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Physiological Ecology of Four Endemic Alabama Species and the Exotic Asiatic Weatherfish, *Misgurnus anguillicaudatus* (Cantor, 1842)

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Abstract

The occurrence of Asiatic Weatherfish, *Misgurnus anguillicaudatus*, in Alabama, a state known for its rich biodiversity, has generated concern among conservation managers. The current study used respirometry techniques to investigate the effects of increasing temperature on four native southeastern fishes (one cyprinid, two percids, and one elassomid) and the non-native *M. anguillicaudatus*. A minimum of five individuals of each species were used, and three experimental temperatures were chosen to represent spring and summer averages of northeast Alabama streams (15, 20, and 25°C). Overall, mean standard metabolic rates (SMRs) for *M. anguillicaudatus* were low (97.01, 127.75, and 158.50 mg O_2 $kg^{-1}h^{-1}$ at 15, 20, and 25°C, respectively); *M. anguillicaudatus* was the only species for which SMR did not significantly increase with temperature (p = 0.467). In contrast, mean SMRs for all native species examined were higher than *M. anguillicaudatus* rates at a given temperature, and mean SMRs for *Cyprinella caerulea*, *Etheostoma brevirostrum*, and *Etheostoma ditrema* exhibited significant increases in SMR when temperatures were increased (e.g. 403.46, 704.42, and 1150.03 mg O_2 $kg^{-1}h^{-1}$ at 25°C, respectively) (p < 0.01). *Elassoma zonatum* displayed highly significant increases in SMR when temperature increased from 15-20°C (p < 0.001). Overall, the abiotic tolerances of *M. anguillicaudatus* may facilitate further establishment that could lead to negative impacts on native species.

Keywords

Physiology, respirometry, temperature adaptation, metabolism, abiotic tolerance, climate change

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INTRODUCTION

Alabama is one of the most biodiverse states in the United States and includes numerous endemic aquatic species. Due to its mild climate and many diverse physiographic regions, the state presents favorable conditions to support native as well as non-native species (Bomford et al., 2010). At least 61 non-native fish species, whether through intentional or accidental introductions, occur in Alabama (Boschung and Mayden, 2004). Many non-native species, such as Grass Carp, *Ctenopharyngodon idella*, and Mosquitofish, *Gambusia affinis*, are considered non-injurious to natives whereas other species, such as Lionfish, *Pterois volitans/Pterois miles*, and Brown Trout, *Salmo trutta*, are well known for their potentially negative impact on southeastern aquatic ecosystems (Côté et al., 2013; McKenna et al., 2013).

The Asiatic Weatherfish, *Misgurnus anguillicaudatus*, known in the aquarium industry as Dojo Loach and Weather Loach, has only recently been reported in Alabama (White and Meade, 2015). The presence of individuals spanning multiple size classes at reported sites suggests successful recruitment is occurring and that the species is established (White and Meade, 2015). At some sites, *M. anguillicaudatus* co-occur with sensitive endemics like the Coldwater Darter, *Etheostoma ditrema* (IUCN: En), and should it continue its range expansion *M. anguillicaudatus* could potentially interact with imperiled populations of Blue Shiners, *Cyprinella caerulea* (IUCN: En), Holiday Darters, *Etheostoma brevirostrum* (IUCN: Vu), and Pygmy Sculpins, *Cottus paulus* (IUCN: Cr).

Impacts to ecosystems are a risk with non-native introductions. Ecological studies pertaining to range expansion and investigations into competition between natives and non-natives are well documented. Less studied are potential abiotic tolerances between species, such as advantages that one species may have during environmental perturbation (Leuven et al., 2011, Walther et al. 2009). One major abiotic factor of interest to aquatic environmental scientists is temperature. As thermal regimes worldwide are altered due to climate change, alterations in water flow, and other anthropogenic influences, native species may be at risk as conditions shift in favor of non-natives (Bunn and Arthington, 2002, Rahel and Olden, 2008, Sorte et. al., 2012, Xenopoulos and Lodge 2006). For example, many native fishes are adapted to tolerating and surviving narrow temperature ranges (Ficke et al., 2007). Comparatively, many invasive fish species tend to be generalists adapted to surviving over wide temperature ranges (Cucherousset and Olden, 2011). Changes in environmental temperatures can result in substantial deviation of physiological homeostasis in stenothermal fishes. Information on adaptive physiology of native and invasive species in response to fluctuating abiotic

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conditions, such as temperature, can be used to assess adaptability, biotic potential (i.e., growth/reproductive success), as well as potential future recruitment or expansion of populations (Wikelski and Cooke, 2006, Sorte et. al., 2012, Diaz et. al., 2007).

