned on and no outside water entered the chamber – allowing mussels to draw down the DO via respiration. As temperature increased during the ramp, the measurement periods were decreased to ensure mussels did not draw DO below 80% oxygen saturation during any given cycle. The flush cycle then pumped fresh water into the chamber, bringing the water back up to ~100% saturation and flushing out metabolic waste. This cycle was repeated throughout the entire 8h diurnal ramp. Respiration rates were calculated during each measurement period by the AutoResp software using the following formula:

$$RMR = ([O2]t0 - [O2]t1) \cdot V/t \cdot 1/WWW$$

Where,

[O2]t0 = DO at time t0 (mg O2/L)

[O2]t1 = DO at time t1 (mg O2/L)

V = respirometer volume – volume of mussel (L)

t = t1 – t0 (hour)

WWW = whole mussel wet weight (g)

To correct for any background oxygen demand from bacteria within the respirometry chambers, the average metabolic rate of the control chambers was subtracted from the metabolic rate of the mussel chambers during each measurement period. Only measurement periods exhibiting a linear relationship between DO and time with an R^2 value > 0.9 were included in

analyses (Chabot et al. 2020). Likewise, only control chamber measurements with an R^2 value > 0.9 were incorporated in the mussel respiration rate corrections.

Clearance Rate

Upon finishing the respirometry portion of the experiment, the six mussels were removed from their chambers and placed along with their PVC cups into 0.75 L beakers. The beakers were partially submerged within the same fiberglass holding tank as a temperature control bath and feeder trough. All beakers were equipped with a pump to flush tank water containing algae feed into the beaker. Beakers were notched at the top to allow water to overflow back into the tank (Figure 1.2). Additional pumps were placed in the tank to keep algae in suspension. Four additional beakers contained no mussels and served as controls to account for any settlement of algae during clearance rate measurement cycles. Mussels were allowed to acclimate to beakers overnight. During this time, temperature controllers were turned back down to the original setting and water temperature gradually declined back down to the original starting temperature associated with the given thermal ramp treatment.

At 08:00 the following morning, clearance measurements began. Starting at 09:00, the temperature was increased by 1°C every 2h, until the 4°C diurnal ramp was complete at 17:00. During each 2h interval, the mussels were provided with a constant source of algae (~30,000 cells/ml) by flushing trough water through the filtration chambers for the first 0.5-1h. This allowed sufficient time for mussels to reach the next temperature in the series and for full feed replenishment within the filtration beakers. During the next 1-1.5h, flush pumps were turned off to allow for measurements of clearance rates. The measurement periods ranged from 1-1.5h to prevent excessive algal cell depletion (i.e. a decrease in absorbance between initial and final measurements of no more than 60%). Two, replicate 15 mL water samples were collected from

each beaker at the beginning and end of the measurement period and algal density (cells/mL) estimated using the spectrophotometer and calibration curve described previously. At the end of each measurement period, the flush pumps were turned back on to replenish the beakers with algae and water from the flush tank. Clearance rate was then calculated using the following equation (Coughlan, 1969):

$$CR = \frac{chamber\ volume \times (ln(initial\ cell\ conc.) - ln(final\ cell\ conc.)) \div time}{whole\ wet\ weight}$$

Absorption Efficiency

Upon completion of the filtration runs, the mussels were gently rinsed and removed from the filtration beakers to allow for fecal collection. Prepared 1µm glass fiber filters (Pall© filters-type A/E) were used for fecal collection and algal trough water samples. The prepped filters were rinsed with DI water using vacuum filtration. The filters were then pre-combusted at 550°C for 1h and cooled in a desiccator for about 15 minutes before weighing filters on an analytical scale to get an initial weight. Filters were placed in aluminum weigh boats, and fecal material from each mussel was carefully collected onto its own corresponding filter. The flush tank samples containing the ending algal concentration were vacuum filtered onto similarly prepped filters. All filters were placed in a drying oven and weighed after fully dried. Then filter samples were combusted at 550°C for 1 h and the ashed weights recorded. Absorption efficiency was calculated using the equation (Conover, 1966):

$$AE = \frac{(F - E)}{[(1 - E)F]}$$

Where,

F = combusted dry weight : dry weight ratio of food

E = combusted dry weight : dry weight ratio of feces

To obtain sufficient feces for analysis, we could only collect feces at the end of each ramp rather than collecting feces at each temperature. Therefore, a single absorption efficiency was calculated for each individual during a thermal ramp.

Calculating Scope for Growth

After all the necessary physiological parameters were measured and calculated, they were converted into energetic units (J/gWWW/h) and used to calculate SFG (Widdows & Staff, 2006):

C = Energy consumed or ingested

C = [maximum clearance rate: L/g/h] × [mg POM/L] × [23 J/mg POM]

where the energy content of particulate organic matter (POM) or algal food is 23J/mg ash-free POM (Slobodkin and Richman, 1961; Widdows et al., 1979).

A = Energy absorbed

A = (C) × food absorption efficiency.

R = Energy respired: (µmoles O2/g/h) × 0.456

where the heat equivalent of oxygen uptake is 0.456 J/µmole O2 (Gnaiger, 1983).

P = Scope for Growth

P = A – R.

Statistical Analyses

Energetic parameters and SFG were calculated separately for each individual mussel using the appropriate parameters measured for each individual and the previously described equations (Excel Microsoft 365, Redmond, WA). For each ramp, we plotted energy ingested, energy absorbed, and respiration rate against temperature using Sigma Plot 13 (2015 Systat

Software Inc. version 13.1). Scope for growth was also plotted against temperature for each ramp and a linear regression fitted through the means (Sigma Plot v. 13.1)

Results

16-20°C Ramp

During the 16-20°C diurnal ramp, clearance rates of individual mussels ranged from 0 to 0.0007 L/gWWW/h. Mean clearance rates showed no significant linear relationship with temperature (Figure 1.3A). Absorption efficiency was estimated at 0.91 ± 0.028 (mean ± SE). Energy ingested by individual mussels ranged from 0 to 0.037 J/gWWW/h. Mean energy ingested showed no significant linear relationship with temperature (Figure 1.3B). Energy absorbed by individual mussels ranged from 0 to 0.034 J/gWWW/h. Mean energy absorbed showed no significant linear relationship with temperature (Figure 1.3C). Energy respired by individual mussels ranged from 0.023 to 0.15 J/gWWW/h. Mean energy respired showed no significant linear relationship with temperature (Figure 1.3C). Scope for growth of individual mussels ranged from -0.15 to -0.018 J/gWWW/h. Mean SFG showed no significant linear relationship with temperature (Figure 1.3D).

20-24°C Ramp

During the 20-24°C diurnal ramp, clearance rates of individual mussels ranged from 0 to 0.002 L/gWWW/h. Mean clearance rates showed no significant linear relationship with temperature (Figure 1.4A). Absorption efficiency was estimated at 0.988 ± 0.001 (mean ± SE). Energy ingested by individual mussels ranged from 0 to 0.081 J/gWWW/h. Mean energy ingested showed no significant linear relationship with temperature (Figure 1.4B). Energy absorbed by individual mussels ranged from 0 to 0.08 J/gWWW/h. Mean energy absorbed showed no significant linear relationship with temperature (Figure 1.4C). Energy respired by

individual mussels ranged from 0.028 to 0.169 J/gWWW/h. Mean energy respired showed no significant linear relationship with temperature (Figure 1.4C). Scope for growth of individual mussels ranged from -0.117 to 0.044 J/gWWW/h. Mean SFG showed no significant linear relationship with temperature (Figure 1.4D).

24-28°C Ramp

During the 24-28°C diurnal ramp, clearance rates of individual mussels ranged from 0 to 0.008 L/gWWW/h. Mean clearance rates showed no significant linear relationship with temperature (Figure 1.5A). Absorption efficiency was estimated at 0.654 ± 0.085 (mean ± SE). Energy ingested by individual mussels ranged from 0 to 0.249 J/gWWW/h. Mean energy ingested showed no significant linear relationship with temperature (Figure 1.5B). Energy absorbed by individual mussels ranged from 0 to 0.163 J/gWWW/h. Mean energy absorbed showed no significant linear relationship with temperature (Figure 1.5C). Energy respired by individual mussels ranged from 0.007 to 0.3 J/gWWW/h. Mean energy respired showed no significant linear relationship with temperature (Figure 1.5C). Scope for growth of individual mussels ranged from -0.214 to 0.019 J/gWWW/h. Mean SFG showed a significant ($p = 0.047$) linear relationship with temperature (Figure 1.5D).

28-32°C Ramp

In the final diurnal ramp from 28-32°C, clearance rates of individual mussels ranged from 0 to 0.002 L/gWWW/h. Mean clearance rates showed a significant ($p = 0.036$) negative linear relationship with temperature (Figure 1.6A). Absorption efficiency was estimated at 0.702 ± 0.084 (mean ± SE). Energy ingested by individual mussels ranged from 0 to 0.061 J/gWWW/h. Mean energy ingested showed a significant ($p = 0.035$) negative linear relationship with temperature (Figure 1.6B). Energy absorbed by individual mussels ranged from 0 to 0.043

J/gWWW/h. Mean energy absorbed also showed a significant ($p = 0.035$) negative linear relationship with temperature (Figure 1.6C). Energy respired by individual mussels ranged from 0.031 to 0.215 J/gWWW/h. Mean energy respired showed a significant ($p = 0.0043$) linear relationship with temperature (Figure 1.6C). Scope for growth of individual mussels ranged from -0.215 to -0.019 J/gWWW/h. Mean SFG showed a significant ($p = 0.0035$) linear relationship with temperature (Figure 1.6D).

Discussion

In this study, we used SFG as a metric of mussel health. We asked whether this metric was sensitive enough to be affected by small temperature changes representative of diurnal increases within the natural habitat of the federally endangered Texas hornshell mussel. Scope for growth has promise as a useful metric of mussel health because it incorporates clearance and respiration rates – both of which are frequently used to assess effects of environmental stressors on filter feeding bivalves (Roberts and Carrington, 2020; Wang et al., 2011; Sara et al., 2008; Widdows and Staff, 1997). However, to-date, few studies have applied SFG models to unionid mussels even though they have been widely used for marine mussels (Luck and Ackerman 2022). Recent studies indicate that it may become increasingly difficult for ectotherms to deal with the increase in daily temperature fluctuations (DTFs) and can negatively affect fitness-related traits such as growth rate and development rate, as well as survival (Verheyen & Stoks 2019). Detrimental effects occur when the range of temperatures encountered during a daily cycle exceeds the optimal temperature, above which performance rapidly falls due to higher allocation of energy to the increased metabolic demands for cell maintenance (Verheyen & Stoks 2019).

