

The effects of temperature and dissolved oxygen on fish respiration determined by enzymatic and organismal techniques.

by

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Abstract

One of the greatest threats to freshwater biodiversity is anthropogenic alteration to habitat. Temperature change is one of the most important anthropogenic impacts, especially for ectotherms that experience the effects of temperature fluctuations more intensely than their endothermic counterparts. Fish biodiversity in Alabama is nearly unrivaled, and to maintain diversity in these systems impacted by anthropogenic stressors, management decisions must be well-informed. Successful management and/or policies are based on information from all relevant aspects of ecosystems, and to that end, in this study I use both game and non-game fish to quantify performance, hypoxia tolerance, and oxygen regulation across a temperature range. I use multiple metrics (metabolic rate, critical oxygen level [DO_{crit}], and regulation index [RI]) to examine temperature effects at the organismal level and use respiratory enzyme assays to quantify thermal performance at the enzymatic level. In addition, I examine the same organismal level metrics in my examination of a potentially compounding stressor: glochidia infestation. Additional stressors may exacerbate temperature fluctuation effects, and a naturally-occurring stressor for some fish species is as a host for parasitic mussels. In an effort to quantify this possible glochidia-induced stress, juvenile bluegill *Lepomis macrochirus* and largemouth bass *Micropterus salmoides* were infested with glochidia and monitored for 11 weeks. At the whole-organism level, metabolic rates were affected by temperature in all non-game species, but temperature effects on DO_{crit} and RI varied. When significant, DO_{crit} increased with temperature while RI was inversely related to temperature. No significant effect of glochidia infestation was found for either gamefish species. At an enzymatic level, enzyme optima and performance were quantified for game and non-gamefish. Performance of enzymes and optimum temperatures varied among species. These results emphasize that fully-informed and comprehensive

management decisions must consider the variable responses to temperature fluctuations across fish genera (including potential mussel hosts). Further, multiple physiological metrics provide a more complete picture, and therefore need to be considered when assessing fish thermal performance.

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1. Introduction

The Southeastern United States, and particularly Alabama, is a region of some of the highest fish and mussel biodiversity in the United States (Stein 2002). Associated with this extreme biodiversity, the region is also a hotspot of species threat and extinction; in the past century, 16 fish species and 45 mussel species in the Southeast have been newly listed as either threatened or endangered, while at least 27 mussel species have been driven to extinction (Boschung and Mayden 2004; Williams et al. 2008). To guard against future adverse effects on diversity and potentially restore imperiled fish and mussel species, causes of decline must be better understood.

Threats to biodiversity are numerous, but one important driver of species imperilment is hydrologic change. Such changes typically involve the widespread distribution of dams (Poff and Hart 2002). Regulations regarding minimum flows and required releases from dams have sometimes been instituted, but rivers and streams may traverse state boundaries complicating management regulations for resident aquatic fauna (Annear et al. 2009). Concerns related to hydrological changes include altered flow regimes, temperature fluctuations, and declining dissolved oxygen (DO, Pringle 2000; Santucci et al. 2005; Jowett and Biggs 2006). Flows that do not mimic natural regime changes (i.e., flows that are too high, too low, too variable) can become disruptive to fish and mussel species whose reproductive cycle depends on seasonal variability of stream/river flow and depth (Weisberg et al. 1990; Freeman et al. 2001). If water release originates from shallow depths in the reservoir, the resulting water input into the stream is warmer, while hypolimnetic release is typically much cooler and may have low DO due to reduced mixing of stratified reservoirs. In streams with smaller or unregulated dams, the effects of altered flow may be reduced in comparison to streams and rivers with large hydroelectric

dams (Poff and Hart 2002; Gangloff 2013); however, these smaller, lowhead dams likely still impact fish, mussel, and macroinvertebrate populations through prevention of upstream migrations or temperature fluctuations due to water forming pools in times of low flow (Freeman et al. 2001; Looy et al. 2014; Tiemann et al. 2016). Reduced flow slows the removal of sediments that could then begin settling into cobble, degrading and reducing availability of habitat for smaller fish or mussels that use crevices of rocks and small boulders (Bednarek 2001). In Alabama, concern over lowhead dams (low-water; 0.4 to 3 m in height) is related to alterations in assemblage structure, habitat degradation, and elevated temperatures that may negatively affect fish and mussel populations (Kingsolving and Bain 1993; Dean et al. 2002; Gangloff et al. 2011; Helms et al. 2011).

The effects of temperature and dissolved oxygen fluctuations on species of recreational and aquaculture importance have been better studied than effects on non-game species (Rummer and Bennett 2005; Powell et al. 2005; Redpath et al. 2010; Lapointe et al. 2014). In fact, while some ecological information exists relative to abundance, preferred habitat, and reproduction for non-gamefish species, little is known about physiological constraints relating to temperature tolerance and dissolved oxygen limits and how these constraints may vary among species. Even less is known about physiological tolerances of freshwater mussel species, and many endangered mussel species of the Southeastern U.S. further require non-game percid and cyprinid hosts facing their own challenges (Layzer and Scott 2006; Williams et al. 2008). Studies pertaining to glochidia infestation of fishes have been performed to identify viable hosts, examine immune system response of the host to infestation, determine optimal load counts on hosts as might be required for culture and reintroduction of threatened mussel species, and quantify host growth rates in response to glochidia parasitism (Moles 1983; Rogers and Dimock 2003; Dodd et al.

2005; Taeubert and Geist 2013). More recent studies have begun to further quantify the effects of glochidia infestation on their host fishes. These studies have been primarily focused on reduced fish activity, host movement suppression, and increased standard metabolic rates of infested hosts albeit without quantifying hypoxia tolerance or regulatory capacity across temperatures (Horky et al. 2014; Slavik et al. 2017; Filippson et al. 2017). Any long-term effects of infestation have been only studied briefly (Kaiser 2005). Given that glochidia parasitism has been shown to cause physiological stress responses in host fish (e.g., enlarged spleen, increased hematocrit), host thermal and hypoxia tolerance may be reduced as a result of glochidia presence (Thomas et al. 2014; Filippson et al. 2017). In the interest of preserving biodiversity for the future, information focusing on mussels, non-game percid and cyprinid species, and glochidia-host interactions may be required for more fully informed policy development and potential future policy change.

One way to measure an organism's response to changes in abiotic conditions is by monitoring an organism's metabolism. Metabolic rates can be quantified to provide physiological data describing an organism's response to changes in temperature and/or dissolved oxygen levels. Using respirometry, oxygen consumption can be measured and, in the absence of activity, it can be used as a measure of resting metabolic rate. In a closed respirometer system, oxygen consumption can be quantified as the organism depletes oxygen inside the chamber. An oxyregulator will consume oxygen at a constant rate until a critical level of dissolved oxygen (DO_{crit}) is reached where maintenance of the constant respiration rate seen at normoxia becomes physiologically disadvantageous to the organism with continued oxygen concentration decline (Richards 2009; McBryan et al. 2013). At this DO_{crit} , the organism will begin conforming—i.e., reducing metabolism and respiration to compensate for declining dissolved oxygen. As

temperature increases, respiration will also be increased due to higher baseline metabolic costs associated with higher temperature (Portner and Grieshaber 1993). In contrast, oxyconformers are distinguished from oxyregulators in that they conform to oxygen concentrations by decreasing respiration rates during normoxia as well as below 4mg/L DO (hypoxia).

The methods for analyzing metabolic rate and DO_{crit} values vary widely throughout the literature and are primarily composed of broken stick models (e.g., hockey stick models or piecewise linear regressions); however, more recent studies have begun to estimate physiological response using nonlinear regression, with one approach being the regulation index (Marshall et al. 2013). Due to both intraspecific and interspecific variability in oxygen consumption and hypoxia tolerance, the regulation index (RI) has been developed to measure regulation ability of organisms that do not exhibit oxygen consumption patterns easily identifiable as regulator or conformer (Mueller and Seymour 2011) (Figure 2.1). RI uses regression to develop an index of regulatory capacity, which can be used to compare regulation potential across temperatures. The regulation index has several advantages, including that it represents a continuum between regulation and conformation, and that it can be quantified using a variety of models.

The electron transport system (ETS) assay is an alternate method of estimating metabolic capacity based on potential maximum enzyme performance at a given temperature (Simcic et al. 2014). Thermal performance curves (TPCs), and specifically ETS TPCs, typically appear unimodal in shape and can be used to measure thermal breadth (width of the TPC) and thermal optima (highest point of the TPC) of maximally performing enzymes across a selected range of temperatures for species of interest (Schulte et al. 2011). By using both a whole animal technique (respirometry) and enzyme level ETS techniques one can quantify oxygen consumption and estimate temperature tolerances of these organisms at the cellular level (Schulte 2015).

In this dissertation, I focused on quantifying the interrelatedness of temperature, dissolved oxygen, metabolism of fish and mussels, and the parasitic relationship of fishes and mussels across three separate but interrelated chapters. Below I describe these chapters.

In Chapter 2, I measured metabolic rates, DO_{crit} levels, and regulation index values for several native fish species across a range of temperatures from a baseline of 18°C up to a sub-lethal temperature. Sub-lethal temperatures were estimated through literature review (Smith and Fausch 1997; Beitinger et al. 2000). Fishes used were non-game species as well as species with an ecological role as potential mussel hosts. I tested the hypothesis that metabolic rate would increase to a maximum followed by rapid decline to biological disfunction. I also tested the hypothesis that DO_{crit} for all species would increase with temperature. Finally, I tested the hypothesis that RIs would decrease for all species with temperature. Closed respirometry was used to determine DO_{crit} and RI values for fishes. Results from this chapter can be used to inform decisions and conservation planning regarding management of habitats in which these habitat specialists and habitat generalists occur.

In Chapter 3, I tested the hypothesis that fishes infested with glochidia, when compared to noninfested fishes, would have lower RI and higher DO_{crit} values due to decreased surface area for oxygen transfer and increased stress due to glochidia presence in the gills. I tested this by infesting bluegill, *L. machrochirus*, and largemouth bass, *Micropterus salmoides*, with glochidia. I also tested the hypothesis that DO_{crit} would be higher and individual RI would be more reduced in infested fish versus uninfested fish. These comparisons between infested fish and uninfested fish examined confounding effects of glochidia infestation and declining DO.

In Chapter 4, I determined optimal temperature ranges for ETS enzyme performance in fish by using ETS assays. In this chapter, I tested the hypotheses that the temperature at

maximum enzymatic performance would differ among species. One major advantage to measuring enzyme activity over whole animal respirometry is that enzyme activity due to any stress of collection or handling would not be apparent on a cellular level until 2-7 days after exposure (Gopalan et al. 1996). By using ETS assays, I could develop and assess a rapid assessment of fish enzyme thermal optima as an alternative to prolonged lab holding periods and physiology experiments in which live animals are subject to stress, infection, etc.

The research described in these chapters allows me to investigate interrelated effects of temperature, hypoxia, and mussel/host ecological interactions on the metabolism of non-game and select game fishes. Conservation and management plans should rely on combined ecological and physiological data to make informed decisions for whole communities of fish and mussels, and the data I present in these chapters will provide needed physiological information on mussel and non-game fish species.

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2. Species-specific dissolved oxygen and temperature requirements for non-game riverine fishes

Abstract

The diverse assemblages of fishes in the Southeastern United States are frequently subjected to stress from temperature extremes and hypoxia due to factors including climate change, drought, and altered flow regimes. Because fish are ectotherms, their biological and chemical processes are dictated by environmental conditions (i.e., temperature as well as oxygen availability); therefore, understanding how fish physiology is affected by temperature and hypoxia at the organismal level is critical in elucidating population level responses. In this study, I quantified the effects of temperature on metabolic rate, dissolved oxygen critical values (DO_{crit}), and regulation indices (RI) for seven native, nongame Alabama fish species using closed respirometry at temperatures ranging from 14-30°C. Significant relationships between metabolic rate and temperature were observed for all species, with the responses best fit by linear or quadratic regressions. Blacktail shiner *Cyrinella venusta* was the only species whose metabolic rate decreased initially with increasing temperature; all others showed increasing metabolic rate with temperature. DO_{crit} increased with temperature only for largescale stoneroller *Campostoma oligolepis*, longjaw minnow *Notropis amplamala*, and creek chub *Semotilus atromaculatus*. RI increased significantly with temperature for all species except longjaw minnow and blacktail shiner. Given the variation among even this group of seven species, it is clear that regulations protecting water quality need to incorporate this diversity. By using multiple metrics to quantify thermal and hypoxia tolerance, better management plans can be developed to include physiological limitations for these or closely-related species.

2.1 Introduction

The Southeastern United States possesses the richest aquatic biodiversity and the highest degree of endemism in North America (Jenkins et al. 2015), but that biological plenty unfortunately also comes with some of the highest imperilment rates on the continent (Warren et al. 1997; MRBMRC 2010). In fact, over 40% of the Federally listed animals in the U.S. Southeast are freshwater mussels, snails, and fishes that occur in Alabama (Warren et al. 1997, Neves et al. 1997). Protecting these imperiled animals requires knowledge as to how multiple uses of waterways impact the risk of imperilment or extinction. Large and small dams are prevalent on the landscape (Graf 1999; Downing et al. 2006), many of which impact downstream water quality in terms of critical levels of temperature and dissolved oxygen (DO; Pringle et al. 2000; Santucci et al. 2005). According to a review of 300 Federal Energy Regulatory Commission (FERC) project records, more than 40% of projects had a specific requirement to maintain DO, 37% had DO monitoring requirements, and 38 and 35% had mitigation recommendations or mandated changes in project operations, respectively (EPRI 1992). The U.S. Fish and Wildlife Service requires information to inform decision making relative to recovery actions, prioritization of stream and river reaches for restoration or reintroduction efforts, and for assistance during permitting where imperiled fishes, snails, and mussels are concerned (MRBMRC 2010).

Maintenance of minimum DO concentration is required for survival of most aquatic organisms. This can be particularly important when stream flows are interrupted by a dam causing DO and temperature fluctuations that sometimes exceed the tolerances of organisms living downstream. High head instream dams are required by their licenses to maintain a minimal level of dissolved oxygen in discharged water, typically determined based on the

temperature and oxygen requirements of largemouth bass *Micropterus salmoides*, channel catfish *Ictalurus punctatus*, or other game fishes (U.S. EPA 1986, as cited by FERC in their 2012 Biological Opinion for the Coosa River). However, while abiotic and biotic requirements of numerous game fishes is well studied (e.g., Beamish 1964a; Rice and Cochran 1984; Redpath et al. 2010; Evans 1984), there is a surprising lack of data regarding critical physiological limits of nongame fishes. As such, threshold values that have been developed based on game fishes may not adequately represent temperature and dissolved oxygen limitations of other ecologically important species. In certain cases where flow from impoundments is reduced, temperature and/or DO concentration may quickly exceed tolerance limits of less-studied species while remaining within accepted bounds determined for better-studied game fishes. Given the potential importance of many nongame fishes as mussel hosts and as important components of the food web, exceeding these limits could clearly have ecological implications for the broader aquatic ecosystem.

An approach that has been commonly used to evaluate hypoxia tolerance of fishes is to quantify respiration rate (MO_2), which reflects the ability of an organism to extract oxygen from the environment required to maintain routine metabolic rate (RMR), as DO changes (Tripathi et al. 2013). Historically, fish had been assumed to fit into one of two discreet categories of oxygen consumption responses to declining DO: oxyregulators (Fry and Hart 1948; Beamish 1964b; Ultsch et al. 1978; Cech et al. 1979; Barnes et al. 2011) or oxyconformers (Barnes et al. 2011; Urbina et al. 2012; Tiffany et al. 2010; Tripathi et al. 2013) (Figure 2.1). Fish that maintain a constant MO_2 across a wide range of dissolved oxygen (DO) levels are considered oxyregulators (Figure 2.1a; Fry and Hart 1948; Beamish 1964b; Ultsch et al. 1978; Cech et al. 1979; Barnes et al. 2011), while those that reduce their oxygen consumption as DO decreases are considered

oxyconformers (Figure 2.1b; Portner et al. 1985; Tiffany et al. 2010; Urbina et al. 2012; Tripathi et al. 2013). In both cases, there exists a critical DO concentration (DO_{crit}), below which the ability of an organism to obtain oxygen from its environment rapidly declines with declining oxygen availability (Jobling 1994; Rogers et al. 2016, Wood 2018). Because a lower DO_{crit} is associated with a greater capacity to obtain oxygen in low DO environments, it is frequently used as an indicator of hypoxia tolerance in fishes (Speers-Roesch et al. 2012, Regan et al. 2019).

Clearly these responses represent the ends of a continuum, with most fishes' responses fall somewhere between conformation and regulation. More recently, as an alternative to categorizing organisms into one of only two distinct metabolic patterns, a regulation index was developed (RI; Mueller and Seymour 2011) that recognizes the continuum of metabolic response patterns. The RI quantifies the relative ability of an organism to regulate oxygen uptake as DO declines to zero, rather than constraining an organism to be defined in a binary way as either a regulator or a conformer (Figure 2.1c). RI is instrumental in describing oxygen uptake strategies. The ability of an organism to regulate oxygen uptake increases with increasing RI decreases with decreasing RI.

In previous work, Hartline et al. (2020) quantified the influence of temperature and dissolved oxygen on oxygen consumption rate, critical oxygen level (DO_{crit}), and the regulation index of five non-game fish species—blacktail shiner (*Cyprinella venusta*), blackbanded darter (*Percina nigrofasciata*), bronze darter (*P. palmaris*), greenbreast darter (*Etheostoma jordani*), and banded sculpin (*Cottus carolinae*). Using closed respirometry, they quantified oxygen consumption as a function of DO concentration at three temperatures. Oxygen uptake patterns for those species represented a continuum between regulation and conformation, and fish ability to regulate was affected differently by temperature among species, declining with increasing

temperature in blackbanded darter, not affected by temperature in bronze darter, greenbreast darter, and blacktail shiner, and increasing with temperature for banded sculpin. In addition, critical oxygen levels increased with temperature for blacktail shiner, greenbreast darter, and blackbanded darter, but did not change with temperature for bronze darter or banded sculpin. This among-species variation further supports the need for additional information concerning effects of low DO across species and that protections based on data from a limited number of taxa may not effectively protect habitat for entire fish communities.

In the current study, I use respirometry to quantify dissolved oxygen uptake across a wider range of temperatures than used by Hartline et al. (2020) for seven nongame fish species in an effort to more explicitly characterize among-species variations, provide a broader database from which to generate predictions, and ultimately generate the rigorous data required for determining suitable conditions in waters below impoundments. Species were all from the Southeastern U.S. and it is my goal that my findings and predictions will assist in decision making for specific projects (i.e., Strategic Habitat Conservation, Alabama's Strategic Habitat Unit-SHU Project), as well as for being applicable to implementation of conservation actions relative to impounded river systems and habitats. I tested the hypothesis that metabolic rate would increase to a maximum followed by rapid decline to biological disfunction. I also tested the hypothesis that DO_{crit} for all species would increase with temperature. Finally, I tested the hypothesis that RIs would decrease for all species with temperature. My overall objective for the proposed research is to quantify oxygen uptake responses to declining dissolved oxygen for several non-game fish species as well as how those responses are affected by temperature.

2.2 Materials and methods

Fish were collected from three Alabama streams—Chewacla Creek (32.556174, -85.468419), Choctafaula Creek (32.466722, -85.641234; sometimes this site is mistakenly identified as Chewacla Creek on digital maps), and Big Wills Creek (34.571256, -85.617058). Largescale stoneroller *Campostoma oligolepis*, blacktail shiner, banded sculpin, rough shiner *Notropis baileyi*, creek chub *Semotilus atromaculatus*, longjaw minnow *Notropis amplamala*, and striped shiner *Luxilus crysocephalus* were collected via seining and backpack electrofishing (LR-24; SmithRoot, Vancouver, WA, USA), held in aerated coolers, and transported to the laboratory where they were placed into holding tanks and acclimated to lab conditions (21°C; 12:12 hr light/dark) for at least ten days before exposure to any temperature change, or respirometry measurements. Temperature was then gradually increased or decreased (1°C per day) until reaching the experimental temperature at which they were to be tested; fish were acclimated to that temperature for an additional ten days prior to respirometry runs. Individual fish were run at only one experimental temperature. Temperatures used during respirometry ranged from a baseline of 18°C to a maximum of 30°C at 3°C increments for all species except banded sculpin. Baseline temperature for banded sculpin was 14°C to a max of 26°C increasing in two-degree increments. Temperatures were chosen based on literature review estimates of sub-lethal temperatures (Smith and Fausch 1997; Beitinger et al. 2000, Hartline et al. 2020).

2.2.1 Respirometry

A respirometry “run” was defined as the duration of time spent inside the respirometer chamber by an individual fish during which temperature, respiration rate, and dissolved oxygen concentration were measured, and from which oxygen uptake and routine metabolic rate were calculated. Fish feeding was suspended for 48 hours prior to a run to allow complete gut

evacuation (Chabot et al. 2016b, Rodgers et al. 2016). Prior to placement in a respirometer chamber, individual fish were weighed (nearest 0.01 g). Eight individual fish were randomly assigned to one of eight glass chambers that were submerged in a large reservoir of dechlorinated tap water. Temperature of the water housing the chambers matched the acclimation temperature of the fish holding tanks, and barriers around the chambers prevented any unnecessary stimulation due to viewing other fish for the duration of a run. Once inside the chamber, an opaque covering was placed over the entire large water reservoir containing the respirometer chambers and fish were allowed to acclimate. Given that acclimation times can be variable both among species and among individuals within species, combined with variation in acclimation times that have been used previously in the literature, I allowed fish to acclimate in the chambers overnight for at least 12 hrs (Chabot et al. 2016b, Rodgers et al. 2016, Chabot et al. 2021).

Each respirometer (Loligo® Systems, Tjele, Denmark) consisted of a flush pump, a recirculating pump, a fiber-optic oxygen probe, and the respirometer chamber (containing the species of interest). Oxygen concentration was measured via the fiber-optic probe. A diel light cycle was maintained which was light during 0700-1900 and dark from 1900-0700. Runs were only performed during daylight hours to minimize any diurnal effects. Fish were placed into the respirometry chamber no later than 1800 on the day prior to the trial. Individual fish were used only once. Fiber-optic probes were calibrated monthly and I completed approximately 96 -120 runs each month. To correct for background respiration caused by bacteria biofilms that could have become established, blank controls were performed for one hour before and one hour after each experimental run.

