DETERMINING THE EFFECTS OF THERMAL INCREASES ON STREAM FISHES OF THE OUACHITA MOUNTAIN ECOREGION

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Abstract: Altered thermal regimes affect stream fishes both directly and indirectly, influencing their distribution, growth, reproduction, and survival. Increasing temperatures and growing demand for freshwater highlight the need to predict stream fish responses to increasing stream temperatures; however, we lack information on the thermal tolerances of many warmwater stream fishes. The objectives of my thesis were to 1) determine critical thermal maxima (CTMax) of 10 stream fishes in the Ouachita Mountain ecoregion, and 2) assess stress response by six stream fishes via whole-body cortisol after prolonged exposure to elevated water temperatures. First, I determined CTMax for 10 stream fishes of the Ouachita Mountain ecoregion (20°C acclimation, 2°C per h heating rate). Critical thermal maxima ranged 34.0-38.3°C among species. Blackspotted Topminnow *Fundulus olivaceus* had the highest CTMax and Orangebelly Darter *Etheostoma radiosum* and Channel Darter *Percina copelandi* had the lowest CTMax. Benthic fishes had the lowest CTMax, and the only surface-dwelling species tested had the highest CTMax. The CTMax values determined in my study are useful for developing proactive conservation and management strategies aimed at maintaining or improving stream-thermal conditions, and predicting species most susceptible to thermal change. Lastly, I measured whole-body cortisol concentrations of six stream fishes of the Ouachita Mountain ecoregion after prolonged exposure to elevated water temperatures. I acclimated fishes to 20.0°C, increased temperature 1°C per d, and exposed fishes to treatment temperatures (control, 27.0°C; experimental, 32.0°C) for 14-d. I hypothesized that fishes exposed to 32.0°C would show increased whole-body cortisol concentrations and that levels of stress response would relate to thermal sensitivity (i.e., CTMax, Chapter 2). I found no significant differences in stress response by fishes between the two treatment temperatures. Results from my stress trials represent basal whole-body cortisol concentrations for all my study species except Highland Stoneroller *Campostoma spadiceum*. Despite apparent tolerance of, or acclimation to, stressful thermal conditions, fishes may suffer from decreased reproduction or growth; thus, future studies should investigate the tertiary stress response to chronic thermal stress in these species. Whole-body cortisol levels from this study may serve as a baseline for monitoring stress in small-bodied stream fishes inhabiting altered stream systems.

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CHAPTER I

INTRODUCTION

The natural flow and thermal regimes shape river ecosystems through abiotic and biotic processes. Both the flow and thermal regimes can be described by the magnitude, frequency, duration, timing, and rate of change of discharge (Poff et al. 1997) or temperature patterns (Caissie 2006). The flow regime moves water through the landscape, transporting sediments and nutrients downstream and across the floodplain (Hynes 1975; Junk et al. 1989). Further, deposition of sediments provides habitat for stream biota (Poff et al. 1997). A range of flow magnitudes is necessary for various biotic processes to occur. For example, over-bank flows are necessary for some stream fishes to successfully reproduce (e.g., floodplain spawning in Common Carp Cyprinus carpio, Jones and Stuart 2009; and Alligator Gar *Atractosteus spatula*, Kluender et al. 2017). Alternatively, low flows control plant diversity in the riparian area of desert streams (Stromberg et al. 2005), and provide suitable rearing habitat for a variety of stream fishes (Holland and Huston 1984; Jurajda 1999; Massicotte et al. 2015). Like the flow regime, the thermal regime controls the suitability of some habitat features (Constantz 1998; Ferencz and Cardenas 2017). The thermal regime determines the distribution and abundance of stream biota via several mechanisms that have been described as lethal, stressing, controlling, limiting, masking, and directing features (Coutant 1976; Rahel and Olden 2008). Just as a range of flow magnitudes is necessary for ecosystem function, a range of temperatures is necessary to support.

completion of the life cycle. For example, age-0 Smallmouth Bass *Micropterus dolomieu* have an optimal growth temperature of 29°C while the adult optimum is 22°C (Whitledge et al. 2006); thus, streams with patchy thermal environments benefit several life stages (Brewer et al. 2007; Brewer 2013). The natural patterns of stream flow and temperature are often disrupted by anthropogenic changes.

Humans alter the flow and thermal regimes of rivers via direct consumption of water, and indirectly by altering flow and thermal patterns through landscape changes. Globally, growing populations are increasing freshwater demand, reducing overall streamflow, and altering discharge timing. In the United States, approximately 870 million m3 of surface water and 280 million m3 of groundwater are consumed annually for public, domestic, agricultural, and industrial uses (Maupin et al. 2014). The withdrawal of groundwater and surface water reduces stream flows (Xenopoulos 2005) and consequently, increases stream temperatures (Caissie 2006). The effects of altered discharge and temperature regimes are exacerbated in urban areas, where nearly half of the world's population resides (Cohen 2003). Urban areas have greater amounts of impervious surfaces and are associated with higher peak flows, truncated baseflows (O'Driscoll et al. 2010), and temperature surges (e.g., runoff-induced temperature surges of 7.4°C lasting 7.6 h, Nelson and Palmer 2007). Damming rivers is another wide-spread practice in North America (~80,000 dams, Graf 2001) that alters flow and temperature regimes. Hydroelectric power generation disrupts the natural frequency and timing of high and low magnitude flows (Harnish et al. 2014). Downstream of flood-control dams, flows are often artificially stabilized or decreased in magnitude (Graf 2006) with corresponding changes to the temperature patterns (Lessard et al 2003). Climate change will exacerbate observed flow and thermal patterns, but the effects of climate change will vary regionally. For example, precipitation in the United States has increased 5-10% since 1900, primarily as extreme events. This increase in precipitation is associated with increasing winter and decreasing summer streamflows (Dore 2005; Olivera and DeFee 2007).

Globally, increases in air temperatures are expected to raise average water temperatures 0.8-1.6°C over the next 100 years (Morrill et al. 2005; Van Vliet et al. 2013). In the United States, Kaushal et al. (2010) found stream temperature increases of 0.009-0.077°C/year associated with increasing atmospheric temperatures. Despite the intertwined relationship between flow and stream temperature, the deleterious effects of altered thermal regimes have largely been overshadowed by flow alteration.

Altered thermal regimes influence the distribution, growth, reproduction, and survival of fishes. The availability of suitable temperatures often limits the distribution of fishes (Comte and Grenouillet 2013; Comte et al. 2014). For example, the natural distribution of riverine Smallmouth Bass of the Ozark Highlands ecoregion is related to changes in springflow volume (Brewer et al. 2007). Similarly, Arkansas Darter *Etheostoma cragini* is associated with spring locations (Miller 1984); however, decreases in groundwater seepage are related to the decline of Arkansas Darter (Eberle and Stark 2000). Both metabolism and fish growth increase with water temperature until a threshold is reached where growth scope is reduced (Wootton 1990; Allan & Castillo 2007). For example, at water temperatures > 30°C, metabolic costs exceed maximum daily consumption for Smallmouth Bass and weight loss is expected (Whitledge et al. 2002). Water temperature also affects the reproductive success of fishes. Temperature is one of many environmental cues that initiates spawning (e.g., Shortnose Sturgeon *Acipenser brevirostrum*, Kynard 1997), but if the timing of these cues is disrupted, spawning may never occur (Auer 2004) or ontogeny may be compromised. For example, embryonic survival rates of Common Bream *Abramis brama* decreased with increasing water temperature (Targońska et al. 2014). Lastly, lower and upper temperature limits dictate where a species can survive (Coutant, 1976; Rahel and Olden 2008). The greater range of Channel Catfish *Ictalurus punctatus* compared to that of Largemouth Bass *Micropterus salmoides* is reflected in its broader thermal tolerance (Currie et al. 1998; Page and Burr 2011). Fishes with lower thermal tolerance are often replaced by more tolerant fishes following drought-induced temperature increases (Magoulick and Kobza 2003; Matthews and Marsh-Matthews 2003). Combined, acutely lethal and chronic stressors shape populations and communities.

Prolonged exposure to various stressors affects cortisol levels, and can negatively affect fish physiology. Production of corticosteroid hormones is a common stress response (Wendelaar Bonga 1997; Mommsen et al. 1999). For example, Pejerry *Odontesthes bonariensis* reared at high temperatures had elevated whole-body cortisol concentrations (Hattori et al. 2014). Mozambique Tilapia *Oreochromis mossambicus* exposed to stressful holding conditions for 24 h showed elevated plasma cortisol and glucose concentrations and mobilization of amino acids. This response may be a mechanism for coping with increased energy demand in stressful conditions (Vijayan et al. 1997). Presence of cortisol promotes the breakdown of skeletal muscle for energy, negatively affecting growth (Van Der Boon et al. 1991). Fish reproduction is also affected by cortisol (Barton and Iwama 1991; Pankhurst and Van Der Kraak 1997; Schreck et al. 2001). For example, Campbell et al. (1994) found that chronic confinement stress was associated with elevated cortisol, decreased egg size, and decreased gamete quality in Rainbow Trout *Oncorhynchus mykiss*. Cortisol may act as an immunosuppressant. Espelid et al. (1996) demonstrated decreased LPS-induced mitogenesis of leukocytes in Atlantic Salmon *Salmo salar* dosed with cortisol, indicating a decreased ability to protect against infections. Similarly, elevated cortisol and decreased disease resistance were simultaneously demonstrated in Fancy Carp *Cyprinus carpio* following crowding stress (Yin et al. 1995). Finally, elevated cortisol is related to a fish's ability to survive chronic stressors. Chronic elevation of plasma cortisol in Brown Trout Salmo trutta and Rainbow Trout was associated with increased bacterial and fungal infections and instantaneous mortality rate (Pickering and Pottinger 1989). Understanding the effects of chronic stress on fishes is useful for the development of stream fish management and conservation strategies.