An important question is whether SFG patterns observed in studies following standard methodology (Widdows and Staff 2006) - where physiological parameters are repeatedly measured at a constant temperature - are similar to patterns when mussels are subjected to increases in temperature representative of natural diurnal cycles. In the case of *P. popeii*, the shape of the relationship between SFG and temperature was similar regardless of whether SFG was measured on individuals exposed to a 4℃ ramp (this study) or measured on separate batches of mussels each exposed to a different, constant temperature (Pieper et al. 2023). In both cases SFG remained temperature independent from 16-24℃, increased with temperature from 24-28℃, and decreased from 28-32℃. However, SFG measured during diurnal temperature ramps were consistently negative for all but a few individuals. This is likely an artifact of the methodology. Under standard methodology, clearance rates are estimated multiple times at the same temperature and only the highest rates are used to calculate energy ingestion (Widdows and Staff 2006). This methodology is performed because mussels periodically close their valves, resulting in a reduced or absent clearance rate during the measurement period. However, when subjecting mussels to a diurnal temperature ramp, only a single clearance rate could be measured at a given temperature due to time limitations. Consequently, energy ingested was almost certainly underestimated during thermal ramps, leading to underestimation of SFG at any single temperature. Despite this limitation the overall patterns remained consistent between the two techniques. Thus, when measuring SFG during a diurnal ramp as we did, it is the pattern (constant SFG, increasing SFG, decreasing SFG) with increasing temperature that is relevant, not the specific values of SFG.

Our findings suggest that during transitional months (i.e. Spring and Fall) diurnal temperature increases from 16-20℃ or from 20-24℃ have minimal effects on *P. popeii* energetic

health. Within this range, energy acquisition as represented by clearance rates and energy absorption remained temperature-independent, resulting in a temperature-independent SFG. As the season progresses, if morning temperatures increase from ~24℃ to an afternoon high of ~28℃, SFG becomes positively related to temperature and THS may obtain increasing energy from feeding activities from morning to afternoon. In this case, diurnal temperature increases may have a net positive effect on THS via increased energy available for growth and reproduction. However, during the hot summer months, this pattern can reverse if morning temperatures start at ~28℃ and continue to climb through the afternoon. Clearance rates and energy ingestion may decline with increasing temperature even as energetic costs continue to increase, compromising the energetic health of THS populations.

The need to maintain suitable flows and reduce the magnitude of daily temperature fluctuations during the hot summer months assumes critical importance in light of the pattern of decreasing SFG above 28℃. USGS stream gage temperatures in the Black River, NM frequently record temperatures approaching or reaching 32℃ during a given year. Pools containing THS may exhibit little to no flow during droughts and/or in hot summer months (USFWS, 2018a; CEHMM, 2017), and it is possible that diurnal temperature ramps reach higher temperatures than those diurnal ramps we have tested. This is of particular concern considering the changing climate and the increasing frequency of extreme weather events.

Throughout the southwestern United States, heat waves are becoming more common, and snow is melting earlier in spring. In the coming decades, predictions show decreased water flow in the Colorado, Rio Grande, and other rivers; increased frequency and intensity of wildfires; and conversions of some rangelands to desert (EPA 2016). Model predictions for the Upper Rio Grande Basin report that annual average high temperature is projected to increase by as much as

3.76°F on average in 2022–2047, up to 6.23°F on average in 2048–2073, and up to 9.33 °F on average in 2074–2099 (Moeser et al. 2021; Dixon et al. 2020). These projected increases in temperatures would increase the rates of evapotranspiration in the region and make drought more likely. Health of *P. popeii* may depend heavily on flowing water during high temperature periods to avoid depletion of energy reserves and contributing to the decline of the only remaining mussel species in New Mexico. This study was conducted in the hopes of providing applicable information to stakeholders invested in the recovery of the Texas hornshell and knowing what the species requires for its future survival.

While *P. popeii* may be experiencing the extreme effects of increasing temperature and decreasing rainfall in the Southwest (IPCC, 2023), all unionids and other ectotherms are at risk due to the increasing stress of extreme weather conditions and concurrent anthropogenic effects (Spooner and Vaughn, 2008; Haag and Williams, 2014; Luck and Ackerman, 2022). Because all unionids are sedentary animals, they are highly susceptible to changes in local environmental conditions such as flow and temperature (Archambault et al. 2014). Our understanding of the threats to mussels and other aquatic organisms is often based on studies looking only at effects of single stressors under controlled conditions. However, in natural ecosystems, effects result from multiple factors occurring simultaneously with complex interactions resulting in positive, negative, synergistic as well as antagonistic interactions (Smith et al., 2015).

While our study examined effects of a single stressor (temperature) on multiple physiological endpoints incorporated into an energetics model, there are many other critical environmental factors that were not considered and should be explored further, such as water velocity and total suspended solids (TSS) concentration. Studies such as Luck and Ackerman (2022) incorporate this multifaceted methodology by investigating how the combined changes in

seasonal temperature, velocity and TSS concentration affect the clearance rates, oxygen consumption, and SFG of *Lampsilis siliquoidea*. Multi-factor models investigating water velocity, TSS, SFG, and DTF to examine the physiological responses of mussels should be further explored and encouraged. These models are essential to effectively advancing recovery efforts of unionids to better understand and conserve these rapidly declining and imperiled taxa.

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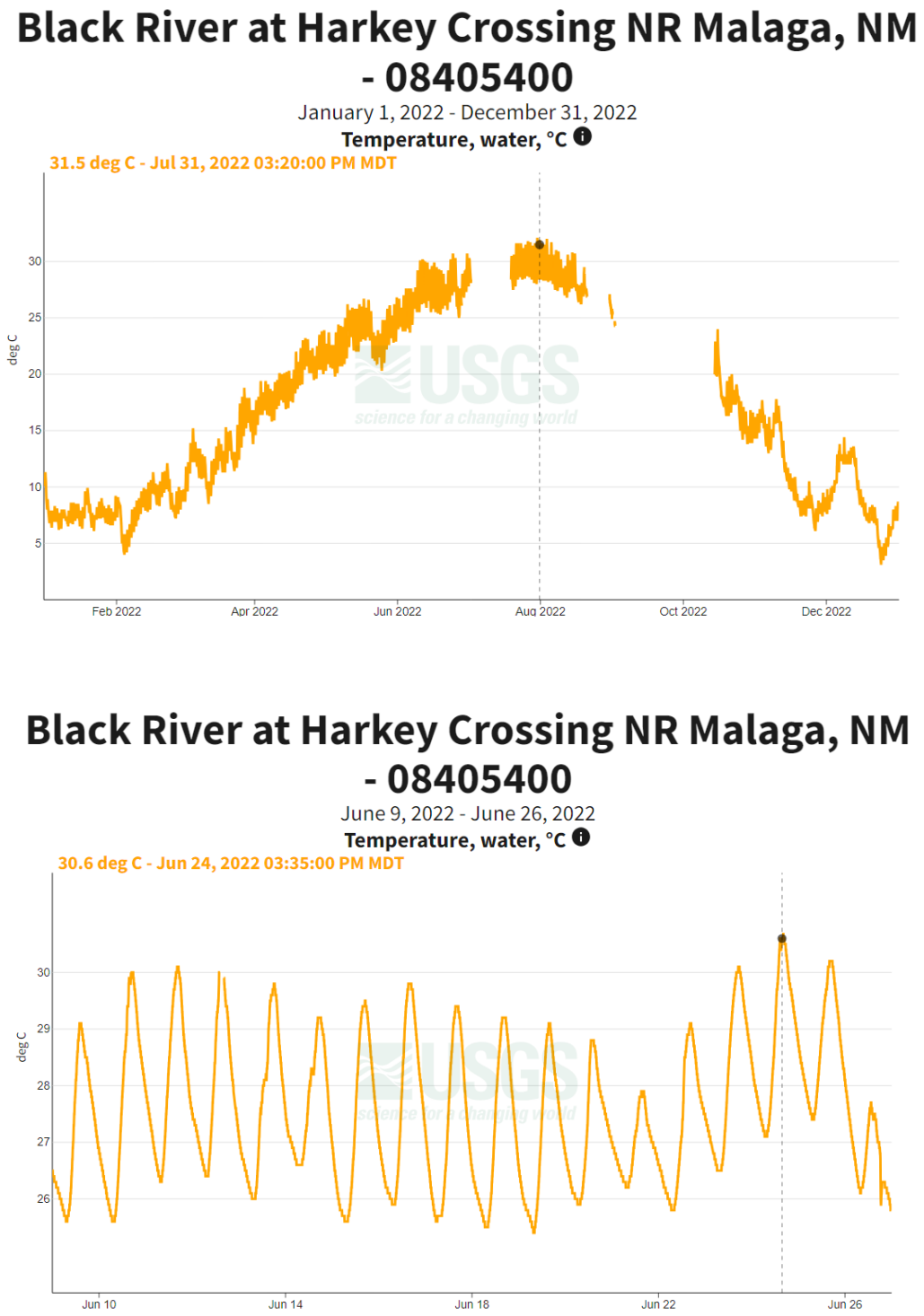
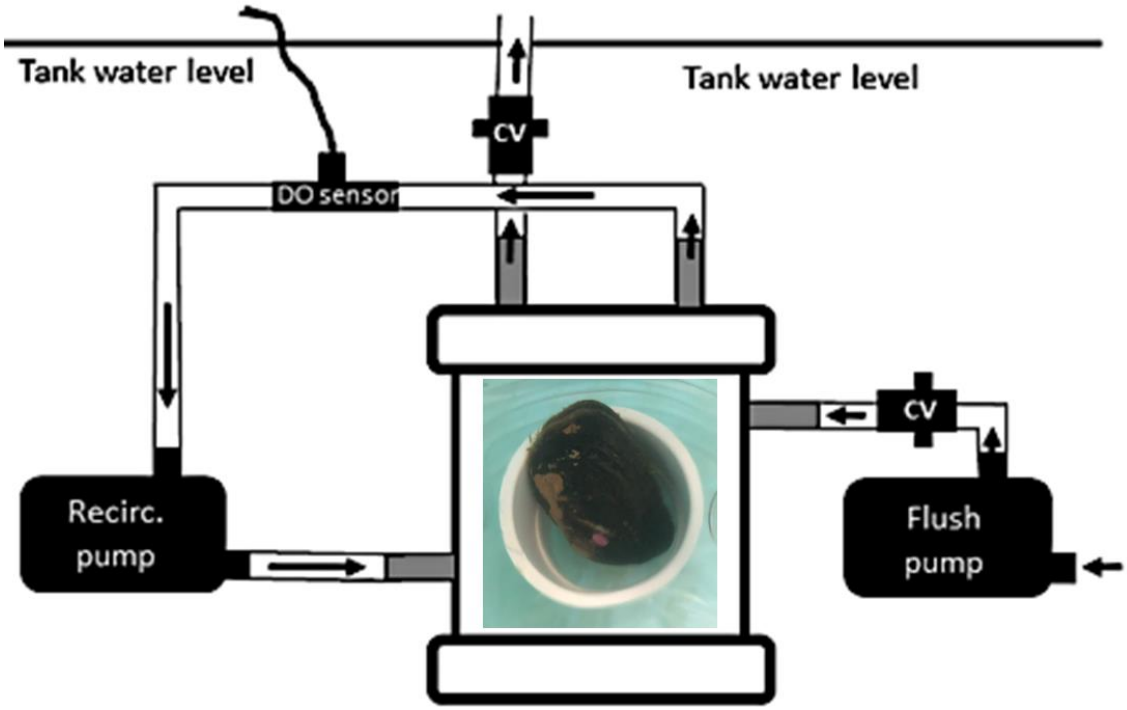