The goal of this study was to examine temperature responses of *M. anguillicaudatus* in comparison to four fishes endemic to Alabama. Specifically, we measured oxygen consumption rates among the species when acclimated to different temperatures to determine if standard metabolic rates were different among the species. Overall, our goal was to use measurements of metabolism as an indicator of thermal performance. Based on the hypothesis that invasive species should be more tolerant to a wide range of temperatures, we would expect that native species should exhibit a greater metabolic response to increases in temperature than *M. anguillicaudatus*.

METHODS

Collection and Handling of Animals

A total of 30 fish, including 6 *M. anguillicaudatus*, 5 *C. caerulea*, 5 *E. brevirostrum*, 5 *E. ditrema*, and 9 *Elassoma zonatum* (Banded Pygmy Sunfish), were collected and used for this project. Due to the imperiled nature of selected species, few individuals of each species were collected.

Fishes were collected during early fall using a combination of electrofishing, dipnetting and/or seining techniques and followed the US EPA rapid bioassessment protocols for fish surveys (Barbour et al. 1999; O'Neil and Chandler 2005). *Elassoma zonatum* and *E. ditrema* were collected from Blue Eye and Goray springs, respectively. *Misgurnus anguillicaudatus* were collected from both sites. *Etheostoma brevirostrum* and *C. caerulea* were collected from Shoal and Choccolocco creeks, respectively. Federal and state permits for the surveying and collecting of aquatic animals are maintained by Dr. Meade. (JSU NIH IACUC #A3680-01; ADCNR collection permit #2010000003168680, USFWS incidental catch permit #TE52836A-0).

In the lab, fish were held in 11.4 L aquariums at 18-20°C with a 12:12 hr light/dark cycle. Fish were fed a variety of food items including commercially prepared feeds (e.g. Omega One™ goldfish pellets, Aqueon™ goldfish pellets, Hikari™ micro pellets, and Tetra™ bloodworms). Once in lab tanks and during acclimation periods, any signs of stress (i.e., abnormal swimming behavior, flashing, etc.) were noted. After an initial 7-10 days of acclimatization to lab conditions, temperatures were decreased/increased at 0.5°C/hr intervals until

reaching experimental temperatures. Experimental temperatures of 15, 20, and 25°C were chosen to reflect approximate middle to high annual summer temperatures observed in regional streams of the Alabama endemics (Figure 1). During acclimation to the 25°C temperature treatment, *E. zonatum* exhibited high mortality above 22°C. Because we did not intend to harm or kill the fish, *E. zonatum* were acclimated to 15 and 20°C only. Fish were maintained for another 14 days at the experimental temperatures before measurements were recorded to ensure acclimation to the desired temperature. Upon completion of the study, fish were acclimated to original environmental temperatures (18-20°C) and released near their approximate original collection site. All animals in this study were collected and handled according to accepted protocols.
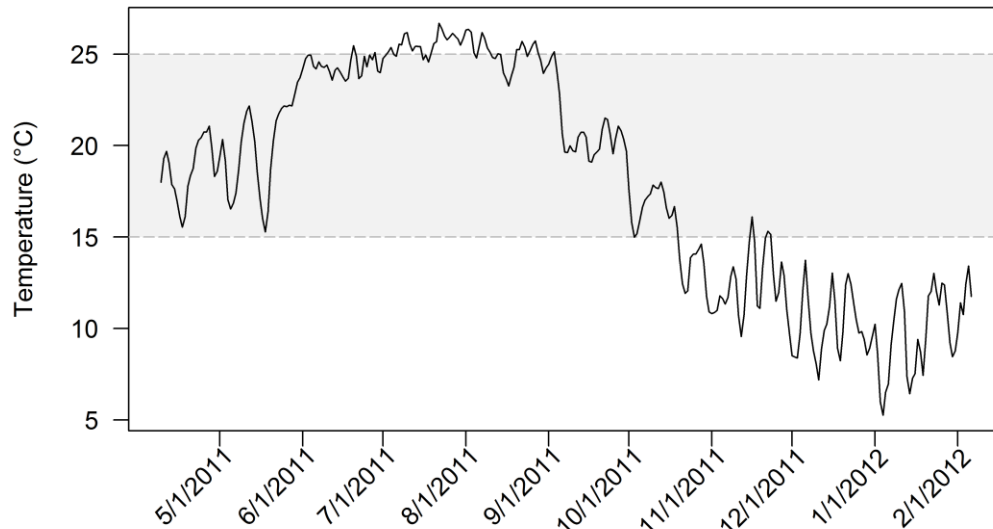