2.2.2 Measures of hypoxia tolerance

Once the acclimation time had elapsed, the respirometer was set to closed so that water recirculated, oxygen was consumed, and oxygen uptake quantified. During this closed period, oxygen was consumed by the fish, and the (declining) oxygen concentration was measured via the fiber-optic oxygen probe. As oxygen concentration declined, fish respiration rates were observed through live monitoring of data through Autoresp software until a concentration of 1 mg/L of oxygen was reached or until fish movement became erratic. The fish was then removed from the chamber and humanely euthanized using a buffered solution of MS-222 (Auburn University Standard Operation Guideline AP-101). To check for carbonic acid and ammonia buildup during the closed experiment, ammonia and pH were tested using aquarium test strips (Tetra®, Blacksburg, VA, USA) immediately following the end of each run. This technique was repeated for a minimum of 6 similarly sized individuals of each species at each temperature. Background respiration was quantified by calculating mean respiration rate inside the glass chamber by averaging pre- and post-run control background respiration ($\text{mg O}_2 / \text{hr}$). This rate was then calculated as a percentage of total respiration inside the chamber during the run and subtracted from the total chamber respiration ($\text{mg O}_2 \cdot \text{chamber}^{-1} \cdot \text{hr}^{-1}$). The chamber respiration rate minus the background respiration was the fish respiration rate ($\text{mg O}_2 \cdot \text{chamber}^{-1} \cdot \text{hr}^{-1}$). This respiration rate was calculated as weight specific metabolic rate by dividing respiration rate by the mass of the fish ($\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$). Three response variables were quantified from the resulting data: normoxic oxygen uptake (MO_2), critical dissolved oxygen concentration (DO_{crit}), and the regulation index (RI). Normoxic MO_2 was calculated as the mean oxygen uptake rate between 5 and 7 mg/L O_2 . DO_{crit} was calculated using the respR function in R to determine the breakpoint from segmented regression. RI was calculated using methods similar to Mueller and

Seymour (2011). Respiration rate was plotted against DO, and data were fit to three curves (exponential rise, quadratic, and segmented regression) using SigmaPlot 14.0 (Systat Software, Inc. San Jose, CA). Oxygen uptake above 6 mg/L O₂ was excluded from RI analyses to avoid bias—oxygen saturation at higher experimental temperatures (≤ 7 mg/L) did not reach concentrations seen at cooler temperatures (> 8 mg/L; Mueller and Seymour 2011). The best fitting model was determined using AIC_C model selection. Using this best fitting model, area under the curve was calculated for the observed data/best fitting model, a horizontal line representing perfect regulation, and a line fit to two points: starting respiration rate and ending respiration rate (linear decline) to represent perfect conformation. These areas were input into the following equation to calculate fish RI as a proportion:

$$RI = \frac{(\text{Observed Area} - \text{Conformation Area})}{(\text{Regulation Area} - \text{Conformation Area})}$$

These three variables were compared across temperatures within species using one-way ANOVAs and Tukey's post hoc adjustments in R version 4.0.3 (R Core Team 2020). I used linear and quadratic regressions to compare response variables across temperatures; linear and quadratic models were compared for best model fit using AIC_C. The model with the best fit (lowest AIC_C) was used in the analysis. Finally, I compared mean normoxic MO₂, DO_{crit}, and RI across species at each temperature using ANOVA with Tukey's post hoc comparisons to quantify differences in metabolic rate among species.

2.3 Results

Fish adjusted quickly to the lab, displaying no behaviors that could be interpreted as induced by stress (e.g., listlessness, erratic swimming, piping, etc.) prior to a respirometry run. Once placed inside the respirometry chamber, fish acclimated to the flow of the flush and recirculating pumps, displaying minimal movement within minutes and well before covering the

basin with the opaque covering. Respirometry was successfully performed on seven species (Table 2.1)

2.3.1 Largescale stoneroller

In contrast to my expectations based on literature values, during acclimation to 30°C. I observed that largescale stonerollers fed less and over time their physical condition visibly declined. During the 2-week acclimation period, I also observed substantial mortality (9 out of 14 individuals died). Given this, I could not perform respirometry for stonerollers at 30°C.

Largescale stonerollers were observed to have the highest mean metabolic rates and the effect of increasing temperature on metabolic rate was significant (ANOVA, Tukey's, $p = 0.004$; Figures 2.2 and 2.9). Metabolic rate significantly increased with temperature (linear regression, $p = 0.005$, $r^2 = 0.215$; Figure 2.2). Largescale stoneroller was one of three species I tested whose DO_{crit} increased significantly with temperature (linear regression, $p = 0.01$, $r^2 = 0.18$; Figure 2.2 and 2.10). Ability of largescale stonerollers to regulate oxygen consumption decreased significantly as temperature increased (linear regression, $p = 0.005$, $r^2 = 0.213$; Figure 2.2).

2.3.2 Blacktail shiner

Blacktail shiner metabolic rate decreased significantly as temperature increased (linear regression, $p = 0.002$, $r^2 = 0.39$; Figure 2.3). Increasing temperature had no effect on DO_{crit} or RI for blacktail shiner (linear regression, $p = 0.95$ and $p = 0.49$, respectively; Figure 2.3).

2.3.3 Striped shiner

Striped shiner metabolic rate was significantly related to temperature in a curvilinear fashion (quadratic regression, $p = 0.003$, $r^2 = 0.66$; Figure 2.4) and was one of the highest rates observed (Figure 2.9). There was no significant response of DO_{crit} to temperature ($p = 0.52$;

Figure 2.4). Striped shiner RI declined significantly as temperature increased (quadratic regression, $p < 0.023$, $r^2 = 0.80$; Figure 2.4).

2.3.4 Longjaw minnow

Metabolic rate of longjaw minnows significantly increased with temperature (quadratic regression, $p < 0.001$, $r^2 = 0.80$, Figure 2.5). Increasing temperatures led to significantly increased DO_{crit} (linear regression, $p = 0.014$, $r^2 = 0.27$, Figure 2.5), while RI was not significantly affected by temperature (quadratic regression, $p = 0.185$, Figure 2.5).

2.3.5 Rough shiner

Increasing temperature had a significant effect on the metabolic rate of rough shiners (quadratic regression, $p = 0.038$, $r^2 = 0.40$; Figure 2.6), and metabolic rate was of an intermediate level among tested species. DO_{crit} did not differ across tested temperatures (linear regression, $p = 0.22$; Figure 2.6). Rough shiner RI had a curvilinear response with temperature, with RI being highest at 18°C, decreasing at 21°C and 24°C, and increasing at 27°C (ANOVA, Tukey's, $p < 0.001$, $r^2 = 0.46$; Figure 2.6).

2.3.6 Creek chub

All response variables were significantly affected by temperature. Metabolic rate curvilinearly increased with temperature (quadratic regression, $p < 0.001$, $r^2 = 0.31$, Figure 2.7). DO_{crit} was significantly affected by increasing temperatures (quadratic regression, $p = 0.033$, $r^2 = 0.14$, Figure 2.7), while RI significantly decreased with increasing temperatures (quadratic regression, $p < 0.001$, $r^2 = 0.36$, Figure 2.7)

2.3.7 Banded sculpin

Similar to largescale stonerollers, substantial mortality of banded sculpins was observed at temperatures above 26°C. Given this, no respirometry was performed for banded sculpin

above this temperature. Banded sculpin metabolic rate increased, and RI decreased as temperature increased (MO_2 : quadratic regression, $p < 0.001$, $r^2 = 0.53$; RI linear regression, $p = 0.01$, $r^2 = 0.13$, respectively; Figure 2.8); however, increasing temperature had no effect on DO_{crit} (linear regression, $p = 0.50$; Figure 2.8).

2.3.8 Across-species comparisons

I was able to compare metabolic rates across species at five temperatures: 18, 21, 24, 27°C, and 30°C (Figures 2.9 and 2.12). At 18°C, there were no significant differences among the six species compared (banded sculpin was not included in the analysis at 18 or 27°C). At 21°C, banded sculpin, creek chub, and longjaw minnow metabolic rates were significantly lower than for largescale stoneroller, with other species being intermediate (ANOVA, $p < 0.001$; Figure 2.12). At 24°C, largescale stoneroller and striped shiner metabolic rates did not differ (ANOVA, $p = 0.99$; Figure 2.12) but were significantly higher than those of blacktail shiner, longjaw minnow, rough shiner, and banded sculpin (ANOVA, $p < 0.001$; Figure 2.12). Banded sculpin metabolic rates were also significantly lower than creek chub (ANOVA, $p < 0.001$; Figure 2.12). At 27°C, creek chub, longjaw minnow, and largescale stoneroller metabolic rates did not differ (ANOVA, $p > 0.05$, Figure 2.12) but were significantly higher than those of blacktail shiner and rough shiner, which did not differ from each other (ANOVA, $p = 0.85$; Figure 2.12). Striped shiner metabolic rates did not differ from any other species (ANOVA, $p > 0.05$; Figure 2.12). Of the three species compared at 30°C, striped shiner metabolic rates did not significantly differ from blacktail shiner or creek chub (ANOVA, $p > 0.05$; Figure 2.12); however, blacktail shiner and creek chub rates did significantly differ (ANOVA, $p = 0.017$; Figure 2.12).

I was able to compare DO_{crit} across species at five temperatures (Figures 2.10 and 2.13). No differences were observed for comparisons at 27 and 30°C (Figure 2.13). At 18°C creek chub

had significantly higher DO_{crit} compared to longjaw minnow ($p = 0.03$), rough shiner ($p = 0.002$), and striped shiner ($p = 0.03$) (Figure 2.13), and largescale stoneroller DO_{crit} was significantly higher than rough shiner ($p = 0.0005$). At 21 and 24°C, largescale stoneroller had significantly higher DO_{crit} than creek chub and banded sculpin ($p = 0.003$; Figures 2.10 and 2.13).

Regulation indices were also compared across species at the five experimental temperatures (Figures 2.11 and 2.14). At 27°C, no significant differences were observed across species ($p = 0.22$; Figure 2.14). At 18°C, striped shiner had a significantly higher RI than largescale stoneroller ($p = 0.2$; Figure 2.14). At 21°C, blacktail shiner had higher RI than rough shiner and largescale stoneroller ($p < 0.001$; Figure 2.14), and creek chub RI was significantly higher rough shiner ($p = 0.006$). At 24°C, blacktail shiner had the highest RI of all compared species ($p < 0.01$; Figure 2.14). Largescale stoneroller had significantly lower RI than creek chub ($p < 0.001$) and banded sculpin ($p = 0.003$; Figure 2.14). At 30°C RI for blacktail shiner was significantly higher than creek chub and striped shiner (ANOVA, $p < 0.01$, Figure 2.14).

Using predicted curves for RI vs temperature, stoneroller and rough shiner had the lowest capacity for regulation (Figure 2.11). Creek chub predicted RI curves remained relatively high and did not decline as quickly as other species (Figure 2.11).

2.4 Discussion

An era of increasing climatic fluctuations is expediting the need to understand fish responses to temperature and dissolved oxygen fluctuations. This demand is furthered by variation in response to warming temperatures. While warm-water species may expand their distribution as cool- and coldwater streams become increasingly warmer, coldwater species are pushed into thermal holdouts that are becoming scarcer (Lyons et al. 2011, Comte 2012, Perrin

2021). With more information on responses to temperature and DO fluctuations, managers can examine streams and assemblages to determine where protections are necessary.

The most extreme detrimental effect of temperature is death. If a fish does not survive exposure to a temperature, that was by definition a lethal temperature. Any fitness related, physiological, or metabolic changes that result from temperature stress that do not explicitly kill the organism are sublethal. Lethality is well studied among fish in the form of LT_{50} , CT_{max} , and CT_{min} experiments (critical thermal maximum and critical thermal minimum; Beitenger et al. 2000, Ali et al. 2019). In these experiments, individuals are acclimated to a temperature and then subjected to increasing or decreasing temperatures (chronic or acute). The point at which 50% of the individuals have died is noted as the LT_{50} , or the median lethal temperature. CT_{max} and CT_{min} are endpoints that are characterized by an individual's loss of muscle control in response to exposure to a temperature (high or low) and usually focus on acute temperature changes. Sublethal effects of high temperatures can include reduced growth rates, changes in respiration, and reduced reproductive output (Dallas and Ross-Gillespie 2015).

Increasing temperatures affect ectothermic fish by increasing energetic demands. Temperature increase equates to higher rates of metabolic function (higher RMR). For fish that require specific temperature ranges for growth or reproduction, higher RMR means an available energy reduction for other activities if those higher temperatures exceed the range for which the fish are adapted. The aerobic scope of the fish is reduced because, even though the RMR has increased, maximum metabolic rate (MMR) will not increase proportionately to support the new demand caused by increasing temperature on routine rate (Farrell 2013, Paschke et al. 2018). Dissolved oxygen availability decreases as temperature increases and, consequently, the ability of a fish to obtain sufficient oxygen is affected. For a fish to sustain aerobic metabolism, enough

oxygen must be consumed in order to meet biological processes. At normoxia (>4 mg/L O_2) for warmwater streams), oxygen is available for regulation of metabolism. When hypoxia begins (<4 mg/L O_2), metabolism must make accommodations for the lower DO supply.

Metabolic rate is often used to quantify performance because it is a measure of biological processes and capacity for growth, reproduction, and other energy flow through an organism (Chabot et al. 2016, Reemeyer and Rees 2020). Per gram metabolic oxygen consumption (MO_2) is particularly important for ectotherms given the important relationship between metabolic rate and temperature. For example, metabolic rates are used when performing bioenergetics analyses. Argued by Ney (1993), one of the problems with the Wisconsin Bioenergetics Model is the practice of species borrowing. When inputting information into bioenergetics models (MO_2) or quantifying response variables (DO_{crit} , RI), having species-specific metabolic rates is very important given that RMR varies across species due to differences in phylogeny, size, activity level and temperature (Clarke and Johnston 1999, Glazier 2005, Chabot et al. 2016) Intraspecifically, individuals with higher RMR may be at a disadvantage compared to individuals with low RMR since individuals with high RMR have higher baseline metabolic demands (Burton et al. 2011).

Largescale stoneroller and creek chub were the only species that I found to have significant relationships for all three metabolic parameters (MO_2 , DO_{crit} , RI) with increasing temperature. Metabolic rates varied by 3-4 times across species, and largescale stoneroller, striped shiner, and creek chub had the highest measured metabolic rates of the seven study species at 24 and 27°C. Anecdotally, it appeared that largescale stoneroller body condition declined during the two-week acclimation to 30°C. At 30°C individuals began to feed less frequently than they had fed at or below 27°C, they appeared to lose weight and were visibly less

robust, and swimming/schooling appeared to be a strenuous activity (some individuals attempted to swim to join conspecifics but would sink and rest on the bottom of the tank). For oxygen uptake, the typical or expected relationship with increasing temperature is an increase in metabolic rate to a maximum temperature followed by a decline at which point the individual fish's metabolism is reduced to compensate for suboptimal temperatures (Portner 2010). I did not observe any such decline in largescale stoneroller due to the inability to acclimate them to 30°C. It is possible that such a reduction in metabolic rate would be observed between upper test temperature (27°C) and 30°C, but that was not tested here. However, I do expect that a critical point for largescale stonerollers to maintain physiological function lies somewhere between 27-30°C. Largescale stoneroller metabolism at these temperatures could be sufficiently high to cause a decline in body condition. Rough shiner and banded sculpin exhibited typical patterns for predicted metabolic rates, increasing with temperature to a point above which metabolic rate declined. While I did observe this more typical pattern in striped shiner and creek chub metabolic rate, there did appear to be a maximum metabolic rate at 24-27°C above which it declined, suggesting that a critical point for striped shiner and creek chub to maintain physiologic function lies somewhere between 27-30°C. Relative to physical condition, if striped shiner and creek chub were to exhibit observable declines in body condition at high temperatures, these symptoms did not present themselves within the two-week acclimation period of this study.

Banded sculpin metabolic rates were low, potentially reflecting its existence as a benthic species as well as its general description of being a coolwater species and preferring cool temperatures (Baltz et al. 1982, Brown 1989). Sculpin do not have to sustain movement through the water column when scouring for forage, instead they seek crevices among cobble and rubble to defend territory. The metabolic rates of rough sculpin at 15 and 20°C (approximately 67 and

192 mg O₂ kg⁻¹h⁻¹ respectively; Brown 1989) were similar to the banded sculpin from my study at 14 and 21°C (approximately 72 and 152 mg O₂ kg⁻¹h⁻¹ respectively). Banded sculpin respiration, like that of striped shiner and creek chub, showed an increase in metabolic rate to maximum followed by a decrease before lethal temperature (as I observed for my sculpin acclimation). Interestingly, the results reported here for banded sculpin differ from those reported in Hartline et al. (2020). Inclusion of the lower 14°C temperature allowed us to quantify the increase in MO₂ that occurred between 14°C and 17°C, likely explaining the unique results found by Hartline et al (2020), where banded sculpin respiration rate decreased and RI increased with increasing temperature between 20-24°C. That is to say, more temperatures in the Hartline et al.'s study would likely have yielded similar results to mine. Acute thermal maximum for mottled sculpin, *Cottus bairdi*, after acclimation at 15 °C was documented by Beitinger et al. (2000), where the endpoint of sculpin thermal tolerance was determined by the onset of muscle spasms at 30 °C (Kowalski et al. 1978). At the same acclimation temperature (15°C), the slimy sculpin, *Cottus cognatus*, exhibited loss of equilibrium between 26.3 and 27.3°C (Otto and O'Hara Rice 1977). More recently, studies have compared thermal maxima for a variety of Southeastern U.S. sculpin species and found similar values to Kowalski et al. (1978) for banded sculpin acclimated to 15°C (Walsh et al. 1997). Similar to my acclimation efforts, Brown (1989) attempted to acclimate three species of sculpin to 27.5°C but saw significant mortality before two weeks had elapsed. Long-term exposure or acclimation of sculpin species to temperatures higher than 26°C may be difficult to achieve based on the findings of these studies.

Another interesting result for banded sculpin was the observed cessation of respiration in some individuals at 14°C. In some trials at 14°C, as dissolved oxygen levels decreased to between 1 and 2 mg/L, banded sculpin were observed to have stopped respiring (both visually as

well as in terms of corrected oxygen consumption); however, prior to this, individuals did not exhibit any visible distress behavior. Instead, sculpin remained at the bottom of the chamber in a manner that was not distinguishable from that occurring at normoxia. Cutaneous respiration has been observed in marine and intertidal cottids (Martin 1991), but even that would not explain the complete lack of oxygen decline below 2 mg/L inside the closed respirometry chamber. My immediate conclusion was that the non-respiring individual had died without exhibiting distress that I observed to be typical for shiners exposed to oxygen levels ≤ 1 mg/L. However, when the chamber was flushed with oxygenated water and the individual removed, the apparently “dead” fish resumed respiration with visible opercular beats. Prior to the flushing, no opercular movement was observed. The cool temperature and low oxygen environment may have induced a torpor-like state in some individuals, allowing them to reduce metabolic activity to survive unfavorable conditions (as seen in *Notothenia coriiceps*; Campbell et al. 2008). These conditions lasted no longer than twelve hours so this could be a response to acute stress of low dissolved oxygen at low water temperature. The behavior was not observed at higher temperatures.

DO_{crit} is used as an indicator of hypoxia tolerance, being that critical point at which a fish’s metabolic rate will become dependent on ambient DO levels (Ultsch and Regan 2019). When DO falls below this critical point, surplus oxygen is no longer available for the process of ATP production through aerobic respiration (Richards 2009). When fish rely on anaerobic respiration (a much less efficient pathway for energy production), toxic byproducts (lactate and excess protons) accumulate much more quickly (Richards 2009). I found DO_{crit} to be positively related to temperature for two species (longjaw minnow, stoneroller), related in a curvilinear fashion for one species (creek chub), but unrelated for the others. Speers-Roesch et al. (2013)

also found inconsistent differences in P_{crit} values among species of intertidal sculpin species (superfamily Cottoidea) but did find P_{crit} useful when measuring glycolysis (anaerobic metabolic pathway). While DO_{crit} has been used as a measure of hypoxia tolerance, based on the relationships between DO_{crit} and temperature I observed and high interspecific variation, DO_{crit} alone is not necessarily the best predictor of hypoxia tolerance (Wood 2018). The benefit and utility of DO_{crit} has been hotly debated, but many of the issues can be resolved with standardization and methods reporting so that investigators can compare data (Wood 2018, Regan et al. 2019).

Regulation indices are used to describe ability to obtain oxygen as DO decreases to conditions approaching hypoxia. It has been suggested that RIs may be used in place of or alongside other metrics to describe hypoxia tolerance; decreasing RI should equate to decreasing hypoxia tolerance (Alexander and McMahon 2004). Regulation index values were negatively related to temperature for two species (stoneroller, banded sculpin), curvilinearly related for three species (rough shiner, striped shiner, creek chub), and unrelated for two species (blacktail shiner, longjaw minnow). Given the range of responses that I found across species, there was no clear overarching consistency among species within any taxonomic families. Turko et al. (2014) found no relationship of RI between control and treatment groups of the mangrove rivulus, *Kryptolebias marmoratus*, an amphibious fish with altered gill morphology; however, when combining hypoxia stress with pharmaceutical pollutant stress in gulf toadfish, *Opsanus beta*, RI of exposed fish was 40% lower than in the control group (Amador et al. 2018). At this time, studies exploring RI remain somewhat limited, but the data from this study combined with others suggest that RI, like DO_{crit} , varies across species and more work is required to more fully quantify regulatory capacity of species under various environmental conditions.

Interestingly, I found that blacktail shiner metabolic rate decreased with increasing temperatures, which was in contrast to results from Hartline et al. (2020). This corresponded to an increase in RI with temperature. These data would suggest that blacktail shiners either favored higher temperatures or were less able to regulate metabolic rate as temperature increased; however, in our study, temperature had no effect on their regulation ability or critical oxygen level, which is similar to Hartline et al. (2020). The actual values of metabolic rate were similar between our study and Hartline et al. (2020), and the overall appearance of no effects of temperature on RI or DO_{crit} are similar between studies, so the reason for the different relationship of metabolic rate with temperature remains unclear. In the generalist species *Fundulus heteroclitus*, gradual acclimation to a broad range of temperatures over 4 weeks did not affect thermal optima or breadth of tolerance in aerobic scope of fish, suggesting that for this species, metabolic rate was only moderately affected by temperature (Healy and Schulte 2012). Decreased metabolic rate of blacktail shiner with increasing temperature could be a result of metabolic depression to decrease metabolic demands, and this response may be an indicator of its adaptive plasticity to changing temperature regimes (Blanchard et al. 2019).

2.4.1 Management implications

Beyond the importance of testing multiple species to effectively represent entire fish assemblages, the results presented here stress the importance of including multiple metrics or endpoints when using physiological data in support of management actions (Wikelski and Cook 2006, Illing and Rummer 2017, Bergman et al. 2019). If we were to rely only on DO_{crit} to support management plans, we could potentially misidentify temperatures or dissolved oxygen levels that are more broadly required for species survival. A significant change in metabolic rate with temperature did not necessarily equate to significant responses of RI or DO_{crit} to

temperature. For example, blacktail shiner metabolic rate decreased significantly with temperature while no effects were observed relative to either DO_{crit} or RI versus temperature. Rough shiner and striped shiner DO_{crit} were not significantly affected by increasing temperature but both their metabolic rate and ability to regulate were significantly affected. Clearly, the response of species to temperature and DO is complex and we are only now beginning to quantify the elements contributing to the larger picture. Considering that some of these species are also hosts for glochidia larvae for freshwater mussels (which have their own responses to fluctuations in water temperature and dissolved oxygen; Williams et al. 2008), the broader system effects become even more complex as mussels who are obligate parasites require host survival (Taeubert and Geist 2013). Additional data are always needed, but in this case, it is of critical importance. Only with additional data can we start to generate the comprehensive and broad-scale view of the dissolved oxygen concentrations that would be required for the long-term survival and success of diverse fishes and the rest of their aquatic communities.