Figure 1.1. USGS streamflow data from the Black River, NM displaying temperature in °C seasonally (top) and diel fluctuations (bottom) experienced by *Popenaias popeii* in this system.



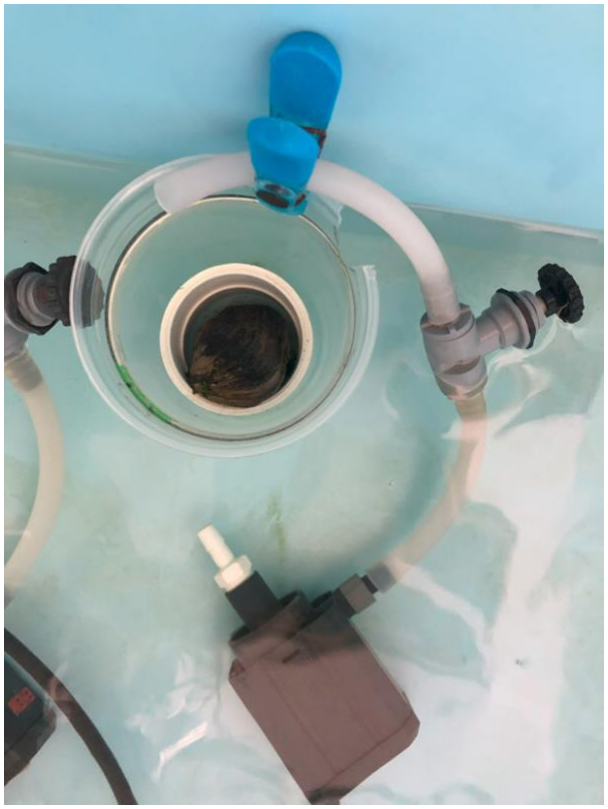


Figure 1.2. Diagram of respiration chamber with associated pumps (top) modified from Haney et. al 2019. Filtration chamber and associated pump for measuring clearance rate (bottom).

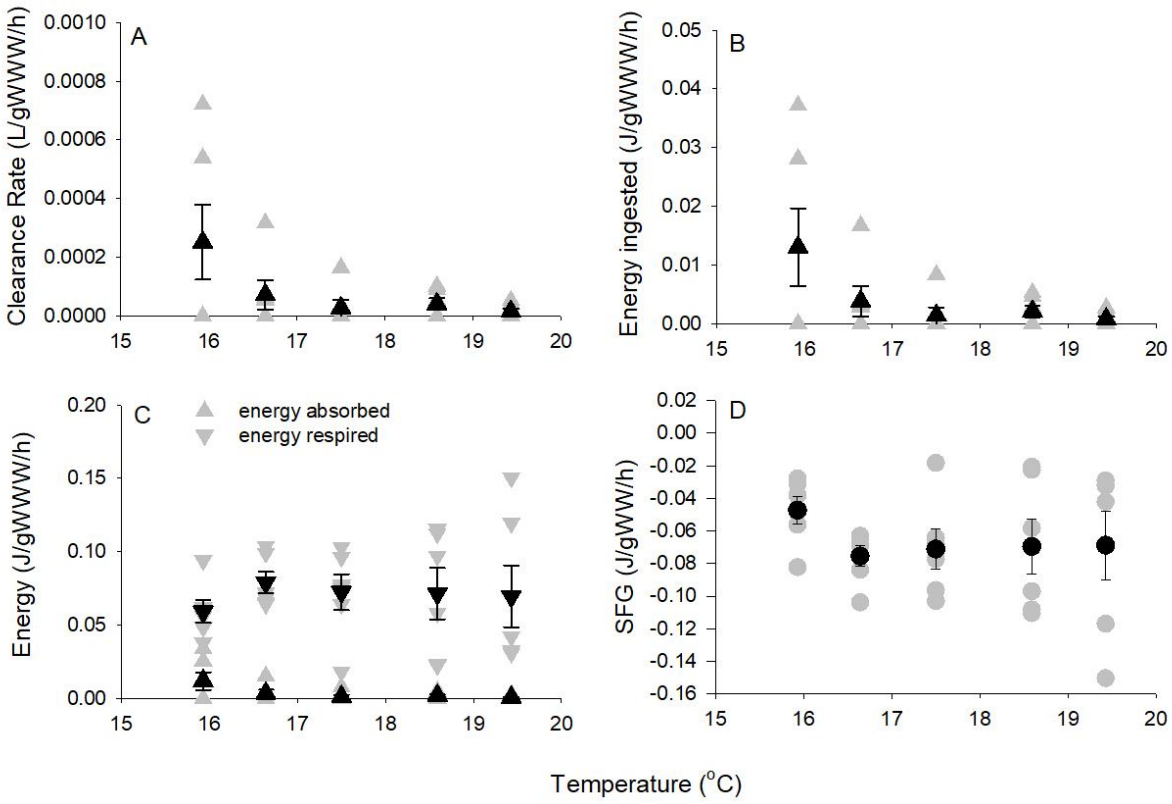


Figure 1.3. Effects of 16-20°C diurnal temperature increase on (A) clearance rate, (B) energy ingested, (C) energy inputs (absorbed) and outputs (respired), and (D) scope for growth.

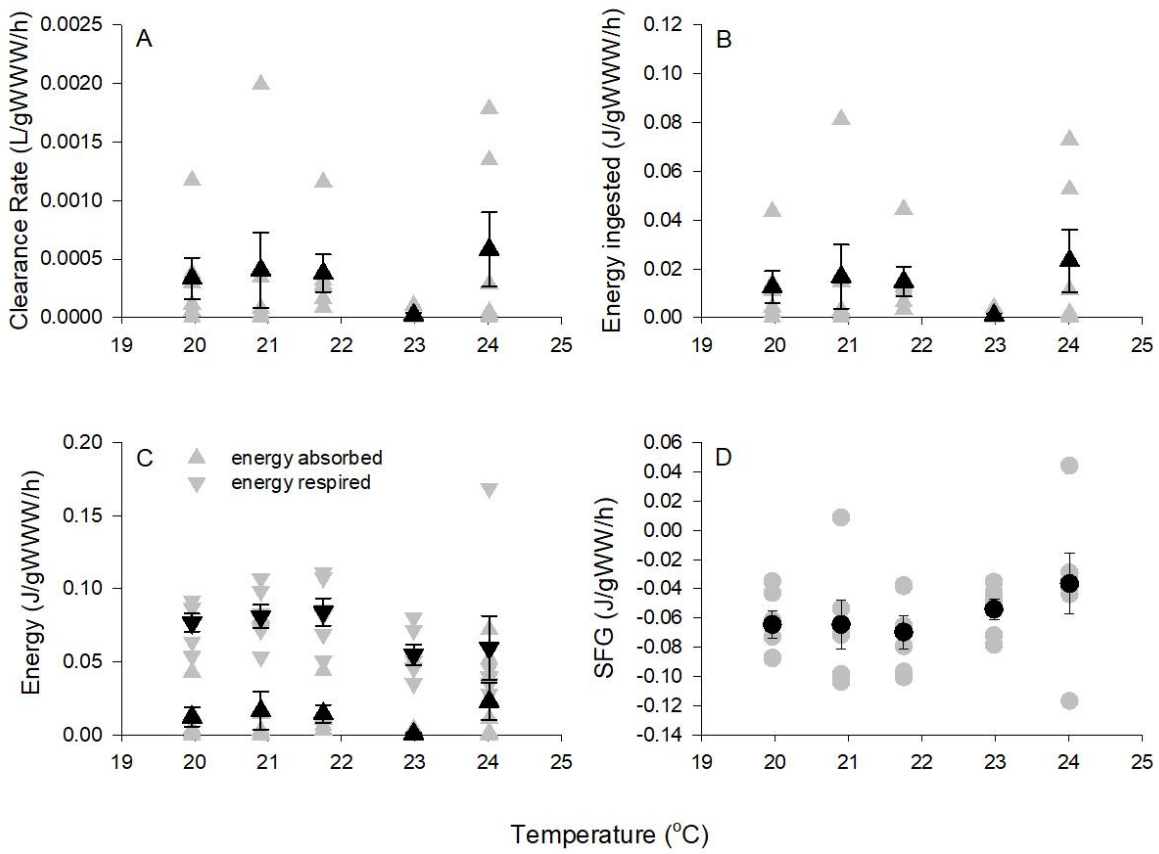


Figure 1.4. Effects of 20-24°C diurnal temperature increase on (A) clearance rate, (B) energy ingested, (C) energy inputs (absorbed) and outputs (respired), and (D) scope for growth.