In order to proactively protect ecosystem health and prevent losses in biodiversity, we must understand the thermal limits of aquatic organisms. Knowing how fishes interact with their thermal environment will enable managers to make appropriate stream water-management decisions (e.g., allocations, water withdrawals, environmental flows, etc.). The goal of my thesis is to provide information on the thermal limits of stream fishes so that managers may develop alternative stream-temperature management strategies. Specifically, my objectives are to 1) determine critical thermal maxima of 10 stream fishes in the Ouachita Mountain ecoregion, and 2) determine if six stream fishes exhibit a stress response via whole-body cortisol after prolonged exposure to elevated water temperatures. Often, stream temperatures exceed the thermal limits of fishes, potentially having lethal consequences. Critical thermal maxima determined in my first objective will serve as conservative estimates of the lethal thermal limits of the species in my study and allow species to be ranked in order of thermal sensitivity. In my second objective, I examine the possibility of sublethal effects of chronic thermal exposure. These data may be used to develop thermal exposure limits and evaluate the benefits of various reservoir releases for mitigation of chronic stress caused by thermal exposure. The usefulness of these data is especially apparent in the Ouachita Mountain ecoregion where extensive hydrologic alterations have biological consequences.

The Ouachita Mountain ecoregion hosts a diverse aquatic fauna, but is also subject to hydrologic and thermal alterations. This ecoregion harbors many endemic species (e.g., Ouachita Rock Pocketbook *Arkansia wheeleri*, Vaughn and Pyron 1995; and Leopard Darter *Percina pantherina*, Dauwalter et al. 2008) and is a hotspot for freshwater biodiversity (Master et al. 1998). Sardis Dam was constructed in 1983 to impound Jackfork Creek, a major tributary of the Kiamichi River (Pyron et al. 1998). This impoundment accounts for approximately 25% of the drainage area of the Kiamichi River (Vaughn et al. 2015). Lack of releases from Sardis Dam and extended periods of drought have caused stream drying, fragmentation, and increased water temperatures in the

Kiamichi River contributing to high mortality rates (Spooner and Vaughn 2000) and reduced reproduction for freshwater mussel fauna (Galbraith et al. 2010). Despite decreased water volume and losses of aquatic biota, additional water diversions from the Kiamichi River to central Oklahoma have been approved (Choctaw Nation of Oklahoma and Chickasaw Nation Water Settlement 2016). Extensive alteration and losses of aquatic biota highlight the importance of characterizing the thermal limits of native fauna in the Kiamichi River.

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CHAPTER II

DETERMINING THE CRITICAL THERMAL MAXIMUM OF FISHES OF THE OUACHITA MOUNTAIN ECOREGION

ABSTRACT

Stream temperatures play a key role in determining the distribution and abundance of stream fishes. Critical thermal maximum (CTMax) effectively evaluates acute thermal tolerance, and is useful for reflecting the sensitivities of stream fishes or a ranking of thermal tolerances. My study objective was to determine CTMax for several stream fishes and determine if habitat use by these fishes was related to their thermal tolerance. I determined CTMax for 10 stream fishes of the Ouachita Mountain ecoregion using an incomplete block design with a survival control. Each block consisted of up to six species, each represented by one individual. I replicated the experiment 10 times for each species. I developed a generalized linear mixed model (GLMM) where CTMax was the dependent variable and my independent variables were species as a fixed effect and trial as a random effect. I used a Tukey Kramer Honest Significant Difference post-hoc test to assess where significant differences within the GLMM occurred. I acclimated fishes in the laboratory to 20.0°C for two weeks, exposed them to a 2.0°C per h water temperature increase, and determined at what temperature individuals exhibited loss of equilibrium. My GLMM was significant for the fixed effect of species and CTMax ranged 34.0-38.3°C among species. Blackspotted Topminnow *Fundulus olivaceus* had the highest CTMax and Orangebelly Darter

Etheostoma radiosum and Channel Darter *Percina copelandi* were the most sensitive to acute thermal increases. Thermal tolerances related to habitat use where benthic fishes had the lowest CTMax, and the only surface-dwelling species tested had the highest thermal tolerance. However, CTMax was not different for many benthic and mid-column fishes: Common Logperch *Percina caprodes*, Dusky Darter *Percina sciera*, Slenderhead Darter *Percina phoxocephala*, Bigeye Shiner *Notropis boops*, Highland Stoneroller *Campostoma spadiceum*, and Steelcolor Shiner *Cyprinella whipplei*. The CTMax values are useful for developing proactive conservation and management strategies aimed at maintaining or improving stream thermal conditions, and predicting species most susceptible to thermal change.

INTRODUCTION

Temperature is a primary factor regulating the distribution and abundance of fishes and is controlled by both natural and anthropogenic landscape features. Some of the natural catchment characteristics that regulate stream temperatures include riparian coverage (Whitledge et al. 2006), spring-flow volume (Nichols et al. 2014), and atmospheric conditions (Johnson et al. 2014). For example, the distribution of riverine Smallmouth Bass *Micropterus dolomieu* of the Ozark Highlands ecoregion is related to changes in spring-flow contributions within a catchment (Brewer et al. 2007), but local groundwater seepage also regulates the use of habitat patches across stream reaches (Brewer 2013). Natural stream fragmentation caused by drought also relates to temperature increases and similar fish-assemblage shifts (Marchetti and Moyle 2001; Matthews and Marsh-Matthews 2003; Mas-Marti et al. 2010); however, human landscape alterations may exacerbate the effects of drought on stream fish populations (Perkin et al. 2015). Many human-induced landscape changes also affect stream temperatures including land-use change to urban (Nelson and Palmer 2007) and agricultural landscapes (Poole and Berman 2001), the construction of dams (Bunn and Arthington 2002), groundwater withdrawals (e.g., Arkansas Darter *Etheostoma cragini*, Eberle and Stark 2000; Arkansas River Shiner *Notropis giardi*, Perkin et al. 2010), and warm-water effluents (Coulter et al. 2014). Both urban and agricultural development tend to increase stream temperatures (Allan 2004). Dams contribute to lower or nonexistent downriver flows (Palmer et al. 2009), increased downstream temperatures (Gu and Li 2002; Norton and Bradford 2009; Olden and Naiman 2010), and result in shifts in fish-assemblage structure (Quist et al. 2005; Freeman and Marcinek 2006; García et al. 2011). Extensive thermal alterations occur within North American streams (Carlisle et al. 2011) and affect stream fishes via several mechanisms.

Alterations to the thermal regime of streams disrupt growth, reproduction, and survival of fishes by increasing metabolic costs, impeding spawning, and ultimately killing fish. For each species of fish, there is an optimal temperature at which maximum growth occurs (Beitinger and Fitzpatrick 1979). Increases beyond the optimal temperature lead to increased energy demand causing slowed growth or weight loss (e.g., Smallmouth Bass, Whitledge et al. 2002; Lumpfish *Cyclopterus lumpus*, Nytrø et al. 2014). Temperature also initiates spawning in several species (e.g., Pallid Sturgeon *Scaphirhynchus albus*, DeLonay et al. 2009; seven Australian fish species, King et al. 2016), influences reproductive effort (e.g., age-1 Pumpkinseed Sunfish *Lepomis gibbosus*, Masson et al. 2015), and reproductive success. For example, truncated warm winters are associated with reduced egg and larval quality and weak recruitment in Yellow Perch *Perca flavescens* (Farmer et al. 2015). Fundamentally, fishes require a particular range of temperatures in order to survive (Coutant 1976; Rahel and Olden 2008; Sandblom et al. 2016). Fish kills are commonly reported by management agencies, particularly during the summer months when water temperatures are high and discharge is low (Bailey 1955; Mundahl 1990; Hupfeld, et al. 2015). In order for fishes to persist, it is important to understand their thermal limits.

Several approaches are used to estimate the tolerances of fishes including statistical techniques, laboratory trials with a terminal or extreme physiological endpoint, physiological response, or genetic expression. Biesinger et al. (1979) used the 95th percentile of mean weekly water temperatures to estimate thermal tolerances of more than 300 North American stream fishes. Similarly, Wehrly et al. (2007) used maximum temperatures over a 1 to 63-d period at their warmest study locations to determine thermal tolerance of Brook Trout *Salvelinus fontinalis* and Brown Trout *Salmo trutta*. More commonly, thermal tolerance is assessed in a laboratory setting following acclimation to some temperature. Subsequent exposure to extreme temperatures occurs immediately (e.g., incipient lethal temperature, Fry et al. 1946), rapidly (e.g., 1-60°C/hr in lethal thermal method, Becker and Genoway 1979; and critical thermal maximum, Cowles and Bogert 1944), or gradually (e.g., 1°C/day in acclimated chronic exposure, Zale 1984; chronic lethal method, Beitinger et al. 2000) and causes loss of equilibrium or death. Rapid heating prevents fishes from acclimating to changing temperatures, whereas more gradual increases allow acclimation (Beitinger et al. 2000). More recent efforts examine exposure to thermal regimes and physiological responses to temperature. For example, Farless and Brewer (2017) examined the importance of daily thermal refugia to overall thermal tolerances of select stream fishes. Alternatively, other investigators use oxygen demand (Pörtner and Knust 2007), maximum heart rate (Ferreira et al. 2014), and cardiac protein abundance (Jayasundara et al. 2015) to evaluate the physiological responses to thermal increases. Lastly, recent efforts are focused on identifying gene expression to thermal increases (e.g., downregulation of protein processing genes in Lemon Damselfish *Pomacentrus moluccensis*, Kassahn et al. 2007; decreased cellular communication gene expression in Antarctic Eelpout *Pachycara brachycephalum*, Windisch et al. 2014). Despite the myriad of techniques available to evaluate thermal tolerances of aquatic organisms, these data are largely unknown for many warmwater stream fishes.

