USE OF NON-LETHAL ENDPOINTS TO ESTABLISH WATER QUALITY REQUIREMENTS AND OPTIMA OF THE TOPEKA SHINER (*NOTROPIS TOPEKA*)

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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USE OF NON-LETHAL ENDPOINTS TO ESTABLISH WATER QUALITY REQUIREMENTS AND OPTIMA OF THE TOPEKA SHINER (*NOTROPIS TOPEKA*)

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ABSTRACT

Water quality influences growth, development, and physiology of aquatic vertebrates. Current criteria on water quality assessments are primarily based on lethal level experiments (e.g. LC_{50} tests), which are poorly suited for assessing optimal water quality conditions or sub-lethal effects of common stressors. Measurements below threshold values may still impede organismal growth and development, especially considering the complex nature of compounding, low-level stressors. This is particularly important to consider for management of an endangered species that is actively cultured for reintroduction to extirpated locations. The endangered Topeka Shiner (*Notropis topeka*) is an ideal example for which this information is needed, as its remaining, stable populations display broad water quality optima and tolerance to naturally occurring stressors. We investigated the effects of dissolved oxygen, temperature (including acclimation), ammonia, nitrite, and chloride on Topeka Shiner using non-lethal

endpoints by: (1) examining *N. topeka's* behavioral responses to a gradual reduction in oxygen, (2) determining thermal optima at different acclimation temperatures using swimming speed, and (3) determining the onset of effect of sub-lethal levels of nitrogenous compounds and chloride concentrations on swimming speed. We determined ASR_{50} and ASR_{90} (i.e. dissolved oxygen concentrations where 50% and 90% of fish use aquatic surface respiration) to occur at 1.65mg/L and 1.08 mg/L of dissolved oxygen, respectively. At 5.52 mg/L of dissolved oxygen, fish vertical position was significantly higher in the water column, presumably in preparation for aquatic surface respiration (ASR). With our thermal swimming tests, the optimum temperature range was determined to be 17.7 to 28.0 °C, while the predicted incipient mortality to high temperature ranged from 33.7 to 40.3 °C, depending on acclimation temperature. Ammonia and sodium chloride significantly reduced swimming speed at concentrations below known LC_{50} values. Other than an initial drop from 0-concentration, nitrite did not reduce swimming speed, even at concentrations higher than known LC_{50} measurements. Although not all stressors were suitable to test with this methodology, emphasis on determining optimal conditions over tolerances, and sub-lethal effects over mortality, assists in selection of sites that have water quality suited for *N. topeka* to thrive after reintroduction.

CHAPTER 1: Introduction and Literature Review

Freshwater systems in North America are experiencing a rapid decline in biodiversity (Richter et al. 1997). Habitat fragmentation caused by anthropogenic activities is among the top threats implicated in an astounding 92% increase in listings of imperiled North American fishes since the last USGS assessment in 1989 (Jelks et al. 2008). Other factors, including habitat destruction, invasive species, altered hydrology, pollution, disease, and over-exploitation account for the imperilment and extinction of 40% of North American fishes (Jelks et al. 2008). Inferior water quality often accompanies these threats in ways that are generally poorly understood; for example, species distributions can be affected not only by physical barriers to dispersal but also stretches of rivers or streams with poor water quality (Bayless et al. 2003; Noatch and Suski 2012). Though less visually dramatic than other forms of habitat degradation, poor water quality can be devastating to the aquatic community by reducing species richness and abundance (Lenat and Crawford 1994). This necessitates examination of how anthropogenic changes in water quality affect the basic physiology and, therefore, survival and fitness, of native stream fishes (Richards et al. 1996; Richter et al. 1997; Wang et al. 1997).

Regulatory agencies have effectively improved stream health nationwide by reducing concentrations of toxic pollutants (Poole et al. 2004). However, standards currently employed to manage our waterways do little to address naturally-occurring regimes associated with dynamic systems. These water quality standards are typically

set as maximum or minimum threshold values, depending on the parameter of interest (e.g. minimum oxygen content, maximum temperature), based on studies of aquatic animal mortality over a range of conditions (e.g. LC_{50} values, estimates of water quality conditions at which animal mortality is 50%). While current standards have led to the reduction of point source pollution and toxin levels in U.S. water bodies, threshold standards based on studies of mortality are not adequately suited to address chronic sub-lethal physiological stressors adversely affecting aquatic organisms (Poole et al. 2004). Further, for some applications, an understanding of threshold values for mortality may be of less interest than understanding water quality optima, or when characteristics of the water body maximize potential fitness of organisms therein. An understanding of sub-lethal stressors and optimal water quality conditions may be of particular interest to managers whose primary objectives are recovering rare and threatened species.

In many cases, recovery plans for threatened and endangered species include propagation and reintroduction into water bodies where the species have been extirpated. Due to the rarity of some endangered and threatened species, reintroduction from captive breeding programs is a widely used method (Snyder et al. 1996). Of the endangered vertebrates, captive breeding management has been recommended for 34% of the 3,550 taxa investigated (Snyder et al. 1996). In addition, 63% of recovery plans for U.S. endangered and threatened species call for reintroduction from captive breeding (Snyder et al. 1996). Snyder et al. (1996) has recognized several common problems with captive breeding, including establishment of

captive populations, poor success in reintroduction, high cost, domestication, preemption of other recovery techniques, disease outbreaks, and maintaining administrative continuity. Despite potential deficiencies in captive breeding programs, propagation and husbandry techniques for stream minnows are well established for the bait industry and transferable to endangered fishes (Shute et al. 2005). Successful reintroduction of endangered stream fish has been demonstrated in Abrams Creek, Tennessee, where four listed fish (i.e. Smokey Madtom, Yellowfin Madtom, Duskytail Darter, and Spotfin Chub) were introduced from captive breeding (Shute et al. 2005). Populations are currently increasing for three of the four species and are comparable in local densities to non-supplemented populations (Shute et al. 2005).

Often, successful recovery plans are linked to the ability to identify important mechanistic linkages between species and environmental conditions within habitats proposed for reintroduction (Foin et al. 1988). Physiology is the connection between specific environmental conditions and behavior and fitness (Horodysky et al. 2015). For reintroduction programs to be successful, managers must demonstrate an understanding of basic physiological optima, sensitivities, and tolerances of the species. American Fisheries Society (AFS) guidelines emphasize that introductions should take place only in sites that fulfill life history requirements for species (AFS 2016). In the case of physical habitat, those requirements are often described; however, water quality optima and tolerances for such factors as temperature, dissolved oxygen, and nutrient concentrations are also critical to understand due to their pervasive effects on fish physiological ecology.

The Topeka Shiner (*Notropis topeka*), native to North American streams in Minnesota, South Dakota, Iowa, Nebraska, Missouri, and Kansas, has experienced population declines and a reduction in range (Hatch 2001). These small cyprinid minnows occupy vegetated upland pools with rocky or sandy bottom substrates (Minckley and Cross 1959; Cross and Collins 1995; Pflieger 1997). A deep-bodied fish with adult lengths less than 75mm (Hatch 2001; Stark et al. 2002), *N. topeka* spawns multiple broods by commensally spawning on Orangespotted Sunfish (*Lepomis humilis*) nests (Hatch 2001; Cross and Collins 1995). Minckley and Cross (1959) first noted a decrease in the abundance and wholesale loss of Topeka Shiner populations; the species is now estimated to occupy only 20% of its former range (USFWS 2001). The Topeka Shiner was federally listed as an endangered species in December of 1988 (USFWS 1988).

As is the case with many endangered fishes (Jelks et al. 2008), an assortment of biological and chemical factors are implicated in the Topeka Shiner's decline including stream channelization, sedimentation, pollution, and nonnative predatory fish (Hatch 2001; MDC 2010). Alterations of stream hydrology and connectivity have prevented upland recolonization after major drought (Winston 2002), and the stocking of largemouth bass and other large piscivorous fish have increased predation pressure on *N. topeka* populations (Gerken and Paukert 2012; Schrank et al. 2001; Knight and Gido 2005). The viability of remaining populations of Topeka Shiner are also threatened by agriculture and road runoff, which can severely alter stream chemistry (Richter et al. 1997). Recent monitoring by Missouri Department of Conservation found that Topeka

Shiner occurrence was negatively related to specific conductance (Novinger et al. 2011), an indicator of pollutants (e.g. increased by chloride ions such as in animal wastes or road treatments; Kelly et al. 2008), and prevailing geologic conditions linked to temperature and evaporative conditions. Additional investigation into the species' physiological tolerances is therefore warranted to not only evaluate the suitability of areas chosen for reintroduction, but also to better understand the mechanisms for population decline.

The draft Topeka Shiner Federal Recovery Plan requires three secure (stable or increasing) populations for all six of its primary recovery units for ten years for the species to be delisted (USFWS 2001). In response, several states in the Topeka Shiner's native home range initiated management plans for reintroduction and improvements in water quality within the species' former range. In 2004, Kansas implemented a recovery plan that focuses on reducing pollution with Topeka Shiner propagation currently underway (KDW&P 2004). With no reintroduction yet attempted in Kansas, reestablishment of additional populations in watersheds historically containing Topeka Shiners is required to delist *N. topeka* as a state endangered species (KDW&P 2004).

The Missouri Department of Conservation (MDC) 10-year recovery plan for Topeka Shiners calls for establishment of seven populations in the state with two extant populations already existing (MDC 2010). Therefore, five additional populations are required through means such as the unlikely discovery of presently unknown populations or the establishment of new populations through reintroductions into historical range. Potential reintroduction sites are organized into tiers based on a

variety of factors (i.e. land ownership, proximity to existing populations, historic range, and condition of stream and watershed) (MDC 2010). In 2013 and 2014, MDC reintroduced Topeka Shiners into three northern Missouri watersheds with a federal status of Non-Essential Experimental Populations (NEP) with additional NEPs in central MO under consideration during the next few years (Graham 2016; D. Novinger, MDC, Nov. 2017, personal communication). Evaluating the existing NEP and selecting watersheds for the future NEP will benefit from a better understanding of physiological optima and tolerances to water quality characteristics.

South Dakota Department of Game, Fish and Parks' management plan focuses on hydrology, geomorphology, and water quality (SD GF&W 2003). By offering financial and technical assistance to landowners to maintain and restore natural flow regimes, channel structure, and reduce non-point source pollution in streams containing Topeka Shiners, South Dakota hopes to enhance amounts of high quality habitats. Managers therefore require a thorough understanding of Topeka shiner optimal water quality characteristics for both setting water quality goals for these restoration programs and for selecting sites for reintroduction and range expansion.

OBJECTIVES

Our project is intended to assist ongoing management and recovery efforts for Topeka Shiners by helping inform reintroduction activity and better understand reasons for population persistence or decline. Specific objectives include: (1) measure hypoxia tolerance based on behavioral cues (e.g. aquatic surface respiration, gill ventilation,

buccal bubble holding, aggression, and water column position) related to a gradual reduction of dissolved oxygen in a controlled setting, (2) use ramped critical swimming speed to measure optima and tolerance to a range of temperatures and acclimations, and (3) use ramped critical swimming speed to determine the onset of stress to sub-lethal levels of ammonia, nitrite, and chloride concentrations. To assist in the selection of reintroduction sites, our experiments will focus on finding an "optimum range" of thermal conditions for the species and determine levels of hypoxia and ammonia byproduct concentrations that correspond with onset of stress rather than mortality.

In the following literature review, we first demonstrate the effects of low dissolved oxygen concentrations on fish and their behavioral responses to hypoxic or anoxic conditions in the water column. Next, we define ramped critical swimming speed as an effective physiological test for measuring metabolic potential. In our thermal review, we show how temperature and acclimation affect the physiology of fishes. Low-level chemical stressors, including dissolved ammonia, ammonia byproducts, and chloride, also disrupt the normal physiological functions of fish. Swimming tests can present a non-lethal alternative to common LC_{50} tests and demonstrate potential for sub-lethal impacts on the species that could affect their overall fitness in a natural environment. The following review material was crucial in development of methodology and fulfilling our project objectives. *N. topeka*'s successful reintroduction into Missouri streams relates to the selection of reintroduction locations increasing individual fitness through optimization of physiological function.

HYPOXIA TOLERANCE

Living in isolated pools that seasonally suffer from desiccation, *N. topeka* must contend with patterns of hypoxia typical of prairie streams (Hatch 2001). Water-breathing fish possess a wide range of evolutionary responses to hypoxia, including both instantaneous behavioral responses (e.g. avoidance) and physiological responses (e.g. increased hemoglobin for oxygen transfer; Timmerman and Chapman 2004). An integrative approach to examining the relative differences among species in hypoxia tolerance is to observe behavioral response to gradual reductions in oxygen in a controlled environment; this incorporates potential for both physiological and behavioral adaptations to low oxygen levels. Species adapted to hypoxia often exhibit behaviors that compensate for lack of oxygen in the water column, ranging from air-breathing to aquatic-surface respiration (ASR). Kramer (1983) defines aquatic surface respiration as a behavioral mechanism by which the fish ventilates its gills at the thin, oxygenated layer near the water surface. In addition to ASR, many species of fishes incorporate buccal bubble holding (holding an air bubble in the mouth and passing water over it to breathe) as a behavioral response to hypoxia, which may provide an additional source of oxygen and improve buoyancy of individuals at the water surface (Chapman et al. 1995; Dwyer et al. 2014).

ASR may allow fish to persist under conditions of low dissolved oxygen; however, this behavior comes at a potential cost (Domenici et al. 2007; Chapman and McKenzie 2009). Loss of schooling formation, coupled with vulnerability to aerial attacks, makes ASR risky and excludes other important behaviors e.g. feeding, reproduction); it is

therefore likely employed only when other physiological or morphological adaptations for extracting oxygen from the water column fail to maintain fish metabolism (e.g. increased hemoglobin content, larger gill surface area, lowered metabolic rate; Domenici et al. 2007). Some species adapt to increased predatory pressure during ASR by altering their behavior in hypoxic environments. Synchronous air breathing (i.e. individuals breathing air as a group or in rapid succession) has anti-predator benefits similar to schooling behaviors (Kramer and Graham 1976).

The initiation of ASR in water-breathing fish is indicative of the oxygen concentrations that fall below the capacity of the fish to extract enough oxygen from the water column to maintain basic metabolic function (Kramer 1983). A species' distribution may be partially determined by its ability to access oxygen from the environment (Mandic et al. 2009). Topeka Shiners were historically found in small headwater streams maintained by groundwater during summer months (Minckley and Cross 1959). However, populations of Topeka Shiners also inhabit and spawn in oxbows and deep pools with heavy sediment loads and surrounding anthropogenic agricultural activities (Hatch 2001). These influences (e.g. nitrogen runoff, sewage discharge) can interact with natural phenomena (e.g. summer desiccations) to exacerbate hypoxia (Chapman and McKenzie 2009). Therefore, it is necessary to examine how Topeka Shiners cope with low levels of dissolved oxygen to better understand habitat suitability and refine ongoing reintroductions.

SWIMMING SPEED

Critical, or maximum, swimming speed is an efficient method to measure physiological responses to changes in water chemistry; it well-represents the metabolic potential of an organism under different conditions and the cost of maintaining homeostasis under stress (Hammer 1995). Metabolic potential, as indicated by critical swimming speed, represents the individual's ability to obtain food, avoid predation, migrate, reproduce, and escape unfavorable conditions (Drucker 1996), and swimming capability is a significant trait affecting Darwinian fitness (Plaut 2001; Plaut and Gordon 1994). Specifically, predator-prey relationships between fish species are highly dependent on locomotion (Webb 1986). In addition, changes in swimming capability of fish provide information on physiological stress that can be directly related to LC_{50} tests without harming the fish (Hammer 1995). For example, *Aphanius dispar* experienced a decrease in critical swimming speed with the addition of water salinity as a stressor (Plaut 2000). Measures of fish swimming performance are individually repeatable, demonstrate maximum aerobic activity, and yield comparable data, thereby providing a useful reflection of basic physiological function of fish (Plaut 2001).