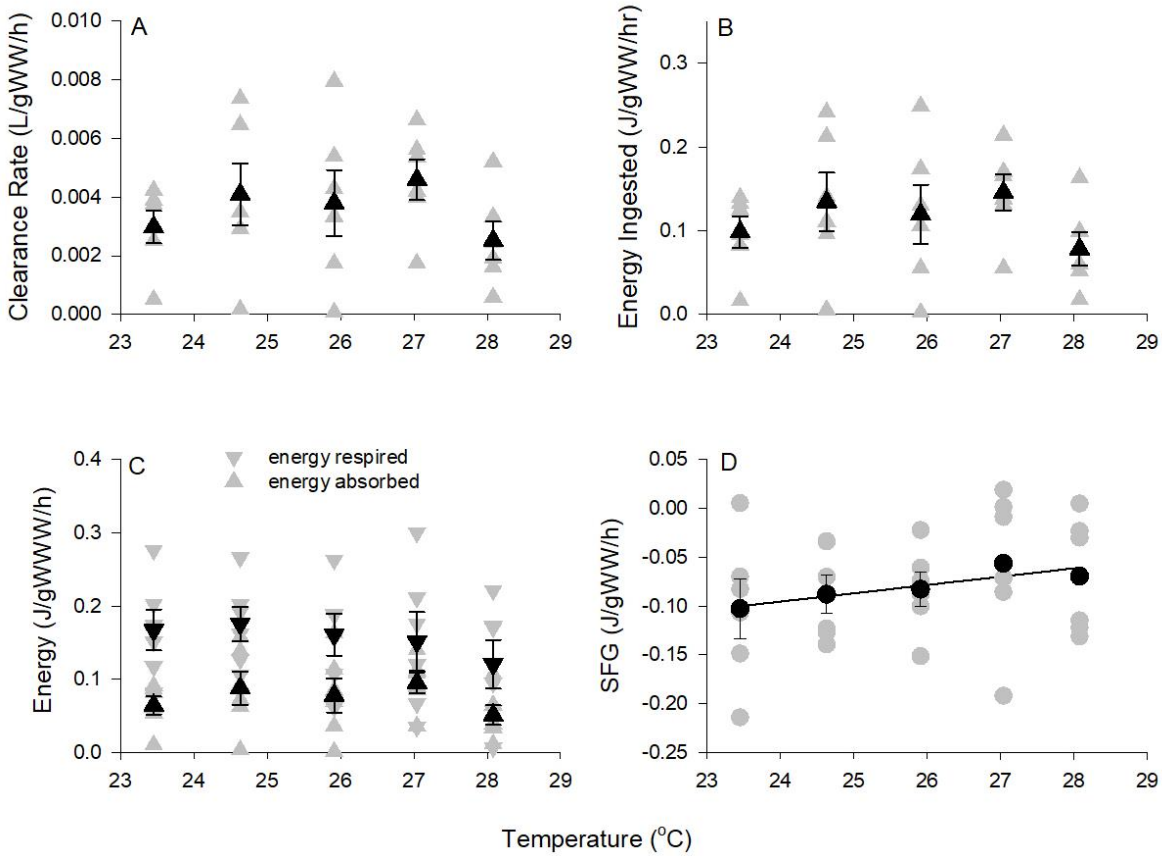


Figure 1.5. Effects of 24-28°C diurnal temperature increase on (A) clearance rate, (B) energy ingested, (C) energy inputs (absorbed) and outputs (respired), resulting in a significant positive linear regression on (D) scope for growth (p=0.047).

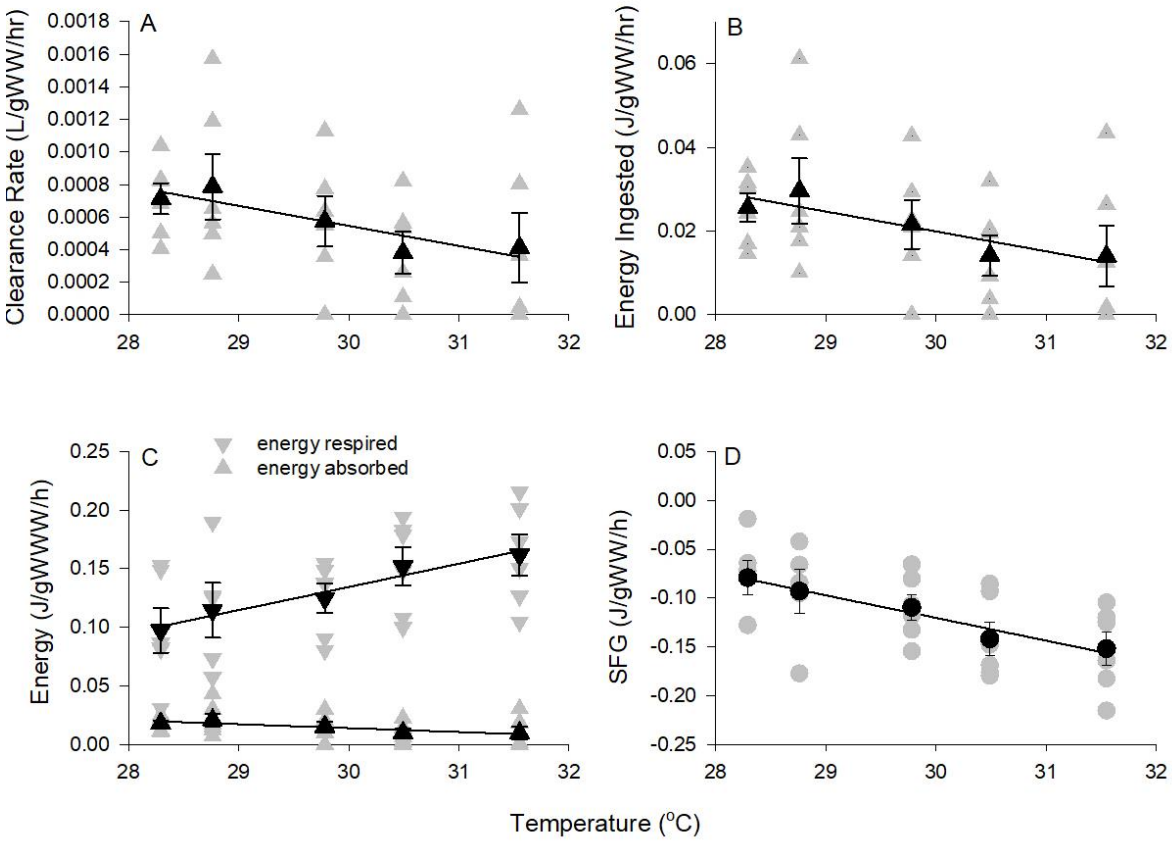


Figure 1.6. Effects of 28-32°C diurnal temperature increase on (A) clearance rate (p= 0.036), (B) energy ingested (p= 0.035), (C) energy inputs (absorbed; p= 0.035) and outputs (respired; p= 0.0043), resulting in a significant negative linear regression on (D) scope for growth (p= 0.0035).

Chapter II: Effects of Acute Thermal Stress on Aerobic Scope of *Popenaias popeii*

Introduction

Texas hornshell (THS) mussel (*Popenaias popeii*) is the only remaining unionid in New Mexico where it occurs in the Rio Grande Basin. This species is federally listed as endangered and protected throughout its US range (83 FR 5720 5735; USFWS 2018a). Given the key role that unionids play in aquatic ecosystems (Spooner and Vaughn, 2008; Haag, 2012), the persistence of the THS is of great importance to the ecology of its native rivers. Increased water withdrawal coupled with increasing surface water temperatures has greatly increased the thermal risk posed to THS as well as their obligate fish hosts: river carpsucker (*Carpiodes carpio*), red shiner (*Cyprinella lutrensis*), and gray redhorse (*Moxostoma congestum*) (CEHMM, 2017; USFWS 2018a; IPCC, 2023).

Extreme temperatures directly impact many organismal functions at all levels of biological organization. These effects range from protein denaturation, membrane instability, cell disruption and organ failure. In fishes, rapid direct thermal impacts are thought to act through three fundamental molecular mechanisms: reaction rates, protein structure, and membrane fluidity (Verbek et al., 2016; Ern et al., 2023). During acute warming, these mechanisms can drive loss of equilibrium and death through various cellular, organ, and physiological pathways such as mitochondrial dysfunction, oxygen limitation, and impacted excitability of cells. Eventually, neural and/or muscular failure occurs and may also lead to loss of homeostasis and subsequent thermal death (Ern et al., 2023).

When fish are exposed to a gradual increase in water temperature over time, they exponentially accumulate physiological manifestations of heat stress. This accumulation of heat stress leads to heat failure at the critical thermal maximum (CT_{max}) - which is manifested in

fishes as a loss of equilibrium (LOE) characterized by rolling, spiral swimming, or turning upside down (Ern et al. 2023). However, in unionids, CT_{max} is not manifested as LOE; instead, CT_{max} is characterized by extreme gaping behavior in which the foot becomes partially or fully extended, the adductor muscles relax, and mantle edges and siphons part and retract (Galbraith et al., 2012). While CT_{max} and associated behavioral endpoints provide useful empirical information regarding the acute upper thermal limits of an individual, they do not identify the specific underlying physiological mechanisms that drive and control thermal tolerance. These mechanisms appear to differ among taxa and life stages. Additional studies are required to determine the limiting physiological mechanisms for organisms of interest (Hussain et al. 2023, Ern et al. 2023).

One potential mechanism that has been proposed to drive thermal tolerance in aquatic ectotherms is aerobic scope. Aerobic scope quantifies the capacity of the cardio-respiratory system to deliver oxygen to tissues for supporting activity, growth, reproduction (Verbek et al. 2016; Ern 2019). Absolute aerobic scope is mathematically defined as the difference between maximum aerobic metabolic rate (MMR) and standard aerobic metabolic rate (SMR) (Verberk et al. 2016). An animal's SMR is the metabolic rate of a sufficiently fasted individual (i.e. in the absence of specific dynamic action) while at rest and represents the basic cost of living (Ern et al. 2014). In fishes, MMR is typically measured as the maximum oxygen consumption rate at the point of exhaustion after intense or prolonged exercise such as swimming against a current or manual chasing (Killen et al., 2017; Rosewarne et al., 2016).