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3. The effects of glochidia infestation on the metabolic rate and hypoxia tolerance of bluegill *Lepomis macrochirus* and largemouth bass *Micropterus salmoides*

Abstract

Gill parasites can negatively affect hosts by altering behavior or causing adverse effects to host physiology. Most unionid mussel larvae (glochidia) are obligate parasites requiring fish hosts, but the literature concerning how these parasites affect their hosts has been contradictory. Here, I test the effects of a glochidia infestation on the resting metabolic rate and hypoxia tolerance of bluegill *Lepomis macrochirus* and largemouth bass *Micropterus salmoides* using glochidia of the southern fatmucket mussel *Lampsilis straminea*. I quantified oxygen uptake, hypoxia tolerance and regulation ability using respiration rate, critical dissolved oxygen values (DO_{crit}), and a regulation index (RI) to compare the effects of glochidia infestation between infested and uninfested fish over 11 weeks after inoculating hosts. Hosts were successfully infested with glochidia at levels that were similar to those seen in wild, naturally-infested individuals. I observed no effects of glochidia infestation on metabolic rate, DO_{crit} or RI of infested versus control fish over the course of the experiment. A significant interaction was observed for glochidia load and bluegill mass. Levels of glochidia on fish gills as might be expected in field conditions may not always induce an organismal-level response or cause host respiratory stress. Preventing respiratory stress caused by infestation could be beneficial for both host and parasite as glochidia survival and dispersal depends on host survival.

3.1 Introduction

Parasite effects on hosts have been relatively well-described in fishes (Barber et al., 2000; Jones, 2001; Buchmann and Lindenstrom, 2002; Mikheev et al., 2010). The negative effects of parasitic infestations can include morphological changes, physiological alterations, and

behavioral shifts (Barber et al., 2000; Iwanowicz, 2011; Crane et al., 2011). Mussels of the family Unionidae produce parasitic larvae (glochidia) that parasitize mostly fish, with host specificity varying among mussel species. Almost all unionid mussels have an obligate parasitic stage that is unable to transform into the juvenile stage without time spent encysted on fish gills, skin, or fins (Haag, 2012). Mussel glochidia are classified as generalist or specialist based on the number of fish species that can serve as host (Williams et al., 2008). Generalist mussel glochidia can successfully transform on multiple host species, whereas specialists have one family of fish or even just a single species on which their glochidia can transform successfully. This usually depends on the mussel species' ability to bypass host immune responses (Haag, 2012).

Research focusing on fish response to glochidia encystment has received increasing attention in recent years (Osterling et al., 2014; Filipsson et al., 2016; Filipsson et al., 2017; Horky et al., 2019). Gill parasitism can affect host fish energetics, reducing a fish's aerobic scope and swimming performance (Hvas et al., 2017). For infested brown trout *Salmo trutta*, high levels of glochidia infestation (≥ 350 glochidia per gram of fish weight) led to reduced swimming performance and increased mortality 48 hours post infestation (Taubert and Geist, 2013). Evidence of negative effects of glochidia infestation can include reduced growth, altered foraging and predator avoidance behaviors, and increased hematocrit (Crane et al., 2011; Filipsson et al., 2017; Chowdhury et al., 2019). Infested fish have also demonstrated reduced tolerance to hypoxia and acute increases in resting metabolic rate (Kaiser, 2005; Methling et al., 2018). Another notable effect of glochidia infestation can include a host immune response. In fact, fish hosts can develop immunity to glochidia such that reinfestation events may yield much lower transformation rates of juvenile mussels for at least 12 months (Rogers and Dimock, 2003; Dodd et al., 2006).

Laboratory infestations of hosts typically require a glochidia bath wherein glochidia are harvested from gravid female mussels and held in suspension while host fish are exposed to the larvae. These baths vary in glochidia concentration and larval attachment to a host (Table 3.1). Bath concentrations used have ranged from 1,500 to 200,000 glochidia/L (Treasurer et al, 2006, Taeubert and Geist, 2013; Osterling et al., 2014, Chowdhury et al. 2019). Osterling et al. (2014) used 30,000 glochidia/L and observed infestation rates of 25.1 ± 2.6 (mean \pm SE) glochidia/g of fish. Taubert and Geist (2013) infested in separate concentrations from 1,500 to 150,000 glochidia/L and observed infestation rates of 6 ± 1 and 906 ± 156 (means \pm SDs) glochidia/g, respectively. Chowdhury et al. (2019) used a bath concentration estimate of 200,000 glochidia/L to reach a mean infestation rate of 138 ± 3 (mean \pm SE) glochidia/g of fish. Treasurer et al. (2006) attempted more “natural” infestations by leaving hosts in aquaria housing gravid mussels overnight and reported infestations of an average of approximately 248 glochidia/g. To preserve host fish health in a conservation aquaculture setting, Taeubert and Geist (2013) suggested using maximum inoculation levels of 5-100 glochidia per gram host wet weight, with the ultimate goal of reintroduction of the threatened freshwater pearl mussel *Margaritifera margaritifera* via release of laboratory infested brown trout *Salmo trutta*. If the hosts were not to be released, but instead maintained in the lab for artificial breeding, they suggested that higher infestation rates (300 glochidia/g wet weight of host) be used. During their trials, mortality occurred at infestation rates of 350 glochidia/g wet weight, and critical swimming speed was inversely related to number of glochidia infested, with high loads yielding reduced swimming performance (Taeubert and Geist, 2013).

In contrast to laboratory inoculations, natural levels of glochidia infestation in wild fish are difficult to determine (Strayer, 2008). Some published accounts of natural infestations per

fish have ranged from 1 to >250 glochidia, and how a “heavy” infestation is defined has depended on the study and fish species (Kneeland and Rhymer, 2008; Filipsson et al., 2016). For example, Filipsson et al. (2016, 2017), who documented glochidia infestation effects on wild caught, naturally infested hosts and observed infestation rates of 1-309 glochidia/fish (0.18 to 22.89/g wet weight of fish), but Kneeland and Rhymer (2008) considered fish heavily infested with >20 glochidia/fish. While “low” infestations rates of glochidia are often considered benign (Barnhardt, 2008; Haag, 2012), instances of host mortality due to parasite load have been observed in smaller hosts (Haag, 2012; Taeubert and Geist, 2013). Successful juvenile metamorphosis of glochidia obviously relies on host survival; as such, minimizing negative effects on a host by mitigating infestation rates would be in the best interest for a female mussel (Methling et al., 2018). In general, natural infestations are lower than laboratory inoculations. Based on the findings of these studies, I define natural infestations of glochidia to be ≤ 300 glochidia/fish. I classify low, medium, and high levels of natural infestations in the methods.

In aquatic systems, hypoxia is an environmental stressor often encountered by both mussels and fish which can lead to shifts in physiology that compromise the health of the organism (Le et al., 2016; Haney et al., 2020, Borowiec et al. 2020). In female mussels, hypoxic events have led to glochidia abortion (Strayer, 2008), and juvenile mussels have been found to be more susceptible than adult mussels to hypoxia and anoxia (Dimick, 2000). In fish, hypoxic conditions have been shown to decrease immune system response, hinder growth, and modify behavior (Cheng et al., 2002; Crane et al., 2011; Abdel-Tawwab et al., 2019).

Few studies have attempted to use respirometry to determine host effects due to glochidia during hypoxic conditions under natural levels of infestation (Kaiser, 2005). In aquatic systems, hypoxia tolerance has been described using the dissolved oxygen critical value (DO_{crit}) which is

the lowest oxygen concentration at which metabolic activity is actively regulated (Regan et al., 2019). In more recent studies, the Regulation Index (RI) has been defined and considered (Alexander and McMahon, 2004; Mueller and Seymour, 2011). The RI quantifies the relative ability of an organism to regulate oxygen uptake as DO declines to zero rather than constraining that organism to be defined as a regulator or conformer (Wood, 2018). “Metabolic regulation” is the ability of a fish’s physiology to maintain constant oxygen uptake as oxygen concentrations decline, whereas “metabolic conformation” is the inability to maintain a constant oxygen uptake rate as ambient oxygen concentrations decline. An organism that always regulates oxygen uptake regardless of oxygen concentration would have an RI value of 1. Conversely, an organism whose oxygen consumption is fully dependent on oxygen concentration would have an RI value of 0. These are hypothetical extremes, and all organisms likely fall somewhere within the continuum between true conformation and true regulation. An organism with an RI value approaching 1 would be considered a strong regulator of oxygen uptake and thus more tolerant of hypoxia; a value approaching 0 would indicate strong metabolic suppression and hypoxia intolerance (Mueller and Seymour 2011). Using RIs has created a more informative profile of aerobic metabolism versus the binary conformation vs. regulation of oxygen uptake inferred by DO_{crit} values.

Here, I use a laboratory setting to quantify the effects of natural levels of glochidial infestation over time on the metabolic function of two common fish host species. Three predictions were made for this study. First, given the presence of encysted glochidia in the gills, infested host fish will have higher respiration rates than uninfested control fish. Second, respiration rates of infested fish will become more similar to those of uninfested fish throughout the duration of an 11-week study due to recovery from the glochidia infestation. And finally,

because of the glochidia infestation, infested fish will exhibit reduced tolerance to declining oxygen and have higher DO_{crit} and lower RIs than uninfested control fish.

3.2 Methods and materials

3.2.1 Glochidia source

Lampsilis straminea, the southern fatmucket, is a unionid native to Alabama. This species is considered a host-generalist species, primarily infesting Largemouth Bass *Micropterus salmoides* and Bluegill *Lepomis macrochirus* as its fish primary and secondary hosts (respectively), although several other hosts have been identified (Brim Box and Williams, 2000; Williams et al., 2008). Time of infestation can last from 3-5 weeks and is temperature dependent (Marwaha et al., 2017).

3.2.2 Animal collection and maintenance

Juvenile bluegill *Lepomis macrochirus* (mean \pm 1 SE, 87 ± 0.73 mm TL, $n = 100$) were obtained from the Eastaboga Fish Hatchery (Calhoun County, AL), and juvenile largemouth bass *Micropterus salmoides* (mean \pm 1 SE, 90.5 ± 0.82 mm TL, $n = 100$) were obtained from American Sport Fish (Montgomery, AL). All fish were transported to the E.W. Shell Fisheries Station (Auburn, AL) and placed into multiple 110 L recirculating aquaria (10 fish/aquarium) for at least 10 days to acclimate to laboratory conditions (21 C; 12:12 hr light/dark). Bluegill and largemouth bass were previously pellet trained and fed readily on Omega® One 1.5 mm sinking goldfish pellets (Omega Sea, Painesville, Ohio, USA). Fish were fed daily. After this initial acclimation, fish were individually placed into 1 L polycarbonate Aquatic Habitats® AHAB tanks (Pentair Aquatic Eco-Systems Inc., Apopka, FL, USA) at a rate of one fish per tank for an additional one-week acclimation prior to inoculation with glochidia. Ten gravid female southern fatmucket *Lampsilis straminea* mussels were collected from resident populations in Auburn

University earthen ponds and held in a 110 L aquarium at the E.W. Shell Fisheries Station until glochidia were removed for inoculation. Mussels were held in water at 17 ± 0.5 C to maintain their glochidia and fed Shellfish Diet® 1800 (Reed Mariculture, FL, USA).

3.2.3 Inoculation

Bluegill (n = 88) inoculation occurred in July 2019 and largemouth bass (n = 88) inoculation occurred in November 2019. One week prior to glochidia removal from the gravid mussels, the mussel tank temperature was increased to the temperature of the AHAB system (21 C) at a rate of 1 C per day. Glochidia were removed from female mussels using a 22-gauge needle and dechlorinated tap water to flush the marsupial gill into a 1000 mL beaker. Ten 0.01 mL subsamples were taken from each brood and averaged to quantify glochidia concentration (glochidia / mL). Glochidia viability was assessed using the salt test (Fritts et al. 2014, Eckert 2018).

Only glochidia from broods with viability higher than 85% were used for inoculation. All glochidia from four female *L. straminea* were combined and diluted to a concentration of 5000 glochidia per liter to inoculate the bluegill in an 80 L bucket for 45 min (Eckert 2018). Glochidia were kept in suspension with multiple airstones and fish movement. The goal infestation rate for my study was to not exceed my definition of a natural infestation rate. Excessive infestations are therefore classified as numbers per fish exceeding > 300 glochidia. I further divided “natural” rates of infestation into 3 subcategories: low (<100 glochidia/fish), moderate (100-200), and high (>200). Non-experimental fish (n = 6, or two per time interval) were removed from the inoculation bath after 15, 30, and 45 minutes to estimate glochidia attachment. These removals and inspections were to prevent excessive infestation of bluegill and largemouth bass, and after 45 minutes, inoculation ended (Table 3.1). After the inoculation bath, bluegill were placed into a

rinse bath for an additional 30 min to allow any unattached glochidia to slough from the fish before being returned to individual AHAB tanks. Prior to the baths, fish were randomly selected to either undergo normal inoculation (n = 66) or an imitation/control inoculation (n = 22). In the control treatment, fish were held in baths containing no glochidia for 45 min, placed into a rinse for 30 min, and returned to individual AHAB tanks.

Glochidia sloughs (unsuccessful transformations) and juveniles (successful transformations) were counted for each fish after being moved into their individual AHAB tank by placing a 150- μ m mesh filter on each AHAB tank through which all outflowing flow-through water passed. Each individual filter was checked daily for blockages and glochidia were counted three times per week under a dissecting scope. Juveniles were differentiated from sloughs by valve or foot movement (i.e., juvenile mussels could be seen moving and opening/closing their valves, and sloughs were empty valves that demonstrated no signs of movement). Counts were performed until the number of juveniles and sloughs reached zero for three consecutive counting days (i.e., one week). Largemouth bass received the same inoculation and control treatments as bluegill. Glochidia transformation success to the juvenile stage was calculated by dividing the number of transformed mussels by the sum of transformed mussels plus sloughs for each individual fish.

3.2.4 Respirometry

To quantify metabolic effects of glochidia infestation on bluegill and largemouth bass, respirometry was performed at 21 C at ten post-inoculation (PI) time intervals—24 hr, 48 hr, 72 hr, 7 days, 14 days, 21 days, 28 days, 35 days, 56 days, and 77 days. A respirometry “run” was defined as the duration of consecutive time spent inside a respirometry chamber by an individual fish during which temperature, respiration rate, dissolved oxygen concentration were

continuously measured, and oxygen uptake and routine metabolic rate were calculated. Each respirometer (Loligo® Systems, Tjele, Denmark) consisted of a glass respirometry chamber (containing an individual fish), a flush pump, a recirculating pump, a fiberoptic oxygen probe, and a temperature probe. Oxygen concentration was measured via the fiberoptic probe connected to an oxygen sensor in the tubing of the recirculation loop of the respirometer. The respirometry system used in this study contained eight individual chamber systems.

Prior to respirometry, six infested fish and two control fish were randomly selected for the trial, and feeding was suspended for 48 hr to allow for gut evacuation. The selected fish were randomly assigned (using a eight-sided die) to one of eight respirometry chambers (to prevent any systematic chamber effects), weighed (nearest 0.01 g), and placed into the designated chamber. Chamber size was chosen so that the ratio of fish volume to water volume was between 1:10 and 1:50 (Svendson et al., 2016). Chambers were all placed in a large water reservoir (~350 L), and barriers were placed between chambers to prevent visual stimulus due to conspecifics; an opaque tarp was placed over the entire water reservoir to reduce any external stimuli. Given that acclimation times can vary across species, among individuals within a species, and among respirometry studies in the literature, fish were allowed to acclimate overnight for at least 12 hr.

After acclimation, closed respirometry was conducted during the daylight period (0700-1900) and continued until oxygen concentration within the chamber was reduced to 1 mg/L. The fiberoptic probe measured oxygen concentration once every second, and calculated an average based on these measurements once every three minutes. To correct for background respiration caused by bacteria that could have become established, blank controls were performed for one hour before and one hour after each experimental run. Background respiration was quantified by calculating mean respiration rate inside the glass chamber by averaging pre- and post-run control

background respiration ($\text{mg O}_2 / \text{hr}$). This rate was then calculated as a percentage of total respiration inside the chamber during the run and subtracted from the total chamber respiration ($\text{mg O}_2 \cdot \text{chamber}^{-1} \cdot \text{hr}^{-1}$). The chamber respiration rate minus the background respiration was the fish respiration rate ($\text{mg O}_2 \cdot \text{chamber}^{-1} \cdot \text{hr}^{-1}$). This respiration rate was converted to weight specific metabolic rate by dividing respiration rate by the mass of the fish ($\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$). To check carbonic acid and ammonia buildup, ammonia and pH were tested using aquarium test strips (Tetra®, Blacksburg, VA, USA) immediately following the end of each run.

3.2.5 Counts of encysted glochidia

Once removed from the chambers, fish were anesthetized in a buffered MS-222 solution and pithed. Gills were removed, and any remaining encysted glochidia quantified under a dissecting microscope. This count was combined with the number of transformations and sloughs for an individual to determine infestation load for each fish.

3.2.6 Analysis

Three response variables were quantified using the respirometry data—routine metabolic rate (RMR), critical dissolved oxygen value (DO_{crit}), and the regulation index (RI). Routine metabolic rate was calculated as the mean rate when oxygen concentration was between 5-7 mg/L O_2 (i.e., normoxia). DO_{crit} was calculated using piecewise regression to quantify the breakpoint in oxygen consumption regressions. RI was calculated as in Mueller and Seymour (2011). All analyses were performed in R version 4.0.3 (R Core Team 2020). Four separate analyses were performed to quantify the effect of glochidia inoculation on the three response variables. Species were analyzed separately, and groups were assumed to have equal variance. An ANCOVA was performed for each of the response variables with glochidia treatment type (inoculated or control) as the categorical predictor and week as the continuous predictor. After

observing that most glochidia had sloughed or transformed by week four for both bluegill and largemouth bass, a two-way ANOVA was performed to compare infestation level within two time periods: glochidia transformation (defined to be inoculation to 3.5 weeks post inoculation) and post-glochidia transformation (defined to be 5 weeks post inoculation to the end of the experiment). These time periods were designated based on the percentage of glochidia fallen from the hosts' gills; I determined that at least 98% of glochidia had transformed or sloughed from fish gills by week 3.5. Infestation level was also divided into three categories treatments (where possible): less than 100, 100 to 200, and greater than 200 glochidia per fish and response variables were compared across these categories for the two time periods. If the two-way ANOVA investigating time period was not significant, another ANOVA excluding time period was performed to compare among infestation levels. Finally, a linear regression was performed to compare glochidia load and fish mass for each species.

3.3 Results

Bluegill (mean wet weight \pm SE = 10.95 ± 0.41 g) and largemouth bass (mean wet weight \pm SE; 7.89 ± 0.22 g) were successfully inoculated with *Lampsilis straminea* glochidia. Mean viability of the mussel broods used for inoculation was 91.5% (Table 3.2). Once placed inside the respirometry chamber, fish quickly oriented to the flow and exhibited no behaviors characteristic of stress (e.g., erratic swimming, listlessness, etc.). Total numbers of glochidia/fish were variable with a mean of 56.2 ± 9.5 (mean \pm SE) for bluegill and 131.2 ± 6.9 (mean \pm SE) for largemouth bass; glochidia/g wet weight was 4.72 ± 0.64 (mean \pm SE) for bluegill (Table 3.3) and 17.48 ± 0.96 (mean \pm SE) for largemouth bass (Table 3.4).

Bluegill and largemouth bass metabolic responses to glochidia inoculation were mixed. ANCOVAs revealed no significant interaction of time post inoculation with any of the response

variables (Figure 3.1). There were no significant comparisons in the two-way ANOVA with time period and infestation level or the one-way ANOVA with infestation level alone for any response variable (Figure 3.2). Finally, regressions of glochidia load (glochidia/fish) versus fish mass for largemouth bass revealed no relationship; however, as bluegill size and glochidia load had a positive relationship. ($p = 0.046$, Figure 3.3).

3.4 Discussion

Understanding the effects of unionid mussel glochidia on their fish hosts is important for conservation of both fish and mussels. My results demonstrate that low (mean 4.72 and 17.48 glochidia \cdot g⁻¹ for bluegill and largemouth bass respectively) infestation rates of glochidia yielded no discernable trends in metabolic rate, DO_{crit}, or RI for inoculated versus control fish. This, however, does not mean that higher levels of glochidia have no effect on host fish, given that a number of studies have documented physiological and organismal effects of infestation across multiple species (Howerth and Keller, 2006; Taeubert and Geist, 2013; Thomas et al., 2014; Methling et al., 2018; Horky et al., 2019).

Infestations of glochidia are usually accompanied by increased hematocrit levels (Thomas, 2014; Filipsson et al., 2017; Marwaha et al., 2019). Hematocrit level is used as a measure of stress in fish and is a measure of oxygen transport capacity in vertebrates (Chen et al., 2004) and an elevated hematocrit is often observed when fish are exposed to low oxygen environments. Marwaha et al. (2019) analyzed hematocrit values from “medium” infested (1-200 per side of fish) and “highly” infested (>200 per side of fish) individual brown trout and found that the medium infested fish hematocrit levels did not significantly differ from those of the uninfested control group. The medium level of infestation in their study was similar to the maximum number of attached glochidia observed in the present study (< 300/fish), though their

experimental fish were smaller ($2.3 + 0.78$ g). If hematocrit levels in bluegill and largemouth bass of this study were similar to Marwaha et al. (2019), we would expect to see minimal or no secondary level physiological or metabolic effects of glochidia inoculation, which was the case.

Physiological responses to glochidia infestation can also include immune responses. Evidence of immune response has been observed in both bluegill and largemouth bass with repeated exposure to glochidia up to a year (Rogers and Dimock, 2003; Dodd et al., 2005). Dodd et al. (2005) used infestation numbers higher than those seen in my study—approximately 36 glochidia/gram largemouth bass weight versus 17.48 ± 0.96 (mean \pm SE) in my study. Rogers and Dimock (2003) observed between 52 and 249 glochidia/fish, the lower end of which was similar to my study: 56.2 ± 9.5 (mean \pm SE)/bluegill. Where investigated, some hosts exhibit specific glochidia antigens that prevent reinfestation for months to years after initial infestation (O’Connell and Neves, 1999).

Organismal level responses include changes in metabolic rate, swimming performance, mortality, or even longer-term effects like reduced growth (Filippsson et al., 2017; Chowdhury et al., 2019). In smallmouth bass *Micropterus dolomieu*, necropsy showed that glochidia infestations at high concentrations caused hemorrhages and lamellar and filament fusion of the gills which most probably contributed to host mortality (Howerth and Keller, 2006). Studies have found that high infestation rates of glochidia on fish can affect respiration, swimming performance, and DO_{crit} (Kaiser, 2005). This may be true for high infestation levels, but levels of infestation used in my study (simulating natural levels) did not lead to significant changes in those response variables. An interesting find by Filippsson et al. (2017) was that there was an initial increase in standard metabolic rate at low levels of infestation (< 5 glochidia per gram wet weight) followed by a decline as numbers on host fish increased. They suggest there is a

compensatory response by fish at higher levels of infestation and that changes in physiology may only be seen at low infestation numbers. I did not see significant shifts in response variables, but I note that my control sample sizes were relatively small ($n = 2$ per test grouping) and high among-individual variation in metabolism may complicate such studies. In the first 72 hours post-inoculation, infested largemouth bass tended to have a higher metabolic rate than controls. While these results were not significant, greater sample sizes could enhance detection of an effect of inoculation during the first three days of a glochidia infestation. Bluegill showed no such trend.