Predicting responses of stream organisms to changing thermal conditions is difficult. First, there is substantial uncertainty inherent in our climate predictions (Shepherd 2014). Current trends suggest extreme weather events (e.g., drought) will be more problematic than annual averages (Cook et al. 2015). However, we also lack information on the thermal tolerances of many warmwater stream fishes (Smale and Rabeni 1995; Lutterschmidt and Hutchison 1997; Beitinger et al. 2000). Use of critical thermal maximum (CTMax) effectively evaluates acute thermal tolerance while requiring a relatively small number of individuals. Therefore, my study objectives were to 1) determine CTMax for 10 stream fishes and 2) assess if fishes could be grouped based on similarities in thermal tolerances related to habitat use. If thermal tolerances of fishes reflect the prevailing conditions of the habitats they occupy, I would expect fishes with similar CTMax to occupy similar environments. My hypothesis was that species inhabiting surface/slack water habitats and pelagic habitats would have higher CTMax compared to species inhabiting more stable thermal environments (i.e., benthos).

METHODS

Study area. Fishes were collected from the Kiamichi and Glover rivers of the Ouachita Mountain Ecoregion, Oklahoma (Figure 1). The ecoregion receives 109-145 cm of precipitation, annually. The lithology of the region is primarily sandstone, shale, and chert resulting in coarse substrates in the headwaters, transitioning to lower gradient streams with finer substrates. The upland portions of the streams are relatively clear during baseflow conditions, but carry high suspended sediment loads during periods of high runoff. The lower portions of the rivers tend to be more turbid (Woods et al. 2005). The discharge patterns of the Kiamichi River are affected by reservoirs at two locations: Sardis Lake on Jackfork Creek, a major tributary, and downstream of the Jackfork Creek confluence, Hugo Lake impounds the mainstem. Mean annual flow in the Kiamichi River is approximately 45 $m^3 \cdot s^{-1}$.

Fish collection and acclimation. Fishes were collected in 2015 and 2016, transported to, and acclimated to laboratory conditions. I collected fishes using a seine (2.44 m in length, 1.83 m in height, with 0.3175 cm diameter mesh) that was pre-soaked in VidaLife (Western Chemical Inc., Ferndale, WA) to minimize handling stress (i.e., reduces friction on the fish). Collected fish were transported in stream water treated with non-iodized salt to 1% (10 g/L) to reduce stress (Swann and Fitzgerald 1992). Fishes remained in hauling containers for up to 12 h until the temperature of the hauling water reached that of the holding tanks, approximately 20.0°C. Fishes were then transferred to 190-L holding tanks covered with a screen on the top. I added airstones to all holding tanks to maintain dissolved oxygen >5 mg/L. Over the first 96 h, fishes were left undisturbed to recover from transportation stress. Brown Trout and Rainbow Trout recover from acute emersion and confinement within 24-48 h (Pickering and Pottinger 1989). Fishes remained in holding tanks where they were acclimated to laboratory conditions over a two-week period.

Following the initial 96 h, fish were fed and water-quality conditions were checked daily. I fed fish flakes (Wardley Advanced Nutrition Perfect Protein Tropical Fish Flake Food, Hartz Mountain Corporation, Secaucus, NJ) and bloodworms (Fish Gum Drops Floating Fish Food Bloodworms, San Francisco Bay Brand, Newark, CA) once daily to satiation (i.e., until fishes ceased eating). Unconsumed food was removed from aquariums daily via siphon. Ammonia, pH, and chloramine were checked twice daily. The temperature of the holding tanks was maintained at approximately 20.0°C. Ammonia was maintained <0.5 ppm. This level was only observed when new fish were added to the holding tanks, and for the first few days of lab acclimation. For the duration of acclimation and experimentation, ammonia was <0.25 ppm, pH was 8-8.5, and chloramine was zero. Water changes of approximately 30% were performed daily after the first 96 h of acclimation.

Critical thermal maximum. I determined the CTMax of ten stream fishes using an incomplete block design with an associated survival control (i.e., the control was not included in the final analyses). Each block consisted of up to six species, each represented by one individual. I replicated the experiment ten times for each species. I set up a system that routed water from a 189.27-L sump to six 37.85-L acrylic aquaria (Figure 2). Two airstones were added to the sump system to maintain dissolved oxygen above 5 mg/L. Water in the sump system was heated with a

5000-W Smartone heater (OEM Heaters, Saint Paul, MN). Because of the incomplete block design, I tested the species in 35 blocks. I randomly assigned species to aquaria, but haphazardly assigned individual fish to each aquarium (one fish per aquarium). I maintained a survival control using a separate sump system where fish experienced the same handling as the treatment fish, but were held at 20.0°C for the duration of the each trial. All fishes were held at 20.0°C for 24 h prior to the start of the experiment to allow acclimation to testing conditions and recovery from handling stress (Hutchison and Maness 1979; Pickering and Pottinger 1989). I only used adults in these trials because they are often less tolerant of higher temperatures (Pörtner and Farrell 2008), and would be more likely to represent thermal population bottlenecks.

I determined the thermal response of ten stream fishes belonging to three habitat guilds (Table 1). These species were chosen based on 1) abundance, 2) ease of capture, and 3) conservation status (i.e., Orangebelly Darter *Etheostoma radiosum*, Oklahoma's Comprehensive Wildlife Conservation Strategy). Habitat guilds were assigned based on descriptions provided in published references (Pflieger 1997; Miller and Robison 2004; Cashner et al. 2010). The "benthic" guild comprised species that primarily occupy the stream bottom. The "mid-column" guild comprised species that primarily occupy the water column. The "surface" guild had only one species that was classified based on its occupancy of the surface of slackwater habitats.

I completed my trials using one critical endpoint, loss of equilibrium (LOE) (Becker and Genoway 1979; Lutterschmidt and Hutchison 1997; Beitinger et al. 2000). During my trials, I increased water temperature 2°C/h until fish experienced LOE. I defined LOE as the point at which an individual lost the ability to maintain dorso-ventral orientation (Becker and Genoway 1979). None of the control fish experienced LOE.

Data analysis. The residuals of the CTMax data were normally distributed and analyzed using a generalized linear mixed model (GLMM). In the GLMM, temperature was the dependent variable and species (e.g., Orangebelly Darter, Steelcolor Shiner *Cyprinella whipplei*, etc.) was assigned as a fixed effect, and trial (i.e., each time a CTMax experiment was performed) was assigned as a random effect. I checked for homogeneity of variance of the fixed effect. The random effect, trial, was treated as a block effect to control for differences among trials that were not directly of interest. The random effect was assumed normally distributed as $N(0, \tau^2)$, where τ^2 is the population variance among levels of the random effect. I assessed significance at $\alpha \leq 0.05$. I then performed a Tukey Kramer Honest Significant Difference (HSD) post hoc test, adjusted for multiple comparisons, to determine where significant differences occurred among species. Analyses were performed using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

The fixed effect in my CTMax model explained how species responded differently to acute thermal stress. Variance of the fixed effect was homogenous (Figure 3). The variance estimate of the random effect was 0. Therefore, accounting for the variation due to trial (i.e., trial random effect) was not necessary. The fixed effect of species was significant in my model ($F_{9, 90} = 30.37$, $P < 0.01$) indicating CTMax was different for some of the 10 species included in the analysis.

The post hoc test I used separated the CTMax of the 10 species into three significant groups (Figure 4). Interestingly, Tukey Kramer HSD groupings did not clearly follow habitat guilds (i.e. benthic, mid-column, and surface) as I hypothesized. Instead, thermal tolerances of fishes that belonged to the benthic and mid-column habitat guilds often did not differ from one another; however, exceptions were the Orangebelly Darter (34.0°C) and Channel Darter *Percina copelandi* (34.1°C) that had lower thermal tolerances when compared to the Bluntnose Minnow *Pimephales notatus* (35.3°C) ($t_{90}=3.99$, $P<0.01$; $t_{90}=3.6$, $P=0.018$, respectively). As expected, the

Blackspotted Topminnow *Fundulus olivaceus* (38.3°C) had a higher thermal tolerance than all other species (t_{90}=9.37, P<0.01).

DISCUSSION

The CTMax data from this study are, to the best of my knowledge, the first records of thermal tolerance of Slenderhead Darter *Percina phoxocephala*, Steelcolor Shiner, Highland Stoneroller *Campostoma spadiceum*, Dusky Darter *Percina sciera*, and Channel Darter. These data provide insight into the thermal ecology of fishes that occupy altered streams and allow us to predict their responses to expected environmental changes (Smale and Rabeni 1995; Ostrand and Wilde 2001). For example, stream temperatures in the Kiamichi River regularly approach Channel Darter's CTMax and further increases may be detrimental.

Critical thermal maxima from my study differed from known values likely due to differences in acclimation temperature, heating rate, and species-specific responses to local environmental conditions. For example, Smale and Rabeni (1995) found CTMax (36.6°C) of Bluntnose Minnow to be much higher than my results (35.3°C) when acclimated to 26°C. Blackspotted Topminnow (38.8°C) were also marginally higher than my results (38.3°C). Mundahl (1990) found a much higher Bluntnose Minnow CTMax (37.9°C) in individuals acclimated to 24°C and heated at rates of 0.5-0.8°C/min. Even when fishes experience the same acclimation temperature and rate of heating, pH and hardness can alter thermal tolerances. For example, Farless and Brewer (2017) found higher CTMax in Orangebelly Darter and Bigeye Shiner *Notropis boops* and lower CTMax in Common Logperch *Percina caprodes* taken from streams with higher pH and hardness than my study. Differences in pH and water hardness are known to alter fishes' gill structure (Platek et al. 2017), respiratory abilities (Miller et al. 2016), and tolerance of ammonia (Baldisserotto et al. 2014) in species-specific ways, all of which may contribute to an individual's effective thermal tolerance (Coutant 1976; Pörtner and Knust 2007; Anttila et al. 2017).