Absolute aerobic scope (AAS) reflects the potential amount of aerobically derived, sustainable energy production that can be allocated to functions beyond body maintenance, such as physical activity, digestion, growth and reproduction (Ern et al. 2014). In studies where AAS

successfully explains thermal tolerance, SMR and MMR initially increase with increasing temperature but MMR peaks sooner than SMR, resulting in a decrease in AAS with further increases in temperature (Hussain et al., 2023). In theory, when MMR declines to the SMR, AAS declines to zero and the organism is no longer able to meet its basic metabolic needs (Verbek et al, 2016; Ern 2019). This critical endpoint may result in the organism reaching its CT_{max} and experiencing a LOE at or within a few degrees of the intersection of MMR and SMR (Figure 2.1A, B).

Standard methods for measuring MMR cannot be used for organisms such as bivalves that will close in response to a disturbance and subsequently enter anerobic respiration. Instead, an enzymatic approach is utilized for estimating MMR via electron transport system (ETS) assay which has been used in various studies investigating thermal tolerance of various invertebrate taxa and fish (Horne et al., 2022; Hussain et al., 2023; Simcic et al., 2014; Westhoff et al., 2021). The ETS assay measures the electron transfer activity of dehydrogenases and cytochromes in the presence of a saturating level of substrates (Maldonado et al. 2012) and estimate the potential metabolic activity (PMA) of an organism (Žagar et al. 2018) by generating thermal performance curves (TPC) to estimate the temperature at which maximum PMA occurs (Westhoff et al. 2021).

Recent studies have shown that estimates of MMR using the ETS assay (MMR_{ets}) may be useful for calculating AAS in aquatic crustaceans such as shrimp (Hussain et al. 2023), linking cellular and organismal responses to acute thermal increases. Using the same conceptual framework as described in Hussain et al. (2023) (Figure 2.1C, D), we investigated whether AAS is a useful concept for understanding the physiological basis of thermal tolerance in *P. popeii* from the Black River population, NM. Physiological responses to acute thermal increases were

evaluated to better understand the tolerance of this species and help inform conservation management decisions for their persistence in their rapidly changing environment.

Methods

Experimental Animals

Fifty adult THS were collected from the Black River near Carlsbad, NM in October 2021 under federal collection permit #TE78507C-0. The total population of the Black River has been estimated at ~48,000 mussels (Inoue et al. 2014). Mussels were primarily found in submerged portions of undercut banks, burrowed in muddy substrate. Adult mussels over 70mm were collected for experiments whereas subadults and juveniles were noted and returned to the substrate. The first day of the collection trip was spent locating and marking areas containing mussels so they could be quickly collected by hand on the second day. Less than 50% of detected mussels were collected from any given site and collections were spread out across three sites. After collection, each mussel was cleaned, photographed, wrapped in moist towels, then placed in coolers with a small ice pack for overnight shipping to the E.W. Shell Fisheries Center (EWSFC) at Auburn University, AL.

Upon arrival at EWSFC, mussels were cleaned, weighed, and marked with FPN glue-on shellfish tags (Hall Print Fish Tags, South Australia) for individual identification. Mussels were held in upweller systems filled with Hard Artificial Freshwater (HAFW) (Smith et al. 1997) and gravel substrate. Automatic feeders (GHL Doser 2.2; GHL International, Germany) connected to each upweller provided a constant algae supply maintained 25,000-35,000 cells/mL of LPB Shellfish Diet (Reed Mariculture Inc., Campbell, CA) stock solution. The algal source for each automatic feeder was a stock solution in 2L beakers, kept in suspension by a stir plate, and placed in a mini fridge to maintain temperature. Stock solutions were replenished weekly and

feeding dosages adjusted to maintain proper algae concentrations. Total ammonia-nitrogen (TAN), nitrate, and nitrite were analyzed twice/week using a YSI 9300 Photometer (YSI Inc., Yellow Springs, OH). A partial water exchange was triggered when TAN increased above 0.1 mg/L.

After acclimation to laboratory conditions for > 2 weeks, 24 mussels were randomly assigned to one of the three assays (CT_{max}, RMR, or ETS). All mussels were acclimated to the starting experimental temperature of 25°C for ≥ 2 weeks prior to running all assays. Immediately before entering experimental assays, mussels were cleaned with a soft bristle brush, towel dried, and whole wet weight recorded.

Critical Thermal Maximum (CT_{max})

Critical thermal maximum assay was conducted on ten THS in a 50 L insulated cooler containing 40 L of HAFW and airstones to maintain ~100% oxygen saturation and water circulation. A 300 W heater bar (Finnex TH-300S, Finnex, Chicago, USA) was used to maintain and adjust experimental temperatures. The ten mussels were placed in 2" PVC cups with gravel to maintain proper orientation within 1 L plastic beakers with fine mesh windows to allow for water circulation. The beakers were arranged such that they were equidistant from the heater bars in the center of the cooler and mussels were acclimated to the beakers for ≥ 12 h at 25°C prior to the start of the experiment (Figure 2.2A).

Starting at 10:00 the following morning, the water temperature was increased at a constant rate (2°C/ hour) until CT_{max} endpoints were achieved for all ten mussels. We monitored mussel behavior every hour until the first visual signs of stress – foot extension – were observed. After mussels began showing signs of stress, they were monitored every 10-15 minutes until reaching CT_{max}. Moderate gaping behavior is natural in mussels, but mussels that experience

prolonged stress exhibit extreme gaping behavior in which their adductor muscles relax, mantle edges and siphons part, and the foot becomes partially or fully extended (Galbraith et al., 2012). Visual observations were recorded along with the temperature at the time of the observed stress response and when CT_{max} was reached. We considered mussels to have reached CT_{max} when all 3 markers described above were observed and mussels were unresponsive to probing.

Resting Metabolic Rate (RMR)

Standard metabolic rate (SMR) requires multiple measurements of respiration at a constant temperature for 24 hours (Chabot et al. 2016), which is unattainable during acute thermal ramps. Resting metabolic rate accounts for low levels of spontaneous activity while still representing the minimum metabolism of an individual in an inactive state (Burton et al. 2011). For our experiment, RMR was measured using intermittent respirometry of six mussels fasted for a 24 h period and used as a surrogate for SMR. The RMR assay was run concurrently with the CT_{max} assay with corresponding thermal increases (2°C/ hour).

Respiration rates for each individual were measured using an 8-chamber optical respirometry system coupled with Auto-Resp software (Loligo Systems, Viborg, Denmark). Chambers were made of acrylic and ranged in volume from 200 to 700 mL. Mussels were paired with an appropriately sized chamber such that the ratio of chamber volume (mL) to mussel whole wet weight (g) was approximately 6.24 mL:g (± 0.82 SE), similar to that of Haney et al. (2020). Each chamber was connected to two Eheim submersible 300 L/h pumps (Eheim GmbhH & Co., Deizisau, Germany). The flush pump circulated fresh, oxygenated water from the trough through the chamber during initial chamber acclimation and during flush cycles. The closed pump circulated water within a closed loop, including the chamber, during measurement periods. A flow-through oxygen cell with an optical dissolved oxygen (DO) sensor was inserted in the

closed pump tubing measures the DO of recirculating water. Two temperature sensors placed one on either end of the experimental tank continually measured the temperature of each run (Figure 2.2B).

Respirometry chambers, pumps, and sensors were submerged in a 400 L rectangular fiberglass tank filled with ~250 L hard artificial freshwater (HAFW). The tank water was aerated to maintain ~100% oxygen saturation. Water temperatures were controlled with a TECO TK-2000 chiller/heater unit (TECO US). To reduce ambient oxygen demand from bacteria, all chambers, tubing, and pumps were submerged in a 3% chlorinated water solution (30 mL bleach/L of tap water) before each trial and rinsed thoroughly. To correct for the background respiration (i.e. bacteria), two respirometry chambers without mussels were included in every experiment, while the other six chambers contained mussels.

On day 0 of each respirometry trial, each mussel was placed in a 2-inch diameter PVC cups for maintaining proper mussel orientation, and each cup placed in an appropriately sized respirometry chamber. Mussels were then held overnight to allow sufficient time to acclimate to the cup and chamber (Haney et al. 2020). Starting at 10:00 on Day 1, the temperature was increased by 2°C every 1h. The experiment was terminated at the temperature at which all individuals in the concurrent thermal tolerance experiment were observed to have reached CT_{max}. During this entire series, we measured respiration using intermittent respirometry with 20 minute measurement periods separated by 20 minute flushing periods. During the measurement periods, only the closed pump was turned on and no outside water entered the chamber –allowing mussels draw down the DO via respiration. As temperature increased during the ramp, the measurement periods were decreased to ensure mussels did not draw DO below 80% oxygen saturation during any given cycle. The flush cycle then pumped fresh water into the chamber,

bringing the water back up to ~100% saturation and flushing out metabolic waste. This cycle was repeated throughout the entire thermal ramp. Respiration rates were calculated during each measurement period by the AutoResp software using the following formula:

$$RMR = ([O2]t0 - [O2]t1) \cdot V/t \cdot 1/WW$$

Where,

[O2]t0 = DO at time t0 (mg O2/L)

[O2]t1 = DO at time t1 (mg O2/L)

V = respirometer volume – volume of mussel (L)

t = t1 – t0 (hour)

WWW = whole mussel wet weight (g)

To correct for any background oxygen demand from bacteria within the respirometry chambers, the average metabolic rate of the control chambers was subtracted from the metabolic rate of the mussel chambers during each measurement period. Only measurement periods exhibiting a linear relationship between DO and time with an R^2 value > 0.9 were included in analyses (Chabot et al. 2020). Likewise, only control chamber measurements with an R^2 value > 0.9 were incorporated in the mussel respiration rate corrections.

Maximum Metabolic Rate (MMR_{ets})

The maximum metabolic rate (MMR_{ets}) was estimated utilizing the ETS assay following procedures as described in Hussain et al. (2023) and Westhoff et al. (2021). After their two week acclimation period, the eight randomly selected mussels were frozen at –80 ºC. The whole wet tissue of each mussel was homogenized on ice with an analytical grinding mill (Item No. 2900001, IKA Works Inc., Wilmington, NC, USA). A subsample of 25% the total homogenized tissue mass of each mussel was diluted with reagent grade deionized water (Cat. No. 9150-1,

Ricca Chemical, Arlington, TX, USA) to obtain a final concentration of 1 mg tissue/mL. The diluted homogenate from each individual was allotted into 20, 2-mL micro-centrifuge tubes and stored at -80 ºC. Homogenate samples were thawed while in an ice-water bath, and ETS activity was measured using methodology originally developed by Packard (1971) and subsequently modified and used for aquatic invertebrates (Hussain et al. 2023; Westhoff et al. 2021). ETS activity (mL O2/gWW/h) was measured at each of 17 temperatures from 9 to 57°C at 3°C intervals to generate a thermal performance curve (TPC) relating ETS activity to temperature as an approximation of MMR.