Swimming speed can be categorized as burst, prolonged, or sustained (Beamish 1978). Burst speed is defined as anaerobic swimming maintained for less than 20 seconds (Beamish 1978). Conversely, sustained swimming is aerobic, lasting between 20 seconds to 200 minutes (Beamish 1978), and ending in fatigue (Jones 1982). Prolonged swimming is described as aerobic swimming lasting six hours without reaching exhaustion (Brett et al. 1958) or lasting longer than 200 minutes (Beamish

1978). Brett (1964) first standardized critical swimming speed (U_{crit}) methodology to provide measures of prolonged swimming speed. The procedure for estimating critical swimming speed is incremental; its intent is to determine how fast a fish can swim until its energetic resources are exhausted. Fish are first introduced to a swim tunnel with an initial starting velocity to acclimate to the new environment. The flow is incrementally increased in steps until the fish is exhausted and can no longer swim. The nature of the U_{crit} value is given with the following formula (Brett 1964, 1967):

$$U_{crit} = U_p + [U_s \left(\frac{T_f}{T_i}\right)] \qquad \text{Equation 1}$$

To calculate the U_{crit} value, one must determine U_p, the speed of the penultimate step, U_s, the interval speed increase, T_f, the fatigue time of last step, and T_j, the step time (Equation 1). Peake et al. (1997) demonstrated that the U_{crit} measurement is highly reproducible, even after brief recovery periods. However, U_{crit} measurements often involve a lengthy test spanning several hours. Jain et al. (1997) experimented with new procedures to shorten test time and estimate U_{crit} with an accelerated stepwise increase in water velocity to 75% of the predicted U_{crit} value, finishing with two or more full swimming intervals before fatigue. The traditional U_{crit} and the new ramp U_{crit} values do not differ significantly from one another (Jain et al. 1997). As long as the faster speed does not require significant white muscle activity and the fish finishes two complete

intervals, the ramped U_{crit} protocol saves time with no cost to accuracy or reproducibility (Figure 1, Jain et al. 1997).

THERMAL ECOLOGY

Given its important role in regulation of poikilothermic metabolism, temperature is frequently the focus of studies investigating freshwater fish ecology (Beitinger et al. 2000). In laboratory studies, two common strategies are employed to discover thermal tolerance maxima and minima. For exposure to extreme temperatures, the upper (warmer) and lower (colder) incipient lethal temperature (ILT) levels are defined as the point where half the exposed population perishes (analogous to the common LC_{50} test). Fry et al. (1942) first used this technique to construct a thermal tolerance polygon, a graphical representation of a fish's thermal ecology and thermal limits. Following incipient lethal temperature levels, Cowles and Bogert (1944) introduced the concept of critical thermal maximum (CTM), later refined by Cox (1974) as an upward or downward progression of temperature from a starting acclimation until the fish's locomotion becomes physically disorganized. From these measurements, a critical thermal maximum relationship can be constructed for different acclimation temperatures. Fangue et al. (2011) demonstrated that the ability to tolerate high temperatures (i.e. CTM experiments) is highly dependent on acclimation temperature, both in a laboratory setting and in the field. While these techniques are suitable for measuring tolerance in fish, little insight is provided for sub-lethal behavior and physiological effects of

suboptimal temperature, and the optimal temperature for growth (given full rations) remains unknown.

Acclimation plays a vital role in any thorough examination of a species' thermal ecology. Holding temperature (i.e. long-term acclimation temperature) can affect the physiological state of ectothermic organisms like fish and alter their thermal optimums (Davis and Parker 1990; Shaklee et al. 2005). Thermal acclimation is defined as a (usually) reversible adjustment in physiology to changes in dominant environmental temperatures (Lagerspetz 2006). Specifically in fish, upper and lower thermal limits are highly dependent on acclimation temperature. Sheepshead Minnows (*Cyprinodon variegatus*) acclimated to different temperatures (i.e. 5°C and 30°C) had significant differences in their thermal maximum and minimum tolerance (i.e. 0.6-34.6°C and 11.3-44.2°C) (Bennett and Beitinger 1997). In a sub-lethal context, fitness reductions are associated with acclimation temperatures that deviate too far from the optima (Leroi et al. 1994). Individuals may have different optimal temperatures that give the greatest fitness benefits, but they still have the capacity to survive over a range of temperatures. The acclimation process thereby allows an individual organism to achieve a steady state of physiological function outside the optimal environmental conditions while receiving a fitness advantage over an organism unable to acclimate (Lagerspetz 2006; Leroi et al. 1994).

Prairie stream fishes are adapted to a highly variable thermal environment; therefore, their tolerance levels are likely not indicative of their thermal optima or their relative capacity for acclimation to dominant thermal conditions (Dodds et al. 2004).

Given that both ambient temperature and acclimation temperature affect swimming ability (Hammer 1995; Plaut 2001), ramped U_{crit} can indicate potential growth for fish acclimated over a range of temperatures. Assuming that the metabolic capacity of the fish corresponds with potential growth under maximum rations, the temperature at which maximum swimming speed is reached for any given acclimation temperature represents the animal's optimal temperature for growth (Green and Fisher 2004). By withholding feeding for at least 48 hours prior to swim tests, the fish will metabolize nutrients (i.e. pre-absorptive state). Therefore, the swim test will reflect metabolic capacity at a predetermined acclimation temperature, without variability introduced by the metabolic costs of digestion. Beamish (1978) showed that salmonid fishes increased swimming speed with temperature to an optimum followed by a decrease. Running thermal profiles at different starting acclimation temperatures, using swimming speed as an indicator of physiological performance, it is possible to construct a maximum swimming speed-temperature relationship that shows both thermal tolerances and optima while accounting for acclimation potential and phenotypic plasticity. Thermal performance curves, built with swimming tests, can give insight to how temperature influences fitness related traits including activity, growth, and metabolic rate (Speers-Roesch and Norin 2016).

NITROGENIOUS CHEMICAL COMPOUNDS AND CHLORIDE ION TOLERANCE

Chemical stressors in water bodies occur naturally, but are often exacerbated by anthropogenic activities. For example, a combination of agricultural runoff and

biological waste may increase the concentration of nitrogenous chemical compounds in aquatic ecosystems. Because of the extensive agricultural land use in *N. topeka's* native range, cumulative point-source and non-point-source loading of nitrogen is a cause of concern (Adelman et al. 2009). Further, runoff, specifically road salt, has dramatically increased concentrations of chloride ions in streams surrounding metropolitan areas (Corsi 2010). The combination of agricultural and urban runoff may increase stress on the already endangered *N. topeka* and drive further declines.

Nitrogen is a naturally occurring compound essential for building amino acids. Its most toxic form, ammonia, can be oxidized by bacteria into nitrate or nitrite. Dissolved in water, ammonia (NH_3) reaches equilibrium with ammonium (NH_4) ions. Ammonium ions can displace potassium ions which depolarizes neurons and activates NMDA (i.e. N-methyl-D-aspartate) type glutamate receptors (Randall and Tsui 2002). This leads to an influx of excessive calcium ions that disrupt osmoregulation and negatively affect the central nervous system. Large doses of ammonia can cause convulsions, coma, and death in fish (Randall and Tsui 2002). In addition, prolonged exposure can cause increased ventilation frequency, reduced appetite, weight loss, and damage to gill tissue (Lang et al. 1987). Formed by oxidizing ammonia, nitrite and nitrate can harm aquatic organisms by converting hemoglobin and hemocyanin into forms unable to carry oxygen (Lewis and Morris 1986; Camargo et al. 2005). As a result, the fish suffers from anoxia and is unable to extract oxygen from the water.

Freshwater systems have experienced an increase in chloride ions from road salt, runoff, agriculture, water treatment facilities, and surrounding geology (Kaushal et al.

2005, Kelly et al. 2008). Chloride is detrimental to the environment because it acidifies streams, mobilizes toxic metals, alters riparian areas, and interferes with natural mixing of lakes (Kaushal et al. 2005). Osmoregulation of sodium chloride is crucial for maintaining hemostasis in fish. In contrast to saltwater fish that uptake water through the gut and excrete salts via gill tissue, freshwater fishes absorb water through the gills and excrete excess water and urea through the kidneys (Maetz 1971). Due to the electrochemical gradients for N^+ and Cl^- between fish plasma and low environmental ion concentration, freshwater fish must uptake these important ions from the surrounding water (Evans et al. 1999). When absorption is greater than excretion, osmoregulation can be disrupted in freshwater fish resulting in stress and death. This is important when considering that Topeka Shiners were more likely found in pools with lower mean conductivity (Novinger et al. 2011).

Given these potential impacts, we propose to examine the effects of nitrogenous compounds and chloride commonly resulting from animal waste and other agricultural and urban activities on the metabolic capacity of *N. topeka* as indicated by deviation from maximum swimming speed for a given temperature. Tolerance information is frequently obtained using lethal methods (e.g. LC_{50}); however Hammer (1995) described swimming speed as a useful, nonlethal alternative. Demonstrating the sub-lethal effects of chemical exposure, U_{crit} may surpass the usefulness of LC_{50} tests by yielding more ecologically relevant information (e.g. concentration of a chemical corresponding with the onset of physiological stress; Plaut 2001). For example, Coho Salmon experienced a significant linear decrease in swimming speed when subjected to sub-lethal ammonia

toxicity (Wicks et al. 2002). Using ramped U_{crit}, we will explore how common chemical pollutants could interfere with physiological performance and by extension growth prior to any lethal effects. However as LC_{50} data are generally more available for fish, it will also be important to validate our findings and provide more comparable data using traditional methodologies for determining tolerance.

CURRENT UNDERSTANDING OF TOLERANCES AND OPTIMA WITH THE TOPEKA SHINER AND CLOSE RELATIVES

The use of model organisms is common practice in laboratory settings. Our use of the Red Shiner (*Cyprinella lutrenisis*) as a model species gave us insight to how *N. topeka* might respond to a variety of conditions in water quality. Red Shiners were chosen over other similarly related species because of their abundance in nearby watersheds and information available in the literature. In some studies, Red Shiners have been used in experiments to compare physiological responses with species scarce in numbers. In thermal based laboratory experiments using Red Shiners, fish acclimated at 25°C had a mean Critical Thermal Maximum (CTM) of 38.99°C (Matthews and Maness 1979). Red Shiners also demonstrated remarkable tolerance to hypoxia. Without the access to surface oxygen, Red Shiners succumbed to hypoxic conditions from 1.50 to 0.85 mg/L (Mathew and Hill 1977).

Much like the Red Shiner, CTM experiments have demonstrated *N. topeka's* elevated tolerance to the isolated effects of high temperature (Koehle and Adelman 2007). At an acclimation temperature of 31°C, *N. topeka* showed maximum growth at

27°C with a CTM at 39°C (Koehle and Adelman 2007). In an effort to quantify Topeka Shiner swimming speed, Adams et al. (2000) measured sustained swimming speed (i.e. >200 min) at water velocities of 30 to 40 cm/s and prolonged and burst swimming speed (i.e. 10 to < 0.1 min) at 40 to 75 cm/s. Due to the selective pressures of low oxygen in prairie streams, the relationship between hypoxia and initiation of ASR is crucial to the management of *N. topeka*. Recent experiments have demonstrated that the Topeka Shiner is well adapted to hypoxia with a LC_{50} value of 1.26 mg/L (Koehle and Adelman 2007). Despite *N. topeka's* high tolerance to hypoxia, growth decreases as oxygen becomes scarce (Koehle and Adelman 2007). If a site proposed for reintroduction chronically falls below oxygen levels where *N. topeka* initiates ASR, the site may be within the range of tolerance for the species, but oxygen conditions could be detrimental to individual fitness and, therefore, overall population health.

Midwestern freshwater fish must also cope with influxes of runoff from roads and agricultural sources. In the summer, these stressors are often exacerbated by the seasonal desiccation of stream beds. Adult *N. topeka's* 96-hour LC_{50} for dissolved ammonia was 21.4 mg/L N (Adelman et al. 2009). The Environmental Protection Agency criteria for total ammonia nitrogen (TAN) is 17 mg TAN/L for acute conditions (i.e. ammonia levels at pH 7 and 20°C that exceed a one-hour average once every three years) and 1.9 mg TAN/L for chronic concentrations (i.e. ammonia levels at pH 7 and 20°C that exceed a 30-day average once every three years) (USEPA 2013). LC_{50} tests on Topeka Shiners resulted in nitrite tolerance of 6.1 mg/L N while nitrate tolerance was 1,559 mg/L N (Adelman et al. 2009).

CONCLUSIONS AND STUDY DESCRIPTION

Water quality influences growth, development, and physiology of aquatic vertebrates. Current criteria on water quality assessments are based on lethal level experiments (e.g. LC_{50} tests), which are poorly suited for evaluating optima or factors that cause low level stress and sub-lethal effects. Water quality characteristics below lethal thresholds may still impede growth and development, especially in the case of compounding low-level stressors. Understanding sub-lethal impacts of water quality characteristics is particularly important when examining the relative suitability of habitats for reintroduction of an endangered species as is currently underway for the federally endangered Topeka Shiner (*Notropis topeka*).

Costly conservation efforts may be misguided without a solid understanding of both physical habitat and water quality requirements of a species proposed for reintroduction. Data on Topeka Shiner optima can also help build better models to predict species abundance and distribution. Additionally, physiological data describing optima may be more beneficial to regulatory agencies setting water quality standards than mortality-based data. Thus, our project objectives are to (1) measure hypoxia tolerance based on behavioral cues related to a gradual reduction of dissolved oxygen in a controlled setting, (2) use ramped critical swimming speed to measure optima and tolerance to a range of temperatures and acclimations, and (3) use ramped critical swimming speed to determine the onset of stress to sub-lethal levels of ammonia, nitrite, and chloride concentrations. A full description of *N. topeka's* physiological ecology will enhance conservation efforts that include selecting the most suitable

reintroduction sites, predicting changes in distribution, and pinpointing causes for ongoing declines. By investigating the effects of temperature, acclimation, hypoxia, chloride, and nitrogenous chemical compounds, we hope to either (1) eliminate concerns about certain water quality conditions in proposed reintroduction sites and (2) demonstrate the harmful nature of particular water quality characteristics to the Topeka Shiner's physiology. Completion of this project will give insight to not only where Topeka Shiners can persist, but also thrive; our focus on optima over tolerance allows selection of the most suitable introduction sites matching its physiological profile. Additionally, the protocols developed can provide a foundation for the study of other small Missouri fishes. These sub-lethal techniques based on obtaining optima can be coupled with standard mortality studies (LC_{50}) to strengthen management and reintroduction practices of endangered fish species.

LITERATURE CITED

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CHAPTER 2: Use of Non-lethal Endpoints to Establish Water Quality Requirements and Optima of the Topeka Shiner (*Notropis topeka*)[1]

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ABSTRACT

Water quality influences growth, development, and physiology of aquatic vertebrates. In particular, the endangered Topeka Shiner (*Notropis topeka*) has broad water quality tolerances and optima. We investigated the effects of dissolved oxygen, temperature (with acclimation), nitrogenous chemical compounds, and chloride on Topeka Shiner using non-lethal endpoints by: (1) monitoring the Topeka Shiner's behavioral responses to a gradual reduction in oxygen, (2) determining thermal optima at different acclimation temperatures using swimming speed as a surrogate measure for metabolic performance, and (3) determined onset of stress to sub-lethal levels of nitrogenous compounds and chloride concentrations with swimming speed. We found ASR_{50} (i.e. dissolved oxygen concentrations where a certain percent of fish use aquatic surface respiration) to be 1.65 mg/L and ASR_{90} to be 1.08 mg/L of dissolved oxygen. Fish position rose to shallower waters at 5.52 mg/L of dissolved oxygen. Swimming tests conducted at three different acclimation temperatures indicated that the optimum temperatures for the species ranged from 17.7 to 28.0 °C, while the predicted 100% mortality ranged from 33.7 to 40.3 °C, depending on the acclimation temperature. Ammonia and sodium chloride reduced swimming speed at concentrations below known LC_{50} values. Other than an initial drop from 0-concentration, nitrite did not reduce swimming speed, however, substantial mortalities occurred at our highest test concentrations. Our results provide insight to not only where Topeka Shiners can persist, but also thrive; our focus on optima and sub-lethal effects over tolerance allows selection of the most suitable reintroduction sites matching the species' physiological

profile; however swimming speed may not be a suitable metric for determining the effects of all contaminants.

INTRODUCTION

Freshwater systems in North America are experiencing a rapid decline in freshwater biodiversity (Richter et al. 1997) due to an assortment of factors, including stream channelization, sedimentation, pollution, and predation by nonnative fishes. Though less visually dramatic than other forms of habitat degradation, poor water quality can devastate the aquatic community and limit the distributions of sensitive species. In particular, stable populations of the federally-listed endangered Topeka Shiner (*Notropis topeka*) have been linked to locations with favorable water quality conditions (USFWS 1988; Bayless et al. 2003; MDC 2010), though they likely possess a broad tolerance to adverse water quality conditions associated with seasonal flow variation. To advance Topeka Shiner conservation and recovery, managers need a better understanding of optimal physiological conditions for the species to better understand threats and declines and to evaluate the suitability of potential reintroduction sites. Studies used to establish water quality standards are often lethal to the study organism, an approach that is poorly suited for evaluating chronic effects levels or optimal conditions (Poole et al. 2004). Conditions below lethal threshold values may still impede growth and development, especially when considering the complex nature of compounding, low-level stressors. Further, for some applications

such as reintroduction, an understanding of threshold values for mortality may be of less interest than understanding physiological optima, or when water quality characteristics maximize potential fitness of organisms.