Small differences in thermal tolerance may have physiological and population-level implications. I hypothesized benthic fishes would have low thermal tolerances because they primarily occupy the streambed where water temperatures typically remain the coolest and most stable (Brown et al. 2005; Schmidt et al. 2006; Seilheimer and Fisher 2010). Alternatively, I expected mid-column fishes to have higher thermal tolerances due to their occupation of warmer, more thermally variable habitats (Webb and Zhang 1999; Caissie 2006). However, differences in CTMax between benthic and mid-column guilds were not significant. The generally lower CTMax observed among darters may have ecological significance. Bioenergetics models for Smallmouth Bass predicted that, at 22°C, a 227-g individual would grow 26% over summer. That same individual would experience 5% growth with a 1°C increase and 5% weight loss with a 2°C increase (Whitledge et al. 2006). Pörtner and Knust (2007) found that increases as little as 1°C above optimal temperature were associated with declines in arterial blood flow in Eelpout *Zoarces viviparus*. Additionally, Eelpout relative abundance sharply decreased with temperature increases of less than 1°C within its pejus range. The sensitivity of aquatic organisms to even slight increases in temperature demonstrates the need to properly manage our water, especially in light of climate change and the prevalence of altered systems.

Fishes in the Kiamichi River currently experience temperatures near their thermal limits and expected increases could be problematic. Increasing atmospheric temperatures will cause a 2-3°C water-temperature increase in the south-central United States over the next 50-100 years (Morrill et al. 2005; van Vliet et al. 2013). An increase of 2-3°C could be detrimental for fish populations in the Kiamichi River, causing stream temperatures (Table 2) to exceed CTMax (Figure 4) for many species in this study. Increasing stream temperatures highlight the importance of thermal refugia such as areas of groundwater upwelling (Brewer 2013). However, dewatering of streams is widespread, increases water temperatures, and reduces suitable habitat for stream fishes (Luttrell et al. 1999; Bonner and Wilde 2000). Dewatering can occur as a result of limited reservoir releases, overexploitation of groundwater and surface water, or extended drought (Muehlbauer et al. 2011). The resulting increases in water temperature stress fish and put them at risk for reduced or delayed reproduction (Tveiten and Johnsen 1999; Auer 2004), increased susceptibility to disease (Yin et al. 1995), weight loss (Whitledge et al. 2002), and even death (Allan and Castillo 2007).

Critical thermal maximum may be useful for developing conservation and management strategies aimed at improving thermal conditions for stream fishes. Thermal tolerance data are useful for ranking species by susceptibility to increases in temperature (Smale and Rabeni 1995; Ostrand and Wilde 2001). Agencies may use CTMax as a target for stream-temperature management, but should remain mindful of the limits and natural plasticity of CTMax (i.e., CTMax is positively related to acclimation temperature and may be a conservative measure of thermal tolerance, Brett 1952; Bennett and Beitinger 1997; Lutterschmidt and Hutchison 1997). Measures should be taken to limit further water-temperature increases (e.g., meeting flow needs, Spooner et al. 2005; Gates et al. 2015; Brewer et al. 2016; limiting groundwater withdrawal, Caissie 2006; Waco and Taylor 2010; Brewer 2013) and protect thermally sensitive aquatic fauna. Stream management could benefit from incorporating temperature-monitoring programs with thermal tolerance of sensitive species as critical values.

Table 1. Critical thermal maxima (CTMax) were determined for fish species that occupy Ouachita Mountain streams. Fishes were acclimated to 20.0°C and exposed to a 2.0°C/hr increase in temperature until loss of equilibrium. Experimental fishes were collected from the Kiamichi River in autumn 2015, spring, and summer 2016. CTMax values were compared among species to see if differences related to habitat use.

Common Name	Scientific Name	Habitat Guild	Typical Habitat	Reference
Bigeye Shiner	*Notropis boops*	Mid-column	Mid-column, run, pool	Pflieger, 1997
Blackspotted Topminnow	*Fundulus olivaceus*	Surface	Surface water, backwaters, edgewaters	Pflieger, 1997
Bluntnose Minnow	*Pimephales notatus*	Mid-column	Mid-column, backwaters, pools	Miller and Robison, 2004
Channel Darter	*Percina copelandi*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Common Logperch	*Percina caprodes*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Dusky Darter	*Percina sciera*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Highland Stoneroller	*Campostoma spadiceum*	Mid-column	Mid-column, riffle, run, pool	Cashner et al., 2010
Orangebelly Darter[1]	*Etheostoma radiosum*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Slenderhead Darter	*Percina phoxocephala*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Steelcolor Shiner	*Cyprinella whipplei*	Mid-column	Mid-column, riffle, run, pool	Pflieger, 1997

[1] Oklahoma Species of Greatest Conservation Concern

Table 2. Maximum temperatures were summarized for six sites along Kiamichi River for 2013, 2014, and 2015. These temperatures approach and often exceed CTM for many fishes in this study.

Site	Year	Maximum Temperature (°C)	Date of Maximum Temperature
Payne Pool			
N 34.42570, W 95.57646	2013	33.68	Aug. 10
	2014	32.56	Aug. 25
	2015	-	-
Payne Riffle			
N 34.42647, W 95.57774	2013	35.56	Jul. 23
	2014	33.57	Aug. 25
	2015	-	-
Pine Spur Pool			
N 34.50702, W 95.50890	2013	34.44	Aug. 7
	2014	-	-
	2015	-	-
Pine Spur Riffle			
N 34.50609, W 95.51008	2013	34.73	Aug. 7
	2014	32.38	Jul. 8
	2015	35.34	Jul. 25
Robins Pool			
N 34.63543, W 95.12070	2013	32.02	Jun. 28
	2014	-	-
	2015	-	-
Robins Riffle			
N 34.63627, W 95.12258	2013	36.28	Jul. 12
	2014	34.47	Aug. 26
	2015	34.20	Jul. 27
Indian Highway Pool			
N 34.65749, W 95.04310	2013	33.03	Jun. 28
	2014	31.84	Jul. 27
	2015	-	-
Indian Highway Riffle			
N 34.65772, W 95.04202	2013	34.55	Jul. 11
	2014	33.33	Aug. 25
	2015	32.25	Jul. 28

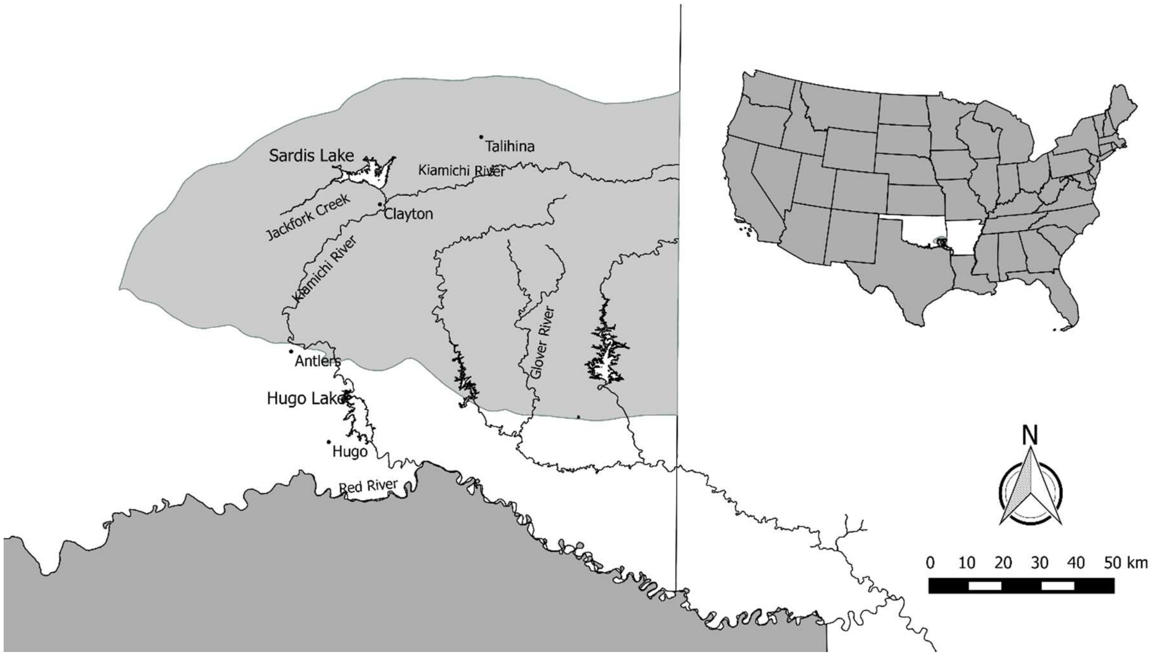

Figure 1. Fishes for CTMax trials were collected from the Kiamichi and Glover rivers of the Ouachita Mountain ecoregion (shaded) in southeast Oklahoma.

Figure 2. Sump system for testing critical thermal maximum (CTM). A pump discharges water into the 37.85-L aquariums and a gravity fed system discharges water into the 189.27-L sump. Water is heated in the sump by a 5000-W Smartone heater (OEM Heaters, Saint Paul, MN).