Much of the research for glochidia infestation effects has focused on salmonids, and centrarchids are relatively tolerant fish by comparison (e.g. oxygen concentrations; Dowling and Wiley 1986). *Margaritifera margaritifera* glochidia are hosted long-term in brown trout— 10 months from late summer to early summer the next year—whereas *Lampsilis straminea* undergoes infestation and transformation in a matter of months on largemouth bass and bluegill. After 3.5 weeks post inoculation, >98% of glochidia had successfully transformed or sloughed from the gills of the host fish. Though we examined this time period in the analysis, we did not observe any significant differences in transformation or post-transformation time periods for the response variables. Longer-term hosting (10 month) may induce physiological change in hosts early in the infestation to compensate for the metabolic demand caused by infestation. The shorter-term infestations (1 month) observed in largemouth bass and bluegill by *L. straminea* probably induces immunological response but these effects may not manifest in metabolic compensation or growth restriction long-term. Other groups like darters, shiners, or sculpins, which are a key host for some of the more threatened mussel genera, may exhibit significant changes in respiration or ability to regulate oxygen consumption when infested (Williams 2008).

Noted in Haag (2012), some *Etheostoma spp.* that become trapped in the valves of female mussels can experience crushing or asphyxiation from the infestation process. I suggest future research focus on these species.

Temperature could also play a factor in response to glochidia infestation. As with other forms of parasitism, hosts experiencing multiple stressors experience more consequences (reduced growth and lethargy) than a single stressor (Hvas et al., 2017). Temperature is one of the most important factors affecting ectotherms. Kaiser (2005), whose mean infestation rate was 632 glochidia/fish (131 glochidia/g), found significant effects on respiration of largemouth bass and DO_{crit} in trials at 22-23°C. My study performed respirometry at 21°C, and though this is only a one-degree difference, the smaller fish (4.8 ± 0.8 g), higher per gram infestation rate, and higher temperature in Kaiser (2005) may have contributed to the results of her study.

I included a time variable to determine if there was a recovery period after which inoculated fish metabolic rate, DO_{crit} , or RI returned to normal or control levels. I did not see any effects of time in this study. Kaiser (2005) used a repeated-measures experimental design, using the same individuals to obtain ventilation and DO_{crit} data over multiple weeks after inoculation. By following the same individuals over time, she was able to look at individual responses to declining oxygen and correct for the high individual variation observed in this study. Thomas et al. (2014) followed glochidia encystment on brown trout for 160 days and noted a significant enlargement of infested host spleens at 30 days post-encystment. Treasurer et al. (2006), who observed initial infestation levels of 1392 ± 641 (mean \pm SD), saw a significant effect of glochidia infestation on growth after 15 weeks but no effect on lactate levels or growth over one year. Their study also focused on the long-term brooder *M. marginifera*.

The low levels of infestation used in this study were used to emulate natural levels of infestation seen in wild fish (Strayer, 2008; Kelly and Watters, 2010). While numerous studies show impacts of glochidia infestation on the physiology and biology of hosts, these studies have used relatively high glochidia loads. The mean glochidia load per fish in this study was below 200. Bluegill are considered a secondary host to *L. straminea* and would explain the lower numbers of infestation. Assuming I successfully mimicked natural levels of glochidia infestation found on wild fish, we would conclude that glochidia do not negatively impact host metabolic rates under these natural conditions. The relationship between unionid mussels and their hosts has existed for millions of years (Strayer, 2008); over this time, hosts should have adaptations to compensate for the presence of glochidia at naturally occurring field levels.

3.5 Literature cited

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4. The impact of acute temperature fluctuation on the potential metabolic activity of seven
Alabama fishes

Abstract

Management of fish populations in systems subject to variable abiotic conditions benefits from an understanding of their underlying physiology and responses to thermal stress. While work with whole organisms can provide insight into how stressors like temperature fluctuations can affect them, electron transport system (ETS) assays can be used to quantify the maximum potential metabolic activity of respiratory enzymes located in the electron transport chain. This activity can be used as an indicator of an organism's maximum attainable metabolic activity, as well as to estimate the optimum thermal temperature (T_{opt}) and optimal breadth of thermal performance ($T_{breadth}$) for respiratory enzymes. I exposed enzymes to acute temperature changes and quantified the effect of temperature on potential maximum metabolic activity and T_{opt} for one game fish species (*Lepomis macrochirus*) and six non-game fish species (*Camptostoma oligolepis*, *Cottus carolinae*, *Cyprinella gibbsi*, *Cyprinella venusta*, *Notropis baileyi*, and *Semotilus atromaculatus*). Acute respiratory enzyme performance across a range of temperatures (11-44°C) differed among species. Optimum temperatures for enzyme activity also varied among species. Relationships for *L. macrochirus*, *C. carolinae*, *C. gibbsi*, *C. venusta*, and *N. baileyi* followed a pattern of steadily increasing enzyme activity to T_{opt} before gradually decreasing with increasing temperature. Enzyme activity for *C. oligolepis* and *S. atromaculatus* gradually climbed to T_{opt} before sharply declining at the highest experimental temperatures. *Cottus carolinae*, a benthic species, had the lowest enzyme activity of all species, suggesting that differences in life history strategy, evolutionary history, morphology, or behavior may manifest at the cellular level. Similarities in enzyme T_{up} and critical thermal maxima found in previous

studies support that ETS assays may be a useful physiological tool for assessing whole organism thermal tolerance.

4.1 Introduction

Abiotic factors play an important role in the physiology of aquatic organisms; in particular, temperature is clearly important in driving ectotherm metabolism (Shaklee et al. 1977, Zagar et al. 2018, Simcic et al. 2014). As ectotherms, fish rely on environmental temperatures for proper metabolic function. Metabolic rate typically increases with increasing temperature up to a maximum metabolic rate (above which it decreases), and the physiology of an individual fish must adjust to accommodate the increased demand for energy at the organismal level caused by increased temperature (Portner and Peck 2010). Given this, an understanding of metabolism is needed in order to understand fish responses to altered temperature regimes.

Given the effect of temperature on fish metabolic rate and ultimately on the aquatic communities in which they live, sharp fluctuations in temperature can be deleterious to fish that rely on more stable regimes (Beachum et al. 2019). Natural fluctuations in temperature (e.g., due to seasonal flooding, diel patterns) are relatively benign when compared to those due to anthropogenic impacts on streams and rivers (Blevins et al. 2013). From agriculture to urbanization, land use changes have led to altered hydrology, and ectotherms are more vulnerable than endotherms to fluctuations in temperature that may be caused by water use (Morrison et al. 2020). Increasing temperature reduces oxygen availability required for aerobic metabolism, can degrade enzymes needed for energy production, and can reduce energy available for growth and reproduction (Clark et al. 2013).

In fish, a complex mix of biological and chemical processes requires a range of temperatures for proper function, and each of these ranges possesses an optimum performance

temperature (Portner 2010). Fish exist within a varying temperature range, but optimal physiological function will vary based on activity and level of organizational complexity. Research in temperature tolerance is usually focused on physiology and functions at the organismal level (e.g., fecundity, growth, and metabolic rate; Simcic et al. 2014, Schulte 2015, O’Gorman et al. 2016, Beachum et al. 2019). Temperature tolerance at the cellular and organ system levels of organization is measured using various assays that are focused on enzyme performance, heart rate, or cortisol levels (Schulte et al. 2011, Farla et al. 2020). Thermal performance and temperature tolerance differ across levels of complexity, with molecular levels exhibiting the highest critical thermal maxima, whole organisms having intermediate levels of thermal maxima, and populations having lower levels of thermal tolerance (Rezende and Bozinovic 2019). Enzyme assays allow examination of the effects of temperature at the cellular level.

Thermal performance curves (TPCs) represent the effects of temperature on biological and physiological process rates (Schulte et al. 2011). In a typical TPC, performance will increase to a maximum or optimum temperature for performance followed by a rapid decline (Angilletta 2006). TPCs, like tolerance, vary across levels of organizational complexity with the cellular or molecular level having higher temperatures for optimum performance (Rezende and Bosninovic, 2019). Like other physiological processes, thermal performance varies across organism life stage and can be impacted by external factors like predator presence altering behavior, reducing foraging success, and energy limitation due to competition for habitat (Sinclair et al. 2016). In addition, shifts in thermal performance are often observed due to changes in acclimation temperature (Fangue et al. 2009, Stitt et al. 2013, Seebacher et al. 2015, McKenzie et al. 2020). These shifts make quantifying thermal optima difficult as there is not a single temperature

optimum for all processes in an individual, or even for the same processes at different acclimation temperatures within an individual, which is why determining maximum metabolic potential of respiratory enzymes is critical to more fully understanding thermal performance.

Respirometry is a popular and relatively well-studied approach for obtaining organismal level metabolic and physiological data (Chabot et al. 2016a). Open flow, intermittent, and closed respirometry have all been used to obtain data to calculate standard, routine, active, and maximum metabolic rates, as well as aerobic scope. These techniques are excellent tools for estimating thermal tolerance and metabolic performance at the organismal level, but studies that use these designs typically require prolonged acclimation times, ample aquarium space to hold organisms, and large sample sizes. Respirometry can also be limited by equipment which can be expensive and be relatively fragile.

Determining metabolic potential is not a new approach; electron transport system (ETS) assays have been used for decades to determine planktonic respiration (e.g., Packard et al. 1971), but their use in fish has only recently increased (Ikeda 1989, 2016, Gopalan et al. 1996, Simcic 2017, Belcher et al. 2020). ETS assays measure the rate-limiting step in the electron transport system: the oxidation of the coenzyme-Q cytochrome b complex. As such, the assay measures the maximum potential metabolic rate that could be achieved by an organism's energy production pathways. Initially used to observe respiration rates in mesopelagic fishes considered too fragile for laboratory acclimation post-collection, potential metabolic activity (PMA) has been used to estimate potential respiration rate in fishes, estimate growth, and quantify metabolic effects of introgression on brown trout *Salmon trutta* and marble trout *Salmo marmoratus* hybridizations (Ikeda 1989, Schmidlin et al. 2015, Simcic et al. 2017, Belcher et al. 2020). By quantifying enzyme activity at various temperatures, TPCs can be developed for the enzyme

complexes in the electron transport system, and these TPCs can be used to estimate the overall metabolic potential of the organism. From this, results could be extrapolated to estimate a species' thermal performance.

The diversity of freshwater fauna of Alabama is unrivaled (Warren et al. 2000, Boschung and Mayden 2004, Jenkins 2015). The Mobile Basin drains an area of approximately 113,000 km² and contains at least 190 native fish species, including 40 Alabama endemics. (Metee et al. 1996). Mussel diversity is also very high in Alabama, with high rates of endemism (Pringle et al. 2000, Gangloff et al. 2006). This diversity is under constant pressure with the expansion of humans and the increased use of natural resources in the state (Calloway et al. 2017). Fish community structure may be altered due to introduced fishes preying upon native species as well as competition between them (Pitt and Witmer 2007). Introduced species that share close common ancestry with native species may hybridize with native species and complicate management of small or fragmented populations of native species (Dowling et al. 2016; Alvarez et al. 2013). Habitat alterations threaten fish and mussel biodiversity as streams and rivers are exploited for agriculture or power (via hydroelectric dams), and as agricultural runoff alters stream conductivity or increases eutrophication/nutrient input and temperature fluctuations (Smith et al. 1999; Nobles and Zhang 2011). These threats could all be further exacerbated by looming climate change (Ficke et al. 2007). As global temperatures rise, biotic homogenization of fishes occurs across climactic zones (Fang and Stefan 1999; Stefan et al. 1999; Mohseni et al. 2003). Species that are physiologically unable to adapt to increasing temperature, subsequent lower oxygen levels, and competition due to habitat homogenization could experience reduced abundance, growth, and reproduction eventually leading to population declines (Buisson and Grenoullit 2009). Endemic fish that are specialized in their habitat requirements may be

particularly susceptible to changes in temperature as their ability to acclimate to increasing temperatures could be limited. Acclimation could also be limited in cases of acute temperature fluctuations if the species of fish is adapted to more stable environments. Based on current trends, determining PMA and TPCs of fishes in Alabama streams could be beneficial to resource managers for identifying those species that are at greatest risk due to changing temperature regimes if a metric is developed using enzyme activity in combination with known metrics (e.g., IBIs).

Here, I expose enzymes of the electron transport system in seven fish species acclimated to a single temperature to a broad range of temperatures to quantify PMA and thermal performance of ETS enzymes. First, I define optimum temperature ranges for enzyme activity in all seven fish species. The optimum range (temperature range for which ETS activity was within 90% of maximum ETS activity) consists of three values: a lower limit, an upper limit, and total breadth of the range between lower and upper limits. Second, I calculate maximum ETS activity and compare optimum temperature for PMA values across among species.

4.2 Methods

4.2.1 Animal collection and maintenance

Creek chub *Semotilus atromaculatus* (n = 8) and banded sculpin *Cottus carolinae* (n = 10) were collected from Big Wills Creek (DeKalb County, AL). Tallapoosa shiner *Cyprinella gibbsi* (n = 9) were collected from Hillabee Creek (Tallapoosa County, AL). Largescale stoneroller *Campostoma oligolepis* (n = 8), rough shiner *Notropis baleyi* (n = 10), and blacktail shiner *Cyprinella venusta* (n = 10) were collected from Choctafaula Creek (Macon County, AL). Bluegill *Lepomis macrochirus* (n = 9) were collected from Auburn Farm Pond S-1 (Lee County, AL). All fish were collected using a combination of backpack electrofishing (Smith-Root LR-24;

Vancouver, WA, USA) and seining. Collected fish were transported in an aerated 18.9 L bucket to the laboratory at the E.W. Shell Fisheries Center where they were placed in 110 L aquaria to acclimate to laboratory conditions (21°C; 12:12 hour light/dark) for at least 10 days. Fishes were fed once daily using Omega® One sinking goldfish pellets (Omega Sea, Painesville, Ohio, USA). Pondmussels *Ligumia subrostrata* (n = 4), kept in tanks located at the E.W. Shell Station, were used as a standard in all assays. After the acclimation period, fishes were humanely euthanized by ice bath (0°C). Our work was conducted under approved Auburn University IACUC protocol 2017-3122. After euthanasia, fish were weighed (nearest 0.1 mg) and measured (nearest mm).

4.2.2 Electron Transport System Assays

A filet of muscle tissue ($\bar{x} \pm SE = 0.150 \pm 0.026$ g) was removed from the scaled, precaudal region of each fish and placed into a scintillation vial for immediate freezing at -80°C. Tissue samples were frozen for a minimum of 24 hrs before ETS assays were performed. After 24 hrs, the plug was removed from the freezer and macerated with a scalpel before being placed into a homogenization vial (Globe Scientific, #6101B, Mahwah, NJ, USA) filled with 1.0 mm glass beads (#11079110; Biospec, Bartlesville, OK, USA) and 4 mL of homogenization buffer. Homogenization buffer was produced using 0.2M sodium phosphate buffer, magnesium sulfate (MgSO₄), polyvinylpyrrolidone (PVP), Triton-X-100, and deionized water.

The tissue inside the vial was homogenized using a bead beater (MiniBeadBeater16, Biospec, Bartlesville, OK, USA) for 1 min, followed by chilling in a -20°C freezer for 2 min. This homogenizing and chilling process was repeated for a total of 4 cycles to minimize enzyme degradation at high temperatures caused by glass bead friction. Vials were centrifuged (Allegra X-30R; Beckman Coulter, Atlanta, GA, USA) at 8500 x g for 4 min at 0°C. The supernatant in

the vials was removed to a 50 mL Falcon® tube, diluted with reagent grade deionized water to reach the final tissue concentration of 0.5 mg/mL, and vortexed for 20 seconds (VMW Analog vortex mixer; Randor, PA, USA). This final concentration of fish tissue was determined during a preliminary dilution experiment to provide excess substrate for the enzyme reaction during the assay (unpublished data). Diluted homogenate was then distributed among 2 mL microcentrifuge vials and stored in a -80°C freezer for no longer than 3 weeks prior to ETS assays.

Vials of all individuals of seven species were randomized (by blocking) into seven groups with 8-9 individual fish each (Table 4.1). After randomizing, each group set contained at least one individual from each species, and tissue from a single, composite sample of *Ligumia subrostrata* (the pondmussel) to serve as a control across ETS groups (i.e., to ensure activity remained stable across runs). Tissue from *L. subrostrata* was obtained from the mussel foot and homogenized following the same methods as described above.

Prior to an assay, frozen homogenate vials were removed from the -80°C freezer and slowly thawed in an ice bath (0°C). The following process describes the ETS assay for a single fish sample. Once thawed, two aliquots of 0.5 mL of homogenate (treated as two pseudo-replicates) were distributed into two test tubes, each containing 1.5 mL substrate solution and 0.5 mL of INT dye (p-Iodonitrotetrazolium Violet tetrazolium salt). Substrate solution contained the necessary substrates (NADH and (NADPH). This 2 mL mixture was then incubated in a water bath (VWR® WB20 Digital Water Bath) for 30 min at one of eleven temperatures from 14°C to 44°C in increments of 3°C (14, 17, 20, 23, 26, 29, 32, 35, 38, 41, and 44°C); the particular temperature that was used was selected randomly. A third test tube containing only 1.5 mL substrate solution and 0.5 mL of INT dye was used as a blank for the two experimental pseudo-replicates. To prevent photodegradation of the INT dye, incubations occurred in the dark. After

30 min of incubation, test tubes were removed from the water baths, and enzyme reactions were halted using a 0.5 mL of stopping solution (1:1 formalin:phosphoric acid). Blanks also received stopping solution. After the reactions were stopped, 0.5 mL of the original homogenate was added to the blank to bring the total volume to 3 mL in all test tubes. The production of formazan was quantified using a spectrophotometer (VWR® UV-1600PC) to measure absorbance at 490 nm. From absorbance, enzyme activity was calculated using the formula (Simcic et al. 2014):

$$\text{ETS Activity (mL O}_2 \text{ g}^{-1} \text{ WW}^{-1} \text{ h}^{-1}) = \frac{\text{Abs}^{490\text{nm}} \times V_r \times V_h \times 60}{V_a \times S \times t \times 1.42 \times 1000}$$

where $\text{Abs}^{490\text{nm}}$ is the average absorption of the two sample replicates (pseudo-replicates), V_r is final volume of the reaction mixture after reaction is halted (3 mL), V_h is volume of the original homogenate from an individual (4 mL), V_a is volume of the homogenate aliquot distributed to the replicate tube before incubation (0.5 mL), S is the mass of the original tissue plug (g), t is the incubation time (30 min), 1.42 is the O_2 conversion factor (unitless), and 1000 is the conversion from uL to mL.

4.2.3 Analysis

To quantify variability in ETS response to temperature on an intra- and interspecific basis, I used generalized additive models (GAMs). For each individual fish, I averaged the two ETS measurements that were taken at each temperature and fit a GAM to the resulting ETS values with temperature as the only variable for smoothing. I used GAMs rather than quadratic regression to relax the assumption that the ETS curve is symmetrical around the temperature at its maximum. For visual purposes only, I average ETS values at a given temperature across individuals within a species to obtain species-specific curves; all formal comparisons among species treated the individual-specific curves as replicates for a given species. For each fitted curve, I extracted four temperature values of interest—temperature at maximum ETS activity to

obtain optimum temperature for enzyme performance (T_{opt}) and the three temperature values associated with the optimum enzyme activity range (T_{low} , T_{up} , $T_{breadth}$)—to quantify optimal enzyme performance. $T_{breadth}$ is the temperature range for optimal enzyme activity (i.e., temperature range where activity is $\geq 90\%$ of maximum enzyme activity). T_{low} is the lowest temperature included in $T_{breadth}$, and T_{up} is the highest temperature included in $T_{breadth}$. I then used one-way ANOVAs to compare T_{up} and T_{low} , $T_{breadth}$, and T_{opt} among species. If statistical significance was found from an ANOVA, Tukey's post-hoc adjustment for multiple comparisons was used to identify which pairwise species comparisons differed significantly.

4.3 Results

Individual fish ETS activity was highly variable within species (Figure 4.1). The species with the highest maximum ETS activity was bluegill ($1.132 \text{ mL O}_2 \text{ g}^{-1} \text{ WW}^{-1} \text{ h}^{-1}$), while banded sculpin had the lowest maximum ETS activity ($0.275 \text{ mL O}_2 \text{ g}^{-1} \text{ WW}^{-1} \text{ h}^{-1}$, Figure 4.2). There were significant differences in T_{opt} and the lower bound of the optimum thermal range for enzymes among species, but there was no difference in the upper bound of the optimum thermal range or breadth of the optimum range across species (Figure 4.3). Mean T_{opt} of largescale stoneroller enzyme activity was the highest among all species tested (34.5°C) and was significantly higher than for blacktail shiner and rough shiner enzymes ($p = 0.002$ and $p = 0.0003$, respectively; Figure 4.3b, Table 4.2). Creek chub, which had the second highest mean optimum temperature for enzyme activity (32.4°C), was significantly higher than for rough shiner (27.5°C) ($p = 0.025$; Figure 4.3b, Table 4.2). Similar results were observed with lower bounds of the optimal enzyme thermal range among species. Largescale stoneroller optimum range mean lower bound was significantly higher than that of blacktail shiner ($p = 0.003$) and

rough shiner ($p = 0.0002$), and creek chub optimum range lower bound was significantly higher than that of rough shiner ($p = 0.007$) (Figure 4.3c, Table 4.2).

4.4 Discussion

Studies quantifying thermal tolerance of fish have typically used whole animal techniques like the incipient lethal temperature and critical thermal methodology, both of which involve exposing acclimated fish to acute temperature changes either by plunging fish into static containers of varying water temperature or increasing temperature linearly until 50% mortality is reached (Beitinger et al. 2000). While these techniques are ubiquitous in the literature, they often require large sample sizes and potentially overestimate thermal tolerance (Maness and Hutchison 1980, Lutterschmidt and Hutchison 1997, Beitinger et al. 2000). Enzyme assays may be able to remedy these issues, if strong correlations occur between CT_{max} and TPC endpoints.