Despite its association in Missouri with good water quality (Bayless et al. 2003), the Topeka Shiner is likely adapted to overcoming challenging conditions over its life history. Living in isolated pools that seasonally suffer from desiccation and high nutrient load, the species must contend with hypoxia typical of prairie streams (Hatch 2001). Water-breathing fish adapted to hypoxia may exhibit behaviors that compensate for lack of oxygen in the water column, such as aquatic-surface respiration (ASR). Kramer (1983) defines aquatic surface respiration as a behavioral mechanism by which the fish ventilates its gills at the thin, oxygenated layer near the water surface. Fish use ASR to persist under hypoxia; however, this behavior comes at a potential cost (Domenici et al. 2007; Chapman and McKenzie 2009). Loss of schooling formation, coupled with vulnerability to aerial attacks, makes ASR risky and excludes other important behaviors (e.g. feeding, reproduction); it is therefore likely employed only when other physiological or morphological adaptations for extracting oxygen from the water column fail to maintain fish metabolism (e.g. increased hemoglobin content, larger gill surface area, lowered metabolic rate; Domenici et al. 2007). A common approach to identify hypoxia tolerance is to observe behavioral response to a gradual reduction in oxygen in a controlled environment (Chapman and Liem 1995); this incorporates potential for both physiological and behavioral adaptations to low oxygen levels.

Not only hypoxia, but other water quality factors, such as temperature and the presence of stressors, can affect fish fitness. Metabolic capacity of a fish is often measured as a surrogate for potential fitness and overall performance. In particular, measures of fish swimming performance are individually repeatable, can demonstrate aerobic capacity, and yield comparable data among individuals, thereby providing a useful reflection of basic physiological function (Plaut 2001). Critical swimming speed measures maximum aerobic capacity in fish by incrementally increasing speed until fatigue; its intent is to determine how fast a fish can swim until its energetic resources are exhausted (Hammer 1995). By using a quicker procedure to estimate critical swimming speed, termed ramped critical swimming speed (Jain et al. 1997), we can determine an optimum range of temperatures for physiological performance, account for the effects of acclimation, and estimate tolerance limits. Thermal performance curves, built with swimming tests, can give insight to how temperature influences fitness related traits including activity, growth, and metabolic rate (Speers-Roesch and Norin 2016). Furthermore, changes in critical swimming speed of fish provide information on physiological stress that can be directly related to mortality-based tests (i.e. LC_{50} tests) without harming the fish (Hammer 1995). We can use this non-lethal approach to identify how low-level concentrations of chloride, ammonia, and nitrite correspond with the onset of physiological stress.

In addition to swimming tests, we also can measure how acute exposure to different test temperatures can affect feeding rates at particular acclimation temperatures (Meeuwig et al. 2004). Results from thermal optimum swimming tests,

predicted lethal limits, feeding optimum, and laboratory observations of behavior originating from different experimental acclimations can be combined into one descriptive figure known as a thermal polygon (Fry et al. 1942; Elliott 1995). This figure can provide managers with descriptive thermal zones detailing growth, tolerance, and incipient lethal levels, which can be used to evaluate habitat for its potential fitness benefits. In addition to thermal experiments, our swimming tests can also demonstrate what concentrations of low-level chemical stressors will first cause a deviation of swimming speed from expected for standard experimental and acclimation temperatures. Demonstrating what sub-lethal factors may cause changes in swimming speed that could be detrimental to the fitness of individuals provides more meaningful information on the ability of fish to cope with stressful chemical conditions.

Costly conservation efforts may be misguided without a solid understanding of both physical habitat and water quality requirements of a species proposed for reintroduction, such as the Topeka shiner. To provide this information, our goal is to explore how water quality conditions influence the physiology and behavior of *N. topeka*. Using non-lethal physiological tests, we plan to (1) observe *N. topeka's* behavioral responses to a gradual reduction in oxygen, (2) determine thermal optima at different acclimation temperatures as indicated by swimming speed, and (3) discover when the onset of stress occurs for sub-lethal levels of nitrogenous chemical compounds and chloride concentrations using swimming speed. Our emphasis on non-lethal experimental endpoints has a twofold advantage of preventing unnecessary stress on fish used in the experiments and providing a more ecologically relevant assessment

of tolerance. This information will yield insight to not only where reintroduced Topeka Shiners can persist, but also thrive; our focus on optima over tolerance allows selection of the most suitable reintroduction sites matching its physiological profile.

METHODS

SPECIMENS

On December 11, 2015, United States Fish and Wildlife Service Neosho National Fish Hatchery provided Topeka Shiners from propagated stocks obtained from Sugar Creek in Harrison County, Missouri. While in transport, the fish were kept in large tanks with temperature control systems and oxygen bubblers, with no mortalities taking place. Upon arrival to the Anheuser-Busch Natural Resources Fish and Growth Laboratory at the University of Missouri, the fish were acclimated to room temperature over four hours and introduced into a 1000-L holding tank maintained at 23 ± 1°C and a 12 hour day and night cycle. Holding tanks were checked three times a week for conductivity, dissolved oxygen, and nitrogenous compounds associated with fish waste to ensure high water quality. After a three-day acclimation period, fish were weighed and measured. We counted 432 Topeka Shiners with a mean weight of 0.22 g and a mean total length of 31 mm (Table 1). Since the fish were mostly juveniles, we spent several months growing the fish with daily morning feedings to satiation with TetraFin fish flakes as recommended by Neosho National Fish Hatchery. Fish were divided into three different size classes (i.e. small, $x < 30$mm; medium, $30 \leq x \geq 40$; and large, $40 < x$) to organize sample groups for each experiment representative of the lab population.

HYPOXIA TOLERANCE

Fish behavioral responses to a gradual decrease in ambient oxygen levels were measured in a specially designed, 150-L tank during n = 10 experiments plus an additional group serving as a behavioral reference (Appendix, Figure A.1). For each experiment, fish were divided into sample groups over a range of sizes representative of the current Topeka Shiner population in the laboratory (n = 10 fish per experiment; Table 1). Because of their endangered status, we were unable to obtain additional animals from the hatchery over the duration of these experiments to control for changes body size in our laboratory population; however, we included fish from all three size classes in each experiment (i.e. small, $x < 30$mm; medium, $30 \leq x \geq 40$; and large, $40 < x$), to avoid size-related bias as much as possible. As such, growth of our experimental fish over the duration of experimentation did not lead to significant differences among hypoxia trials in mean total length and weight (i.e. ANOVA: mean total length $F = 0.49$, $p = 0.87$; ANOVA: mean weight $F = 0.59$, $p = 0.80$; Table 1). The sample groups were first held under dissolved oxygen conditions reflecting saturation at ambient temperature and atmospheric pressure (> 8 mg/L) in 37-L aquaria to prevent acclimation to hypoxia. After introduction to the test tank, a 30-minute behavioral acclimation allowed fish to adjust to the new environment. Four bubblers, two located at each end of the tank, were set behind plastic mesh screens to minimize disturbance to the experimental animals. During behavioral acclimation, the bubblers administered pressurized atmospheric gas to the hypoxia chamber. At initiation of the experiment, nitrogen gas was introduced through the bubblers to displace oxygen in the water from > 8.00 mg/L

to < 0.20 mg/L over the timeframe of approximately two hours. A small, submersible pump circulated the water to ensure the tank was well-mixed. When nitrogen was no longer effective in reducing oxygen levels in the water, 8 to 10 g portions of sodium sulfite were added directly into the tank above the bubblers (i.e. avoiding areas of the test tank that fish occupied) to bind any residual oxygen in the system (Kramer 1983). A YSI EcoSense ODO 200 probe, located in the center of the tank, continuously monitored oxygen levels (i.e. with precision at ±1.5% of reading or ±0.15 mg/L; whichever is greater). Topeka Shiner's behavior and oxygen concentration were concurrently monitored and recorded during experimentation using video cameras hidden behind a blind. If an individual lost equilibrium, it was quickly removed from the experimental tank and placed in well-oxygenated water to recover. The experiment was concluded 15 minutes after concentrations reached < 0.20 mg/L of dissolved oxygen.

A two-camera system (i.e. GoPro Hero 4 cameras) was used to track fish position in the tank over the duration of the experiment and later analyzed using VidSync, software for measuring individual fish coordinates in three dimensions (Neuswanger et al. 2016). Data were collected at predetermined oxygen concentrations from video footage: water oxygen content in mg/L, percent of fish using ASR, 3-D coordinates of fish within the tank, and loss of equilibrium or death of individual fish. In addition, observations of any unusual behaviors such as jumping (flight attempts) and aggression were noted in the video footage.

SWIM TUBE PROTOCOL

The primary goal of the swim tube protocol was to determine the ramped critical swimming speed (ramped U_{crit}) of individual fish. With the swimming tube (Appendix 1, Figure A.2), it was important to standardize and/or control for the following: (1) holding stress (e.g. short-term acclimation to experimental conditions); (2) flow characteristics (e.g. uniform, steady flow); (3) metabolic state of the fish (e.g. empty stomach, long-term acclimation or holding temperatures); (4) temperature; (5) and water quality (e.g. normoxia). To ensure all fish were in a similar metabolic state, individuals were fasted a minimum of 48 hours prior to testing in the swim tunnel (Matthews and Maness 1979). For our thermal experiments, it was necessary to developed two procedures for short-term acclimations (i.e. acclimations lasting < 12 hours) to minimize thermal shock and avoid loss of equilibrium. Because fish retain gene expression from long-term acclimation to daily temperature fluctuations (Podrabsky and Somero 2004), we could acclimate fish to the swim tube experimental temperature without changing the physiology from long-term acclimations. For swimming speed tests where the difference between long-term acclimation temperature (i.e. two-week long physiological acclimation) and the experimental temperature was < 15 °C, an acclimation of one hour was implemented to avoid loss of equilibrium. For swimming speed tests where the difference between long-term acclimation and experimental temperatures was > 15 °C, a 12-hour (i.e. overnight) acclimation was employed. The acclimation protocol was designed to minimize physiological stress related to rapid

changes in temperature while avoiding initiation of full physiological acclimation to the experimental temperature treatment.

After introduction to the swim tunnel, electric barriers were activated and held at low voltage (6-8 V) (Ward 2002), and the fish were given an additional 30-minute behavioral acclimation in the tube with no flow. This time allowed fish to recover from handling stress and behaviorally acclimate to the novel swim tube environment. Following this, using the standardized ramped U_{crit} protocol (Jain et al. 1997), water velocity was increased by 3 cm/s every 5 minutes until velocity was at 75% of the estimated U_{crit} value obtained from pilot runs with Red Shiners and data from previous work (Appendix 2, Figure A.4; Adams et al. 2000). After ramping the speed to 75% of the estimated U_{crit}, velocity in the swimming tube was increased by 3 cm/s every 20 minutes (Figure 1). The incremental increase in swimming speed, or "step size" (Figure 1), was based on the average body size of Topeka Shiners in the laboratory population (i.e. study population average of Topeka Shiners body size 3.1 cm, SD 0.5 cm; Table 1). For the ramped U_{crit} test, it was important to ensure that at least two twenty-minute intervals were completed to accurately determine a ramped U_{crit} value (Jain et al. 1997). The test ended when a fish was fatigued and unable to leave the rear electrical barrier after 10 seconds. After the flow was cut off, fish were removed from the swimming tube to recover and acclimated back to their holding temperature.

EVALUATION OF TEMPERATURE OPTIMA VIA SWIMMING PERFORMANCE

Temperature tolerances and optima were measured using ramped U_{crit} as an indication of metabolic capacity. Sample groups of fish from each size class (n =10 fish for 15 experiments, for a total of 150 fish) were subdivided into three different holding temperatures for a two-week acclimation period based on previous temperature experiments on specific growth of Topeka Shiners (Koehle and Adelman 2007; Adams et al. 2000). Similarly, Matthews and Maness (1979) used a two-week acclimation period for experiments involving CTM measurements on Red Shiners. Each sample group was comprised of fish representative of the lab population such that the range and average size of individuals varied as little as possible among treatments (Table 1). Swimming speed was later standardized by fish total length to account for differences among sample groups due to growth and small laboratory population size. Five groups were acclimated to 10°C in an Environmental Growth Chamber (i.e. model number W2WH-150-TAC-001), while a second Environmental Growth Chamber (i.e. model number W2WH-150-TAC-001) acclimated five additional groups to 30°C. Another five groups were held and acclimated to room temperature (23°C) over a two-week period. For each acclimation temperature, fish were tested for ramped U_{crit} at 5, 10, 17, 23, 30 and 35 ± 1°C experimental temperatures, except in cases where acclimation differences were too extreme. Water temperature in the swimming tube was adjusted with an AquaEuroUSA Model: MC-1/4HD chiller or with a 2500W Process Technology Immersion Heater. Sample groups from thermal experiments underwent long-term acclimation and were tested concurrently; sequential experiments were staggered by acclimation

temperature through time to avoid the confounding effects of growth with experimental or acclimation temperatures. This process was repeated until all fish were tested.

ESTABLISHMENT OF FEEDING LIMITS

We further explored thermal, sub-lethal endpoints by performing a feeding limit test on Topeka Shiners acclimated to room temperature (23 ± 1°C) for a two-week period. We chose fish from our 3 size classes for multiple temperature treatments (n = 10; 23, 30, 35, and 38 ± 1°C; ANOVA: mean total length among treatments $F = 0.55$, $p = 0.18$; mean weight among treatments $F = 0.54$, $p = 0.65$, Table 1) that matched predicted thermal tolerance values from prior swimming tests at 23 ± 1°C. Fish were placed in a 150 L tank for a two-hour acclimation period, while the ambient temperature was slowly raised to the test temperature using 2500W Process Technology Immersion Heater. A small, submersible pump was placed in the tank to circulate the water for uniform heating. For n = 10 fish for each test temperature, fish were fed 0.2 g of TetraMin fish flakes in the experimental tank. The ration was chosen based on daily observations from feeding n = 10 fish at room temperature. Topeka Shiner successful feeding strikes were recorded over a 5-minute period. It was important that the food was completely suspended in the water column because the Topeka Shiners did not strike food that was floating at the surface or resting on the bottom substrate. Trials were video recorded for later review in VidSync to count the number of successful feeding attempts for each test temperature. When an individual made a successful

feeding attempt, we would mark it with a symbol within VidSync, which compiled these data for analysis.

TOLERANCE TO NITROGENOUS COMPOUNDS AND CHLORIDE

Using ramped U_{crit} as an indication of metabolic capacity and fitness, we examined how the Topeka Shiner responded to select chemical concentrations below lethal levels. However, before we could explore sub-lethal effects, we determined traditional, acute lethal tolerance to chloride, ammonia, and nitrite by researching literature and, when information was not available, conducting LC_{50} experiments. We found LC_{50} values for ammonia and nitrite from previous experiments performed by Adelman et al. (2009), but no information on Topeka Shiner chloride tolerances. For chloride, we tested for lethal concentrations before we estimated sub-lethal effects. For this mortality-based experiment, Topeka Shiners were acclimated to room temperature (23 ± 1°C) for two weeks and placed in 37 L aquaria set at nine chloride concentrations (i.e. 0, 2000, 3000, 4000, 6000, 8000, 10000, 12000, and 14000 mg/L NaCl) with other factors held constant. Because of our limited number of specimens, we reused fish from thermal swimming tests for our LC_{50} test. Fish were given 2 months of rest after swimming tests before sorting by length into 9 groups (n = 10; ANOVA: mean total length $p = 0.47$, $F = 0.97$, ANOVA: mean weight $p = 0.48$, $F = 0.96$; Table 1) based on the average size of the Topeka Shiner lab population. Fisher BioReagent reagent grade sodium chloride was used for LC_{50} trials. After the groups were introduced into each treatment to initiate the trial, the number of dead fish was assessed at regular time

intervals by experimental chloride concentration (e.g. 0, 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hr).