Statistical Analyses

The CT_{max} assay data was analyzed using Kaplan-Meier survival analyses to determine the mean temperature of THS thermal endpoints. Statistical significance was set at p-value < 0.05, and data were presented as the mean ± SD. Survival analyses were performed in SigmaPlot 13 (2015 Systat Software Inc. version 13.1). All other statistical analyses were conducted using R software for windows (version 4.1.1; R Core team, 2021).

A thermal performance curve was created by fitting a smoothing spline (SS) model to RMR_{ets} data (y-axis) at tested incubation temperatures (x-axis). The selection of the smoothing parameter (λ) was based on the restricted maximum likelihood (REML) method to ensure the compromise between the smoothness of the function and the lack of fit (Berry and Helwig, 2021). The fitted SS models were used to predict MMR_{ets} at temperatures from 9 to 57°C with an interval of 0.001°C. Predicted MMR_{ets} curves were fitted with 95% confidence intervals (95% CI) and standard errors (SE) around the predictions via bootstrapping (Efron and Tibshirani, 1993) and implemented in the boot package (version 1.3–28; Canty and Ripley, 2021). Data were

resampled with replacement 1,000 times, with the SS model re-fitted to these data each time, and the 95% CIs were determined from the 2.5th and 97.5th percentiles.

Similarly, SS models with the REML method were applied to RMR data to create a TPC. The fitted SS models were used to predict RMR across the tested respirometry temperature ranges with an interval of 0.001°C, and bootstrapping was used to create 95% CIs and standard errors (SE) of predicted RMR curves. At any temperature, the AAS range consisted of a central estimate (predicted mean MMR – predicted mean RMR; Clark et al., 2013), an upper boundary (upper 95% CI of MMR – lower 95% CI of RMR), and a lower boundary (lower 95% CI of MMR – upper 95% CI of RMR). Data for MMR, RMR, and AAS were presented as mean ± SE (95% CI).

Results

P. popeii began displaying initial behavioral signs of acute thermal stress at 38.9°C as evidenced by foot extension. Three mussels reached CT_{max} at 43°C and seven at 43.1°C as defined by four concurrent behavioral responses: foot extension, gaping, mantle retraction, and unresponsiveness to probing (Galbraith et al. 2012). The mean temperature at which 50% of mussels exhibited CT_{max} was 43.07 ± 0.015 SE.

Resting metabolic rate increased slowly from 0.005 to 0.007 mL O_2/gWW/h as the temperature rose from 25 - 34°C. This was followed by a rapid increase in RMR from about 35 - 40°C, with RMR peaking at 0.013 ± 0.002 mL O_2/gWW/h (95% CI: 0.009 – 0.018). The mussels' first behavioral indicator of thermal stress – foot extension – was observed near peak RMR. Resting metabolic rate subsequently declined rapidly until mussels reached their CT_{max} (Figure 2.3).

Maximum metabolic rate, as measured by the ETS assay displayed three peaks with increasing temperature (Figure 2.4). The first and highest peak (ETS_{max}) occurred at 28.75°C with an estimated value of 0.960 ± 0.034 (95% CI: 0.893 – 1.028) mL O_2/gWW/h. The second, intermediate peak (ETS_{peak2}) occurred at 38.81°C with an estimated value of 0.882 ± 0.038 (95% CI: 0.808 – 0.956) mL O_2/gWW/h. The third and lowest peak (ETS_{peak3}) occurred at 50.89°C with an estimated value of 0.483 ± 0.037 (95% CI: 0.409 – 0.557) mL O_2/gWW/h. The first behavioral indicator of thermal stress – foot extension – occurred at ETS_{peak2} while CT_{max} occurred approximately midway between ETS_{peak2} and ETS_{peak3}. The ETS_{max} occurred while RMR was rising only very slowly with temperature, whereas ETS_{peak2} occurred when RMR was also peaking (Figures 2.3 & 2.4).

Because MMR_{ets} was typically 1-2 orders of magnitude higher than RMR (Figure 2.5A), estimates of AAS were always ≥ 98% of MMR_{ets}. Thus, the relationship of AAS and temperature was primarily driven by ETS activity and nearly identical to MMR_{ets} within the calculable temperature range (Figure 2.5B). Estimates of AAS never approached zero within the temperature range tested, due to MMR_{ets} never decreasing at a fast enough rate to intersect with RMR. The minimum AAS value (0.538, 95% CI: 0.466 - 0.610 mL O_2/gWWW/h) occurred at 43.37°C when MMR_{ets} was 927 times higher than RMR.

Discussion

In this study, we explored the effects of acute thermal stress on the federally endangered *P. popeii* and whether aerobic scope is a useful approach to understanding the physiological basis for thermal tolerance of unionid mussels. Given that standard methodology to measure MMR in fish (Killen et al., 2017; Rosewarne et al., 2016) cannot be used for mussels that simply close, rather than exhibit an active escape response to disturbance, we evaluated the use of the ETS

assay as an alternative estimate MMR and AAS. The ETS assay has been shown to be a useful way to calculate AAS in marine shrimp – linking cellular and organismal responses to tolerance of acute thermal stress (Hussain et al. 2023). We found that unlike shrimp, MMR_{ets} was 1-2 orders of magnitude higher than RMR in THS. This resulted in an AAS that was only minimally affected by changes in RMR and essentially matching the MMR_{ets} and temperature relationship. As temperatures increased, AAS remained high and did not decline to near zero when THS reached CT_{max}. Use of the MMR_{ets} assay does not appear to be a useful method for estimating AAS in unionid mussels such as THS. Additional alternatives for measuring MMR in bivalves need to be tested and validated before we can accurately assess the usefulness of AAS as physiological driver of acute thermal tolerance in bivalves.

While AAS, as estimated using MMR_{ets}, was not applicable for explaining thermal tolerance in THS, the TPC generated through ETS assay may provide useful insight into thermal tolerance of aquatic ectotherms at the organismal level. Thermal performance theory suggests an ectotherm's response (metabolic rate, growth, or fitness) to temperature will increase until a peak is reached, after which the organism will suffer physiological stress and ultimately death (Westhoff et al. 2021). In crayfish, the TPC for ETS enzyme activity is theorized to serve as a useful framework to link thermal endpoints and estimate the transition from optimal to stressful temperatures. Thermal preference and optimal temperature estimates for crayfish consistently fell below ETS_{max} whereas CT_{max} estimates fell above ETS_{max}. Consistent location of thermal endpoints on ETS enzyme activity TPC suggests these curves may serve as a useful first step to predict general optimal and stressful temperature ranges for crayfish (and potentially other aquatic ectotherms) with optimal temperatures occurring below ETS_{max}, and lethal temperatures occurring above ETS_{max} (Westhoff et al. 2021).

At the organismal level, optimal temperatures for scope for growth can be estimated from bioenergetics models (Whitledge and Rabeni 2002) and have been shown to be several degrees lower than the temperatures that support maximum ETS activity in crayfish (Simcic et al. 2014). In our previous studies on THS scope for growth (SFG), we determined peak SFG to occur around 28°C (Pieper et al. 2023) which would fall below the observed ETS_{max} on our TPC (Figure 2.4). The first indicator of thermal stress (foot extension) occurred on the ETS_{peak2} which also provides useful information for determining physiological effects from the TPC. We also determined that CT_{max} for THS occurs at 43.1°C and indeed falls above the ETS_{max} and ETS_{peak2} on the TPC. Locations of SFG and CT_{max} on the ETS thermal performance curve for THS were similar to those for crayfish (Simcic et al. 2014; Westhoff et al. 2021), where SFG occurred at temperatures a few degrees lower than ETS_{max} and CT_{max} occurred a few degrees higher than ETS_{max}.

Thermal performance curves of RMR may also be useful predictors of SFG and $CT_{max,}$ with SFG occurring while RMR is increasing at a relatively slow rate with temperature, foot extension occurring at RMR_{peak}, and CT_{max} occurring as RMR rapidly declines after the peak. This provides a useful link between physiological effects of thermal stress as well as observable effects such as foot extension. If these patterns between physiology, behavior, and various indicators of optimal and stressful temperatures are consistent across a range of unionid species, it would allow for rapid assessment of optimal, stressful, and lethal thermal ranges via a non-lethal (i.e. RMR) or a single assay (i.e. ETS). This would be incredibly valuable for threatened and endangered species where large numbers of animals cannot be collected and used in multiple, and often time-consuming experimental assays. Single assays that require a minimal number of animals could provide baseline information on species' thermal performance, which

ultimately can inform management decisions, environmental regulation, and may predict how a species will respond to altered temperatures from climate change (Westhoff et al. 2021).

Although we utilized a lethal methodology to estimate CT_{max} in this study, some organisms can recover after reaching CT_{max} if they are quickly returned to lower temperatures once they exhibit loss of equilibrium (Ern et al. 2023). The heat stress experienced during CT_{max} trials does not appear to cause any lasting harm in many species, as indicated by zebrafish (*Danio rerio*) resuming normal swimming within seconds and feeding within minutes of being returned to control temperatures, and growth being unaffected by weekly CT_{max} trials (Ern et al. 2023). Further investigations considering the survival of recovered organisms post CT_{max} studies may inform species management decisions for salvaging animals during thermally critical scenarios such as droughts and high temperatures. Future studies utilizing the CT_{max} assay would benefit by exploring this recovery option to test whether CT_{max} of unionids is recoverable and can be a non-lethal study, which may be useful for assessing thermal stress in federally and state protected species.

Energetic (RMR) and behavioral (valve closure) endpoints are useful when assessing impacts of warming temperatures on unionid mussels (Haney et al. 2020). The observational determination of CT_{max} in unionids through extreme gaping (Galbraith et al. 2012) is useful in determining this thermal endpoint, but measurable data such as valveometry may be a useful avenue for quantifying the amount of gape a mussel is displaying. Valveometry would also include the response of valve closures to thermal stress which is not considered when measuring RMR versus temperature and only considering CT_{max} as the thermal endpoint. Although RMR generally increases with temperature, suggesting that mussels need to increase their food intake to keep up with increasing energy demands, many bivalves exhibit an increased frequency of

valve closure as temperatures warm (Haney et al. 2020). These closure periods have been interpreted as a mechanism to offset thermally induced increases in energy demand (Anestis et al., 2007) since aerobic respiration essentially ceases during valve closure. Utilizing valveometry for a quantifiable measurement of gape and valve closures would provide valuable, concurrent data to compliment CT_{max} observations and determine if a measurable amount of gaping in unionids corresponds to certain aspects of their CT_{max} endpoints such as extreme foot extension and retraction of siphons, or even valve closures as a pre-determining response prior to the lethal effects of reaching CT_{max}.