Here I quantified the optimum ranges for enzyme activity of the seven study species. Similar species to those in my study have had thermal tolerances quantified through incipient lethal temperature and critical thermal maximum studies (using loss of equilibrium and onset of muscular spasms as endpoints). Interestingly for banded sculpin, bluegill, creek chub, and largescale stoneroller, the T_{up} (the upper bound of the optimum temperature range) for PMA in my study is similar to the critical thermal maxima (CT_{max}) found in the literature for these or similar species (Table 4.3). I found T_{up} for bluegill enzymes to be $34.7^{\circ}C$. CT_{max} for bluegill acclimated from ~ 20 to $26^{\circ}C$ ranged from 31.5 to $37.5^{\circ}C$ (Beitinger et al. 2000). Beitinger et al. (2000) reviewed studies for critical thermal maxima and reports on genera *Cottus*, *Campostoma*, *Cyprinella*, *Notropis*, and *Semotilus*. The CT_{max} for mottled sculpin *Cottus bairdii* acclimated to $15^{\circ}C$, determined by the onset of muscle spasms, was $30.9^{\circ}C$, and slimy sculpin *Cottus cognatus gracilis* acclimated at $20^{\circ}C$ displayed loss of equilibrium at $29.4^{\circ}C$ (Otto and O'Hara Rice 1977,

Kowalski et al. 1978). The T_{up} for banded sculpin enzymes in my study was 36.7°C. Central stoneroller *Campostoma anomalum* acclimated to 23°C displayed loss of equilibrium at 35.8°C with reported CT_{max} as high as 38°C (Chagnon and Hlohowskyj 1989, Mundahl 1990, Frenette et al. 2019). Largescale stoneroller enzymes here had a T_{up} of 37.9°C. Red shiner *Cyprinella lutrensis* is a widely distributed member of the *Cyprinella* genus; individuals acclimated to 20 and 22°C experienced loss of equilibrium at 34 and 36.2°C, respectively (Carrier and Beitinger 1988). Upper optimum range temperature for enzyme activity was 32.8 and 35.6°C for two members of the same genus—blacktail shiner and tallapoosa shiner, respectively. For creek chub *Semotilus atromaculatus* acclimated to 26°C, CT_{max} was documented at 35.7°C, and the creek chub enzymes in my study had T_{up} of 36.2°C (Smale and Rabini 1995). CT_{max} within the *Notropis* genus was less clear upon review of literature. While rough shiner is in this genus, few examples of closely related species exist. Beitinger et al. (2000) lists 11 *Notropis* species with CT_{max} values ranging from 31.8 to 38.6°C after acclimation to temperatures ranging from 15 to 26°C. Rough shiner in my study had a T_{up} of 33.7°C and the widest overall $T_{breadth}$. In order to further assess the relationship between CT_{max} and T_{up} , combination studies determining CT_{max} and ETS activity should be performed and compared within species.

I found among-species differences in T_{opt} and T_{low} , but no differences in T_{up} or $T_{breadth}$. No significant difference in upper bound of the optimum range of enzyme activity may suggest that enzyme performance at T_{up} and $T_{breadth}$ is similar across these species. Phylogenetics and thermal tolerance are related, with thermal tolerance being similar among species with close evolutionary relationships (Comte and Olden 2016). Comte and Olden (2016) found that species that experienced similar temperature ranges had similar thermal tolerances. Here I chose species that occupy similar thermal niches, and five of the seven species are members of Leuciscidae.

With the exception of banded sculpin, the species in this study could be considered thermal generalists, able to occupy a wide range of temperatures without deleterious effects to their physiologies (Seebacher et al. 2015). Additional comparisons of species with more restricted thermal niches will be required to explore the generality of these findings.

Adverse outcome pathways is a conceptual framework that was developed to extrapolate molecular markers to biological and population level responses for ecotoxicology studies (Ankley et al. 2009, Leist et al. 2017). The above examples suggest that enzyme T_{up} may indicate that there is a predictive physiological marker (i.e., enzyme activity) for organismal CT_{max} . Merging enzyme or cellular level responses to temperature shifts and individual level responses could be instrumental in the development of broadly applicable management criteria or regulations concerning species of interest. One reason that enzyme thermal optima vary from organismal optima is due to level of complexity. Enzymes and cellular components are more tolerant than the whole organism (Portner 2002, Verberk et al. 2016). Acute enzyme response to temperature variation likely varies from the response to chronic exposure much in the same way that whole organism acclimation protocols yield variations in response to acute and chronic temperatures (Sandblom et al. 2014, Haney 2019). Chronic acclimation of enzymes is more relevant for slower changes in temperature (like warming global temperatures) while responses to acute changes in water temperature, such as hyper- or hypolimnetic release, are better quantified using the approach in this study. With acute exposure, enzymes have not degraded or acclimated to the temperature challenge. Thermal performance curves of enzymes that have undergone acclimation were found to have inconsistent results across select mussel species; two populations of mussels that underwent acclimation to temperatures before performing ETS assays had increased T_{opt} and PMA compared to unacclimated enzymes, whereas other

populations were unaffected or exhibited declines in PMA (Haney 2019). These differences among populations could indicate that thermal performance varies across populations within a species, as seen in fish. Beachum et al. (2019) documented variations in thermal tolerance across northern and southern populations of bluntnose minnow *Pimphales notatus* with southern populations determined to be more sensitive to temperature shifts than northern populations. Heat-shock proteins (HSPs) are evolutionarily conserved in fish, and they function as chaperones to damaged proteins caused by temperature extremes (Basu et al. 2002). Between northern and southern populations of common killifish *Fundulus heteroclitus*, southern populations had greater thermal tolerance, possibly due to better regulation of HSP gene regulation at temperature extremes (Fangue et al. 2006). This within-species variation in thermal tolerance must be considered when performing enzyme assays and drawing conclusions for optima for diverse fish species.

Finally, some improvements could be made to the approach used in my study. Similar to Haney (2019), acclimation to each temperature, emulating chronic temperature exposure, in the enzyme assays would show the long-term effects of temperature shifts. Enzyme acclimation to temperature can take 2-7 days before degradation or acclimation is complete (Gopalan et al. 1996). Such an approach would complement the acute responses I determined here. Another way to improve the enzyme TPCs is to perform assays at lower temperatures to compare critical thermal minima (CT_{min}) at temperatures below 14°C, which was the lowest assessed in my study. Lower temperatures can sometimes prove more detrimental to fish survival as fish are less likely to escape colder temperatures than warmer temperatures (Beitinger et al. 2000). Metabolic responses to temperature can be viewed as both a constraint and a benefit. As a constraint, increasing temperature would increase standard metabolic demands for basic functions of the

organism and reduce aerobic scope, while elevated metabolic rate would increase potential for growth, activity, and reproduction (Verberk et al. 2016). Enzyme assays are one tool of many for quantifying PMA and responses to temperature exposure. Enzyme optimal thermal temperatures are likely to be higher than organismal optimal temperatures. Previous studies on crayfish support these findings that CT_{max} seems to fall near T_{up} (Westhoff et al. 2021). Knowing where these organismal level endpoint fall on the enzyme thermal performance curve can be useful for developing a metric that determines organismal and species performance. If we attempt to extrapolate enzyme performance data to make population level assessments, we must remember that the population will have reduced tolerance to stressors compared to lower levels of complexity. Management and conservation of species and rich biodiversity will require multidisciplinary approaches to be successful.

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Table 2.1. Metabolic rate ($\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$), DO_{crit} , and RI (means \pm SE) for all species at each temperature for which respirometry runs were performed.

Species	Temp	Metabolic Rate	DO_{crit}	RI
Largescale Stoneroller	18	222.24 ± 41.91	3.22 ± 0.41	0.497 ± 0.06
	21	349.78 ± 38.80	3.57 ± 0.39	0.450 ± 0.05
	24	356.26 ± 30.55	4.58 ± 0.36	0.233 ± 0.05
	27	466.26 ± 20.62	4.69 ± 0.14	0.334 ± 0.05
Blacktail shiner	18	248.58 ± 50.08	2.86 ± 0.45	0.526 ± 0.15
	21	248.90 ± 22.32	2.69 ± 0.18	0.708 ± 0.03
	24	186.61 ± 24.63	2.70 ± 0.16	0.757 ± 0.03
	27	135.25 ± 23.27	4.17 ± 0.97	0.420 ± 0.12
	30	149.48 ± 15.86	2.02 ± 0.41	0.666 ± 0.05
Striped shiner	18	171.47 ± 34.88	1.99 ± 0.07	0.824 ± 0.05
	21	236.24 ± 43.72	3.25 ± 0.48	0.588 ± 0.08
	24	373.81 ± 51.70	3.59 ± 0.51	0.414 ± 0.06
	27	352.34 ± 42.61	4.16 ± 1.92	0.224 ± 0.11
	30	246.15 ± 25.57	2.50 ± 0.44	0.270 ± 0.04
Longjaw minnow	18	147.1 ± 20.53	1.97 ± 0.24	0.627 ± 0.05
	21	144.5 ± 6.44	2.12 ± 0.27	0.548 ± 0.07
	24	255.18 ± 24.02	2.72 ± 0.51	0.421 ± 0.05
	27	515.43 ± 64.48	3.01 ± 0.29	0.514 ± 0.10
Rough shiner	18	167.63 ± 22.95	1.73 ± 0.21	0.688 ± 0.02
	21	256.26 ± 22.15	2.56 ± 0.29	0.358 ± 0.05
	24	159.05 ± 21.69	2.26 ± 0.27	0.340 ± 0.06
	27	172.93 ± 50.37	2.61 ± 0.57	0.465 ± 0.13
Creek chub	18	204.40 ± 22.32	3.38 ± 0.34	0.607 ± 0.09
	21	196.98 ± 15.77	2.23 ± 0.21	0.614 ± 0.05
	24	292.75 ± 8.45	2.52 ± 0.23	0.540 ± 0.03
	27	510.24 ± 63.41	2.92 ± 0.29	0.583 ± 0.02
	30	323.04 ± 40.40	3.33 ± 0.53	0.264 ± 0.05
Banded sculpin	14	71.75 ± 14.21	2.49 ± 0.47	0.609 ± 0.09
	18	110.03 ± 10.91	3.12 ± 0.33	0.451 ± 0.04
	21	152.21 ± 7.12	2.08 ± 0.24	0.533 ± 0.05
	24	145.04 ± 1.95	2.33 ± 0.41	0.436 ± 0.03
	26	148.52 ± 18.36	4.54 ± 1.13	0.314 ± 0.09

Table 3.1. Literature review of glochidia infestations. Error estimates (\pm SE or SD) for fish size (g), glochidia/fish, and glochidia/g vary across studies and are listed in column “x”. “Bath” infestations describe fishes inoculated via baths with extracted glochidia set to specific concentrations (concentrations of bath not included here). “Simulated natural” infestations are performed in the laboratory with mussels and hosts placed in the same tanks to simulate natural conditions for infestation. “Wild” denotes wild mussels infesting wild fish *in situ*.

Paper	Year	fish spp.	fish size (mean \pm x)	glochidia / fish (mean \pm x)	glochidia / g (mean \pm x)	x	Infestation method	mussel spp.
Treasurer et al.	2006	<i>Salmo salar</i>	5.6 \pm 1.5	1392 \pm 641	248.57 [†]	SD	bath	<i>Margaritifera margaritifera</i>
Chowdhury et al.	2019	<i>Salmo trutta</i>	57.2 [†]	7889 \pm 390	138 \pm 3	SE	bath	<i>M. margaritifera</i>
Taeubert & Geist et al.	2013	<i>S. trutta</i>	64.8 [†]	58747 \pm 4793	906 \pm 156	SD	bath	<i>M. margaritifera</i>
Taeubert & Geist et al.	2013	<i>S. trutta</i>	61.8 [†]	21833+5378	353 \pm 91	SD	bath	<i>M. margaritifera</i>
Taeubert & Geist et al.	2013	<i>S. trutta</i>	60.4 [†]	6823+1320	113 \pm 20	SD	bath	<i>M. margaritifera</i>
Taeubert & Geist et al.	2013	<i>S. trutta</i>	59.7 [†]	358+106	6+1	SD	bath	<i>M. margaritifera</i>
Marwaha et al.	2019	<i>S. trutta</i>	2.3 \pm 0.79	4.47	1.94 [†]	SD	bath	<i>M. margaritifera</i>
Marwaha et al.	2019	<i>S. trutta</i>	9.8 \pm 3.5	212.8	21.71 [†]	SD	bath	<i>M. margaritifera</i>
Osterling et al.	2014	<i>S. trutta</i>	2.16 \pm 0.28	54.22 [†]	25.1 \pm 5.7	SD	bath	<i>M. margaritifera</i>
Dodd et al.	2006	<i>Micropterus salmoides</i>	13.4 \pm 4.0	480 \pm 139	35.82 [†]	SD	bath	<i>Lampsilis reeveina</i>
Castrillo et al.	2019	<i>S. salar</i>	3.1	1633.70 [†]	527 \pm 144	SE	bath	<i>M. margaritifera</i>
Kaiser*	2005	<i>M. salmoides</i>	4.3 \pm 0.7	632.4 \pm 208.3	131.7 [†]	SD	bath	<i>L. reeveina</i>
Methling et al.	2018	<i>Rhodeus ocellatus</i>	0.99 \pm 0.22	-	-	SD	bath	<i>Sinanodonata woodiana</i>
Thomas et al.	2014	<i>S. trutta</i>	-	100.7 \pm 18.62	-	SE	simulated natural	<i>M. margaritifera</i>

Table 3.1. *Continued*

Paper	Year	fish spp.	fish size (g)	glochidia / fish (mean \pm x)	glochidia / g (mean \pm x)	x	Infestation method	mussel spp.
Thomas et al.	2014	<i>S. trutta</i>	-	150.9 \pm 2.94	-	SE	simulated natural	<i>M. margaritifera</i>
Thomas et al.	2014	<i>S. trutta</i>	-	36.7 \pm 7.94	-	SE	simulated natural	<i>M. margaritifera</i>
Thomas et al.	2014	<i>S. trutta</i>	-	54.1 \pm 7.66	-	SE	simulated natural	<i>M. margaritifera</i>
Filipsson et al.	2017	<i>S. salar</i>	16.5 \pm 1.2	181.5 [†]	11	SE	wild	<i>M. margaritifera</i>
Kelly and Watters	2010	<i>Dorosoma cepedianum</i>	-	1-5	-	-	wild	multiple
Kelly and Watters	2010	<i>Ictalurus punctatus</i>	-	1-15	-	-	wild	multiple
Kelly and Watters	2010	<i>Aplodinotus grunniens</i>	-	1-140	-	-	wild	multiple
Kelly and Watters	2010	<i>Lepomis humilis</i>	-	3-8	-	-	wild	multiple
Kelly and Watters	2010	<i>L. macrochirus</i>	-	5-41	-	-	wild	multiple
Kelly and Watters	2010	Hybrid <i>Morone</i>	-	1	-	-	wild	multiple

*unpublished thesis

†calculated from data within paper

Table 3.2 Bluegill and largemouth bass at 15 minute intervals during inoculation taken to count glochidia attachment; n = 2 for each time interval.

Species	time in bath (min)	weight (g)	glochidia / fish	glochidia / g
largemouth bass	15	8.19	13	1.59
		6.77	26	3.849
	30	7.01	52	7.42
		5.23	68	13.00
	45	9.38	137	14.61
		7.54	120	15.92
bluegill	15	12.00	5	0.42
		8.40	2	0.24
	30	9.73	29	2.98
		11.22	47	4.19
	45	7.59	120	15.81
		8.30	61	7.35

Table 3.3. Viability of *Lampsilis straminea* (n = 4) used in the inoculation of fish hosts, weight of each individual also included.

species	mussel	weight (g)	% viability
bluegill	1	33.13	94.4
	2	32.42	96.4
	3	28.7	92.1
	4	31.6	91.2
	5	25.85	95.1
		mean	93.8
largemouth bass	1	37.04	87.9
	2	33.7	89.0
	3	28.15	93.4
	4	27.15	95.7
			mean

Table 2.4. Bluegill metabolic rate ($\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$), DO_{crit} , RI, glochidia/g wet weight of host, and glochidia per fish (means \pm SEs) grouped by both time post-inoculation and by control versus treatment (infested) groups.

Time	Group	Metabolic Rate	P_{crit}	RI	Glochidia / g	Glochidia / fish
24 h	Control	163.06 \pm 41.57	1.87 \pm 0.35	0.466 \pm 0.29	-	-
	Infested	132.72 \pm 11.92	1.92 \pm 0.21	0.653 \pm 0.04	11.61 \pm 1.56	157.2 \pm 35.8
48 h	Control	211.72 \pm 29.73	3.70 \pm 1.74	0.626 \pm 0.16	-	-
	Infested	161.967 \pm 13.24	1.69 \pm 0.13	0.698 \pm 0.07	5.40 \pm 1.97	65.7 \pm 36.5
72 h	Control	113.02 \pm 5.72	1.33 \pm 0.04	0.748 \pm 0.004	-	-
	Infested	126.90 \pm 13.58	3.06 \pm 1.16	0.554 \pm 0.07	4.82 \pm 0.91	40.8 \pm 7.3
1 week	Control	154.65 \pm 12.04	1.51 \pm 0.08	0.662 \pm 0.07	-	-
	Infested	109.85 \pm 9.12	1.13 \pm 0.08	0.567 \pm 0.08	6.11 \pm 1.85	65.3 \pm 21.3
2 weeks	Control	108.11 \pm 20.33	1.51 \pm 0.17	0.694 \pm 0.06	-	-
	Infested	153.23 \pm 23.59	2.14 \pm 0.78	0.478 \pm 0.09	3.88 \pm 1.67	41.5 \pm 17.7
3 weeks	Control	125.37 \pm 20.72	1.39 \pm 0.13	0.730 \pm 0.10	-	-
	Infested	113.36 \pm 11.82	1.52 \pm 0.30	0.509 \pm 0.09	2.39 \pm 1.13	30.7 \pm 16.9
5 weeks	Control	142.41 \pm 3.30	1.75 \pm 0.14	0.479 \pm 0.12	-	-
	Infested	111.53 \pm 15.16	1.70 \pm 0.12	0.601 \pm 0.09	0.89 \pm 0.36	11.5 \pm 5.5
8 weeks	Control	150.80 \pm 56.74	3.47 \pm 2.15	0.358 \pm 0.09	-	-
	Infested	75.26 \pm 9.18	1.51 \pm 0.08	0.603 \pm 0.07	2.69 \pm 1.15	37.0 \pm 16.9
				Mean	4.72 \pm 0.64	56.2 \pm 9.5

Table 3.5. Largemouth bass metabolic rate ($\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$), DO_{crit} , RI, and glochidia/g wet weight of host, and glochidia per fish (means \pm SEs) grouped by both time post-inoculation and by control versus treatment (infested) groups.

Time	Group	Metabolic Rate	P_{crit}	RI	Glochidia / g	Glochidia / fish
24 h	Control	192.17 \pm 2.55	1.72 \pm 0.07	0.294 \pm 0.12	-	-
	Infested	248.47 \pm 17.57	2.16 \pm 0.15	0.340 \pm 0.10	20.61 \pm 1.72	151.8 \pm 20.8
48 h	Control	227.15 \pm 49.91	1.78 \pm 0.07	0.623 \pm 0.18	-	-
	Infested	149.37 \pm 29.38	1.77 \pm 0.12	0.543 \pm 0.09	20.08 \pm 2.92	146.7 \pm 11.1
72 h	Control	112.10 \pm 24.41	1.69 \pm 0.09	0.306 \pm 0.06	-	-
	Infested	187.72 \pm 31.71	1.80 \pm 0.12	0.649 \pm 0.07	22.43 \pm 2.54	138.3 \pm 14.1
1 week	Control	248.74 \pm 112.04	2.29 \pm 0.28	0.545 \pm 0.24	-	-
	Infested	175.79 \pm 27.40	2.29 \pm 0.45	0.590 \pm 0.07	24.06 \pm 4.22	178.8 \pm 38.9
2 weeks	Control	135.35 \pm 6.62	1.55 \pm 0.06	0.701 \pm 0.21	-	-
	Infested	158.48 \pm 23.00	2.10 \pm 0.39	0.750 \pm 0.05	18.77 \pm 1.76	133.8 \pm 17.2
3 weeks	Control	-	-	-	-	-
	Infested	121.91 \pm 15.39	2.31 \pm 0.19	0.624 \pm 0.06	20.63 \pm 0.65	166.2 \pm 11.0
3.5 weeks	Control	190.40 \pm 18.01	1.83 \pm 0.16	0.688 \pm 0.10	-	-
	Infested	180.54 \pm 31.80	2.65 \pm 0.37	0.648 \pm 0.04	18.66 \pm 2.27	138.0 \pm 16.1
5 weeks	Control	167.11 \pm 41.30	2.08 \pm 0.08	0.714 \pm 0.08	-	-
	Infested	188.30 \pm 30.03	2.57 \pm 0.40	0.639 \pm 0.04	11.88 \pm 2.29	84.3 \pm 18.4
8 weeks	Control	122.03 \pm 3.78	2.19 \pm 0.03	0.676 \pm 0.04	-	-
	Infested	203.32 \pm 26.58	3.11 \pm 0.61	0.601 \pm 0.04	9.36 \pm 0.78	95.5 \pm 14.7
11 weeks	Control	195.02 \pm 69.21	2.09 \pm 0.07	0.603 \pm 0.03	-	-
	Infested	178.99 \pm 26.56	2.56 \pm 0.40	0.676 \pm 0.04	8.84 \pm 1.03	83.8 \pm 8.8
				Mean	17.48 \pm 0.96	131.2 \pm 6.9

Table 3.1 Grouping of ETS assay sets. Assays at all temperature incubations (11 - 44°C) were performed on Group A before beginning assays for Group B. Group B assays were performed after all assays for Group A were complete but before Group C were performed, etc. All groups contained a *Ligumia subrostrata* control sample; this sample was not numbered. Abbreviations for species are as follows: blacktail shiner (BTS), banded sculpin (SCP), bluegill (BGL), creek chub (CRC), largescale stoneroller (SRL), rough shiner (RHS), Tallapoosa shiner (TPS).

<i>Group A</i>		<i>Group B</i>		<i>Group C</i>		<i>Group D</i>		<i>Group E</i>		<i>Group F</i>		<i>Group G</i>	
Species	Fish #	Species	Fish #	Species	Fish #	Species	Fish #	Species	Fish #	Species	Fish #	Species	Fish #
CRC	2	BTS	2	SRL	7	SRL	4	SCP	4	SCP	2	CRC	3
RHS	1	BTS	6	SCP	5	BTS	8	CRC	4	SRL	8	RHS	6
BTS	3	TPS	4	BGL	8	TPS	6	BGL	5	BGL	9	TPS	10
SCP	6	RHS	3	CRC	5	RHS	9	TPS	7	RHS	2	SCP	10
BTS	5	RHS	7	TPS	10	SCP	8	SRL	6	BGL	6	SCP	7
RHS	8	BGL	2	RHS	4	TPS	3	BTS	10	CRC	6	BGL	7
TPS	9	SRL	5	BTS	9	BGL	4	SRL	2	BTS	7	SRL	1
SRL	3	SCP	1	SCP	3	CRC	1	RHS	5	CRC	7	BTS	1
BGL	1			CRC	8	TPS	2	BTS	4	TPS	8		
control	-	control	-	control	-	control	-	control	-	control	-	control	-

Table 4.2. Optimum temperature (T_{opt}), lower (T_{low}) and upper (T_{up}) bounds of optimum thermal range for enzyme activity, breadth of optimum range, and 95% confidence intervals (in brackets) for all seven study species.