For chemical swimming tests, conductivity and pH were controlled in the swimming tube with a combination of 7.5 pH buffer (MicroLife 7.5 pH Buffer), deionized water, and dechlorinated tap water. For our treatment chemicals used to dose the swimming tube, we used solid reagent-grade ammonium chloride (Sycamore Life Sciences RDCA0430-500B1), sodium nitrite (Sigma 52252), and sodium chloride (Fisher BioReagents BP-358-10). We first dissolved solids in a 1 L beaker before adding the solution into the swimming tube. We reached target test concentrations by continuously monitoring chemical levels with a Hydrolab DS4x Multiparameter Data Sonde as the dissolved solution was slowly added. If water chemistry changed during or between tests, we would add small amounts of deionized water or dissolved chemical to adjust concentrations. Fish from earlier hypoxia test (i.e. 150 individuals) were given 2 months rest after hypoxia trials before being sorted by length into 15 groups (n = 10; 5 groups for each chemical test) based on the average size of the Topeka Shiner lab population (Table 1). The first chemical treatment used for ramped U_{crit} starting treatment was set at LC_{25} to identify sub-lethal effects on swimming speed for chloride, ammonia, and nitrite. Based on the swimming response of the fish, the remaining treatment concentrations were determined, with the focus on finding the lowest, sub-lethal concentrations that result in a decrease in swimming speed. Experiments were conducted consecutively due to the finite supply of chemicals and deionized water. As described above, final swimming speed results were standardized by total fish length to

account for differences in size. Deviation from maximum potential swimming speed at similar acclimation temperatures (23 ± 1°C) determined the concentration of the chemical corresponding to the onset of metabolic stress.

DATA ANALYSIS

To evaluate the response to hypoxic conditions, we measured the percentage of fish in each test (i.e. n = 10 tests) using ASR at set oxygen concentrations (e.g. 8.00, 7.00, 6.00, 5.50, 5.00, 4.50, 4.00, 3.75, 3.50, 3.25, 3.00, 2.75, 2.50, 2.25, 2.00, 1.75, 1.50, 1.25, 1.00, 0.75, 0.50, and 0.25 mg/L O_2). We then created a binomial linear model in R studio program (R Core Team 2015; Venables and Ripley 2002) (i.e. n = 100 fish) by categorizing fish into two groups based on use or non-use of ASR at each oxygen concentration. Next, we calculated ASR_{90} and ASR_{50} from the binomial model as a measure of hypoxia tolerance (Chapman et al. 1995). We determined goodness-of-fit for the binomial model using the Hosmer and Lemeshow Test (Lele et al. 2014). For our position analysis, we used Vidsync, software that triangulates fish position from a two-camera perspective, to calculate three-dimensional coordinates of fish in our test tank (Neuswanger 2016). For each of our selected oxygen concentrations, we used Vidsync to find coordinates of fish from all 10 hypoxia treatments and the normoxia behavioral reference test. We then fit a polynomial regression model to the position data and used 95% confidence intervals to calculate oxygen values corresponding to when fish initiated a change in aquarium position (R Core Team 2015; Wickham 2009). Equal variance was tested with Bartlett's Test. The final test served as a behavioral reference to confirm

that ASR percentages and vertical fish positions remain constant under normoxia (i.e. > 8 mg/L dissolved O_2)

From our combined thermal swimming speed experiments, we constructed a thermal performance curve (best-fit polynomial model using R^2 and adjusted R^2 values; Condon et al. 2010; Table 2) for each acclimation temperature using R statistical package (R Core Team 2015; Wickham 2009). The peaks of the curves represented the temperatures at which maximum growth under full rations would be expected. After the regression equations were computed, we calculated the temperature that had the highest predicted maximum swimming speed. Through extrapolating the regression lines to higher test temperatures, we also predicted what temperatures would result in 100% mortality for each acclimation temperature. We then performed Bartlett's Test on our treatments to test for equal variances. Feeding trials did not require statistical analysis for interpretation but were visually assessed for indication of pattern in number of attempts at food across the four experimental temperatures.

For the NaCl LC_{50} test, we used R studio to calculate the LC_{50} value (R Core Team 2015; Venables and Ripley 2002). To determine goodness of fit, A p-value was calculated for the binomial distribution using the Hosmer and Lemeshow Test (Lele et al. 2014). For all chemical swimming tests, we calculated best-fit polynomial regression lines in R Studio with 95% confidence intervals (R Core Team 2015; Wickham 2009; Table 2). We found when the chemical concentration first corresponded with a significant decrease in swimming speed by comparing 95% confidence intervals along

the mean to the 95% confidence around 0 concentration. Bartlett's Test was used to test for equal variances.

RESULTS

During the hypoxia tolerance experiments, Topeka Shiners initiated ASR at 2.75 mg/L O_2 and at 0.50 mg/L O_2, all fish were using ASR (Figure 2, 3; ASR_{50} = 1.65 mg/L O_2; ASR_{90} = 1.08 mg/L O_2; Table 2, 3). At 5.52 mg/L O_2, fish initiated a rise to the water surface, and variation in water position visibly decreased (Bartlett's Test K^2 = 3520, $p < 0.001$; Figure 4). During the gradual reduction of dissolved oxygen, Topeka Shiner activity appeared to become more lethargic, presumably to reduce metabolic oxygen demands. Loss of equilibrium and deaths (n = 21) occurred at concentrations ≤ 0.45 mg/L with the majority occurring at concentrations below 0.25 mg/L (Table 4). A one-way ANOVA test indicated that Topeka Shiners that lost equilibrium or died were significantly larger in weight ($F = 24.7$, $p < 0.001$) and total length ($F = 15.5$, $p < 0.001$) than individuals surviving the hypoxia trials. No aggression, buccal bubble holding, synchronized schooling, or flight attempts were observed during the tests. Gill ventilation rate was not successfully measured because Topeka Shiner labored breathing manifested as gill "fluttering," exceeding the frame rate and resolution of video capture.

Fish acclimated to 10°C had a predicted maximum ramped critical swimming speed of 5.2 body lengths/sec at 17.7°C with a predicted mortality of 100% at 33.7°C (Figure 5). Topeka Shiners from this group had the slowest ramped critical swimming

speeds. Variance among individual trials was the greatest at highest test temperatures considerably greater than the acclimation temperature (Bartlett's Test $K^2 = 85.9$, $p < 0.001$; Appendix 2, Figure A.1). Fish acclimated to 23°C had the highest predicted maximum swimming speed of 8.6 body lengths/sec at 25.7°C and a predicted mortality of 100% at 37.5°C (Figure 6), showing similar patterns in among-individual variance between test temperatures (Bartlett's Test $K^2 = 59.6$, $p < 0.001$; Appendix 2, Figure A.2). Finally, Topeka Shiners acclimated to 30°C had a predicted maximum swimming speed of 6.7 body lengths/sec at 28.0°C and the highest predicted temperature for 100% mortality at 40.2°C (Figure 7), again with an increase in variance among individuals at the highest test temperatures (Bartlett's Test $K^2 = 62.5$, $P < 0.001$; Appendix 2, Figure A.3). Based on our swimming speed tests, Topeka Shiners demonstrated maximum metabolic activity between 17.7 and 28.0°C, depending on acclimation, with a lethal limit of 33.7°C to 40.3°C (Table 3; Figure 8). In general, Topeka Shiners showed signs of stress at test temperatures above their acclimation temperature including behaviors such as excessive gill ventilation, venturing into electrical barriers, and searching the top of the swimming tube for an escape. These behaviors may account for the increased variation among individuals at the warmest test temperatures.

In our feeding tests, we demonstrate how feeding strikes over time can differ with experimental temperature. Fish acclimated to 23°C exhibited the most feeding attempts over a 5 minute period at the 30°C test temperature (Figure 9). The final trial at the highest test temperatures (i.e. 38°C) resulted in a dramatic reduction in the

number of feeding attempts, accompanied by 100% mortality immediately following test conclusion (Figure 9).

Swimming speed was predicted to first deviate from what was expected at 0-concentration at 7.6 mg/L ammonia with one mortality occurring at 10 mg/L and three occurring at 50 mg/L (Figure 10). Variance in swimming speed increased with increased concentration of ammonia (Bartlett's Test $K^2 = 179$, $p < 0.001$). Nitrite did not reduce swimming speed beyond an initial drop from 0-concentration conditions (Figure 11). Regardless of experimental nitrite concentrations, fish exhibited similar swimming speeds; however, 7 mortalities were observed at 15 mg/L and 8 at 20 mg/L. Although a Bartlett's Test revealed differences in variance among samples, variance did not increase with chemical concentration ($K^2 = 93.5$, $p < 0.001$). For sodium chloride, for which there were no tolerance values in the literature for Topeka shiner, we experimentally determined the LC_{50} value to be 3942 ppm to inform swimming tests. Swimming speeds were comparable in our first four treatments and first decreased significantly from expected (speed at 0 ppm) at a concentration of 1993 ppm sodium chloride (Figure 12, Table 3). As with ammonia, variance among groups increased with the concentration of sodium chloride (Bartlett's Test $K^2 = 673$, $p < 0.001$).

DISCUSSION

We anticipated, for our study, that Topeka shiners would display broad water quality tolerances for naturally occurring stressors typical of the harsh prairie environment in which they evolved (Smale and Rabeni 1995). However, because

Topeka shiners are slated for reintroduction into new habitats with the goal of recovery, acute tolerance was of secondary interest than information on water quality characteristics that allow individuals to thrive. Our focus on water quality optima and the sub-lethal impacts of stressors allows selection of the most suitable watersheds for reintroduction and leads to a greater understanding of the Topeka Shiner's physiological profile.

For our experiments, Topeka Shiners demonstrated anticipated tolerance to hypoxic water quality conditions, comparable to other species of *Notropis* (Gee et al. 1978; Smale and Rabeni 1995; Ostrand 2001). This corroborates past research showing that Topeka Shiners can tolerate periods of hypoxia, though with considerable reductions in growth rate and increased vulnerability to predation (Domenici et al. 2007; Koehle and Adelman 2007). While Topeka Shiner can persist through periods of acute hypoxia, chronic hypoxia may prove more problematic. We found that, at intermediate levels of oxygen (5.52 mg/L), Topeka Shiners significantly altered their position closer to the water surface, where they may be more vulnerable to aerial predation. In past studies, growth of Topeka shiners slowed when held at dissolved oxygen concentrations below 4 mg/L (Koehle and Adelman 2007). In our hypoxia trials, we found that larger Topeka Shiners were more likely to lose equilibrium or die during hypoxia experiments, presumably due to higher metabolic demands that could not be met via obtaining oxygen at the air-water interface (Small et al. 2014). This may be of some concern for larger adult Topeka Shiners that have reached sexual maturity encountering acute hypoxia. Our mortalities and losses of equilibrium occurred at dissolved oxygen levels ≤

0.45 mg/L, below a measured 96 hours LC_{50} of 1.26 mg/L of dissolved oxygen (Koehle and Adelman 2007). Current standards set by the Missouri Department of Natural Resources (MDNR) require warm-water fisheries to have a minimum of 5 mg/L of dissolved oxygen (MDNR 2014). This standard might not be completely protective as we observed negative behavioral impacts occurring at 5.52 mg/L, supporting higher dissolved oxygen criteria. If reintroduction sites chronically fall below these levels, or result in prolonged periods of ASR for Topeka Shiner, it could jeopardize reintroduction attempts by lowering individual fitness, even if hypoxia does not directly lead to individual mortality.

In addition to hypoxic conditions, prairie environments bring extreme fluctuations in stream temperature, forcing fish to either find thermal refugia or persist via thermal tolerance (Mundahl 1990). Our predicted upper CTM for Topeka Shiners was 33.7 to 40.3°C, depending on their long-term acclimation temperatures (i.e. test temperatures 10, 23, 30 ± 1°C). Similar results were found in past studies when Topeka Shiners measured a CTM of 39°C at a 31°C acclimation (Koehle and Adelman 2007). Many minnow species demonstrate high critical thermal maxima (CTM), with the Red Shiner and Plains Minnow having similar measures of CTM to the Topeka Shiner (Matthews and Maness 1979; Ostrand and Wilde 2001; Smale and Rabeni 1995). In addition to tolerance, fish thermal performance curves are important to explore because of the correspondence with optimum temperature for activity, growth, and metabolic rate (Kellogg and Gift 1983; Speers-Roesch and Norin 2016). In our ramped critical swimming speed tests, Topeka Shiner predicted maximum metabolic activity for

our 10, 23, and 30 ± 1°C acclimation temperatures measured 17.7, 25.7, and 28.0 °C, respectively. This closely matches previous findings, indicating Topeka Shiner maximum growth rate (i.e. % growth per day) occurs when acclimated to approximately 27°C (Koehle and Adelman 2007).

To visually describe our findings on Topeka Shiner thermal ecology, we combined our thermal data, predicted values, feeding test results, and observations from acclimation into three zones describing growth, feeding, and mortality based on the fish acclimation and ambient temperature (Figure 13), though some components of the figure (i.e. dashed lines), particularly thresholds at lower temperatures, remain hypotheses. The lethal zone is defined as any ambient temperature that lies above the predicted incipient lethal levels from the swimming test (Figure 13). Our incipient lethal level curve closely matched results that showed Topeka Shiner CTM of 39°C when acclimated to 31°C (Koehle and Adelman 2007). The region that defines fish stress is located above the maximum ramped critical swimming speed and below the predicted lethal level. Maximum growth and reproduction of Topeka Shiners is expected to occur in the optimum zone. This zone represents not only temperatures that result in the highest metabolic potential of the species, but also the adaptive potential of the Topeka Shiner to adjust to different ambient temperatures based on acclimation temperatures. The green point at 30°C indicates the test temperature with the highest feeding intensity for 23°C acclimated Topeka Shiners (Figure 9, 13). Complete mortality from the 38°C feeding test closely matched our predicted incipient lethal limit of 37.5°C from ramped U_{crit} tests with Topeka Shiners acclimated to the same temperature (i.e. 23°C).

More feeding tests performed at different acclimation temperatures can further define a region of reduced growth. Overall, our results indicate the adaptability Topeka Shiners have to changes in acclimation and experimental temperatures.

Naturally-occurring chemical stressors in stream environments can exacerbate already stressful conditions such as hypoxia or high temperatures. Deviation of the maximum swimming speed from standard conditions yielded information on the negative consequences of excessive concentrations of nitrogenous chemical compounds that may be introduced into streams from animal waste, application of chemical fertilizers, or other anthropogenic sources. These compounds concentrate during periods of drought when prairie stream habitats may be reduced to isolated pools (Ostrand and Wilde 2001), or increase during runoff events from numerous non-point sources (Carpenter et al. 1998). High salt concentrations, which can occur naturally in inland systems, particularly in closed watersheds (Kelly et al. 2008), can also increase artificially as a result of road or agricultural runoff (Corsi et al. 2010) and influence abundance of less tolerant species (Ostrand and Wilde 2001).

Our acute swimming speed experiments with ammonia revealed a gradual reduction in swimming speed with increased concentration, as expected. At 7.6 mg/L ammonia, ramped critical swimming speed first significantly deviated from 0 concentration tests, a value considerably below the know LC_{50} value of 21.4 mg/L for adult Topeka Shiners (Adelman et al. 2009). Current Missouri water quality standards set by MDNR (2014) have acute total ammonia for cool and warm-fisheries restricted to below 19.9 mg N/L (assuming pH = 7.5), while chronic conditions are restricted to 2.6

mg N/L (assuming pH = 7.5). Our results indicate that acute increases in ammonia concentration measuring below current standards can have substantial sub-lethal effects on Topeka Shiner physiology. In addition, experiments predicting Topeka Shiner embryo-juvenile maximum acceptable toxicant concentration demonstrates vulnerability to low total ammonia concentrations (Adelman et al. 2009). Current standards for acute and chronic ammonia concentrations might therefore be inadequate to designate or protect suitable areas for Topeka shiner reintroduction.

Unlike ammonia, increased concentrations of nitrite did not consistently reduce swimming speed at concentrations above 10 mg/L. Instead, concentrations of 15 and 20 mg/L were associated with mortality after termination of the experiments. We concluded that our swimming speed tests did not adequately measure the immediate metabolic effect of nitrite on Topeka Shiners. For future studies on nitrite, longer sustained swimming speed, more detailed critical swimming speed treatments above 10 mg/L, or traditional measures of tolerance would be more appropriate for this particular contaminant. The manifestation of gill irritation and other soft tissue damage from nitrite may take longer to elicit an effect than a two-hour swimming test allows. Nitrite uptake causes methemoglobin (hemoglobin that cannot unbind to oxygen) to form in the blood, from which we would anticipate an eventual, if not immediate, reduction in oxygen-carrying capacity in the blood (Lewis and Morris 1986). The constant conversion of methemoglobin back to hemoglobin can take 24 to 48 hours (Lewis and Morris 1986). Post-experimental mortalities indicate that, after gradually absorbing nitrite during swimming tests, fish may have been unable to absorb oxygen for post-experiment

recovery. We conclude that ramped critical swimming speed is not a useful metric for measuring the effect of toxic stressors when the onset of stress and the physiological pathway of removal or compensation are delayed. A longer, sustained swimming test or an examination of growth under chronic exposure may be better suited to capture the onset of stress at sub-lethal levels of nitrite concentrations.