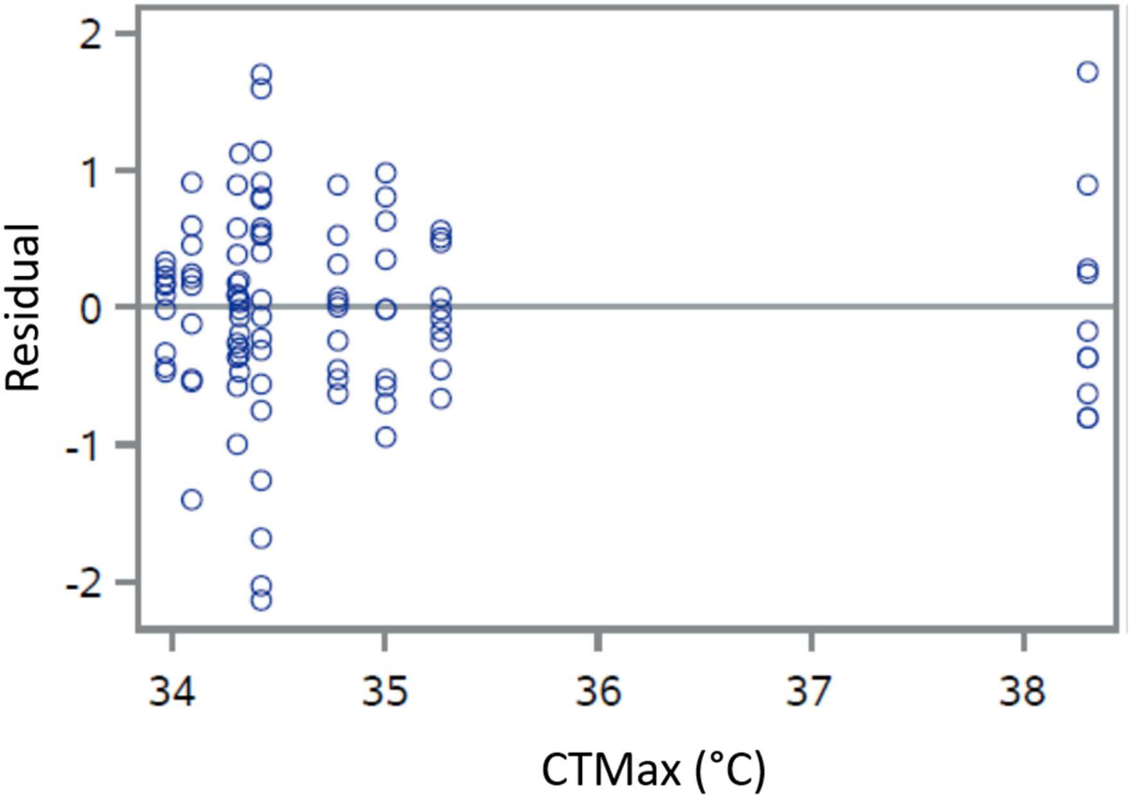

Figure 3. Plot of residuals against critical thermal maximum (CTMax) from trials on 10 fishes: Bigeye Shiner *Notropis boops*, Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Common Logperch *Percina caprodes*, Dusky Darter *Percina sciera*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, Slenderhead Darter *Percina phoxocephala*, and Steelcolor Shiner *Cyprinella whipplei*. Fish were acclimated to 20.0°C and exposed to a 2.0°C/hr increase in temperature until loss of equilibrium. Experimental fishes were collected from the Kiamichi River in autumn 2015, spring, and summer 2016.

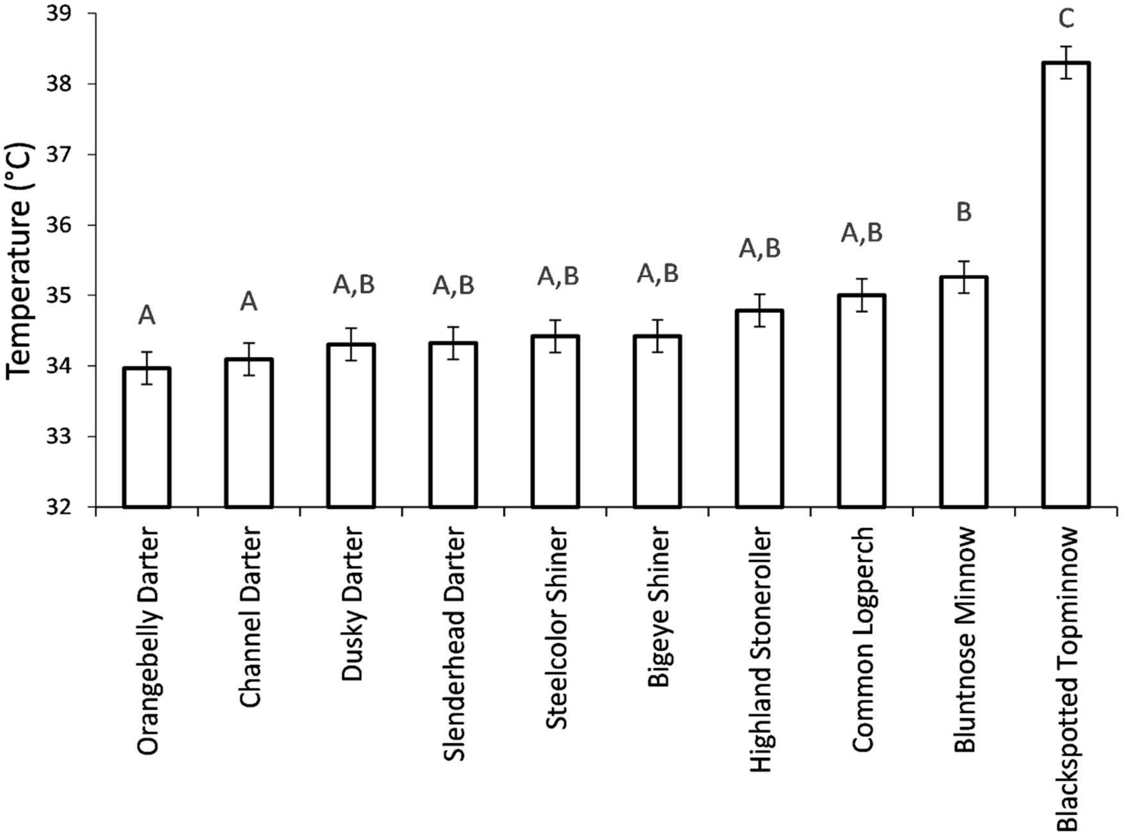

Figure 4. Average (± standard error) critical thermal maximum (CTMax) from trials on 10 stream fishes: Bigeye Shiner *Notropis boops*, Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Common Logperch *Percina caprodes*, Dusky Darter *Percina sciera*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, Slenderhead Darter *Percina phoxocephala*, and Steelcolor Shiner *Cyprinella whipplei*. Fish were acclimated to 20.0°C and exposed to a 2.0°C/hr increase in temperature until loss of equilibrium. Experimental fishes were collected from the Kiamichi River in autumn 2015, spring, and summer 2016. Letters over each bar represent groupings from Tukey Kramer Honest Significant Difference post hoc analysis.

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APPENDICES

Appendix 1. Data analysis code for critical thermal maximum (CTMax) for 10 stream fishes: Bigeye Shiner *Notropis boops*, Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Common Logperch *Percina caprodes*, Dusky Darter *Percina sciera*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, Slenderhead Darter *Percina phoxocephala*, and Steelcolor Shiner *Cyprinella whipplei*. Fish were acclimated to 20.0°C and exposed to a 2.0°C/hr increase in temperature until loss of equilibrium. Experimental fishes were collected from the Kiamichi River in autumn 2015, spring, and summer 2016. Analyses were performed using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, NC).

```
PROC GLIMMIX data=jra plots=residualpanel(conditional marginal);

CLASS species trial;

MODEL temperature = species / ddfm=kr;

RANDOM trial;

RANDOM residual / subject=trial*species type=un;

LSMEANS species/ pdiff adjust=tukey lines;

run;

quit;
```

Appendix 2. Mean (°C) and standard error (SE) of critical thermal maximum (CTMax) for 10 stream fishes: Bigeye Shiner *Notropis boops*, Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Common Logperch *Percina caprodes*, Dusky Darter *Percina sciera*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, Slenderhead Darter *Percina phoxocephala*, and Steelcolor Shiner *Cyprinella whipplei*. Fish were acclimated to 20.0°C and exposed to a 2.0°C/hr increase in temperature until loss of equilibrium. Dissolved oxygen was maintained >5 mg/L, ammonia was <0.25 ppm, and pH was 8-8.5. Experimental fishes were collected from the Kiamichi River in autumn 2015, spring, and summer 2016.

Common Name	Scientific Name	Mean CTMax (°C)	SE
Bigeye Shiner	*Notropis boops*	34.4	0.23
Blackspotted Topminnow	*Fundulus olivaceus*	38.3	0.23
Bluntnose Minnow	*Pimephales notatus*	35.3	0.23
Channel Darter	*Percina copelandi*	34.1	0.23
Common Logperch	*Percina caprodes*	35.0	0.23
Dusky Darter	*Percina sciera*	34.3	0.23
Highland Stoneroller	*Campostoma spadiceum*	34.8	0.23
Orangebelly Darter	*Etheostoma radiosum*	34.0	0.23
Slenderhead Darter	*Percina phoxocephala*	34.3	0.23
Steelcolor Shiner	*Cyprinella whipplei*	34.4	0.23

Appendix 3. Summary of critical thermal maxima (CTMax) of Bigeye Shiner *Notropis boops*, Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Common Logperch *Percina caprodes*, and Orangebelly Darter *Etheostoma radiosum* from published references. Where reported, each entry includes acclimation temperature, rate of heating, test endpoint (OS = onset of muscular spasms, LOE = loss of equilibrium), dissolved oxygen, ammonia, and pH. Dashes (-) indicate information not reported.

Species	CTMax (°C)	Acclimation temperature (°C)	Heating rate (°C/h)	Endpoint	Dissolved oxygen (mg/L)	Ammonia (µg/L)	pH	Reference
Bigeye Shiner	35.3	20	2	LOE	>6	0	7.8-8.0	Farless and Brewer, 2017
Blackspotted Topminnow	38.8	26	2	LOE	-	-	-	Smale and Rabeni, 1995
Bluntnose Minnow	31.9	15	60	OS	10.1-10.5	<0.5	7.8-8.5	Kowalski et al., 1978
	31.3	11	60	LOE	-	-	-	Hockett and Mundahl, 1988
	37.9	24	30.0-48.0	LOE	>2.4	-	-	Mundahl, 1990
	36.6	26	2	LOE	-	-	-	Smale and Rabeni, 1995
	33.7	10	60	OS	-	-	-	Lutterschmidt and Hutchison, 1997
Common Logperch	34.2	20	2	LOE	>6	0	7.8-8.0	Farless and Brewer, 2017
Orangebelly Darter	34.5	20	2	LOE	>6	0	7.8-8.0	Farless and Brewer, 2017

CHAPTER III

DOES PROLONGED EXPOSURE TO ELEVATED WATER TEMPERATURES INCREASE CORTISOL IN STREAM FISHES?