Figure 1. Mean daily temperatures of Choccolocco Creek (site 33.789747, -85.659987) from April 9, 2011 through February 6, 2012. The shaded area denotes the selected treatment range for this experiment.

Respirometry

Weight-specific standard metabolic rates (SMRs) were recorded for each fish at the experimental temperatures using an intermittent flow respirometer (AR1500, Loligo Systems™, Tjele, Denmark). Temperature treatments were repeated on individuals to quantify within individual variation in metabolic responses to increasing temperatures. Fish were isolated and feeding suspended 24hr prior to recordings to normalize metabolic rates due to digestive condition. Immediately prior to measurements, fish were weighed and placed inside the sealed respirometry chamber (0.294 L). Data collection began immediately after a short flush cycle (60-90 s). The recording cycle was set for 30 min and measurements were recorded at normoxic oxygen tensions (>15 kPa pO_2) for a minimum of four

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cycles or until similar and stable oxygen consumption rates were recorded and for two cycles thereafter. Values prior to stabilization were not used in analyses. To account for possible diurnal changes, SMRs were recorded between 0900 and 1600 hour in the light. SMRs are reported as $\dot{M}O_2$ (mg O_2 consumed/kg^{-1} hr^{-1}) and mean values were determined for a minimum of five fish from each species at each temperature.

Analysis

We used a linear mixed effects model (Zuur et. al., 2009) to investigate differences in species-specific metabolic responses to increasing temperature using the R package "nlme" (Pinheiro et. al., 2016). Species and temperature were treated as fixed effects and individual was treated as the random factor nested within each species. Each individual was informed by three replicated metabolic trials at each temperature. We included an interaction between the fixed effects of species and temperature to test the hypothesis that different species have different metabolic responses to increasing temperature. Two random effect structures were investigated: (1) random intercepts for each individual and (2) random intercepts and slopes for each individual. These two models were compared with a likelihood ratio test. A significance level of 0.05 was used for all tests of significance.

Sensitivity

It was important to investigate the sensitivity of our observed qualitative differences in metabolic rates to potential biases associated with use of a small sample size for our analysis. The primary concern with a small sample would be that the detected metabolic responses were systematically different from the true population in such a manner that we were led to draw spurious conclusions (e.g., detected different metabolic responses to temperature between species that were more extreme than the true differences). By conducting a sensitivity analysis, we could determine how biased the data obtained from the small sample included in the analysis would need to have been from the true population for the same qualitative conclusions to no longer be drawn. This can be conducted by assuming the true metabolic response to temperature (slope in the repeated measure ANCOVA model) for the non-native *M. anguillicaudatus* was X% steeper than detected and each of the native species' metabolic responses were Y% shallower than detected. Null hypothesis testing can then be conducted assuming these "true" effects from the sensitivity analysis. A conservative approach to this analysis would be to use the same variance of the effects that were estimated from the real data (i.e., smaller variances would likely result in rejection of the null hypotheses). If it is determined that the qualitative inferences are the same across a reasonably wide range of X and Y percent biases as those made using the

estimates from the sample data, it can then be concluded that the inferences drawn here are robust to inaccuracies inserted into the analysis by the small sample.