In our ramped critical swimming speed tests, Topeka Shiners decreased in swimming speed with an increased concentration of chloride, deviating significantly at concentrations considerably lower than our estimated lethal concentrations (1993 ppm versus 3942 ppm). A similar onset of effect was observed with the Fathead Minnow (IC_{25}, i.e. the concentration at 25% inhibition = 1810 ppm; Corsi et al. 2010), although the Fathead Minnow has a higher LC_{50} tolerance of 7650 ppm (Adelman et al. 1976). Headwater streams in Missouri that are home to the Topeka Shiner can achieve 100 mg/L of dissolved chloride ion (Bayless et al. 2003). As it follows, MDNR (2014) standards for acute chloride toxicity are 988 mg/L and are 610 mg/L for chronic toxicity, assuming a hardness of 400 mg/L. Streams can experience an artificial increase in chloride levels from chronic road salt runoff that is detrimental to community structure, diversity, and productivity (Corsi et al. 2010). These concentrations can often be exceeded in urban environments or heavy-traffic roads with substantial runoff (Gardner and Royer 2009). Chronic, persistent chloride concentrations may be of concern to the Topeka Shiner and can be addressed in future studies.

Many state agencies are invested in recovery plans to manage the federally endangered Topeka Shiner (KDW&P 2004; MDC 2010; SD GF&W 2003). Specifically, the

Missouri Department of Conservation is investigating several watersheds for suitability for reintroduction of the Topeka Shiner into its former range (MDC 2010). For these plans to be successful, an understanding of mechanistic linkages is needed between species' physiology and environmental conditions (Foin et al. 1988). Differences among watersheds in water quality characteristics are likely to be subtle, and existing information based on mortality studies is too coarse to differentiate what locations have optimum water quality conditions for reintroduced populations of Topeka Shiner. Water quality information provided in this study can provide more stringent, data-based criteria for aiding managers in identifying which sites have water quality characteristics most likely to maximize individual fitness. In addition to physical characteristics of the sites, this information can be integrated into a decision framework for prioritization of reintroduction sites that insure not only survival, but also maximize potential growth and reproduction of stocked individuals. Finally, information on water quality optima provided herein could inform ongoing efforts to predict the species current and future distribution (Wall et al. 2004).

Water quality optima and tolerances of the Topeka Shiner can be used in conjunction with information on other species in the system to promote recovery and persistence. Topeka Shiners are nest associates of the Orangespotted Sunfish (*Lepomis humilis*) and the Green Sunfish (*Lepomis cyanellus*) (Kerns 1983; Pflieger 1997). These fish are remarkably tolerant of hypoxic conditions when compared to other common headwater stream fish (Smale and Rabeni 1995). In addition, The Orangespotted Sunfish and Green Sunfish have similar maximum thermal levels when compared to the Topeka

Shiner (Smale and Rabeni 1995). This suggests that habitat that temporarily experiences hypoxic conditions and high temperatures could still support not only the Topeka Shiner but also its nest associates.

In contrast, the relatively high tolerance of the Topeka Shiner could provide opportunities for managers to reduce harm from its predators or competitors whose range of water quality tolerances may not be as great by selecting reintroduction sites that are temporarily stressful. Populations of Largemouth Bass (*Micropterus salmoides*) originating in small stream impoundments are a known threat to the species (Hatch 2001), pushing Topeka Shiners into less favorable habitat for foraging and reproduction (Knight and Gido 2005). These areas, though less favorable from a water quality standpoint, may serve as important refugia from a relatively intolerant predatory species (e.g. Chapman et al. 1995, 1996). The Largemouth Bass is considerably less tolerant to hypoxia and hyperthermia than the Topeka Shiner and its nest associates (Smale and Rabeni 1995). Although low oxygen conditions can negatively affect Topeka Shiner behavior and growth (Koehle and Adelman 2007), it can also prevent exotic invasions of Largemouth Bass into headwater streams while still permitting the shiner and its nest associates to persist.

The methodology presented in this study reflects our desire to understand water quality conditions promoting survival and maximizing growth and reproduction of the Topeka shiner. While our hypoxia tests followed a standard methodology, our novel use of the software, Vidsync, to analyze position and behavior is a relatively new approach (Neuswanger et al. 2016). By carefully tracking fish position to a gradual decrease in

dissolved oxygen, we can quantify behavioral reactions such as movement toward the surface before the initiation of ASR. This methodology enables a detailed exploration of the initiation of stress and behavioral changes associated with hypoxia. While we tracked fish position in our experiments, the video apparatus coupled with the analytical prowess of Vidsync yielded greater information on the behavioral changes occurring with the gradual oxygen reduction of a hypoxia test and, for some species, may be able to capture additional individual data more accurately (e.g. gill ventilation rate, heart rate, schooling formations, etc).

The use of swimming speed to measure physiological potential of fish is common practice (Hammer 1995; Plaut 2001). This non-lethal approach for determining optima can yield more ecologically relevant data compared to tolerance studies relying on mortality as an experimental endpoint (i.e. LC_{50} experiments) (Plaut 2001). Ramped critical swimming speed methodology was successful for testing effect concentrations in our case, with the exception of nitrite. For nitrate, which we did not test in this study, the amount required to illicit a likely physiological response was impractical to obtain in our 40 gallon swimming apparatus. Despite these limitations, our non-lethal methodology may be useful for other species of conservation concern. Physiological profiles can be created with relatively low numbers of specimens that would otherwise be impossible with lethal studies. However, swimming experiment methodology may require alteration for those species whose morphology and behavior discourages mid-water swimming (e.g. benthic fishes; Baltz et al. 1982).

Our experiments show, that for some stressors, physiological performance (i.e. active potential for growth and reproduction) is negatively influenced at concentrations far below threshold values obtained through traditional methods. Overall, information on sub-lethal optima yields a more complete and potentially more useful understanding of a species' ecology than traditional tolerance criteria emphasizing lethal and acute effects. Additionally, information of this kind supports management that incorporates the temporal and spatial nature of water quality regimes that drive species distributions in headwater streams (Pool et al. 2004). Regime-based standards, dynamic standards based on the seasonal changes in discharge, coupled with sub-lethal physiological data, could facilitate successful species recovery and environmental protection of our streams, and, ultimately, provide more protection for species like the Topeka Shiner.

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Chapter 3. OVERALL STUDY CONCLUSIONS

Water quality has a profound influence on aquatic vertebrates. Existing standards assess water quality frequently through lethal experimentation, which gives little information regarding optimal conditions. Data describing sub-lethal and optimal condition are ecologically relevant assessments of physiology, especially when considering species of conservation concern. Results from this study demonstrate how single low-level effects of natural stressors can influence *N. Topeka's* physiology. Our study on the federally endangered Topeka Shiner presents non-lethal approaches to finding optima and sub-lethal effects to common water quality conditions as an alternative to more traditional mortality studies. A combination of both optimum physiological conditions and tolerance is important to consider when developing management plans to differentiate watersheds for species reintroduction, compare population genetics related to differences in tolerance and optima, and predict distribution and abundance.

Although tolerant to low oxygen conditions commonly encountered in prairie streams, Topeka Shiners significantly changed position in the water column before initiation of aquatic surface respiration. Temporary declines in dissolved oxygen may in fact help reintroduced populations by selecting against larger, intolerant predators. However, acute hypoxia can also differentially affect adult Topeka Shiners and reduce reproductive success. Chronic hypoxic areas may be of greater concern in most streams limiting growth and reproduction.

Our acclimation and temperature studies based on sub-lethal swimming speed methodology set the framework for *N. topeka's* thermal tolerance profile. The optimum range of temperatures exhibits the important role of acclimation with determining the adaptive potential of Topeka Shiners. Using our thermal data, managers can find suitable reintroductions sites that have thermal regimes within the Topeka Shiners adaptive potential for thermal acclimation and tolerance. Further research into critical thermal temperatures, feeding limits using additional acclimation temperatures, and supplementary physiological tests for determining optimal thermal conditions would create a more accurate, explanatory thermal tolerance profile.

In this study, swimming speed proved to be an informative, non-lethal physiological test compared to traditional experiments based on mortality. Our study explored commonly occurring chemical stressors and demonstrated that ammonia and sodium chloride have significant physiological effects below known LC_{50} values. The sub-lethal effects of nitrite on Topeka Shiners requires further investigation with different swimming studies or mortality based experimentation. While most chronic standards are protective for ammonia and sodium chloride, agricultural and urban environments can cause acute influxes of pollutants that have sub-lethal impacts affecting growth and reproduction.

Future studies of Topeka Shiner physiology would be informed through incorporation of compounding effects of several water quality conditions. When multiple stressors are combined, they may result in unexpected effects on physiology. Further experimentation on these complex relationships will result in a holistic

understanding of the Topeka Shiner's optimal water quality conditions and tolerance. Also, water quality studies on different life stages could potentially reveal possible vulnerabilities throughout the Topeka Shiner's life history. In this respect, managers could focus on important life history stages and select appropriately protective management techniques. Incorporating our non-lethal approaches for determining physiological optima with traditional mortality based studies can create more ecologically relevant data. Such data will help agencies better manage and conserve threatened or endangered species.

Table 1: Topeka Shiner weights and lengths for experiments.

Description	Date	Number	Ave. Length (mm)	Std. Dev. (mm)	Ave. Weight (g)	Std. Dev. (g)
Fish Starting Size	12/16/15	432	31	5	0.22	0.14
Hypoxia Tests						
Test 1	4/29/16	10	41	5	0.60	0.35
Test 2	5/23/16	10	43	6	0.61	0.25
Test 3	6/21/16	10	44	6	0.73	0.42
Test 4	6/22/16	10	43	8	0.77	0.55
Test 5	6/24/16	10	46	7	0.88	0.40
Test 6	7/5/16	10	45	7	0.81	0.44
Test 7	7/7/16	10	45	6	0.82	0.37
Test 8	7/8/16	10	44	5	0.71	0.31
Test 9	7/14/16	10	45	6	0.74	0.43
Test 10	7/15/16	10	44	7	0.90	0.55
Behavioral Reference	6/21/17	10	51	10	0.76	0.33
Feeding Tests						
Test 1 (22°C)	9/29/16	10	44	4	0.74	0.20
Test 2 (30°C)	9/30/16	10	45	4	0.86	0.34
Test 3 (35°C)	10/3/16	10	48	6	0.92	0.45
Test 4 (38°C)	10/24/16	10	44	4	0.81	0.26

LC50 NACL						
NaCl 0mg/L	11/30/16	10	48	6	0.86	0.38
NaCl 2000 mg/L	11/30/16	10	48	6	0.89	0.40
NaCl 3000 mg/L	11/30/16	10	44	4	0.61	0.15
NaCl 4000 mg/L	11/30/16	10	47	5	0.87	0.42
NaCl 6000mg/L	11/30/16	10	47	6	0.84	0.44
NaCl 8000 mg/L	11/30/16	10	46	7	0.81	0.45
NaCl 10000 mg/L	11/30/16	10	46	4	0.74	0.27
NaCl 12000 mg/L	11/30/16	10	47	4	0.74	0.28
NaCl 14000 mg/L	11/30/16	10	50	5	1.00	0.33

Thermal Tests						
Acclimation 10°C, Test 5°C	3/1/2016 - 6/22/2016	10	42	6	0.68	0.34
Acclimation 10°C, Test 10°C	3/2/2016 - 6/21/2016	10	42	4	0.66	0.23
Acclimation 10°C, Test 17°C	2/5/2015 - 6/16/2016	10	39	3	0.47	0.12
Acclimation 10°C, Test 23°C	2/9/2016 - 6/6/2016	10	39	4	0.48	0.19
Acclimation 10°C, Test 30°C	2/11/2016 - 6/3/2016	10	39	3	0.48	0.16
Acclimation 23°C, Test 10°C	3/4/2016 - 5/23/2016	10	40	5	0.52	0.19
Acclimation 23°C, Test 17°C	2/1/2016 - 5/27/2016	10	37	3	0.38	0.11
Acclimation 23°C, Test 23°C	1/28/2016 - 5/17/2016	10	42	6	0.60	0.26
Acclimation 23°C, Test 30°C	3/28/2016 - 5/19/2016	10	38	5	0.38	0.19
Acclimation 23°C, Test 35°C	2/23/2016 - 5/23/2016	10	40	4	0.56	0.15
Acclimation 30°C, Test 10°C	3/3/2016 - 6/14/2016	10	39	4	0.48	0.16
Acclimation 30°C, Test 17°C	2/2/2016 - 6/16/2016	10	39	3	0.50	0.16
Acclimation 30°C, Test 23°C	1/25/2016 - 6/8/2016	10	39	3	0.47	0.11
Acclimation 30°C, Test 30°C	2/15/2016 - 6/10/2016	10	42	3	0.55	0.15
Acclimation 30°C, Test 35°C	2/18/2016 - 6/13/2016	10	40	4	0.54	0.17

Chemical Tests						
NaCl 5000 ppm	7/6/2016 - 7/12/2016	10	43	6	0.75	0.39
NaCl 2500 ppm	7/12/2016 - 7/15/2016	10	43	8	0.76	0.44
NaCl 1250 ppm	7/15/2016 -7/25/2016	10	55	4	0.67	0.21
NaCl 625 ppm	7/25/2016 - 8/5/2016	10	46	6	0.86	0.45
NaCl 315 ppm	8/1/2016 - 8/5/2016	10	47	6	0.85	0.36
NaNO3 20 mg/L	9/30/2016 - 9/6/2016	10	44	8	0.84	0.62
NaNO3 15 mg/L	10/7/2017 - 11/3/2016	10	47	5	0.90	0.28
NaNO3 10 mg/L	10/13/2016 - 11/3/2016	10	48	8	1.00	0.48
NaNO3 5 mg/L	10/24/2016 - 10/31/2016	10	46	5	0.88	0.33
NH3 50 mg/L	1/30/2016 - 2/3/2017	10	46	6	0.75	0.27
NH3 25 mg/L	2/6/2017 - 2/22/2017	10	45	4	0.60	0.11
NH3 15 mg/L	2/22/2017 - 3/1/2017	10	47	4	0.71	0.23
NH3 10 mg/L	3/2/2017 -3/17/2017	10	48	6	0.73	0.4
NH3 5 mg/L	3/17/2017 - 3/29/2017	10	47	7	0.75	0.38

Table 2: Regression analysis used for Topeka Shiner physiological tests.

Acclimation Temperature (°C)	Regression	Equation	P Value	Multiple R Squared	Adjusted R Squared
10	2nd poly	$y = -2.016e\text{-}2(x^2) + 7.136e\text{-}1(x) - 1.124$	< 0.0001	0.463	0.440
23	3rd poly	$y = -2.228e\text{-}3(x^3) + 1.232e\text{-}1(x^2) - 1.758(x) + 10.209$	< 0.0001	0.663	0.641
30	3rd poly	$y = -1.003e\text{-}3(x^3) + 5.092e\text{-}2(x^2) - 4.933e\text{-}1(x) + 2.859$	< 0.0001	0.443	0.407

Chemical Trial	Regression	Equation	P Value	Multiple R Squared	Adjusted R Squared
Ammonia	2nd poly	$y = 1.221e\text{-}3(x^2) - 1.421e\text{-}1(x) + 7.386$	< 0.0001	0.554	0.538
Nitrite	3rd poly	$y = -9.240e\text{-}4(x^3) + 3.87e\text{-}2(x^2) - 5.008e\text{-}1(x) + 7.58$	0.0009	0.296	0.250
Sodium Chloride	3rd poly	$y = 2.046e\text{-}10(x^3) - 1.541e\text{-}6(x^2) + 1.471e\text{-}3(x) + 7.525$	< 0.0001	0.645	0.626

Hypoxia Trials	Regression	Equation	P Value	Multiple R Squared	Adjusted R Squared
Fish Position	3rd poly	$y = -1.502e\text{-}1(x^3) + 2.634(x^2) - 16.023(x) + 50.338$	< 0.0001	0.477	0.476
Control Fish Position	Linear	$y = -1.51e\text{-}2(x) + 4.471$	< 0.0001	0.019	0.010
Percent ASR	Binomial	O2 = -3.851, Intercept = 6.352	< 0.0001	-	-

Lethal Trials	Regression	Equation	P Value	Multiple R Squared	Adjusted R Squared
Sodium Chloride LC_{50}	Binomial	NaCl = -2.390e-2, Intercept = 94.56	< 0.0001	-	-

Table 3: Effects table of physiological optima and tolerances for the Topeka Shiner.