ABSTRACT

Fish respond to stressors in many ways, including changes in behavior, physiology, and abundance. Climate change and alteration of freshwater systems threaten to increase stream temperatures to stressful levels. Measuring the primary stress response in thermally sensitive fishes may indicate sub-lethal thermal stress. My objective was to determine if six stream fishes exhibit a stress response after prolonged exposure, 14 d, to elevated water temperatures. I measured whole-body cortisol concentrations of six stream fishes of the Ouachita Mountain ecoregion: Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, and Steelcolor Shiner *Cyprinella whipplei*. I used a generalized linear mixed model (GLMM) to analyze whole-body cortisol concentrations following a split-plot design with trial as a blocking factor, sump as the whole plot and aquarium as the subplot. Whole-body cortisol concentration was the dependent variable, temperature, species, and temperature-species interaction were fixed effects, and sump and trial were random effects in my model. I used a Tukey Kramer Honest Significant Difference post-hoc test to assess where significant differences within the GLMM occurred. I acclimated fishes in the laboratory to

20.0°C. I randomly assigned temperature treatments to sumps. Within each sump, I randomly assigned species to aquaria and placed three individuals in each. I exposed fishes to treatment temperatures (control, 27.0°C, average summer water temperature; experimental, 32.0°C, average maximum summer water temperature in Kiamichi River, OK) for 14 d. I hypothesized that fishes exposed to 32.0°C would show increased whole-body cortisol concentrations and levels of stress response would relate to thermal sensitivity (i.e., CTMax, Chapter 2). I found no significant differences between treatment temperatures. Results from my trials represent basal whole-body cortisol concentrations for all my study species except Highland Stoneroller. Despite apparent tolerance of or acclimation to stressful thermal conditions, fishes may suffer from decreased reproductive abilities or growth; thus, future studies that investigate the tertiary stress response to chronic thermal stress in these species would be beneficial. Whole-body cortisol levels from this study may serve as a baseline for monitoring stress in small-bodied stream fishes inhabiting highly altered systems.

INTRODUCTION

Fish exhibit a complex stress response which can be observed via behavior and at multiple organizational levels (i.e., physiologic, and population). Upon perceiving a stressor (e.g., predators, temperature, etc.), fish exhibit changes in behavior designed to avoid or mitigate the effects of the stressor. For example, Stewart et al. (2014) found that larval Zebrafish *Danio rerio* use the lateral line to detect predator-generated changes in water velocity and respond with a burst movement away from the stimuli. Brook Trout *Salvelinus fontinalis* and Rainbow Trout *Oncorhynchus mykiss* seek out areas of groundwater discharge to maintain cooler body temperatures (Baird and Krueger 2003). Physiologically, exposure to stressors causes immediate spikes in production of stress hormones (e.g., cortisol, Pickering and Pottinger 1989; Grutter and Pankhurst 2000; adrenaline, Iwama et al. 1989; Reid et al. 1998). Catecholamines (e.g., adrenaline and noradrenaline) are secreted by the chromaffin cells of the head kidney in response to severe acute stress. Production of adrenocorticotropic hormone and melanophore-stimulating hormone by the pituitary gland in response to stress induces cortisol secretion by the interrenal cells of the head kidney (Wendelaar Bonga 1997). Production of heat shock proteins increases in of proteins and inhibition of cell death by repairing stress-induced cell damage (Roberts et al. 2010). Ultimately, unavoidable and chronic stress requires energy devotion to both cellular repair and organism survival (Schreck 2000), and negatively affects fish fitness (e.g., decreased disease resistance, Magnadottir 2010; decreased reproduction, Pankhurst and Munday 2011); therefore, stress may affect entire populations, limiting distributions and decreasing abundance (Pörtner and Knust 2007; Schreck 2010). Stress is inevitable for fishes, and its effects are largely determined by the duration of exposure.

Cortisol production follows exposure to both acute and chronic stressors and affects the physiological processes of fishes. Production of corticosteroid hormones is a common stress response in fishes (Wendelaar Bonga 1997; Mommsen et al. 1999). Cortisol promotes the breakdown of skeletal muscle for energy, thereby negatively affecting growth (van der Boon et al. 1991). For example, Walking Catfish *Clarius batrachus* treated with cortisol showed reduced short-term growth and protein content in the brain, liver, and skeletal muscle suggesting use of stored energy in response to stress (Tripathi and Verma 2003). Just as stress-induced cortisol diverts energy away from growth, it also reduces reproductive investment. The effect of corticosteroids on fish reproduction varies by species and developmental stage (Barton and Iwama 1991; Schreck et al. 2001). For example, oocyte growth in Mozambique Tilapia *Oreochromis mossambicus* was negatively related to increasing cortisol levels (Foo and Lam 1993); however, Channel Catfish *Ictalurus punctatus* showed no reproductive suppression by cortisol (Small 2004). Increased cortisol related to thermal stress exposure also relates to masculinization in fishes (e.g., larval Pejerrey *Odontesthes bonariensis*, Hattori et al. 2009). Finally, elevated cortisol may cause weakened immune responses (e.g., resistance to bacterial infections, Yin et al. 1995; Binuramesh et al. 2005), and in some cases, lead to increased mortality rates (Pickering and Pottinger 1989). The established relationships among stressors and cortisol suggest measurement of cortisol may be useful in determining non-lethal responses to chronic environmental stressors.

Fishes are exposed to a myriad of environmental stressors; however, the coupled stressors of altered flow and thermal regimes are considered some of the most pressing (Poff et al. 1997; Caissie 2006) and often interactive. Flow alteration affects many of the major rivers across the United States (Graf 2001), and the downriver systems are often thermally impaired (Graf 2006). Combined with projected thermal increases due to climate change (2-3°C by 2050 in south-central United States, Morrill et al. 2005; van Vliet et al. 2013), managers are increasingly tasked with determining options that are available to offset the effects of these thermal alterations to allow the persistence of organisms (e.g., selective withdrawal from reservoirs, Rheinheimer et al. 2014; riparian forest replanting Kristensen et al. 2015). The Kiamichi River of southeast Oklahoma is a major center of aquatic biodiversity, but experiences similar stressors with two major impoundments and downriver thermal impairment (see also introduction chapter). Although mortality has been documented for both freshwater mussels and fishes of the Kiamichi River due to stream drying and thermal stress (Galbraith et al. 2008; Galbraith et al. 2010), the stress responses of more mobile fishes would provide a predictive indicator of thermal stresses prior to death. Therefore, the objective of this chapter was to determine if six stream fishes (Table 1) exhibit a stress response via whole-body cortisol after prolonged exposure to elevated water temperatures. My hypotheses were that 1) whole-body cortisol of fishes would be inversely related to their thermal tolerance (i.e., high CTMax would result in lower cortisol and vice versa); and 2) fishes exposed to the warmer thermal treatment would have higher levels of whole-body cortisol than those exposed to my control temperature.

METHODS

Study area. Fishes were collected from the Kiamichi River of the Ouachita Mountain Ecoregion, Oklahoma (Chapter Two, Figure 1). A detailed description of the study area is provided in Chapter Two. Briefly, the Kiamichi River traverses two major and humid ecoregions (109-145 cm of precipitation, annually). Average summer temperatures range 19-34°C (Woods et al. 2005). Maximum water temperatures at eight sites ranged 32.0-36.3°C during summer 2013 (Chapter Two, Table 2). Discharge patterns of the Kiamichi River are affected by two major reservoirs. Sardis Reservoir impounds Jackfork Creek and reduces the overall flow in the downriver Kiamichi River by approximately 25% (Vaughn et al. 2015). This area has been subject to several recent droughts (Arndt 2002), and drought frequency is expected to increase in the future (Seager and Vecchi 2010). Stream drying and elevated water temperatures in the Kiamichi River, due to flow alterations, threaten imperiled endemic species (Schaefer et al. 2003; Galbraith et al. 2008).

Fish collection and acclimation. Fishes were collected, transported, and held using the methods described in Chapter Two. I collected fishes in 2016-2017. Briefly, fishes were collected using a seine and immediately transported to the laboratory, and acclimated to laboratory conditions. Fishes remained in hauling containers for up to 12 h until the temperature of the hauling water reached that of the holding tanks (~20.0°C). Fishes were then transferred to 190-L holding tanks covered on the top with a screen. I added airstones to all holding tanks to maintain dissolved oxygen >5 mg/L. Over the first 96 h, fishes were left undisturbed to recover from transportation stress. Fishes remained in holding tanks where they were acclimated to laboratory conditions over a two-week period.

Following the initial holding period, fishes were fed, and water-quality conditions were checked daily. I fed fish flakes (Wardley Advanced Nutrition Perfect Protein Tropical Fish Flake Food, Hartz Mountain Corporation, Secaucus, NJ) and bloodworms (Fish Gum Drops Floating Fish Food Bloodworms, San Francisco Bay Brand, Newark, CA) once daily to satiation (i.e., until fishes ceased eating). Approximately six hours after daily feeding, I removed the unconsumed food from the aquaria via siphon. Ambient temperature was maintained at approximately 20.0°C. Ammonia was maintained <0.5 ppm. This level was only observed when new fish were added to the holding tanks, and for the first few days of lab acclimation. For the duration of acclimation and experimentation, ammonia was <0.25 ppm, pH was 8-8.5, and chloramine was zero. Water changes of approximately 30% were performed daily after the first 96 h of acclimation.

Whole-body cortisol concentration. I determined whole-body cortisol concentration of six stream fishes in response to thermal exposure using a split-plot design that was blocked by trial. I used a 2x6 factorial treatment structure with two levels of temperature (27.0°C and 32.0°C) and six levels of species (Table 1). I set up four identical sump systems that routed water from a 189.27-L sump to six 37.85-L acrylic aquaria (Chapter Two, Figure 2). Two airstones were added to each sump system to maintain dissolved oxygen above 5 mg/L. Water in each sump system was heated with a 1700 W Smartone heater (OEM Heaters, Saint Paul, MN). I randomly assigned temperature treatments to sumps (whole plots). Within each sump, I randomly assigned species to aquaria (my sub-plots). I used 27.0°C as the control temperature because it is commonly experienced in my study area during the summer. The control temperature was below the thermal tolerance of all my study species (Chapter Two, Figure 4). I used 32.0°C as the experimental temperature because it was 2.0°C less than CTMax of the most thermally-sensitive species (Chapter Two, Figure 4), but was anticipated to be stressful to stream fishes. Each temperature-species combination was replicated 10 times.