RESULTS

Respirometry

Once placed in the respirometry chamber, fish were typically active as they acclimatized to their new surroundings. Metabolic rates of most fish stabilized within 2 or 3 cycles (i.e., within 30-60 min) after placement in the chambers. Species- and individual-specific SMR mean responses to increasing temperature are presented in Figure 2. The random slope and intercept model was selected for inference over the random intercept-only model as the likelihood ratio test suggested strongly that it was justified (χ^2 = 53.24, df = 2, p < 0.001). SMRs for the four native species were more sensitive to increasing temperatures than for the *M. anguillicaudatus*. The only species for which the effect of temperature on SMR was not significant was *M. anguillicaudatus* (p = 0.467; Figure 2), whereas all native species had effects that were significantly greater than zero (maximum p = 0.01, *C. caerulea*; Figure 2, Table. 1). Species-level fixed effects are presented in Table 1, which shows both the significance in the hypothesis test for zero effect and for significance in difference in effects between *M. anguillicaudatus* and each native species. The effect of temperature on SMR was smallest for *M. anguillicaudatus* (mean = 6.15 $\dot{M}O_2$/1°C, SE = 8.43) and largest for species *E. zonatum* (mean = 197.57 $\dot{M}O_2$/1°C, SE = 8.05).

Sensitivity

After the sensitivity of the null hypothesis tests to small sample size was conducted, it was clear that our analysis was generally robust to a wide range of biases in the estimated effects (Figure 3). For the species comparison of B − A, the results were never significant as the interaction effect was assumed smaller (all combinations were dark grey), which would be expected as the originally estimated effect was insignificant. For the species comparison of E − A, we found that even if we underestimated the slope for species A by 100%, we would have needed to have overestimated the species E slope 82% to no longer make the same conclusion at α = 0.05 and by 84% at α = 0.1. As this was the strongest difference we detected, the biases would need to have been smaller for the same conclusions to have not been made. In each of the comparisons, the conclusions were more sensitive to biases in the estimated temperature effect for the native species than the non-native species, as the slopes of the former were larger and thus a percent decrease went a longer way in reducing the interaction effect to zero than did the same percent reduction in the native species.