Species	Temperature (°C)						Breadth	95% CI
	T_{low}	95% CI	T_{opt}	95% CI	T_{up}	95% CI		
largescale stoneroller	29.3	[27.2, 31.5]	34.5	[32.2, 36.7]	37.9	[35.4, 40.4]	8.5	[6.3, 10.8]
creek chub	27.8	[25.7, 30.0]	32.4	[30.2, 34.6]	36.2	[33.7, 38.7]	8.4	[6.1, 10.6]
tallapoosa shiner	26.4	[24.4, 28.4]	32.0	[29.9, 34.1]	35.6	[33.4, 38.4]	9.1	[7.0, 11.2]
banded sculpin	26.4	[24.5, 28.3]	31.0	[29.0, 33.0]	35.3	[33.1, 37.6]	9.0	[7.0, 11.0]
bluegill	26.0	[24.0, 28.0]	30.3	[28.2, 32.4]	34.3	[32.0, 36.6]	8.3	[6.2, 10.4]
blacktail shiner	23.6	[21.7, 25.5]	28.1	[26.1, 30.1]	32.8	[30.6, 35.0]	9.2	[7.2, 11.2]
rough shiner	22.5	[27.2, 31.5]	27.5	[25.5, 29.5]	33.7	[31.5, 36.0]	11.3	[9.3, 13.3]

Table 4.3. T_{up} of ETS activity from the present study compared to CT_{max} values published in peer-reviewed journals.

Species from ETS	T_{up} (°C)	Published CT_{max} (°C)	Species in literature
<i>Campostoma oligolepis</i>	37.9	35.8 - 38	<i>Campostoma anomalum</i>
<i>Cyprinella venusta</i>	32.8	34 - 36.2	<i>Cyprinella lutrensis</i>
<i>Cyprinella gibbsi</i>	35.6		
<i>Notropis baileyi</i>	33.7	31.8 - 38.6	<i>Notropis</i> spp.
<i>Lepomis macrochirus</i>	34.7	31.5 - 37.5	<i>Lepomis macrochirus</i>
<i>Semotilus atromaculatus</i>	36.2	35.7	<i>Semotilus atromaculatus</i>
<i>Cottus carolinae</i>	36.7	30.9	<i>Cottus bairdi</i>
		29.4	<i>C. cognatus</i>

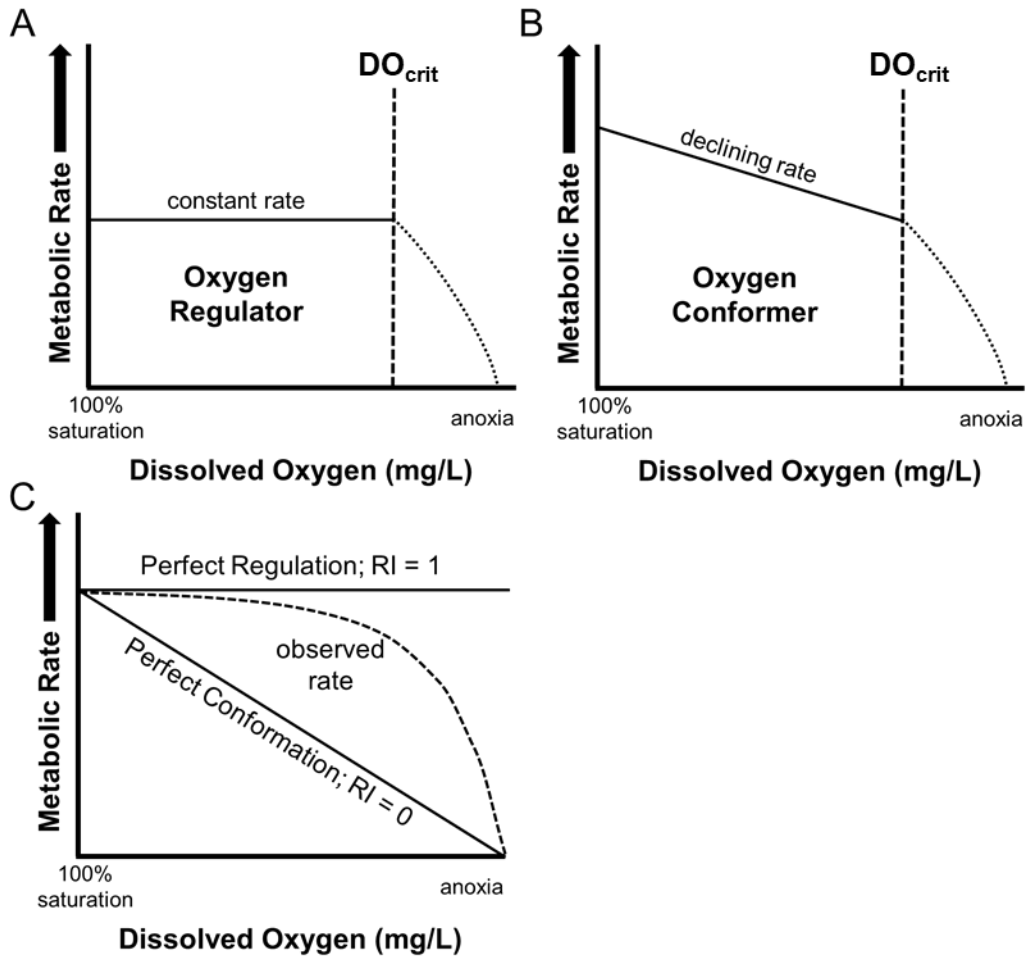


Figure 2.1. Oxygen uptake patterns graphed as a function of DO of (A) an oxyregulator and (B) an oxyconformer. DO_{crit} is defined as the DO threshold below which respiration rates show a marked change in slope. (C) Oxygen uptake patterns ($\dot{M}O_2$) graphed as a function of DO indicating the range of values of the Regulation Index (RI; Mueller and Seymour 2011), with solid lines indicating either perfect regulation ($RI = 1$) or perfect conformation ($RI = 0$); the dashed line indicates an intermediate RI value.

Largescale Stoneroller

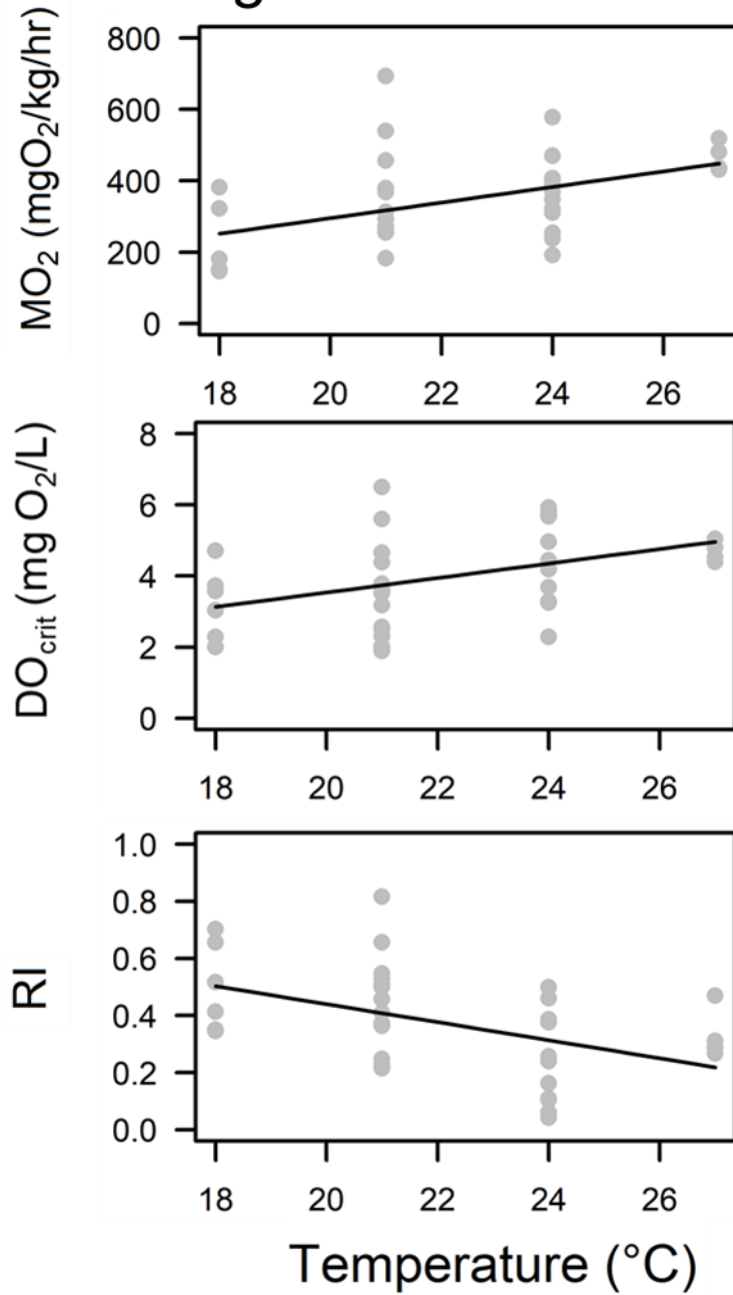


Figure 2.2. Metabolic rate, DO_{crit}, and RI of largescale stoneroller across a temperature range from 18 to 27°C. Fitted lines indicate significant relationships.

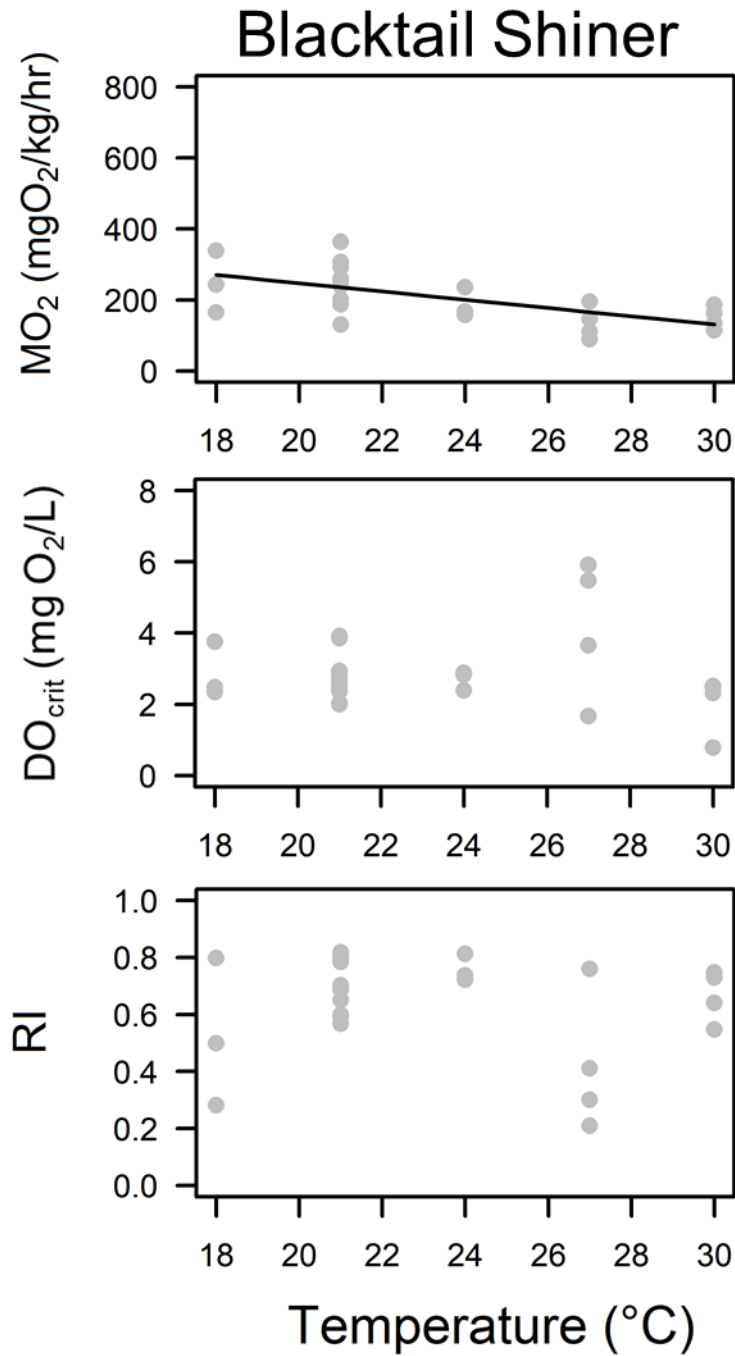


Figure 2.3. Metabolic rate, DO_{crit}, and RI of blacktail shiner across a temperature range from 18 to 30°C. Fitted lines indicate significant relationships,

Striped Shiner

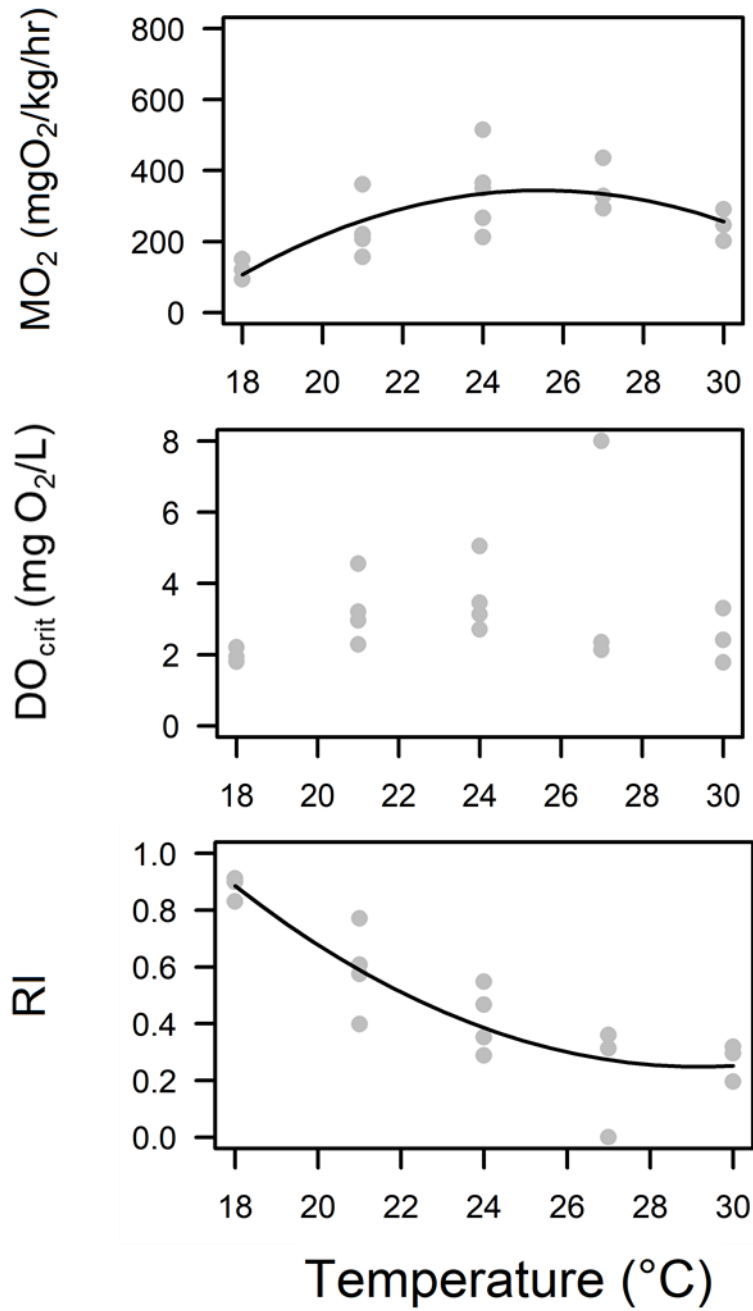


Figure 2.4. Metabolic rate, DO_{crit}, and RI of striped shiner across a temperature range from 18 to 30°C. Fitted lines indicate significant relationships.

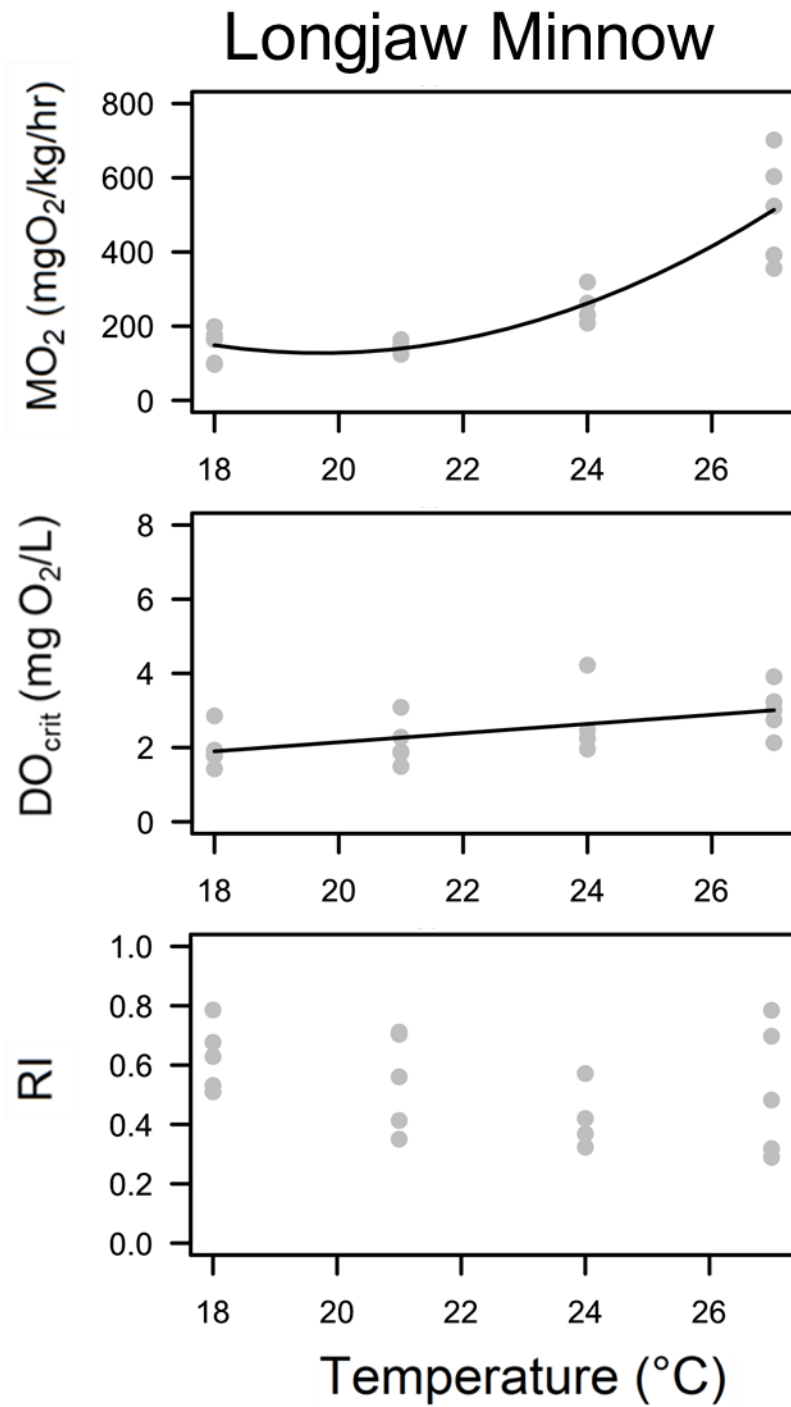


Figure 2.5. Metabolic rate, DO_{crit} , and RI of longjaw minnow across a temperature range from 18 to 27°C. Fitted lines indicate significant relationships.

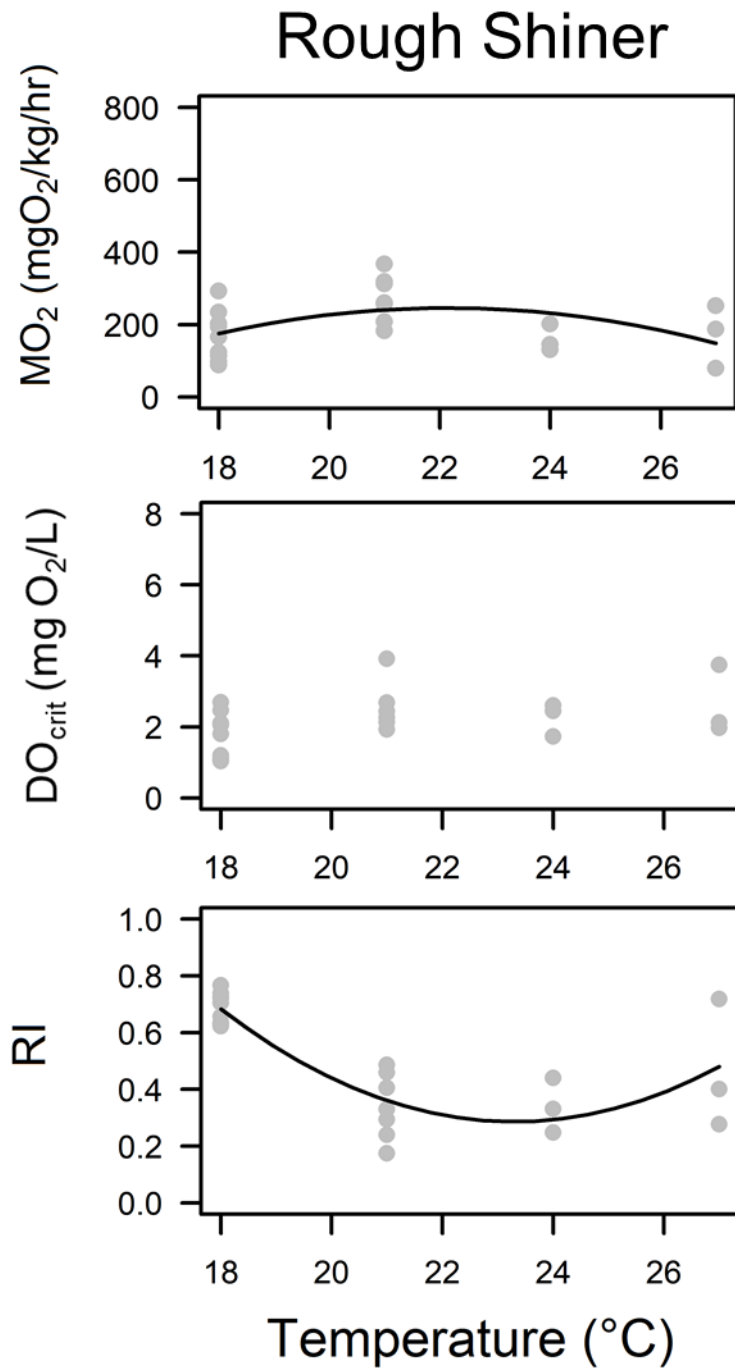


Figure 2.6. Metabolic rate, DO_{crit} , and RI of rough shiner across a temperature range from 18 to 27°C. Fitted lines denote significant relationships.