Fishes were assigned to treatment aquaria, and then acclimated to the new conditions prior to starting each trial. I randomly assigned species to each of six aquaria in each sump system for each trial, and then I haphazardly selected three individual adult fish (psuedoreplicates) to place in each aquarium. I only used adults in these trials because they are often less tolerant of higher temperatures (Pörtner and Farrell 2008), and would be more likely to represent thermal population bottlenecks. All fishes were held at 20.0°C for 24 h prior to the start of the experiment to allow acclimation to testing conditions and recovery from handling stress (Hutchison and Maness 1979; Pickering and Pottinger 1989).

I used a 12h:12h diel cycle to gradually heat each sump to its treatment temperature and maintained a 2.5 °C nightly refuge during the trials. During each trial, I increased water temperature 2.5°C over 12 h (0700-1900), daily, and decreased water temperature 1.5°C over 12 h (1900-0700), nightly. The net water temperature increase was 1.0°C/d until the treatment temperature of 27.0°C (control) or 32.0°C (experimental) was reached. All sumps were provided with a 2.5°C nightly (1900-0700) thermal refuge, but returned to the treatment temperature each day. I maintained each sump at this thermal regime for 14 d. After 14 d at the treatment temperatures, I sacrificed all fishes by freezing them in liquid nitrogen. The fish samples were then stored at -80°C until homogenization.

To quantify whole-body cortisol, I weighed and homogenized individuals, extracted cortisol, and performed an enzyme-linked immunosorbent assay (ELISA). I measured whole-body cortisol because sampling blood in my study fishes was impractical and holding water was shared among species in each trial (Zuberi et al. 2014; Belanger et al. 2016). Fish samples were weighed (0.001 g), partially thawed, and homogenized in 1x phosphate buffered saline (PBS) (1-part fish tissue, 5-parts 1x PBS). I combined 1 mL of homogenate with 5 mL diethyl ether in a glass centrifuge tube and vortexed for 1 min to extract cortisol. I then centrifuged samples at 3,500 rpm for 5 min and removed the organic layer containing cortisol. I repeated the extraction process three times for each sample. Following extraction, diethyl ether was allowed to evaporate overnight in a fume hood, leaving behind only proteins. I reconstituted samples with 1 mL of 1x PBS and incubated them overnight at 4°C. I performed ELISAs according to manufacturer's instructions to determine cortisol concentrations using a human salivary cortisol kit (Salimetrics LLC, College Station, PA). Each kit included cortisol standards, blanks, and high and low controls. I assayed samples in triplicate. I used a Cytation 5 cell imaging multi-mode reader (Biotek U.S., Winooski, VT) with Gen5 software (version 3.03, Biotek U.S., Winooski, VT) to measure sample optical density. I quantified whole-body cortisol concentrations of my samples using a 4-parameter sigmoid minus curve fit based on optical density of cortisol standards. High and low controls included in the kit verified values for standards. Cortisol concentrations were normalized by weight of the whole-body sample and reported as absolute cortisol concentrations (ng/g body weight). Values of psuedoreplicates were averaged to represent conditions in each aquarium.

Data analysis. I used a generalized linear mixed model (GLMM) to analyze the whole-body cortisol concentrations following a split-plot design with trial as a blocking factor, sump as the whole plot and aquarium as the subplot. In my model, whole-body cortisol concentration was the dependent variable, and temperature, species, and the temperature-species interaction were fixed effects. I checked for homogeneity of variance of the fixed effects. I used sump and trial as random effects in my model to control for differences among sumps and trials that were not directly of interest. The random effects, sump and trial, were assumed normally distributed as $N(0, \tau^2)$, where τ^2 is the population variance among levels of sump and $N(0, \beta^2)$, where β^2 is the population variance among levels of trial. I performed a Tukey Kramer Honest Significant Difference (HSD) post hoc test when an effect was significant. I assessed significance at $\alpha \leq 0.05$. All analyses were performed in SAS (version 9.4, SAS Institute, Cary, NC).

RESULTS

Assumptions of normality and homoscedasticity were not met by my model. Natural-log transformation of whole-body cortisol concentrations improved skewness. However, unequal variances of the fixed effects were still apparent and thus, were modeled to account for heteroscedasticity.

Whole-body cortisol concentrations varied among the species I examined, but not between the two treatment temperatures. The fixed effect of species was significant in my model ($F_{5,\ 36.86}$ =

62.46, $P < 0.01$) indicating a significant difference in stress response for at least one species. Interestingly, the fixed effect of temperature ($F_{1,\ 17.57} = 0.84$, $P = 0.37$), and the interaction of the fixed effects were not significant ($F_{5,\ 36.86} = 0.55$, $P = 0.74$).

Results from Tukey Kramer HSD indicated there were differences in whole-body cortisol concentrations among species (Figure 1). Highland Stoneroller *Campostoma spadiceum* had the highest cortisol concentration (67.61 ng/g body weight at the treatment temperature, 56.38 ng/g body weight at the control temperature) regardless of temperature (P = <0.01). Channel Darter *Percina copelandi* had the lowest cortisol concentration (1.64 ng/g body weight at the treatment temperature, 2.07 ng/g body weight at the control temperature), significantly different from Steelcolor Shiner *Cyprinella whipplei* ($P = 0.02$) and Bluntnose Minnow *Pimephales notatus* ($P = 0.04$), but not significantly different from Orangebelly Darter *Etheostoma radiosum* ($P = 0.79$) or Blackspotted Topminnow *Fundulus olivaceus* ($P = 0.46$). Cortisol concentrations in all other species were statistically similar among one another (Figure 1), and ranged 3.45-9.12 ng/g body weight in treatment fishes and 3.04-5.55 ng/g body weight in control fishes.

DISCUSSION

Contrary to my expectations, cortisol concentrations did not follow the pattern of CTMax among the six fishes studied. Channel Darter had the lowest cortisol concentration at both treatment temperatures (Figure 1), and the second lowest CTMax among the ten species I tested (Chapter Two, Figure 4). Highland Stoneroller had the highest cortisol concentration (Figure 1), and the fourth highest CTMax among the ten species I tested (Chapter Two, Figure 4). These data show that basal cortisol concentrations do not depend on acute thermal sensitivity (e.g., CTMax); instead, they are species-specific. For example, Channel Catfish acclimated to 20°C have greater basal cortisol concentrations (50 ng/mL, Strange 1980) than Rainbow Trout reared at 13-14°C (1.6 ng/mL, Barton 2000) despite the much higher CTMax of Channel Catfish (Beitinger et al.

2000). Higher cortisol levels are frequently associated with occupation of higher temperatures and attendant increases in metabolic demand (Davis 2004; Fanouraki et al. 2007; Fanouraki et al. 2011). Koban et al. (1991) suggest that higher basal levels of heat shock proteins, components of the cellular stress response, allow thermally tolerant species to cope with rapid temperature increases. Species-specific stress responses may be partly explained by seasonal or life-history stage (Pankhurst 2011). For example, European Perch *Perca fluviatilis* show increased basal cortisol during prespawn (Noaksson 2005), but Black Bream *Acanthopagrus butcheri* do not (Haddy and Pankhurst 1999).

The cortisol concentrations associated with Highland Stoneroller suggested this species exhibited a stress response higher than basal levels and equal in magnitude at each treatment temperature. I attribute this level of stress to species-specific intolerance of confinement in the laboratory setting. Previous studies have shown that confinement can cause increased levels of plasma cortisol (Clearwater and Pankhurst 1997; Pottinger 2010; Murray et al. 2017). Due to their exaggerated stress response in captivity, it may be advisable to avoid using Highland Stoneroller to determine sources of stress in a laboratory setting. For the same reason, the reliability of lab-determined CTMax for Highland Stoneroller and Central Stoneroller *Campostoma anomalum*, a closely related species, may be questionable. Often, simultaneous exposure to multiple stressors results in synergistic effects (Folt et al. 1999; Pörtner 2010); therefore, in the absence of confinement, actual thermal tolerance is likely to be higher than my data indicated.

Interestingly, none of the species examined in this study exhibited stress responses to the experimental temperature. With the exception of Highland Stoneroller, whole-body cortisol levels among the species in this study were similar to basal levels found in other studies (e.g., 5-20 ng/g body weight for three diminutive stream fishes, Sutherland et al. 2008; Li et al. 2009). The similarity of cortisol concentrations in my study to basal levels in previous studies suggests that all my study species except Highland Stoneroller tolerate laboratory conditions well, and did not exhibit a stress response to the two thermal treatments.

My study species did not exhibit stress responses to 14 d at the treatment temperature I tested, and this may relate to acclimation abilities or having a daily thermal refuge. In order to mimic conditions actually experienced in the Kiamichi River, I exposed fishes to a slow heating rate with daily refuge for 14 d. Slower heating rates are more natural, but can allow acclimation to occur (Lutterschmidt and Hutchison 1997). For example Hancock's Blenny *Acantemblemaria hancocki* had higher thermal tolerance at a heating rate of 1°C/h compared to faster rates (Mora and Maya 2006), suggesting acclimation at slower heating rates. Similarly Pumpkinseed Sunfish *Lepomis gibbosus* acclimated to 10°C showed higher thermal tolerance at a heating rate of 1°C/h compared to 6°C/h (Becker and Genoway 1979). The net temperature increase of 1.0°C/d in my study likely allowed acclimation; therefore, fishes tolerated higher temperatures. This rate of increase was observed in the Kiamichi River during June 2013. In addition to a slow rate of increase, a nightly refuge of 2.5°C was provided throughout the trials to mimic natural conditions in the Kiamichi River. Periodic exposure to high temperatures followed by access to a thermal refuge is known to increase thermal tolerance in ectotherms (Oliver and Palumbi 2011; Sgro 2016; Farless and Brewer 2017). For example, Zebrafish *Danio rerio* reared at variable temperatures (i.e., daily thermal refuge) had a higher thermal tolerance than those reared at constant temperatures (Schaefer and Ryan 2006). However, persistent exposure with no refuge may not allow organisms to harden to stressors (Schreck 2000). I exposed fishes to treatment temperatures for 14 d because high temperature events commonly last this long in the Kiamichi River. Maximum daily temperatures from multiple sites on the Kiamichi River in summer 2013 were ≥30.0°C for 14 d.