Swimming Trials

Acclimation Temperature (°C)	P Value	Predicted Optimum (°C)	Predicted 100% Mortality (°C)
10	< 0.0001	17.7	33.7
23	< 0.0001	25.7	37.5
30	< 0.0001	28.0	40.3

Chemical	P Value	LC_{50}	Effect Detected
Ammonia	< 0.0001	*21.4 mg/L	7.6 mg/L
Nitrite	0.0009	*6.1 mg/L	4.7 mg/L
Sodium Chloride	< 0.0001	3942 ppm (P < 0.0001)	1993 ppm

Behavioral Trials

Hypoxia	P Value	Effect Detected	ASR_{50}	ASR_{90}
Fish Position	< 0.0001	5.52 mg/L	-	-
Percent ASR	< 0.0001	-	1.65 mg/L	1.08 mg/L

*Value obtained from Adelman et al. 2009.

Table 4: Loss of equilibrium and death from 21 Topeka Shiners as a result of oxygen deprivation. Test number, oxygen concentrations at loss of equilibrium or death, weight, and length are shown for each fish.

Hypoxia Test Number	Dissolved Oxygen (mg/L)	Weight (g)	Length (mm)
1	0.22	1.78	58
2	0.23	0.62	43
3	0.21	1.81	57
4	0.45	1.63	53
4	0.22	1.56	55
4	0.21	1.34	52
5	0.22	1.74	56
6	0.43	0.55	39
6	0.21	0.45	39
7	0.22	1.39	52
7	0.21	1.50	58
7	0.21	1.08	51
7	0.21	0.49	40
8	0.21	1.11	49
8	0.12	0.82	45
8	0.22	1.04	50
8	0.19	0.90	46
8	0.19	0.38	35
8	0.19	0.70	41
10	0.20	2.24	60
10	0.20	0.84	44

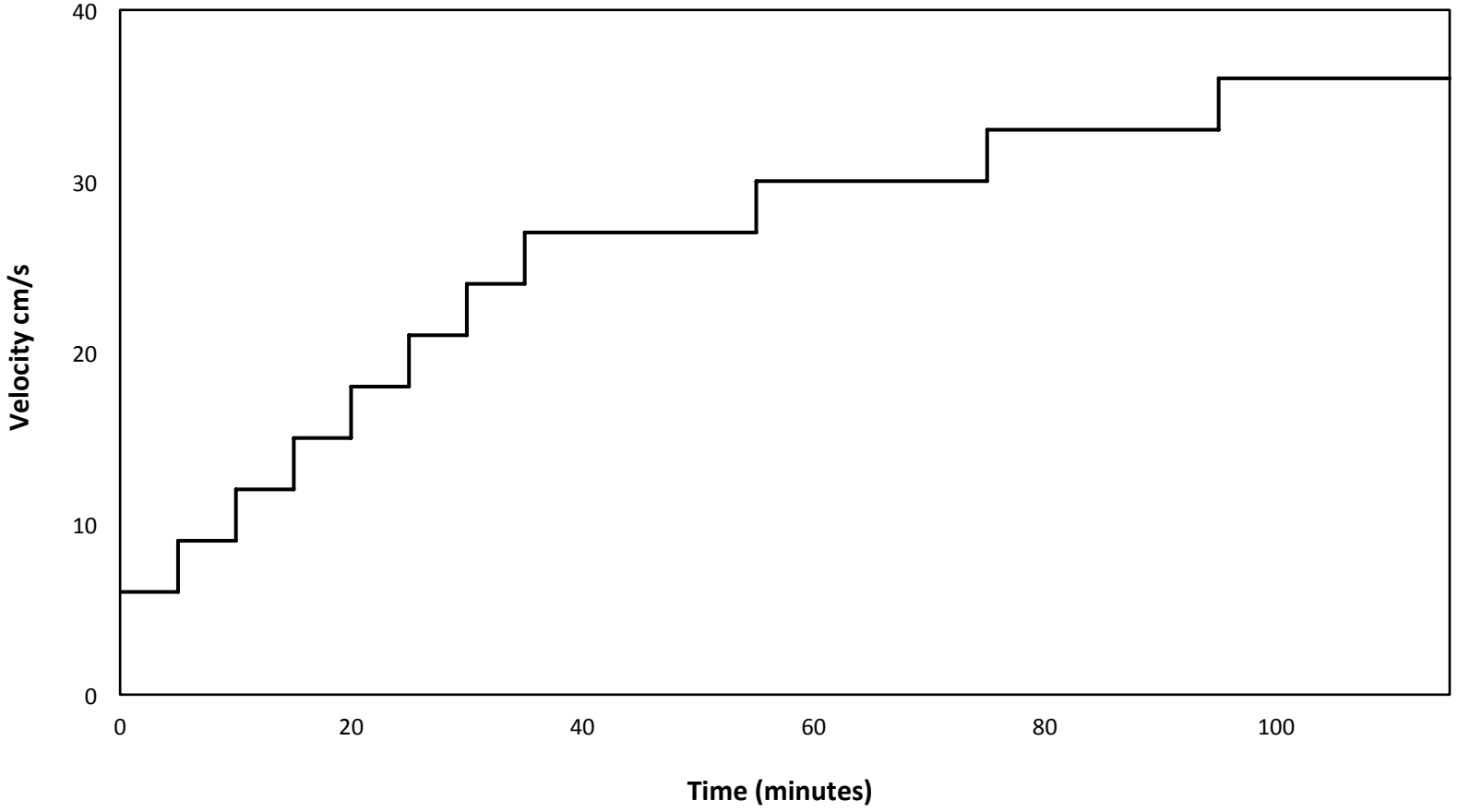

Figure 1**:** Target velocities within the swim tube over the course of a single run for obtaining ramped critical swimming speed (ramped U_{crit}) for a fish acclimated and swam at 23±1°C, assuming a critical swimming speed of around 33 cm/s.

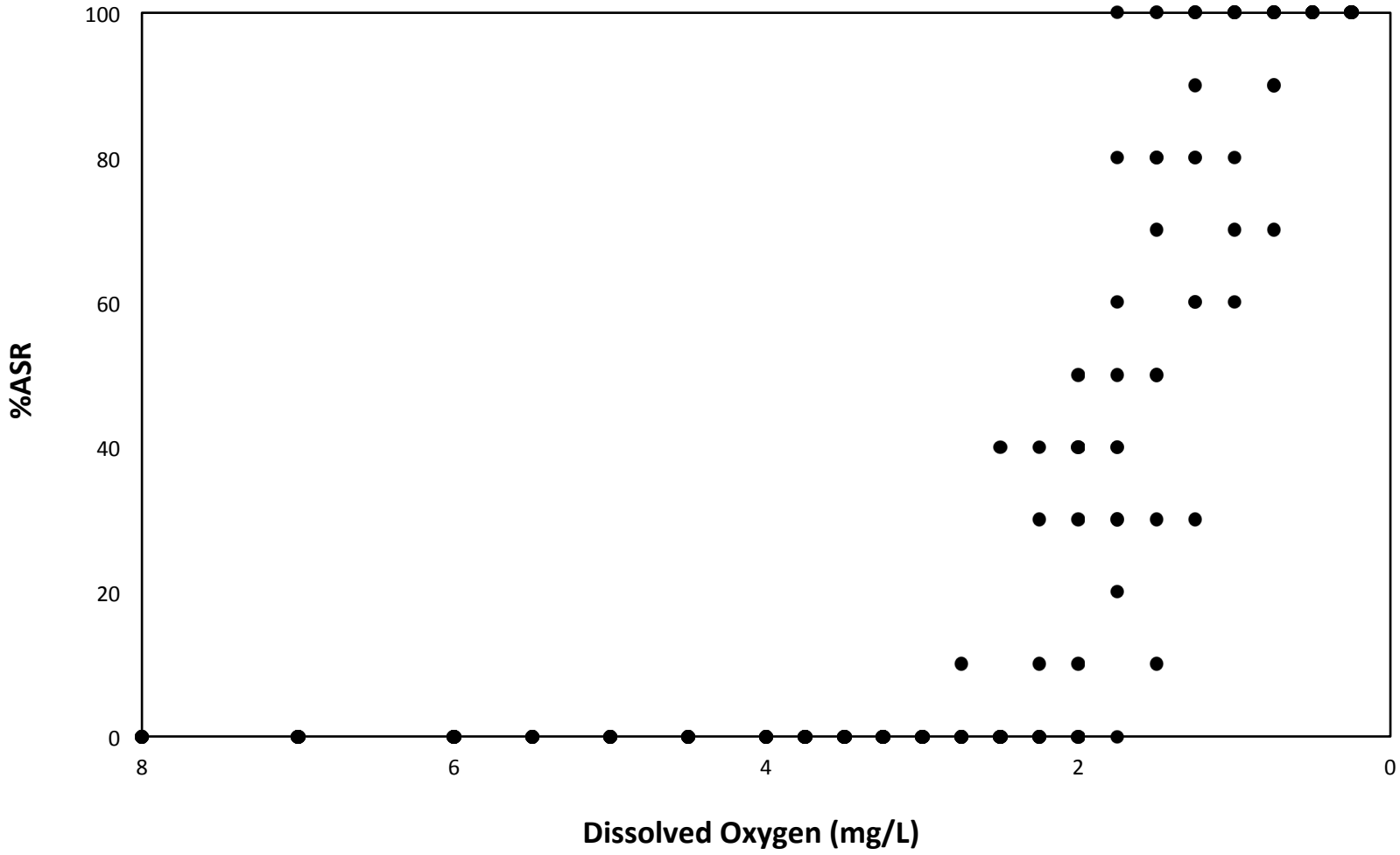

Figure 2: Percent of Topeka Shiners using aquatic surface respiration (ASR) over a range of experimental oxygen concentrations. Each point represents n = 10 individuals for a single trail. Data from all 10 trials, excluding the control trial, are plotted (n = 10 fish per trial).

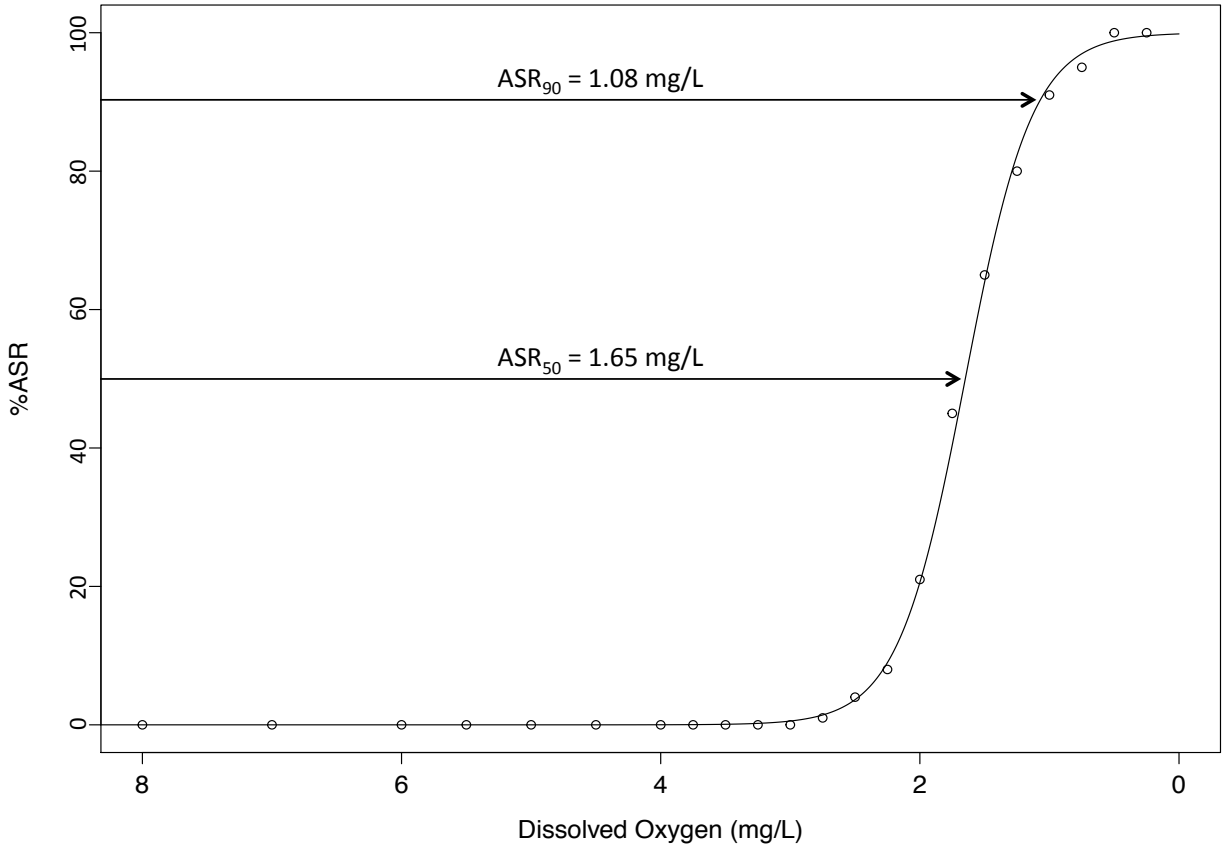

Figure 3: Binomial curve summarizing the response of Topeka shiner to a gradual reduction in oxygen over the course of n = 10 experiments. Each point represents the percentage of Topeka Shiners using Aquatic Surface Respiration (ASR) for all experiments.

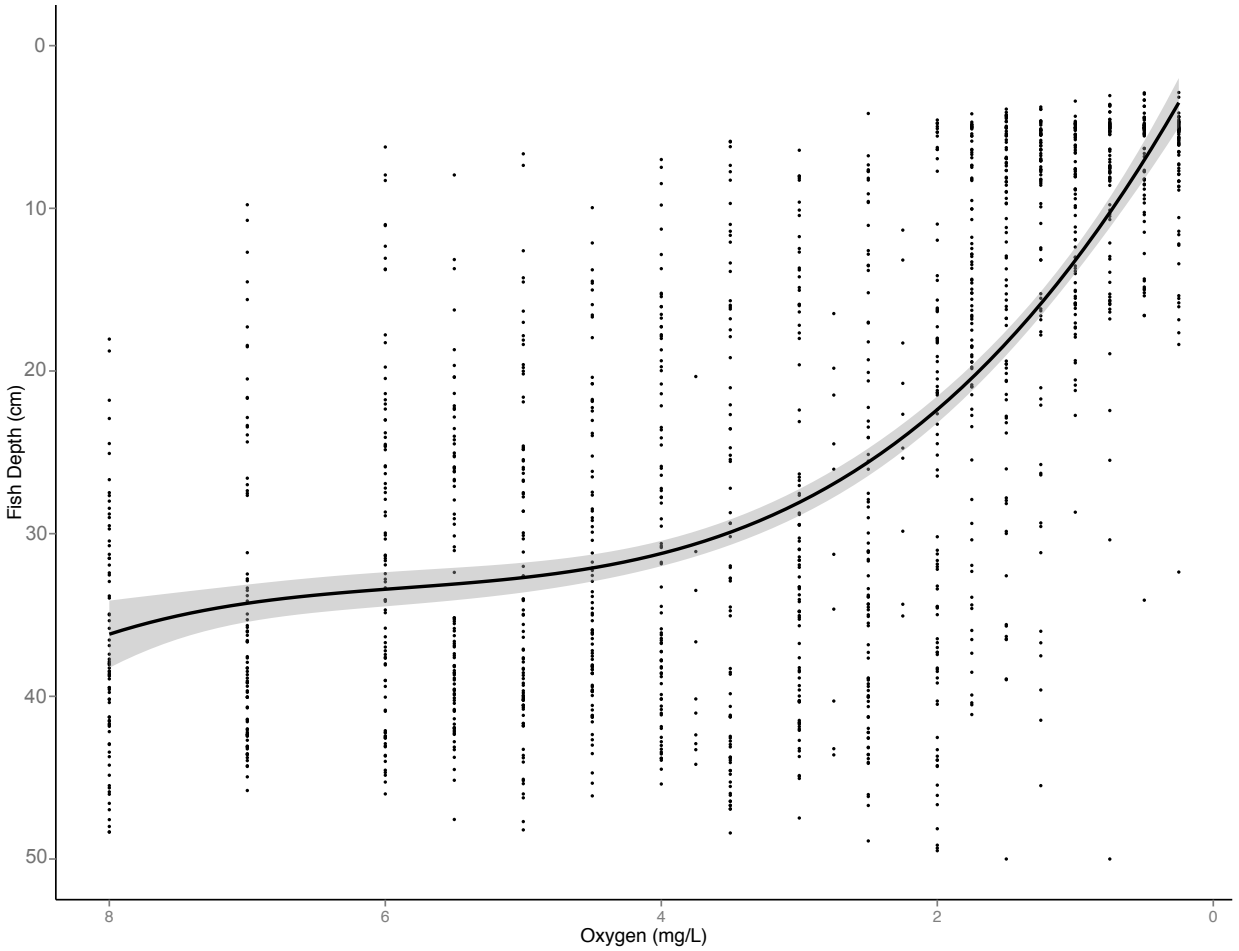

Figure 4: Polynomial regression of average depth of Topeka Shiner in experimental tanks over a range of oxygen concentration with a 95% confidence interval. Each point represents individual observations in position for n = 100 individuals for n = 10 trials at selected oxygen concentrations.