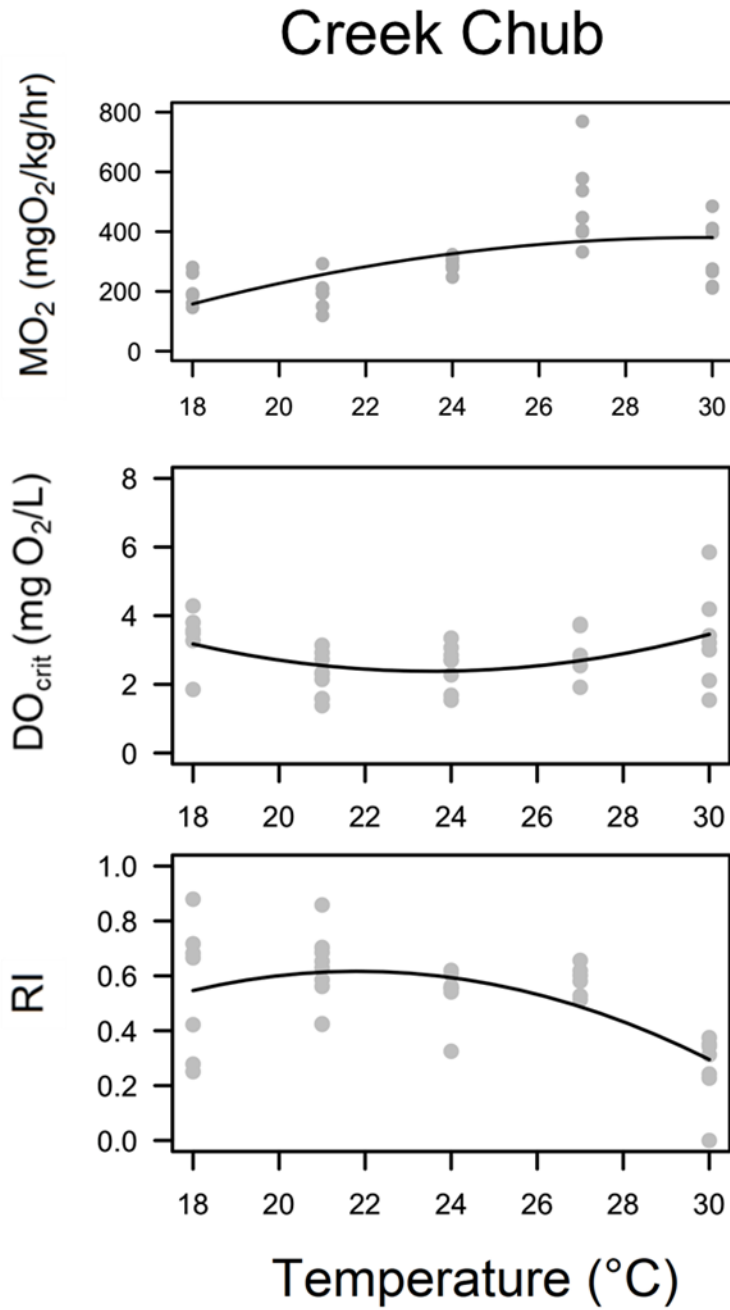


Figure 2.7. Metabolic rate, DO_{crit} , and RI of *creek chub* across a temperature range from 18 to 30°C. Fitted lines indicate significant relationships.

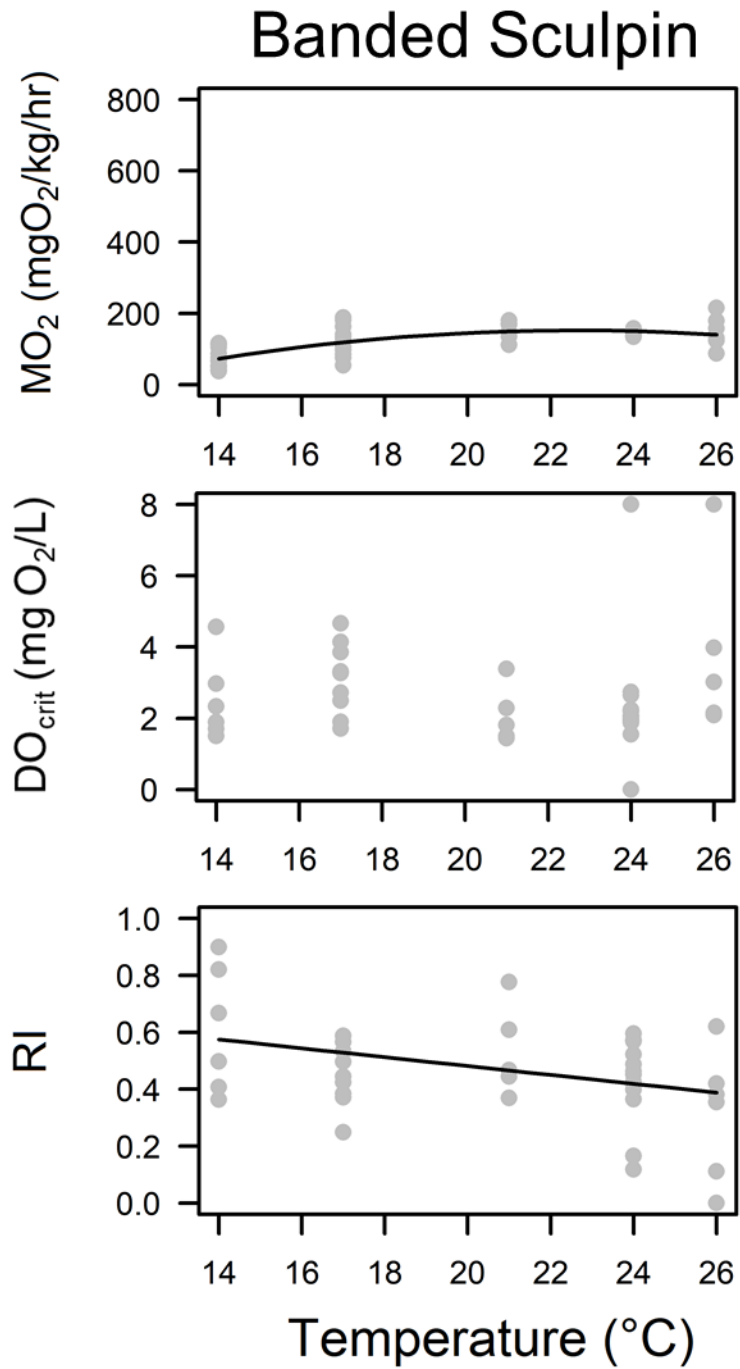


Figure 2.8. Metabolic rate, DO_{crit} , and RI of banded sculpin across a temperature range from 14 to 26°C. Fitted lines indicate significant relationships.

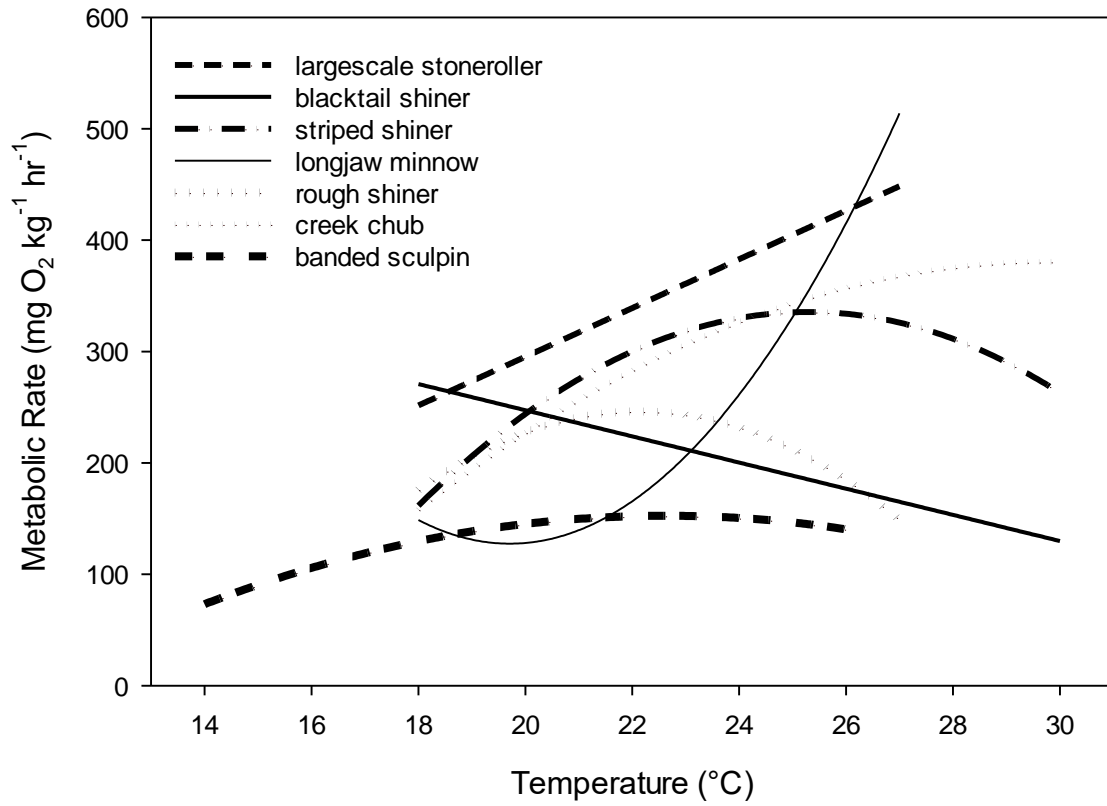


Figure 2.9. Predicted relationships between metabolic rate and temperature (14-30°C) for those species with significant relationships.

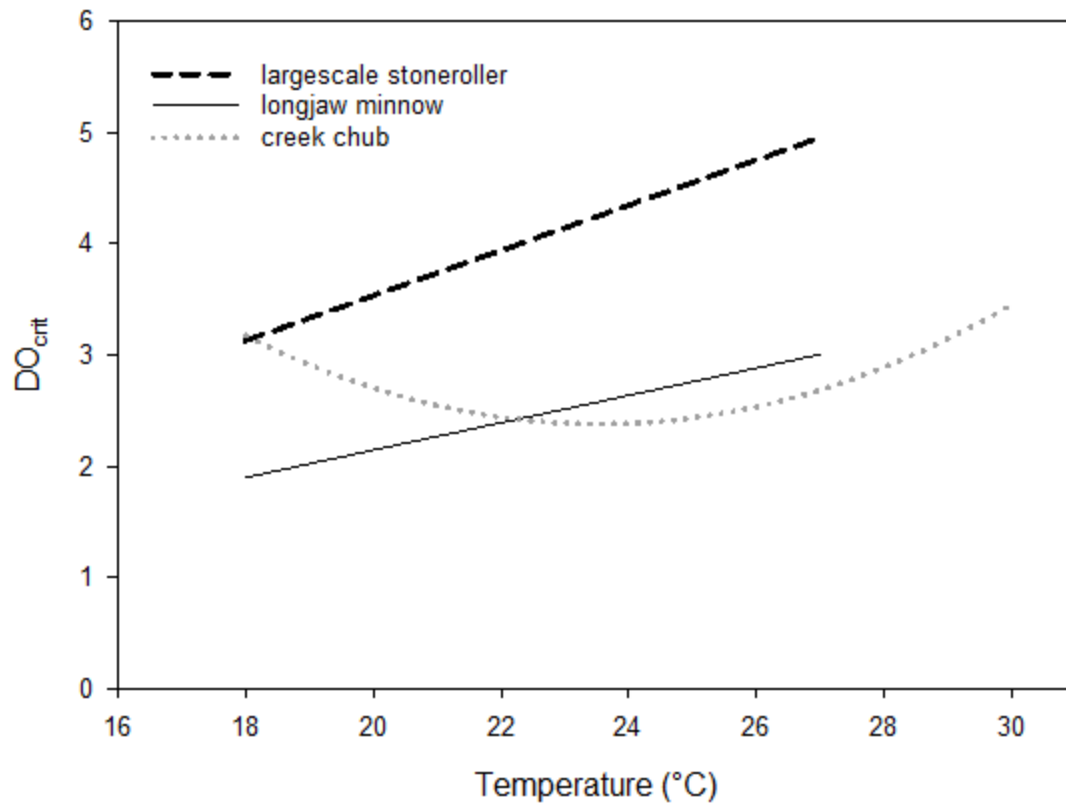


Figure 2.10. Predicted relationships between DO_{crit} and temperature (18-30°C) for those species with significant relationships.

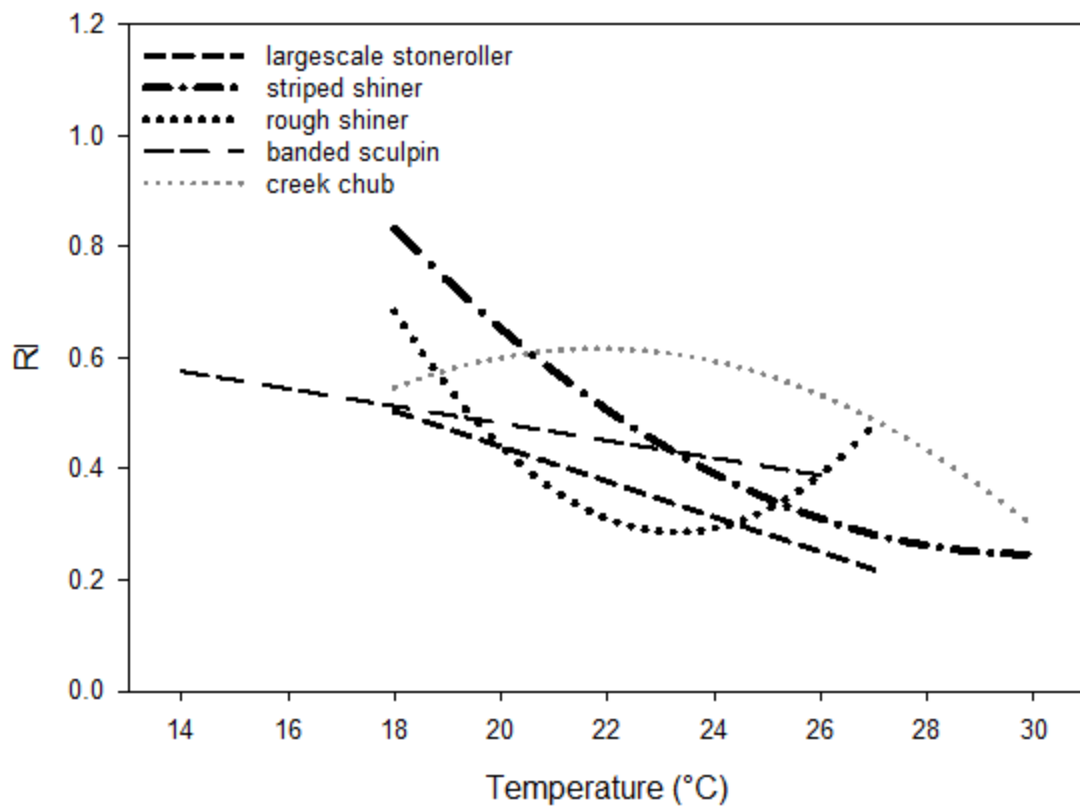


Figure 2.11. Predicted relationships between RI and temperature (14-30°C) for those species with significant relationships.

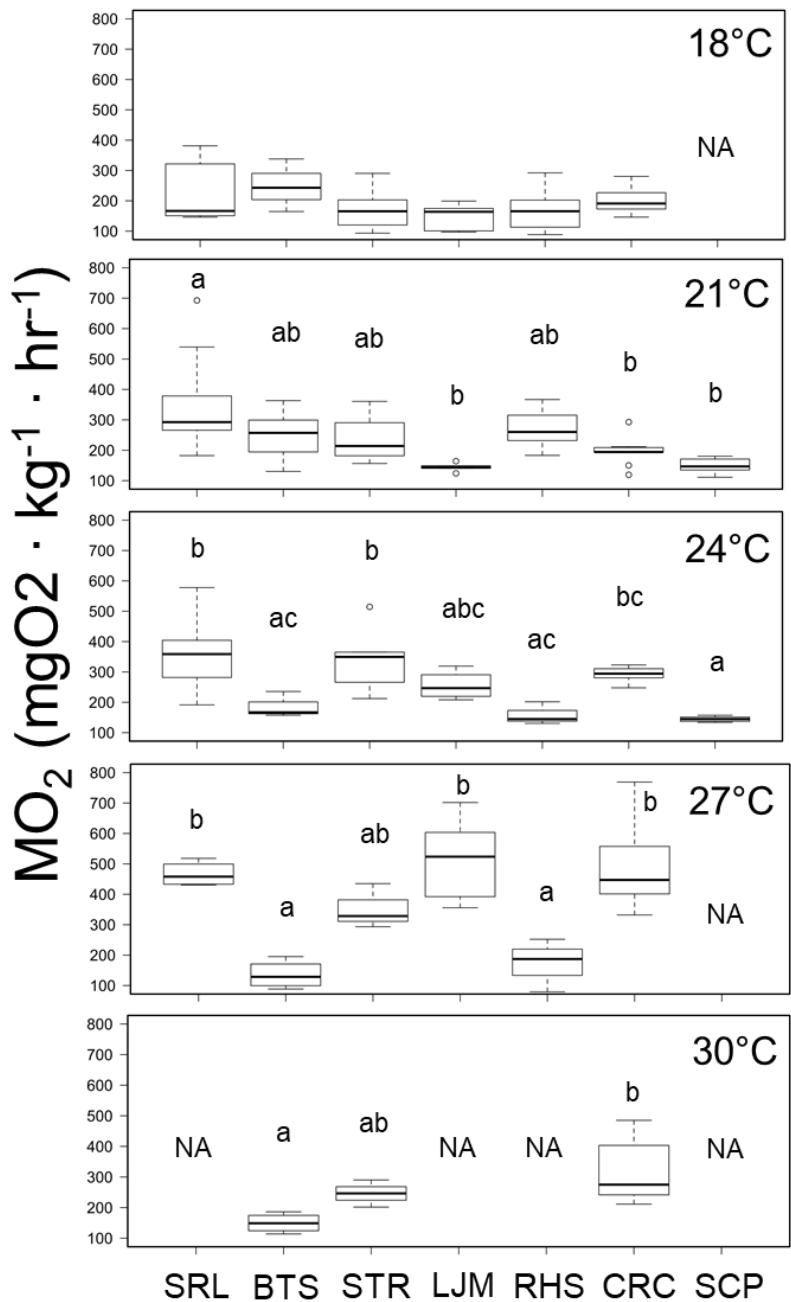


Figure 2.12. Metabolic rate of largescale stoneroller (SRL), blacktail shiner (BTS), striped shiner (STR), longjaw minnow (LJM), rough shiner (RHS), creek chub (CRC), and banded sculpin (SCP), compared at five temperatures. “NA” denotes data for a species at a temperature that were unavailable. Bars within a panel with different letters indicate significant differences in metabolic rates between species at that temperature.

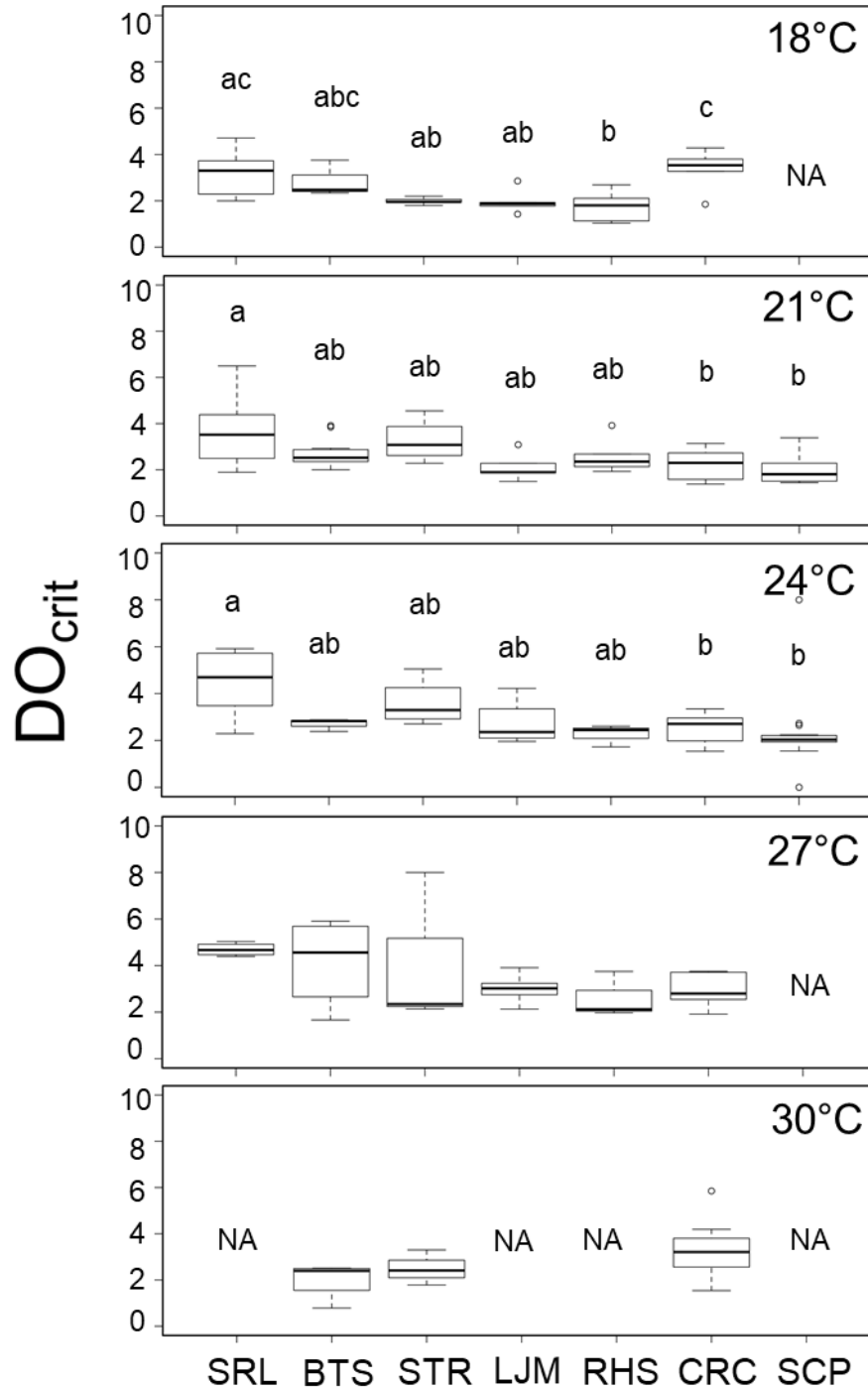


Figure 2.13. DO_{crit} of the seven study species (abbreviations as in Figure 2.12) compared at five temperatures. “NA” denotes data for a species at a temperatures that were unavailable. Bars within a panel with different letters indicate significant differences in DO_{crit} between species at that temperature.