I chose the experimental temperature for my study based on CTMax determined after acclimation to 20.0°C. Higher acclimation temperatures tend to increase CTMax (Lutterschmidt and

Hutchison 1997; Zhang and Kieffer 2014; Moyano et al. 2017). For example, with every 1°C increase in acclimation temperature from 20-30°C, Largemouth Bass *Micropterus salmoides* and Channel Catfish CTMax increased 0.32°C and 0.4°C, respectively (Currie et al. 1998). It is likely that species in my study are tolerant of higher temperatures than their CTMax after acclimation to 20.0°C. Hence, fishes may not exhibit a stress response until temperatures closer to the ultimate lethal temperatures. However, attempts to use 34.0°C as the experimental temperature resulted in death in Channel Darter. This may be due to an inability to adapt to the accumulation of stress (Schreck 2000). For example, Coho Salmon *Oncorhynchus kisutch* showed variable mortality in response to repeated thermal exposure among stocks and their hybrids (Campbell et al. 1998).

Whole-body cortisol concentrations from my study may serve as a baseline for monitoring stress in small-bodied stream fishes inhabiting highly altered systems. Stressful conditions in the Kiamichi River caused by flow and thermal alterations may result in community-level problems (e.g., reproduction, Schreck et al. 2001; Schreck 2010; disease resistance, Binuramesh et al. 2005; changing species interactions, Gilman et al. 2010). For example, stressful temperatures alter the ecosystem services provided by freshwater mussels and species composition of mussel beds (Spooner and Vaughn 2008). Elevated cortisol would indicate stressful conditions and the need for alternative management strategies (e.g., meeting flow needs, Spooner et al. 2005; Gates et al. 2015; Brewer et al. 2016; limiting groundwater withdrawal, Caissie 2006; Waco and Taylor 2010; Brewer 2013). Monitoring sublethal stress in fishes would allow for proactive rather than reactive stream-temperature management. Future studies interested in the primary stress response should measure cortisol at multiple intervals of thermal exposure (e.g., 2-7 d) to observe when peak stress and subsequent acclimation occur. Basal cortisol levels from this study suggest that individuals may have adapted to and regained the ability to perform basic functions in stressful conditions. Despite tolerance of stressful conditions, fishes may suffer from decreased reproductive abilities (Schreck 2000; Schreck et al. 2001) or growth (Wendelaar Bonga 1997);

thus, it would be interesting to investigate the tertiary stress response to chronic thermal stress in these species. Investigating the tertiary stress response may highlight the importance of maintaining suitable thermal conditions; however, it does not allow proactive management. Instead, managers should consider monitoring primary and secondary stress responses.

Table 1. Whole-body cortisol concentrations from chronic thermal stress trials were measured on six fishes: Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, and Steelcolor Shiner *Cyprinella whipplei*. Fishes were expected to exhibit stress responses associated with habitat guilds defined by documented habitat use. Experimental fishes were collected from the Kiamichi River in autumn 2016 and spring 2017. Fish were acclimated to laboratory conditions of 20.0°C and exposed to a 1.0°C/d increase in temperature until reaching the treatment temperatures (i.e., 27.0°C control; 32.0°C experimental). Fish remained at treatment temperatures for 14 days, but were all provide a thermal refuge of 2.5°C each night during trials.

Common Name	Scientific Name	Habitat Guild	Typical Habitat	Reference
Blackspotted Topminnow	*Fundulus olivaceus*	Surface	Surface water, backwaters, edgewaters	Pflieger, 1997
Bluntnose Minnow	*Pimephales notatus*	Mid-column	Mid-column, backwaters, pools	Miller and Robison, 2004
Channel Darter	*Percina copelandi*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Highland Stoneroller	*Campostoma spadiceum*	Mid-column	Mid-column, riffle, run, pool	Cashner et al., 2010
Orangebelly Darter[1]	*Etheostoma radiosum*	Benthic	Benthic, riffle, run, pool	Miller and Robison, 2004
Steelcolor Shiner	*Cyprinella whipplei*	Mid-column	Mid-column, riffle, run, pool	Pflieger, 1997

[1] Oklahoma Species of Greatest Conservation Concern

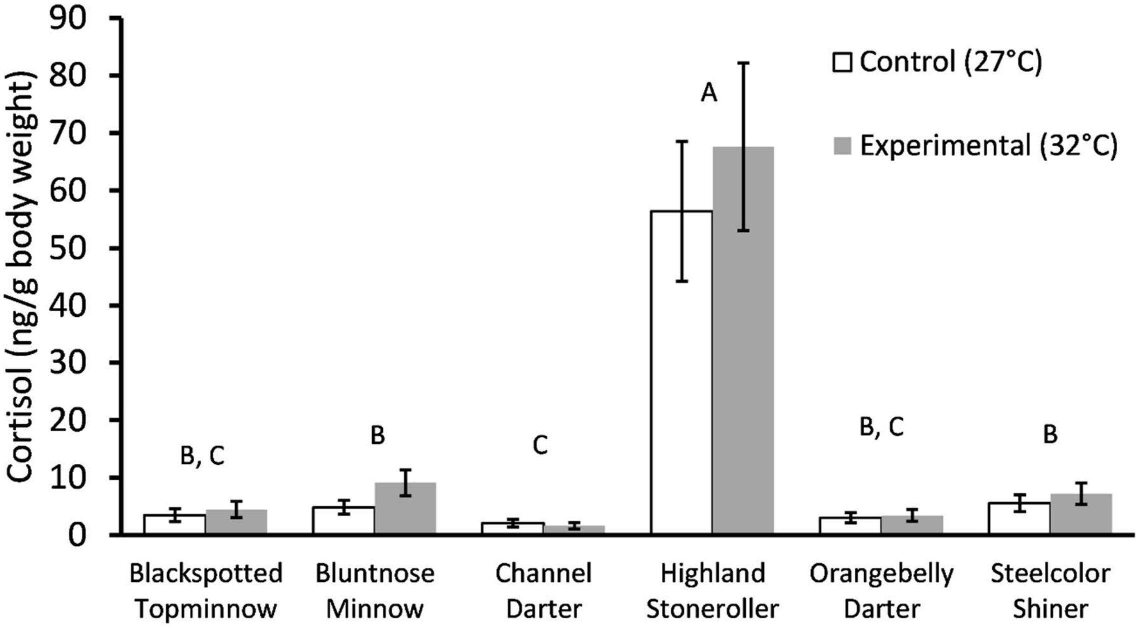

Figure 1. Average (± standard error) whole-body cortisol concentrations from chronic thermal stress trials on six stream fishes: Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, and Steelcolor Shiner *Cyprinella whipplei*. Experimental fishes were collected from the Kiamichi River in autumn 2016 and spring 2017. Fish were acclimated to laboratory conditions of 20.0°C and exposed to a 1.0°C/d increase in temperature until reaching the treatment temperatures (i.e., 27.0°C control; 32.0°C experimental). Fish remained at treatment temperatures for 14 days, but were provided a thermal refuge of 2.5°C each night during trials. Letters over each bar indicate species differences in whole-body cortisol concentration from the Tukey Kramer Honest Significant Difference post-hoc analysis.

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APPENDICES

Appendix 1. Data analysis code for whole-body cortisol concentrations from chronic thermal stress trials on six stream fishes: Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, and Steelcolor Shiner *Cyprinella whipplei*. Experimental fishes were collected from the Kiamichi River in autumn 2016 and spring 2017. Fish were acclimated to laboratory conditions of 20.0°C and exposed to a 1.0°C/d increase in temperature until reaching the treatment temperatures (i.e., 27.0°C control; 32.0°C experimental). Fish remained at treatment temperatures for 14 days, but were provided a thermal refuge of 2.5°C each night during trials. Analyses were performed using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, NC).

```
logcortisol=log(cortisol);
PROC GLIMMIX data=jra plots=residualpanel(conditional marginal) pconv=0.0002;
CLASS temperature trial sump species;
MODEL logcortisol = temperature|species / ddfm=kr;
COVTEST / wald;
RANDOM trial trial*temperature*sump;
RANDOM residual / group=species;
LSMEANS species*temperature/ pdiff adjust=tukey lines;
LSMEANS species/ pdiff adjust=tukey lines;
run;
run;
quit;
```

Appendix 2. Mean (ng/g body weight) and standard error (SE) of whole-body cortisol concentrations for six stream fishes: Blackspotted Topminnow *Fundulus olivaceus*, Bluntnose Minnow *Pimephales notatus*, Channel Darter *Percina copelandi*, Highland Stoneroller *Campostoma spadiceum*, Orangebelly Darter *Etheostoma radiosum*, and Steelcolor Shiner *Cyprinella whipplei*. Experimental fishes were collected from the Kiamichi River in autumn 2016 and spring 2017. Fish were acclimated to laboratory conditions of 20.0°C and exposed to a 1.0°C/d increase in temperature until reaching the treatment temperatures (i.e., 27.0°C control; 32.0°C experimental). Fish remained at treatment temperatures for 14 days, but were provided a thermal refuge of 2.5°C each night during trials.

		Control (27.0°C)		Experimental (32.0°C)	
Common Name	Scientific Name	Mean (ng/g body weight)	SE	Mean (ng/g body weight)	SE
Blackspotted Topminnow	*Fundulus olivaceus*	3.47	1.10	4.46	1.41
Bluntnose Minnow	*Pimephales notatus*	4.84	1.20	9.12	2.27
Channel Darter	*Percina copelandi*	2.07	0.68	1.64	0.54
Highland Stoneroller	*Campostoma spadiceum*	56.38	12.17	67.61	14.59
Orangebelly Darter	*Etheostoma radiosum*	3.04	0.89	3.45	1.01
Steelcolor Shiner	*Cyprinella whipplei*	5.55	1.45	7.20	1.88

VITA

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