The study of biological responses may assist in detecting early warning signs before the occurrence of mortality, provide a method to study the effects of sublethal stressors, and aid the evaluation of population condition in response to translocation and restoration efforts (Curley et al. 2021). Living sedentary lifestyles leaves mussels vulnerable to thermal stress (Spooner & Vaughn, 2008). Given that unionids are already living close to their upper thermal tolerances in some systems (Pandolfo et al., 2010; Ganser et al., 2015), thermal stress is common for unionids when temperatures peak throughout the year. Mussel communities in medium to large streams may be particularly susceptible to low oxygen events during low-flow periods and severe droughts (Gagnon et al., 2004), which is especially the case for THS that are subject to low or no flow pools within the river that can arise during droughts or in hot summer months (USFWS, 2018a; CEHMM, 2017). Assessing impacts of low oxygen concentrations and increasing temperatures has been identified as a priority research item for the conservation of freshwater mussels (Haney et al. 2020; Ferreira-Rodriguez et al. 2019). On a global scale, freshwater biodiversity is rapidly declining, with extinction rates five times higher in freshwater than in terrestrial systems (Dudgeon et al., 2006). Thermal tolerance assays are critical to determining

what unionids are capable of surviving in their rapidly changing environment, and exploring new avenues for assessing sublethal responses to thermal stress should be encouraged to minimize the further impacts posed to this imperiled taxa.

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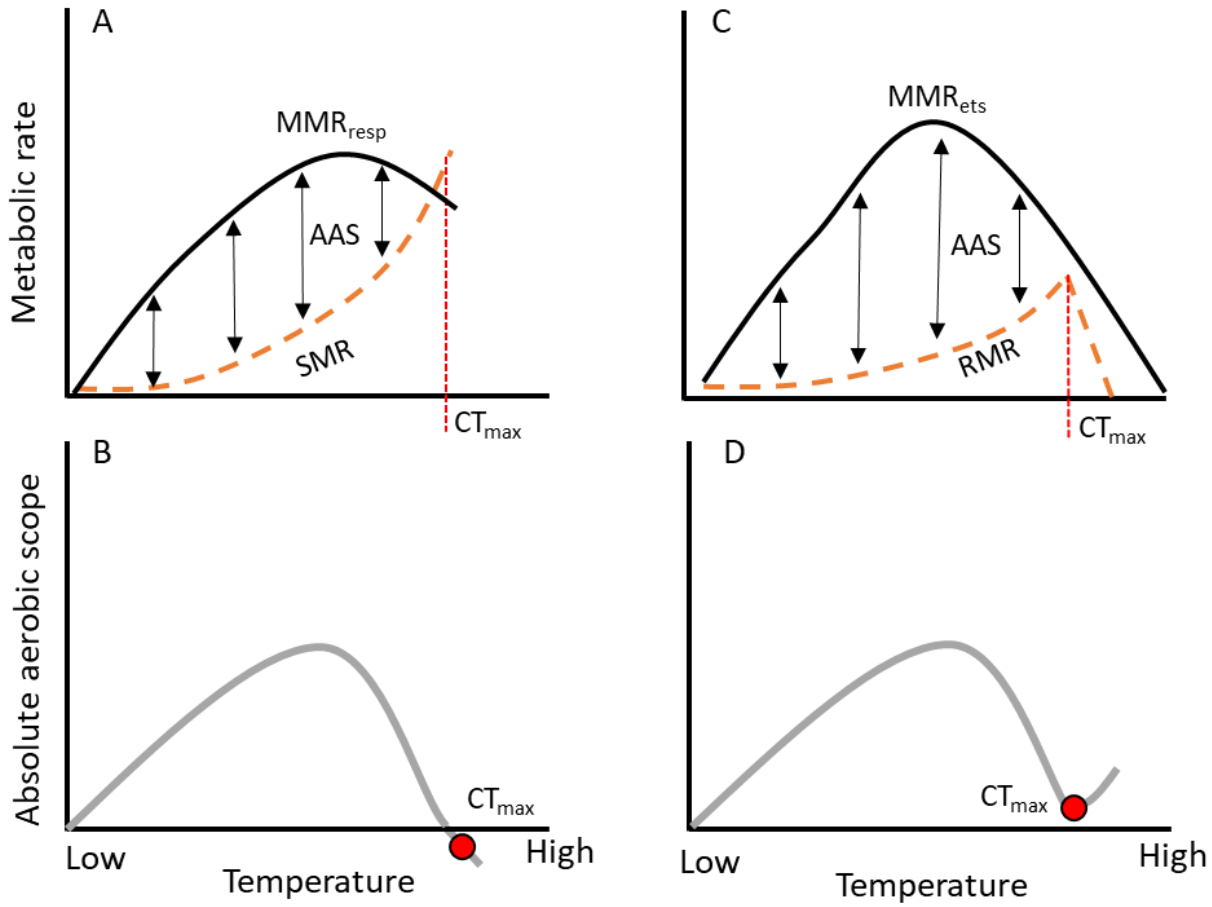

Figure 2.1. (A) The traditionally measured relationship between SMR, MMR_{resp} and temperature. (B) The difference between SMR and MMR known as absolute aerobic scope modified from Verbek et al. 2016. (C) Hypothesized relationship of MMR_{ets} using RMR as a proxy for SMR and its relationship with temperature. (D) The difference between RMR and MMR_{ets} shown as AAS with CT_{max} hypothesized to occur around AAS = 0 modified from Hussain et al. 2023.

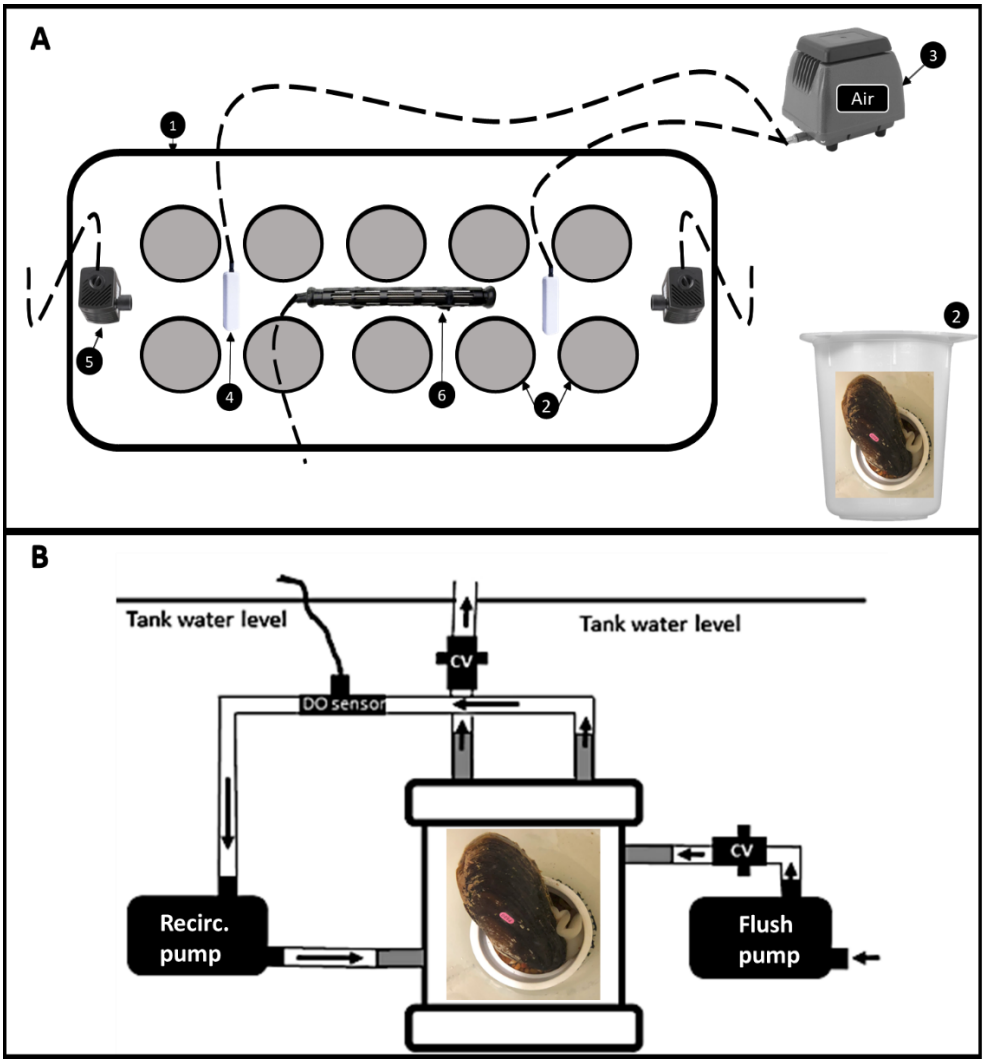

Figure 2.2. (A) Diagram of the CT_{max} assay modified from Hussain et al. (2023) showing (1) insulated tank containing (2) 1-L mesh-sided beakers containing one experimental animal per beaker, (3) an air pump and (4) airstones, (5) water circulation pumps, and (6) an aquarium heater. (B) Diagram of the intermittent respirometry system adapted from Haney et al. (2020) where mussels were placed within an acrylic respiration chamber and a flush pump pumped oxygen saturated water through the chambers during flush cycles and the recirculating (recirc.) pump circulated water through the chamber during measure cycles. An optical dissolved oxygen (DO) sensor within the recirculation tubing measures oxygen. Arrows indicate the direction of water flow.