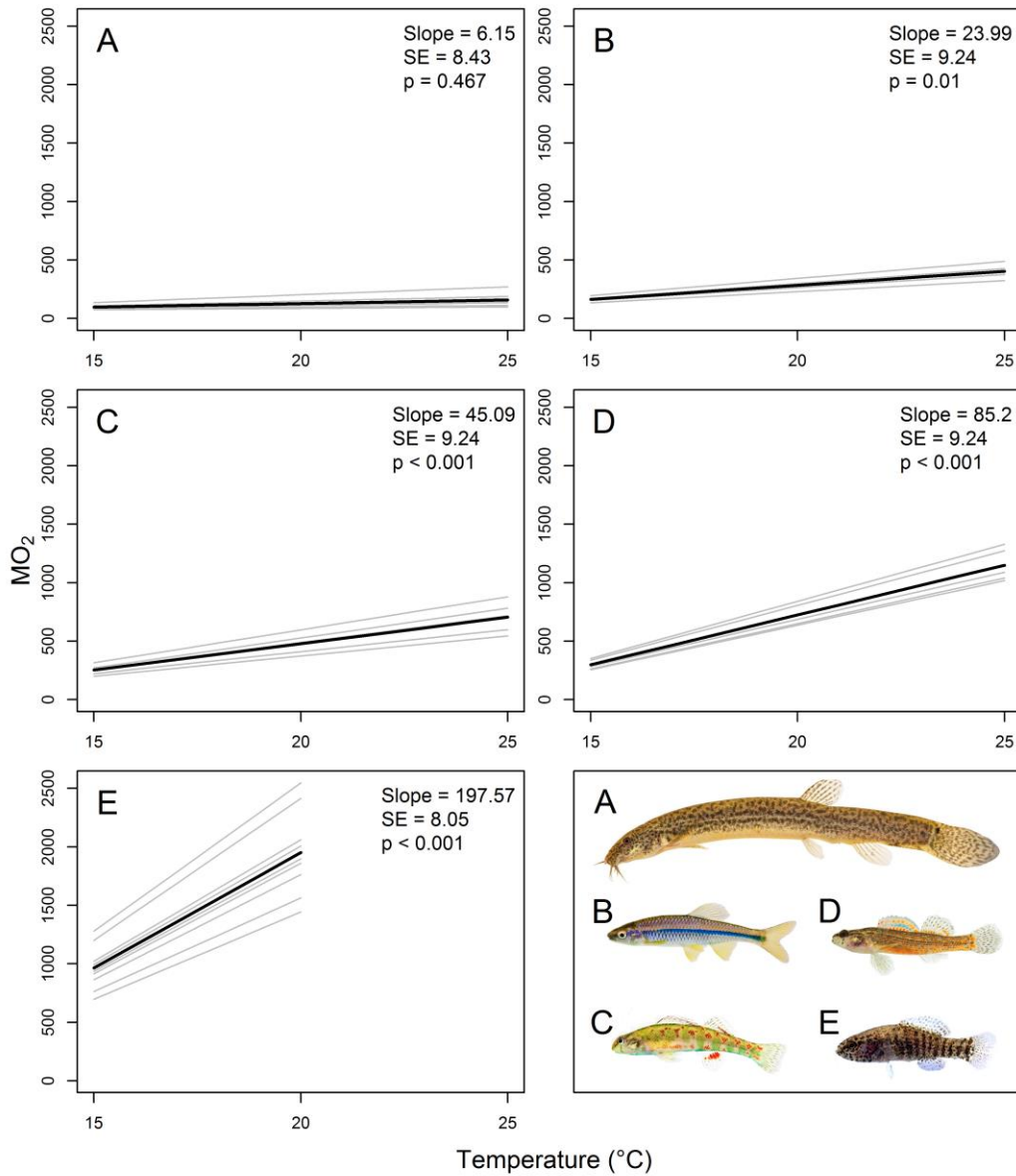Figure 2. Species specific SMRs (shown here as $\dot{M}O_2$: mg O_2 consumed/kg^{-1} hr^{-1}) responses to increases in temperature: A) *M. anguillicaudatus* B) *C. caerulea* C) *E. brevirostrum* D) *E. ditrema* E*) E. zonatum*. Black lines indicate fixed effects (species means), and grey lines are individual random effects. P-values test for differences in zero effect. Species images are not to scale.

Table 1. Fixed effects of 1°C increase in temperature on the metabolic rate of each of the five species tested. The column titled "Slope" is the slope of each species-specific line (*p*-value tests if different than zero slope) and the column titled "Difference in Slope" is the difference in slopes between species A and other species tested. Significance codes are: ** $0.001 < P \leq 0.01$ and *** $P \leq 0.001$.