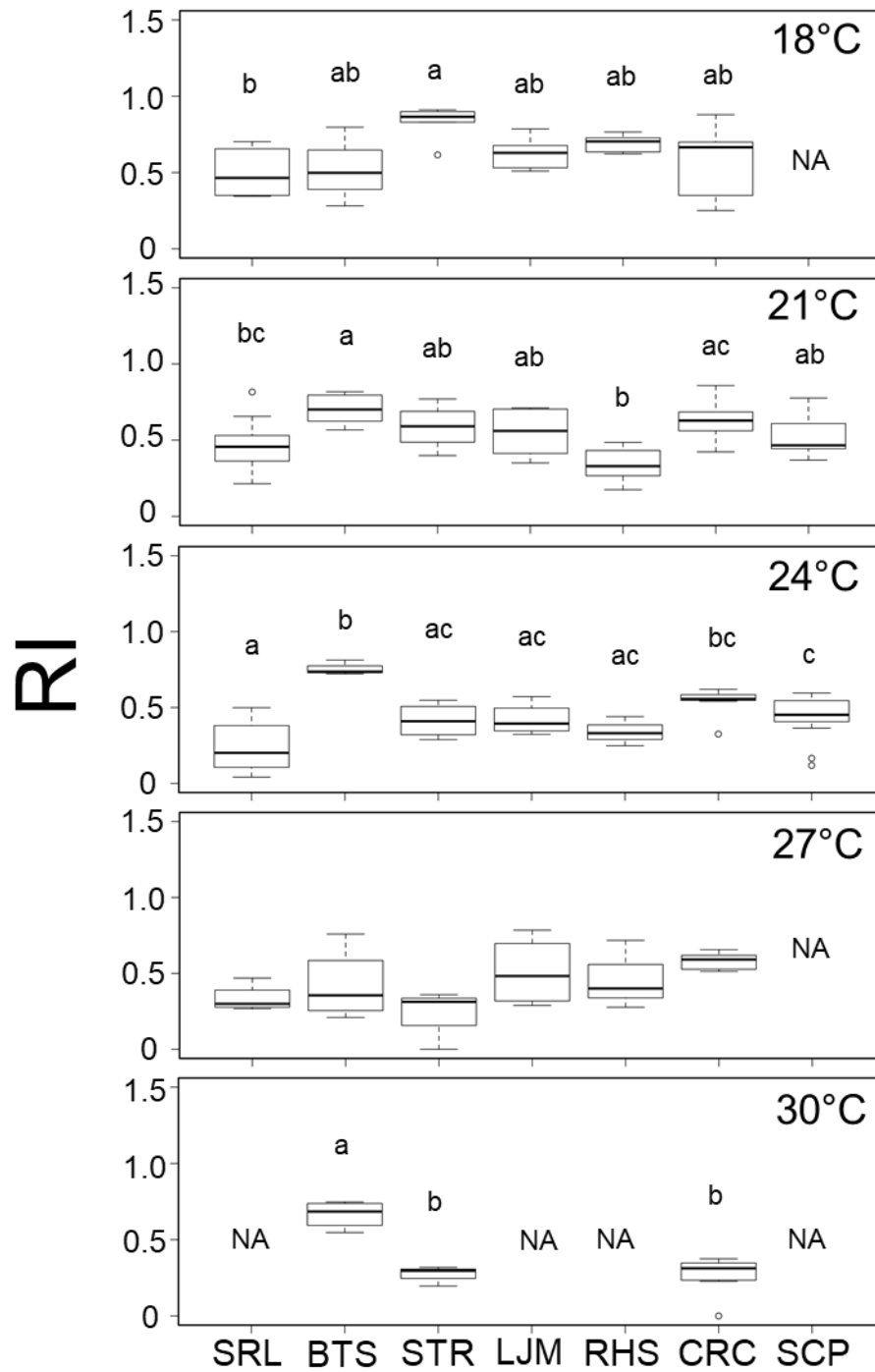


Figure 2.14. Regulation index of the seven study species (abbreviations as in Figure 2.12) compared at five temperatures. “NA” denotes data for a species at a temperature that were unavailable. Bars within a panel with different letters indicate significant differences in RI between species at that temperature.

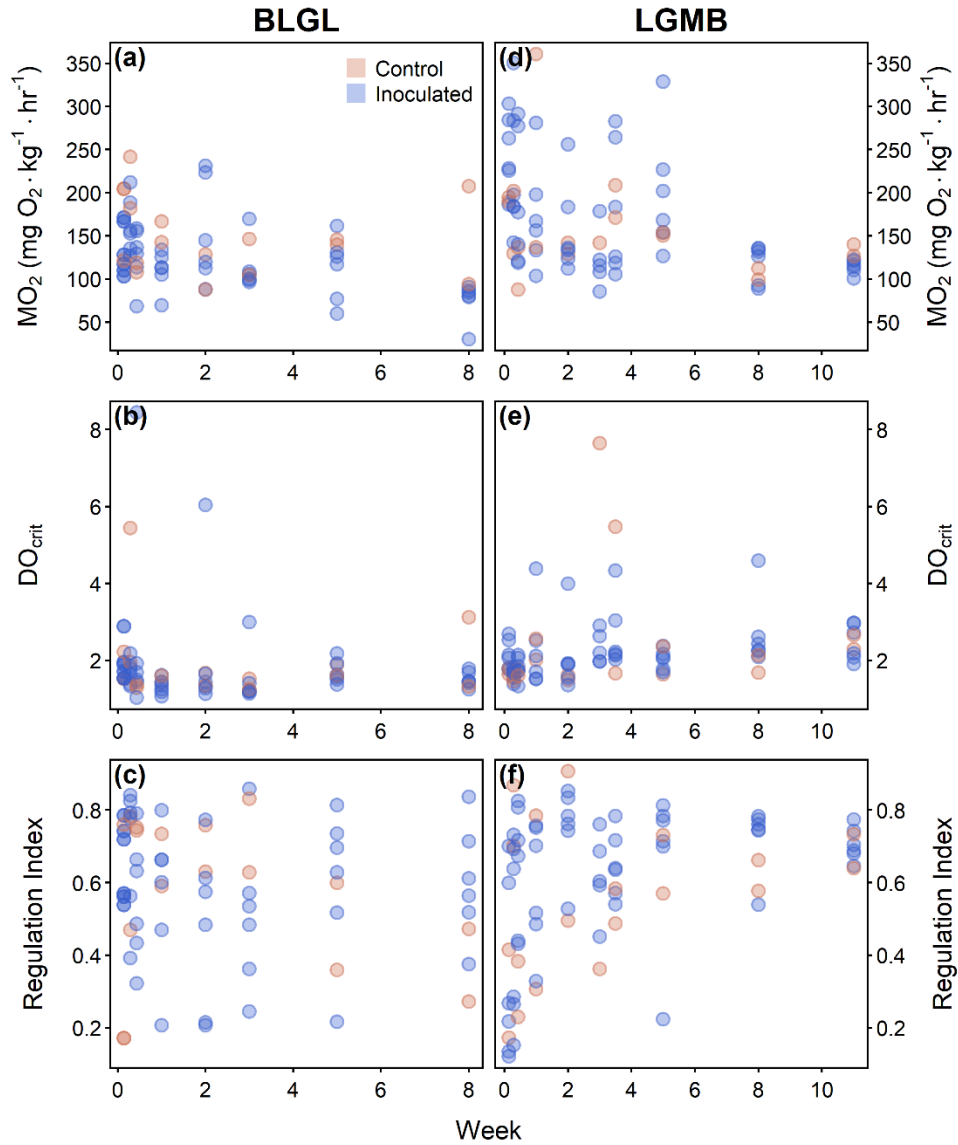


Figure 3.1. The three response variables for bluegill (BLGL) and largemouth bass (LGMB) as a function of time past inoculation for control fish (red) and fish inoculated with glochidia (blue). Time by response variable slopes did not differ between control or treatment fish for any group.

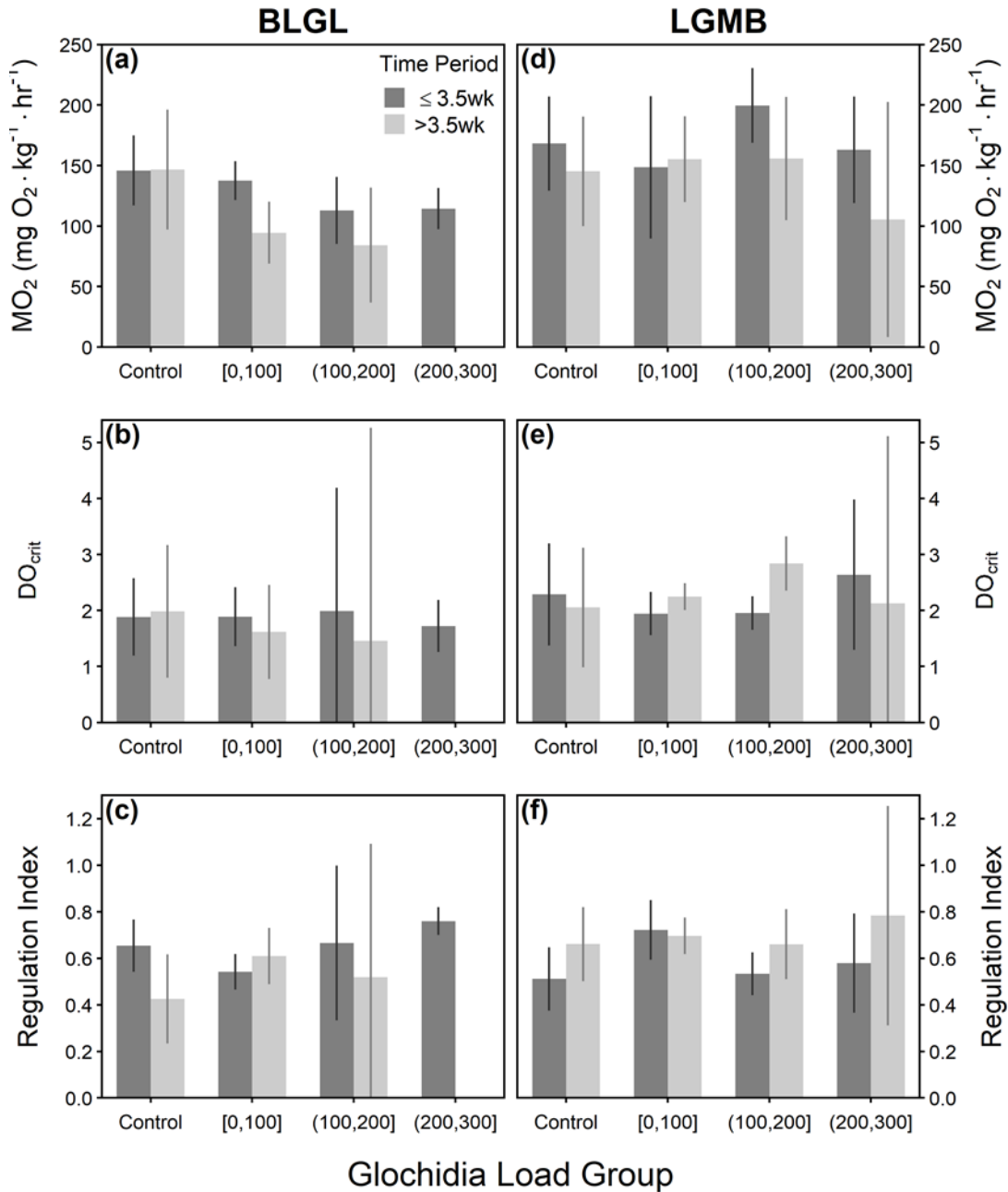


Figure 3.2. The three response variables for bluegill (BLGL; a, b, c) and largemouth bass (LGMB; d, e, f) for the transformation time period (dark bars) and the post-transformation time period (light bars) as a function of glochidia load divided into 3 groups (0-99, 100-200, and >200 glochidia per fish).

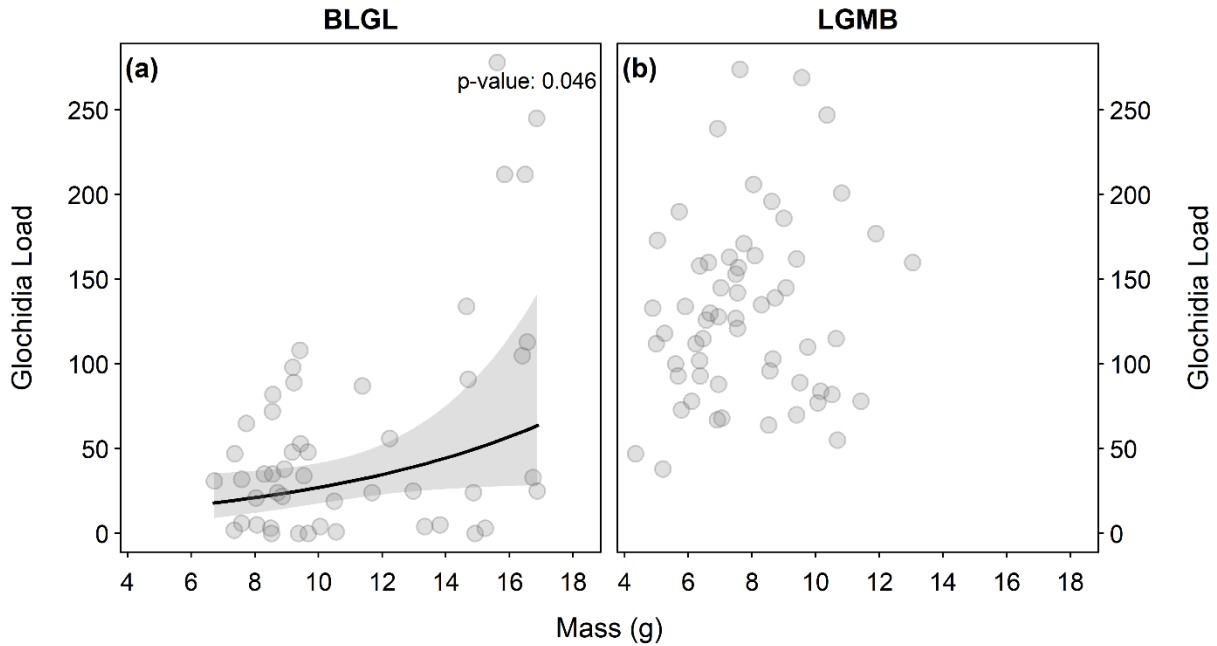


Figure 3.3. Regression of glochidia load (glochidia/fish) versus mass for bluegill (BLGL; a) and largemouth bass (LGMB; b). Data for bluegill were log-transformed for analysis due to a violation of assumptions but are displayed on the natural scale. The line represents a best fit for the significant relationship and the shaded area represents the 95% confidence interval.

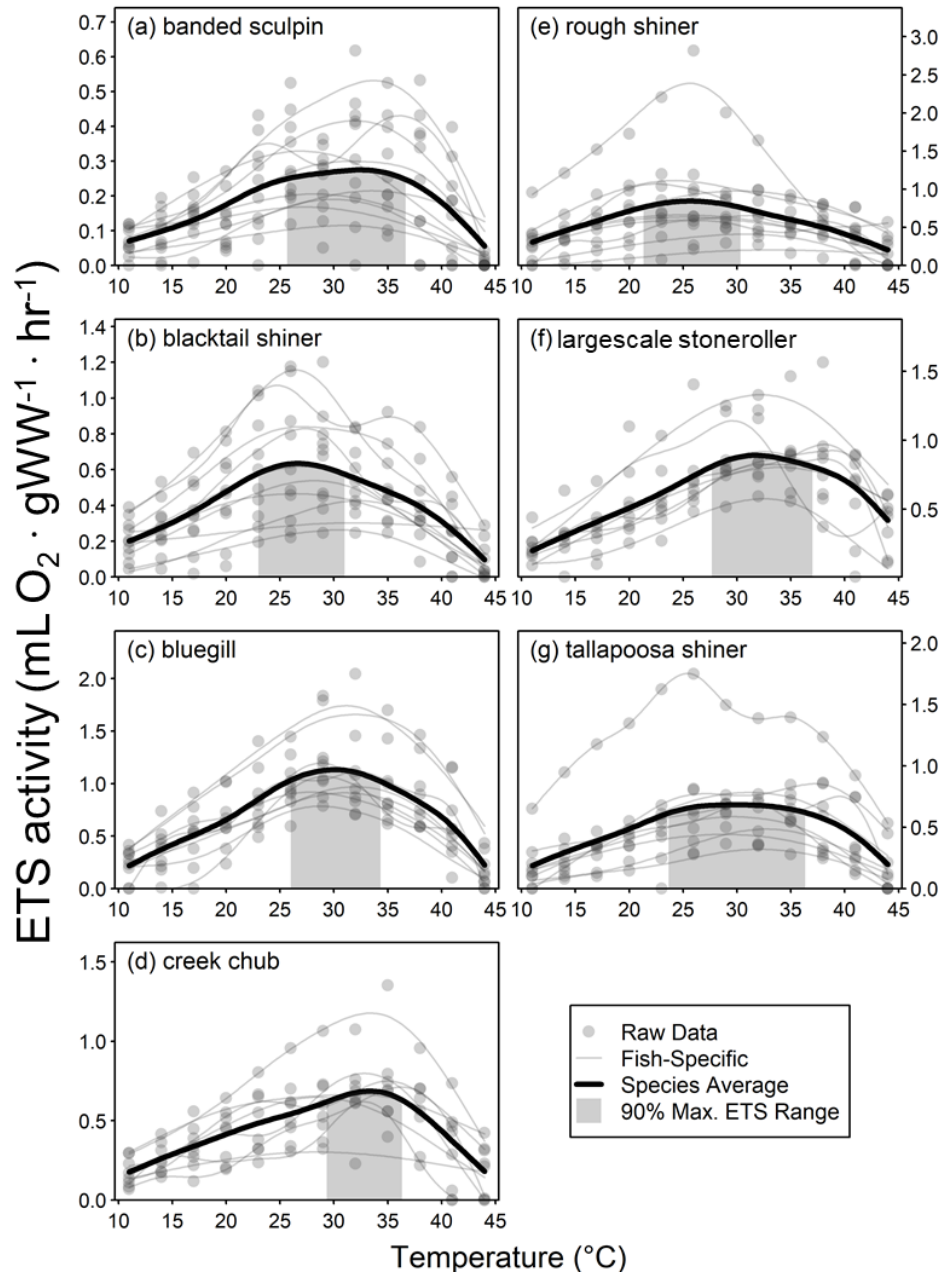


Figure 4.1. ETS activity as a function of temperature for both individuals and species. Circles represent raw data from enzyme assays for individual fish and grey lines represent individual fits for ETS activity. The black line represents the average curve within a species. The shaded area underneath the average curve is the optimum thermal range or T_{breadth} for enzyme activity (defined as 90% of the maximum ETS value) for a given species. Axes vary across plots to show within-species individual variation.

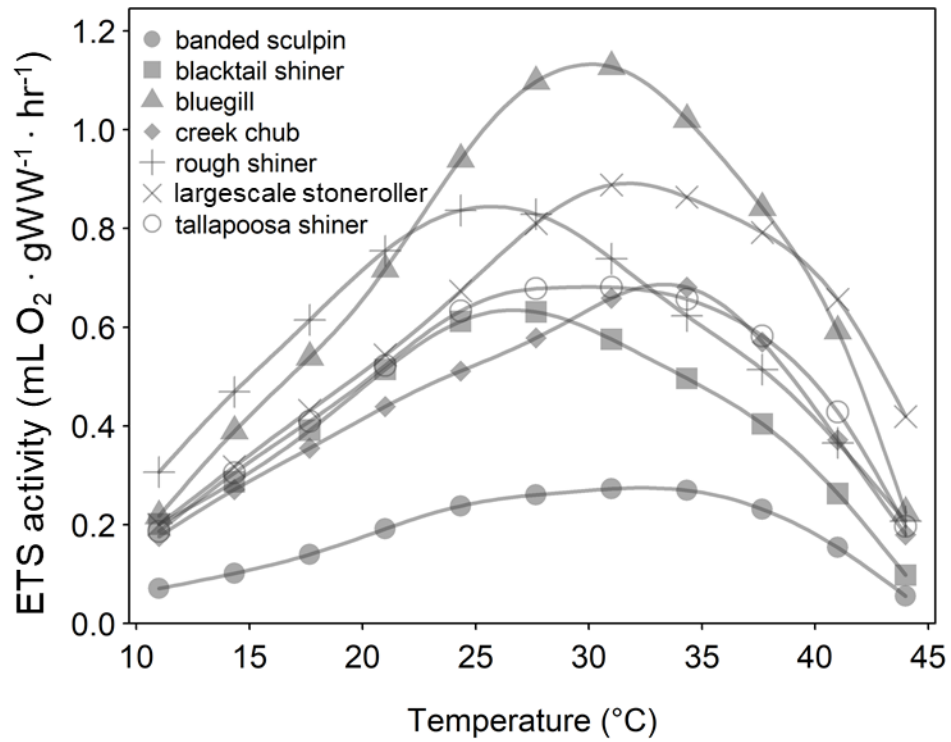


Figure 4.2. The averages curves of ETS versus temperature for each species. Symbols indicate the mean ETS activity for each temperature within a species.

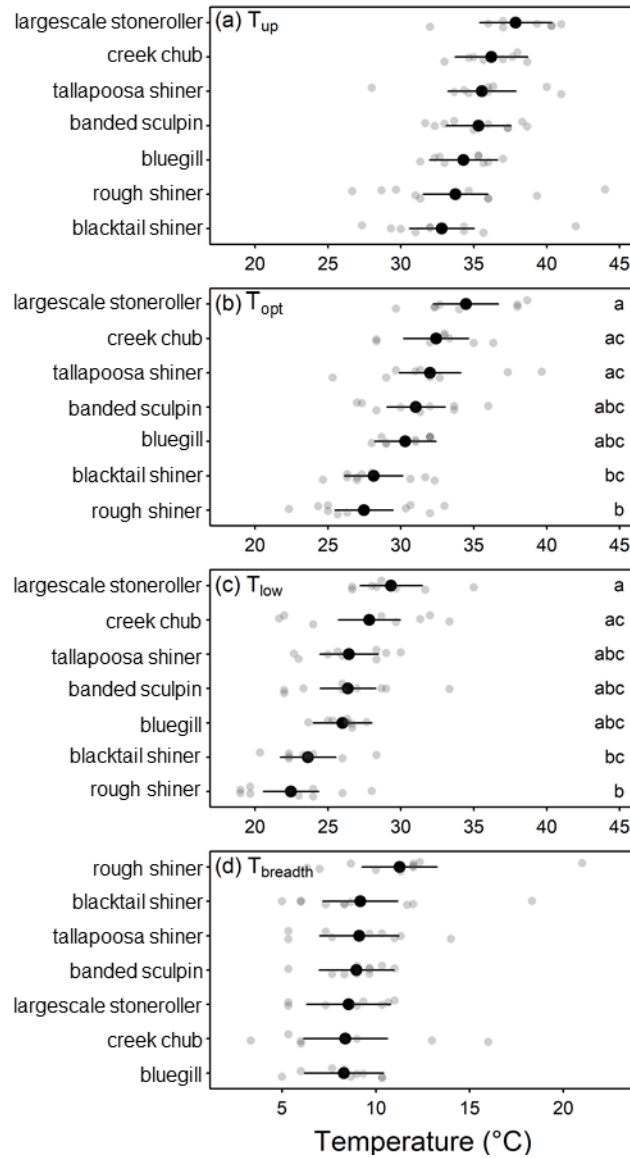


Figure 4.3. Mean and 95% confidence intervals for (a) upper limit of the optimum enzyme activity range (T_{up}), (b) optimum temperature for enzyme activity (T_{opt}), (c) lower limit of the optimum enzyme activity range (T_{low}), and (d) breadth of the optimum range for enzyme activity for each species ($T_{breadth}$). Bars with different letters within a panel indicate significant differences among species for that response variable.