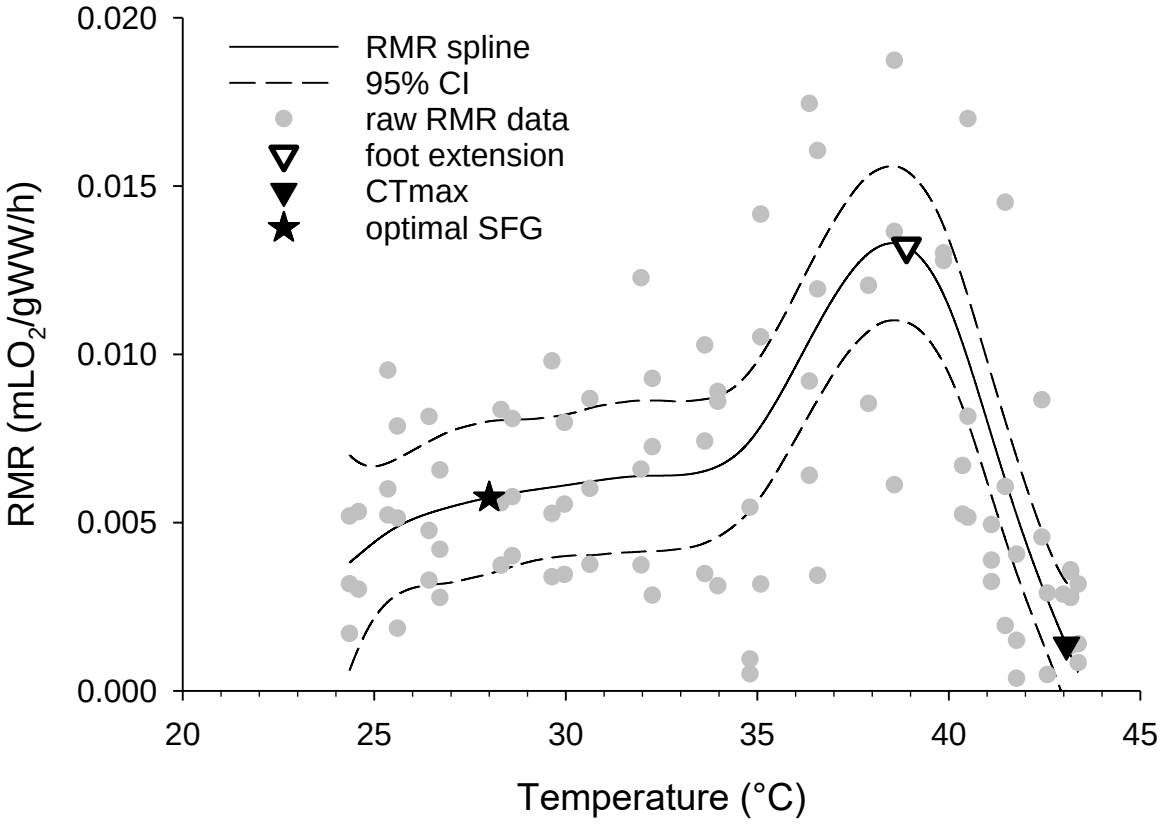

Figure 2.3. Relationship between individual RMR and temperature using a smoothing spline model and 95% confidence intervals. The optimal SFG and thermal stress endpoints (foot extension and CT_{max}) are indicated on the curve.

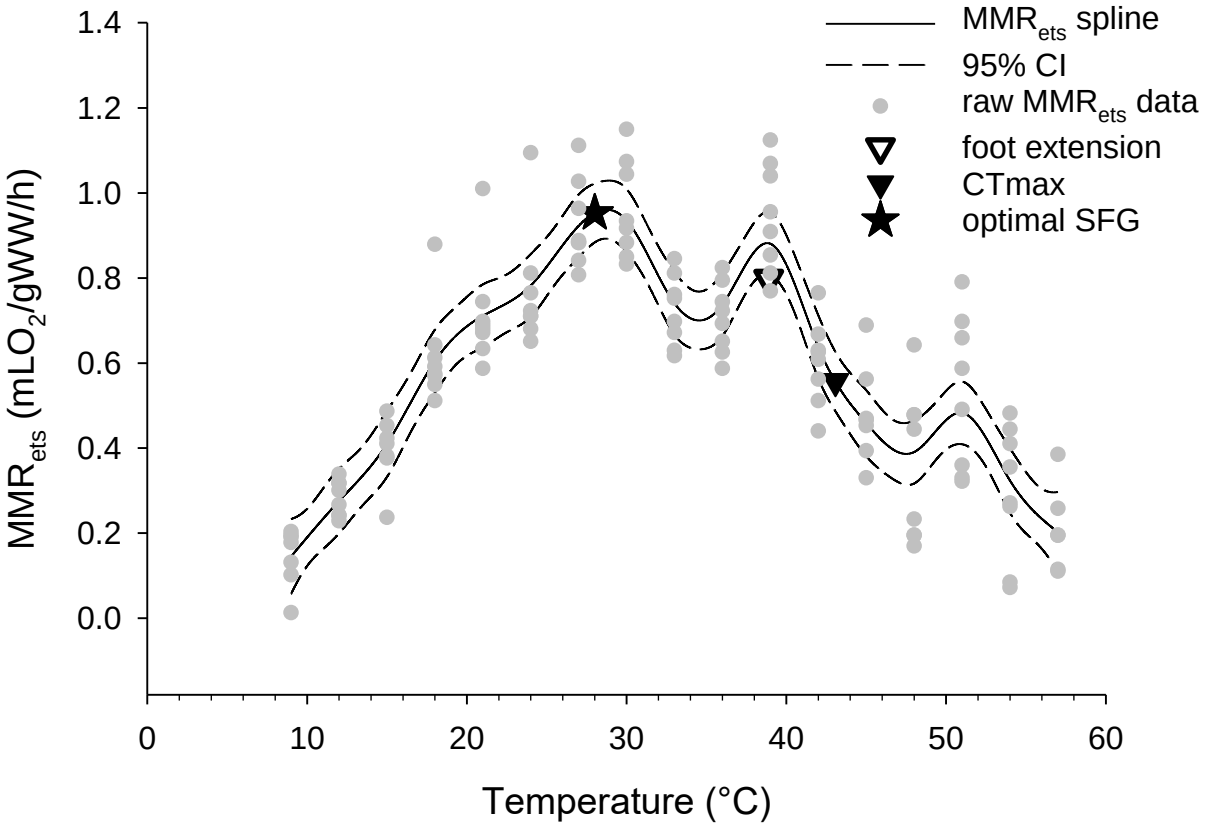

Figure 2.4. Relationship between individual MMR_{ets} and temperature using a smoothing spline model and 95% confidence intervals. The optimal SFG and thermal stress endpoints (foot extension and CT_{max}) are indicated on the curve.

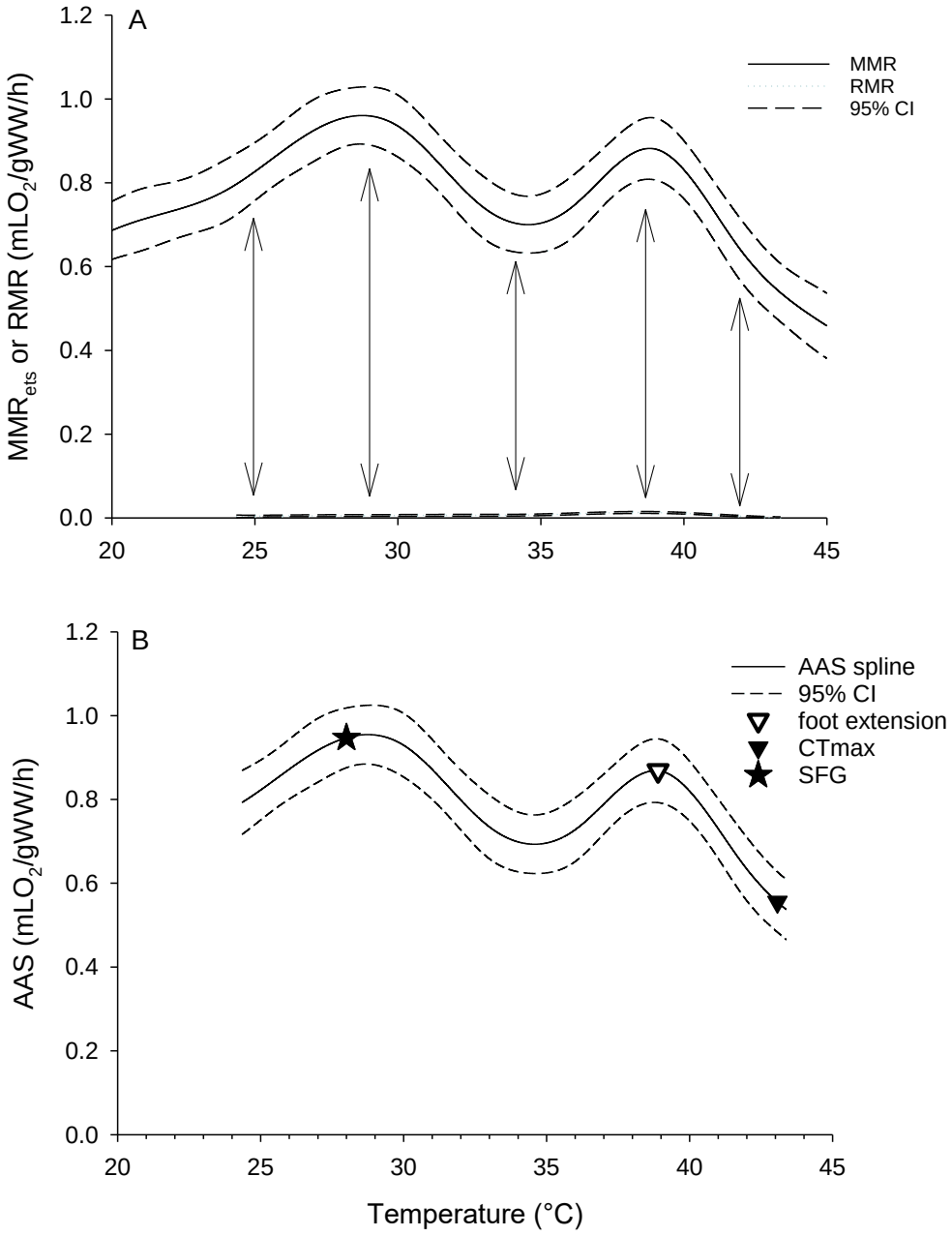

Figure 2.5. (A) Relationship between MMR_{ets} activity, RMR, and temperature and (B) relationship between AAS, CT_{max}, and temperature for *P. popeii* with optimal SFG and thermal stress endpoints (foot extension and CT_{max}) indicated on the AAS curve.