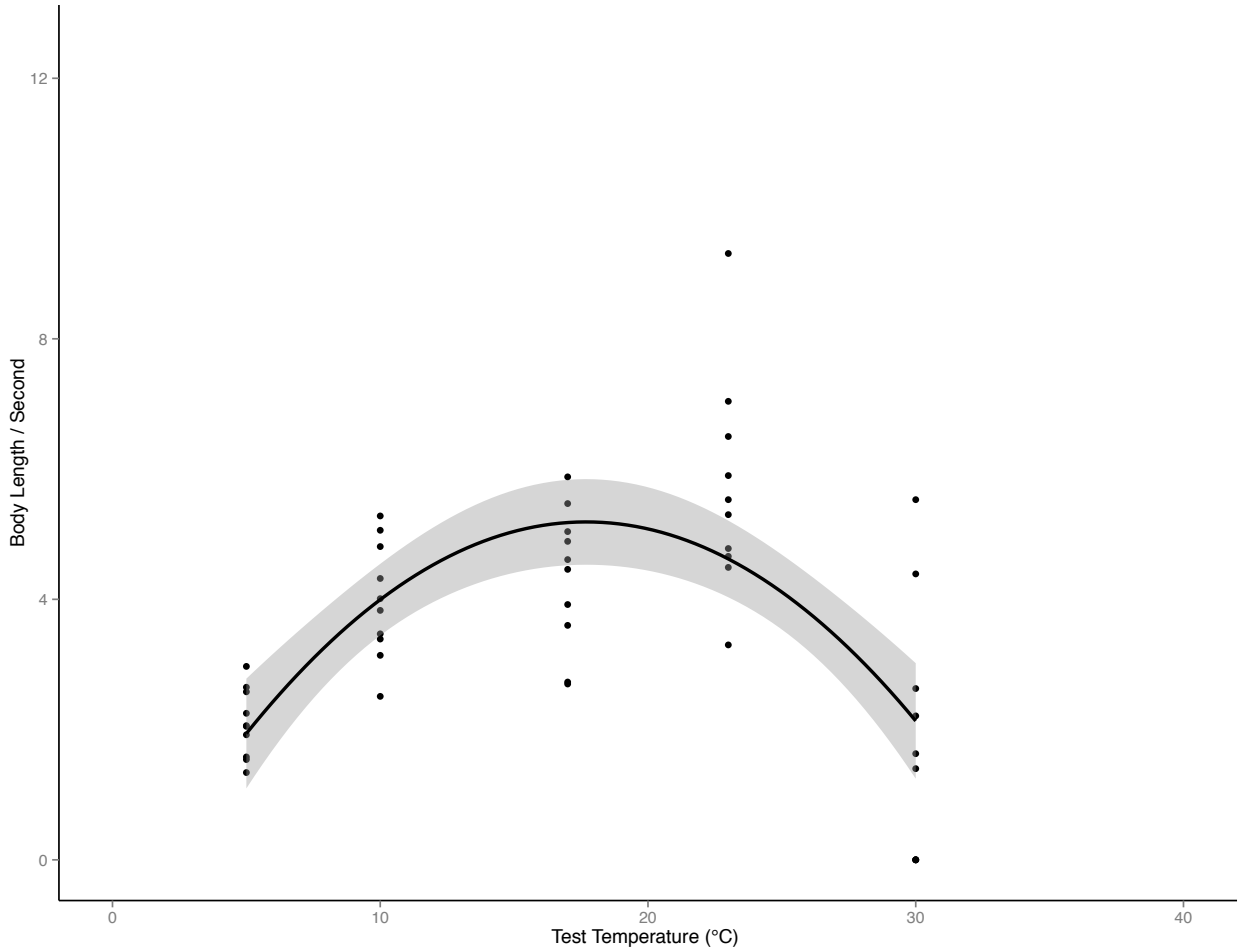

Figure 5: Polynomial regression of ramped critical swimming speed (ramped U_{crit}), standardized by body length of Topeka Shiners acclimated to 10°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. Two mortalities were observed at 30°C.

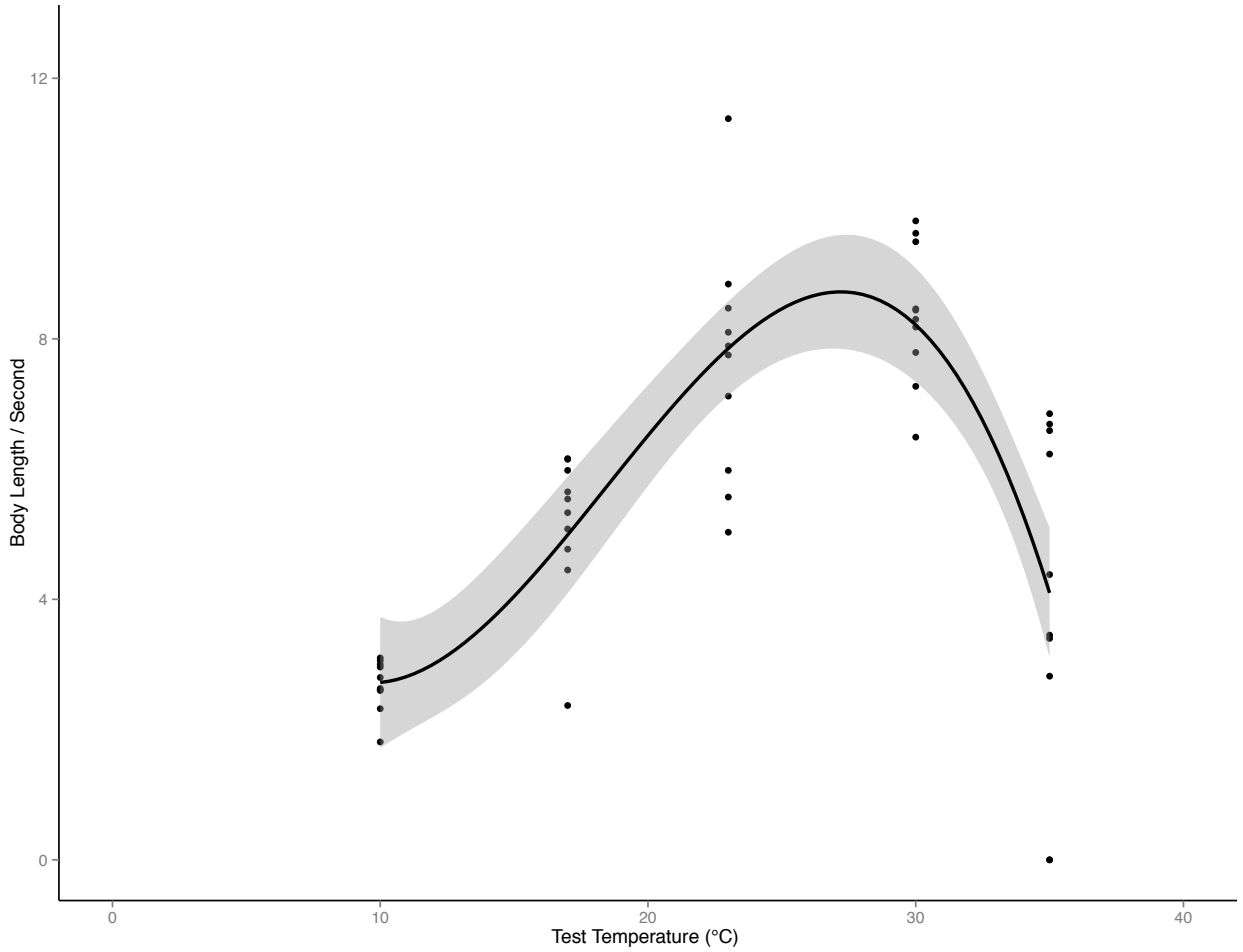

Figure 6: Polynomial regression of ramped critical swimming speed (ramped U_{crit}), standardized by body length of Topeka Shiners acclimated to 23°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. Three mortalities were observed at 35°C.

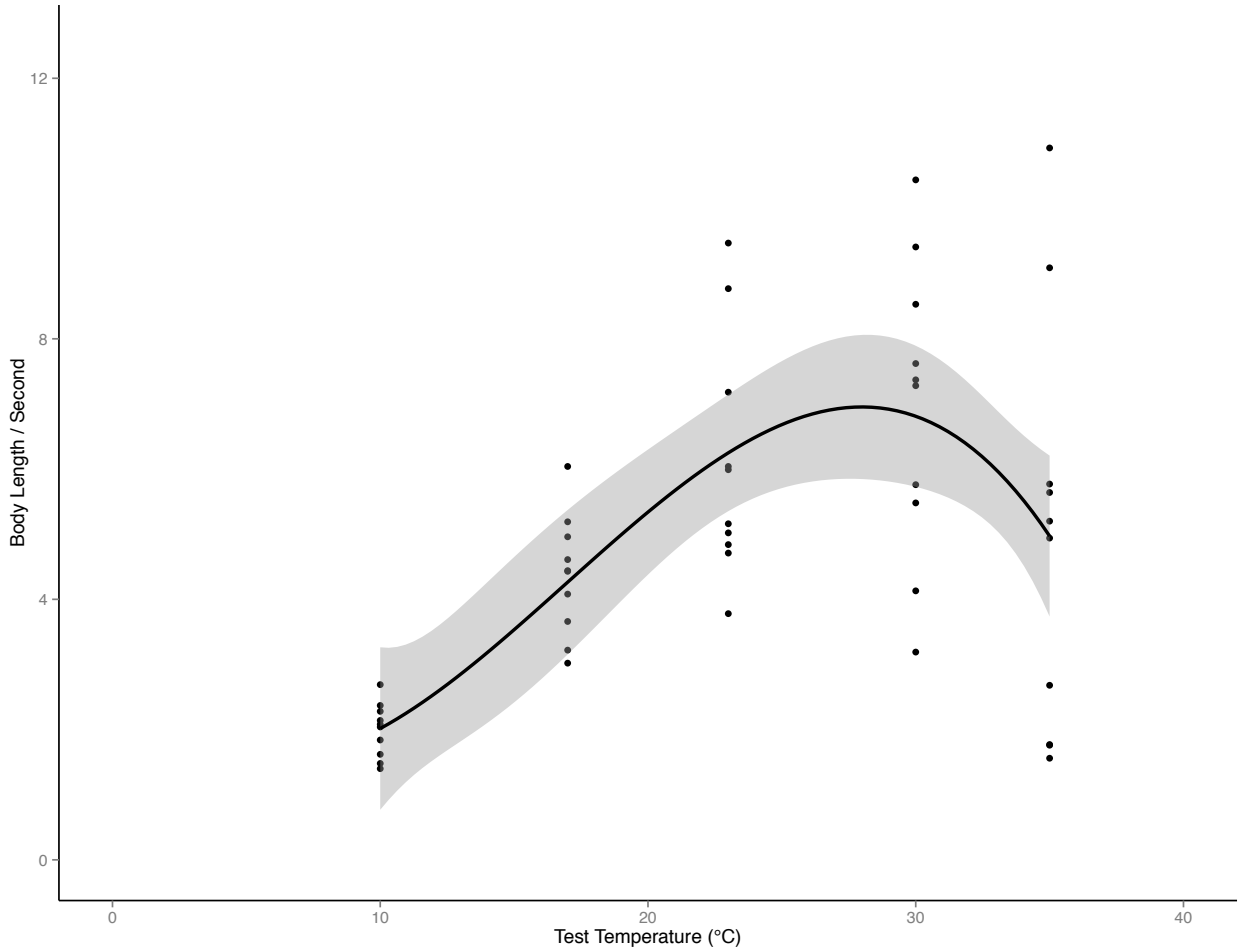

Figure 7: Polynomial regression of ramped critical swimming speed (ramped U_{crit}), standardized by body length of Topeka Shiners acclimated to 30°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. No mortalities took place during experimentation.

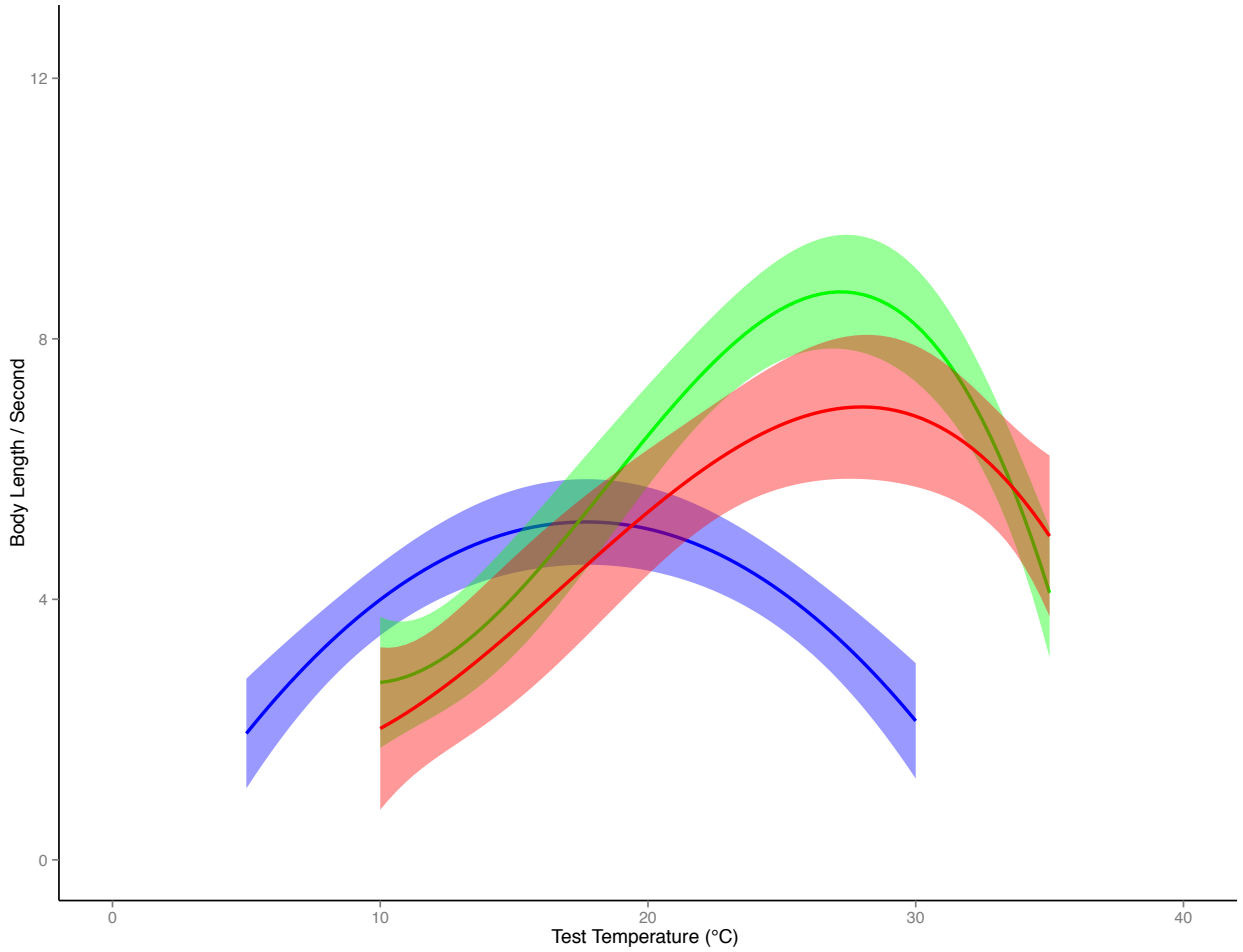

Figure 8: Polynomial regression of ramped critical swimming speed (ramped U_{crit}), standardized by body length of Topeka Shiners acclimated to 10°C (blue), 23°C (green), and 30°C (red) and tested over a ranged of experimental conditions. The shaded color zone represents the 95% confidence interval around the similar color line.