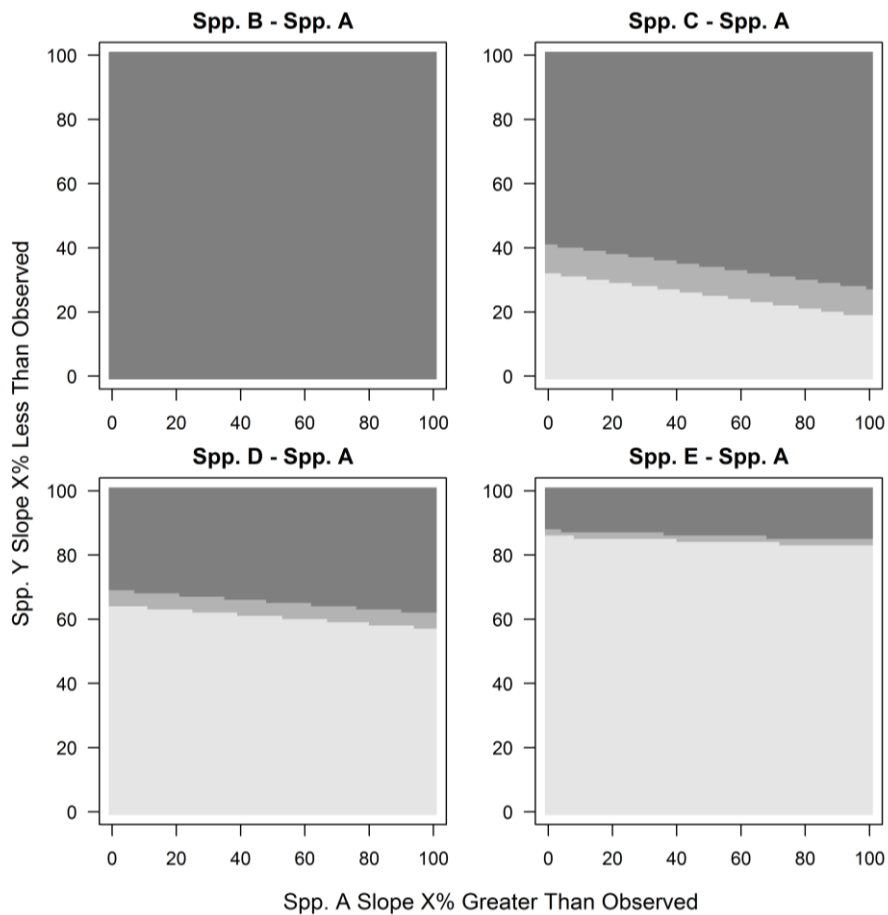| Species	Slope	Difference in Slope
M. anguillicaudatus	6.15 (8.43)	-
C. caerulea	23.99 (9.24) **	17.84 (12.51)
E. brevirostrum	45.09 (9.24) ***	38.94 (12.51)**
E. ditrema	85.20 (9.24) ***	79.05 (12.1)***
E. zonatum	197.57 (8.05) ***	191.42(11.66)***

Figure 3. Surface plots showing the hypothetical *p*-value that would be obtained from null hypothesis tests assuming biases of various sizes in the respective species' metabolic responses to increasing temperature. The lightest grey regions indicate *p*-values significant at $\alpha = 0.05$, the medium grey regions indicate *p*-values significant at $\alpha = 0.1$, and the darkest grey regions indicate *p*-values greater than 0.1. The y-axis in each plot indicates the percent positive bias assumed for our sample in each of the four native species (B – E) and the x-axis indicates the percent negative bias assumed for the sample of the non-native species (A). Species letters are the same as in Figure 2.

DISCUSSION

Metabolic data from this study on *M. anguillicaudatus*, in comparison to native endemics, revealed two major points. First, SMRs of *M. anguillicaudatus* were lower at any given temperature compared to Alabama endemics, and were, overall, lower at their highest acclimation temperature than most natives at their lowest acclimation temperature. *Misgurnus anguillicaudatus* is considered a benthic species and has been reported as relatively docile in captivity (Logan et al., 1996). As such, its resting metabolic rate at any temperature might reflect its fossorial lifestyle in comparison to more active Alabama endemic species. Second, *M. anguillicaudatus* appear to tolerate increased temperatures and may exhibit an adaptive advantage over native Alabama species, particularly spring-dwelling species such as the *E. zonatum* and *E. ditrema*. Based on these results, increased environmental temperatures would favor the invasive, eurythermal *M. anguillicaudatus* as energy demands are substantially increased in stenothermal endemics. If environmental resources decline concomitantly with increased temperatures, the adaptive advantage of *M. anguillicaudatus* would intensify (Sorte et. al., 2012, Urquhart and Koetsier, 2014). Although an oxy-caloric equivalent is not available for the endemics or *M. anguillicaudatus* from this study, it is evident based on observed activity levels that the daily caloric needs of endemics are much higher than *M. anguillicaudatus* at any temperature examined. Shiners exhibited active schooling behavior, and darters would dart around the tank; *M. anguillicaudatus* was observably inactive until food presentation. Overall, at higher environmental temperatures we might expect reduced aerobic scope for our endemic species in comparison to the non-native *M. anguillicaudatus* unless natives successfully adapt aerobic scopes to include higher temperatures (Currie and Schulte, 2014, Nati et. al., 2016).

Over recent decades, numerous reports have been released pertaining to climate warming and its current and predicted effects on aquatic ectotherms (Portner et al., 2002, Clark et al., 2013). In the face of climate change, Portner and Farrell (2008) summarize the thermal window requirements for proper metabolic functions of fishes, stating that species with "wider windows," species with wider thermal performance breadth, will fare better in the face of change. Simply put, increases in temperature to non-optimal levels alter energy allocation of organisms away from reproduction and growth and, to extremes, could negatively affect recruitment and community structure (Barrionuevo and Fernandes, 1998, Leuvin, 2011). Aquatic animals tend to respond negatively to increasing temperatures, and eurythermal invasives appear to have a greater ability to respond to warming (Sorte et. al., 2012).

Regarding invasive species, Cucherousset and Olden (2011) mention adverse effects to population and community structure, even changing demographics and community structure of an affected ecosystem. In Europe, established exotic species have prevented full recovery of native species in areas of thermal pollution, and decreased overall biodiversity-related stream status (Leuvin 2011). While no competition has yet been observed, any direct effect the presence of *M. anguillicaudatus* may have on unique or currently threatened endemic Alabama species could be compounded by abiotic tolerances and their ability to readily adapt to fluctuating environmental conditions, specifically temperature. Long-term successes of non-natives are more likely to occur in altered ecosystems, and maintaining thermal regimes for natives in the face of change could be possible in altered ecosystems through restoration and management (Bunn and Arthington, 2002, Lawrence et. al., 2014). As the *M. anguillicaudatus* invasion is still in its infancy, and based on the small sample sizes in this study, more information is needed to assess the impact on Alabama ecosystems.

The sensitivity analysis revealed that, although our sample sizes were small, our conclusion that the selected native species were less tolerant to increases in temperature compared to the non-native *M. anguillicaudatus* was generally robust to biases due to non-representative sampling. We found that our conclusions were robust to biases ranging from 18% to 82% for the native species and up to 100% (and higher) for the non-native *M. anguillicadatus*. These high values suggest that our conclusions are valid even if the samples that were used were systematically biased. That being said, we encourage one to consider the sampling process that gave rise to the observations used in this study. Fish were sampled using primarily backpack electrofishing. It is difficult to imagine a reasonable explanation for why the small number of fish of each species included in the study would represent a systematically biased sample from the population of responses. While electrofishers are known to be size/species/habitat selective, it is highly unlikely that the electrofisher would select for individuals with systematically higher or lower metabolic responses (slopes). Nonetheless, even if the sampling instrument did collect a biased sample, collecting more fish would not fix this potential bias.

Important to our discussion is the data we collected on the *C. caerulea* and *E. brevirostrum*, from the Choccolocco Creek watershed. Choccolocco Creek is home to numerous imperiled species, including the aforementioned species, and is part of the region in North America with the highest aquatic biodiversity and number of imperiled species (O'Neil and Chandler 2005). While some studies involve intense sampling techniques could cause mortalities to sensitive species (either intentional or accidental), the use of intermittent flow respirometry is a non-

lethal method of obtaining valuable physiological data. Although not ideal, when certain species are unobtainable or collection of a species is discouraged, the use of sister taxa may be explored. Currently, respirometry equipment is available to measure a range of metabolic rates directly in the field (e.g. Foxbox™, Sable Systems International), and by removing transportation to a laboratory setting from the experimental methods, investigators could eliminate the need to acclimate individuals to artificial temperatures or any handling stress due to transportation to a laboratory by collecting data *in situ* (Farrell et al, 2003). Better representation of diel and seasonal fluctuations of abiotic factors using these methods could give scientists an alternative view of multiple influential factors on the physiology of aquatic animals. In any case, understanding those physiological and environmental adaptations of species will help us to better predict the impacts of global change on future populations, both native and non-native, and help define management options for an ecosystem.

ACKNOWLEDGEMENTS

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