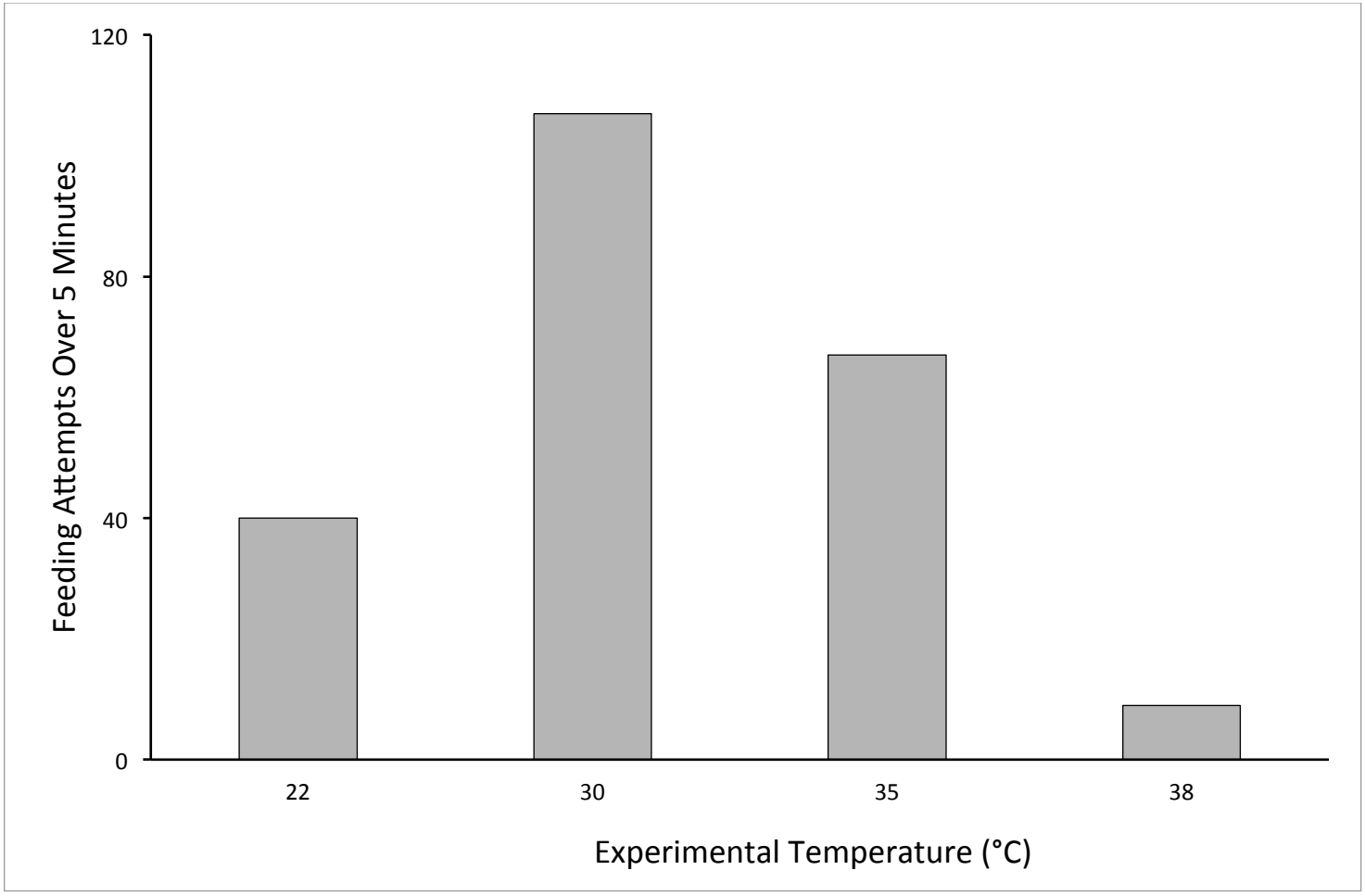

Figure 9: Number of feeding attempts observed over 5 minutes for Topeka Shiners acclimated to 23°C and exposed to a range of experimental temperatures (n = 4 trials, 10 Topeka Shiners per trial).

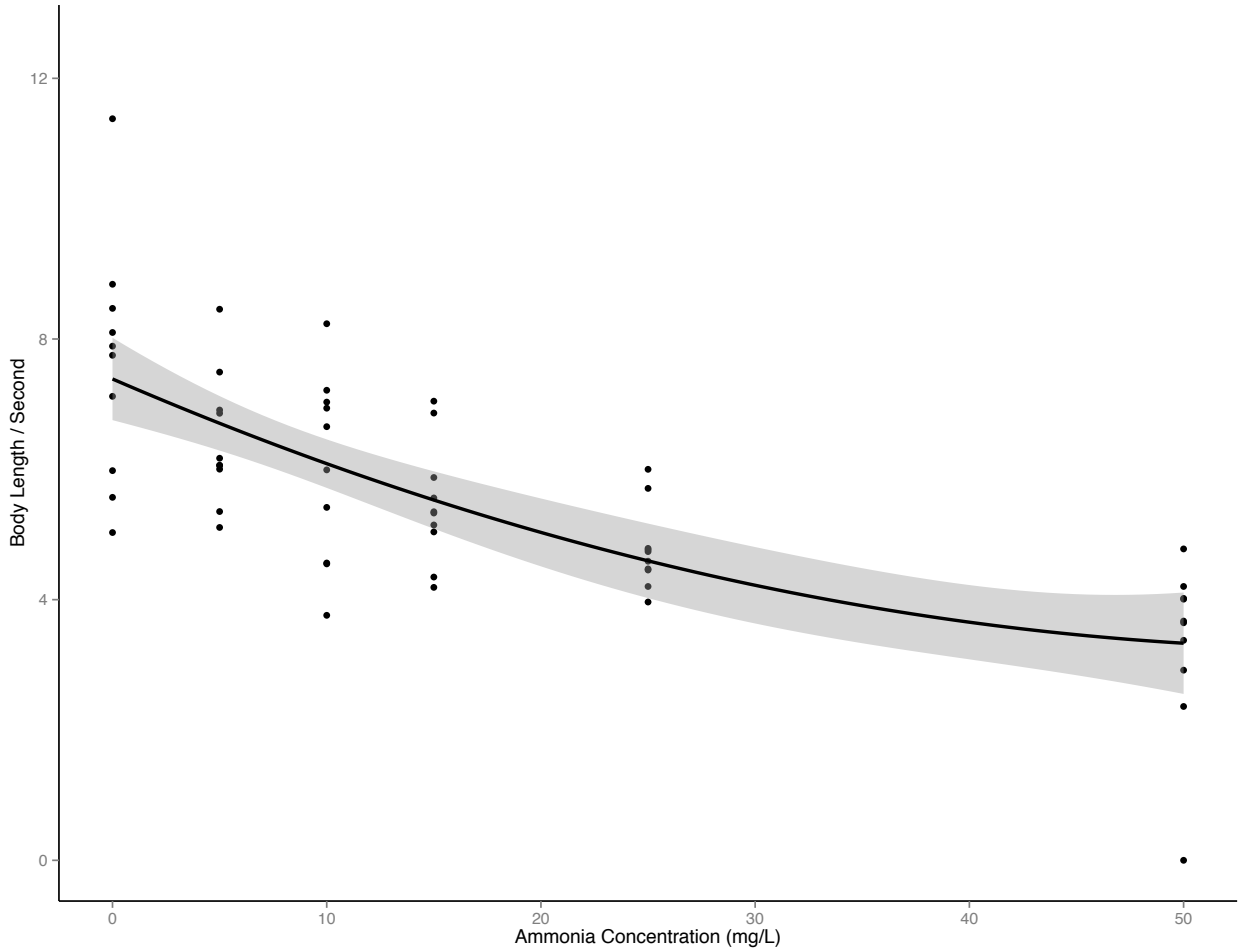

Figure 10: Ramped critical swimming speed (ramped U_{crit}) of Topeka Shiners exposed to a range of ammonia concentrations. A polynomial regression is shown with the 95% confidence interval highlighted in grey. One mortality was observed at 10 mg/L and three at 50 mg/L. Known LC_{50} is at 21.4 mg/L (Adelman et al. 2009).

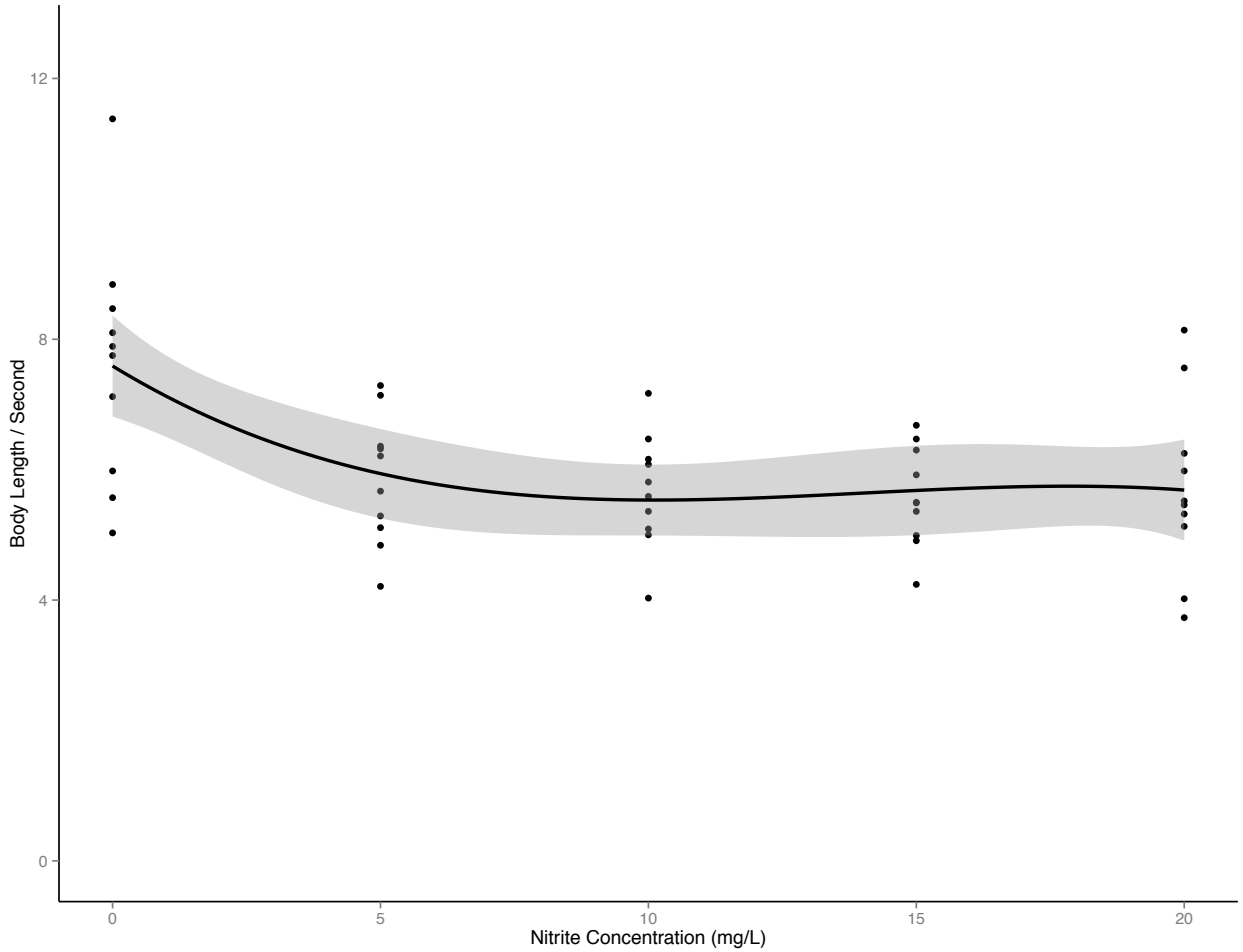

Figure 11: Ramped critical swimming speed (ramped U_{crit}) of Topeka Shiners exposed to a range of nitrite concentrations. A polynomial regression is shown with the 95% confidence interval highlighted in grey. Seven mortalities were observed at 15 mg/L and eight at 20 mg/L. Known LC_{50} is at 6.1 mg/L (Adelman et al. 2009).

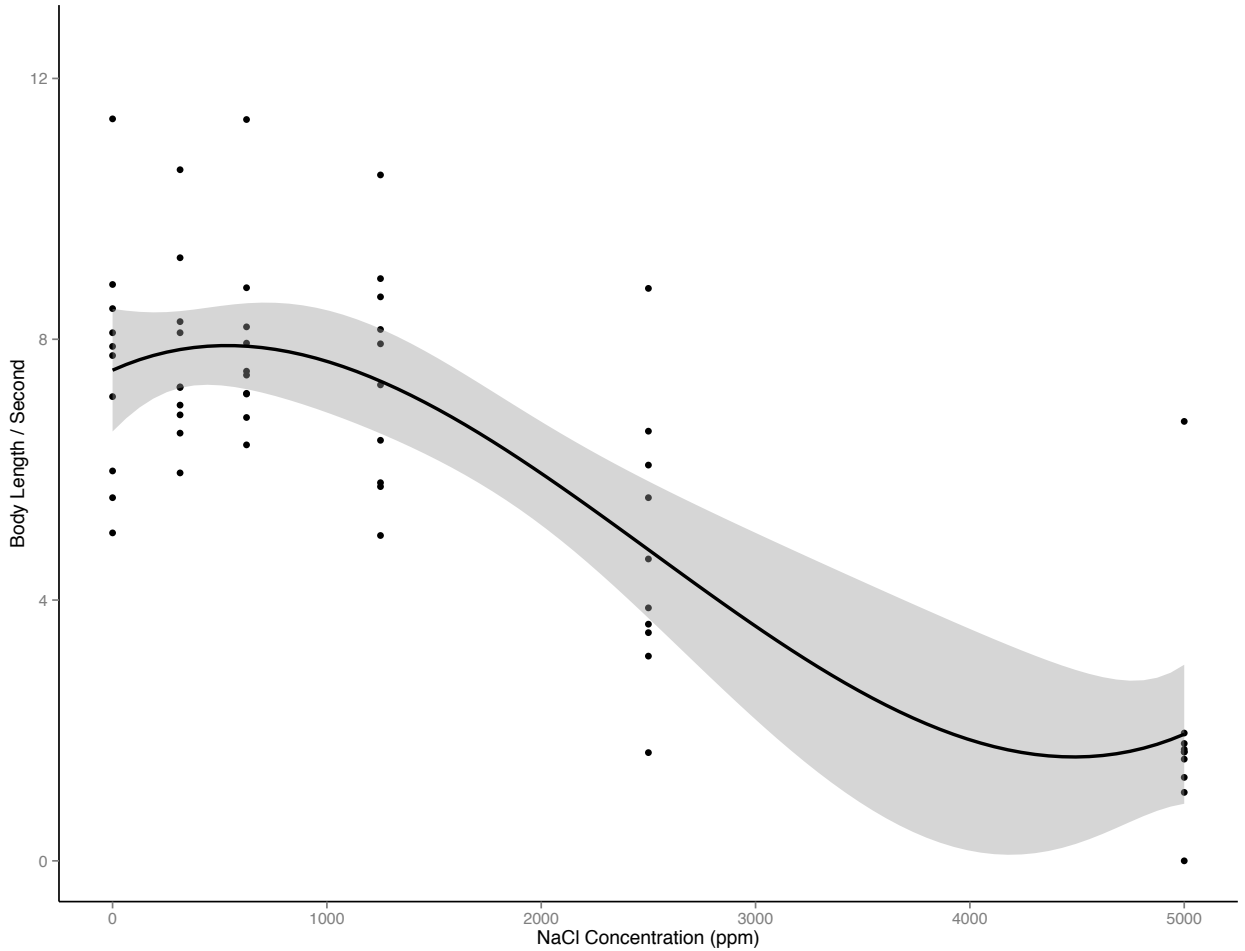

Figure 12: Ramped critical swimming speed (ramped U_{crit}) of Topeka Shiners exposed to a range of chloride concentrations. A polynomial regression is shown with the 95% confidence interval highlighted in grey. Two mortalities were observed at 5000 ppm. LC_{50} was calculated to be 3942 ppm.

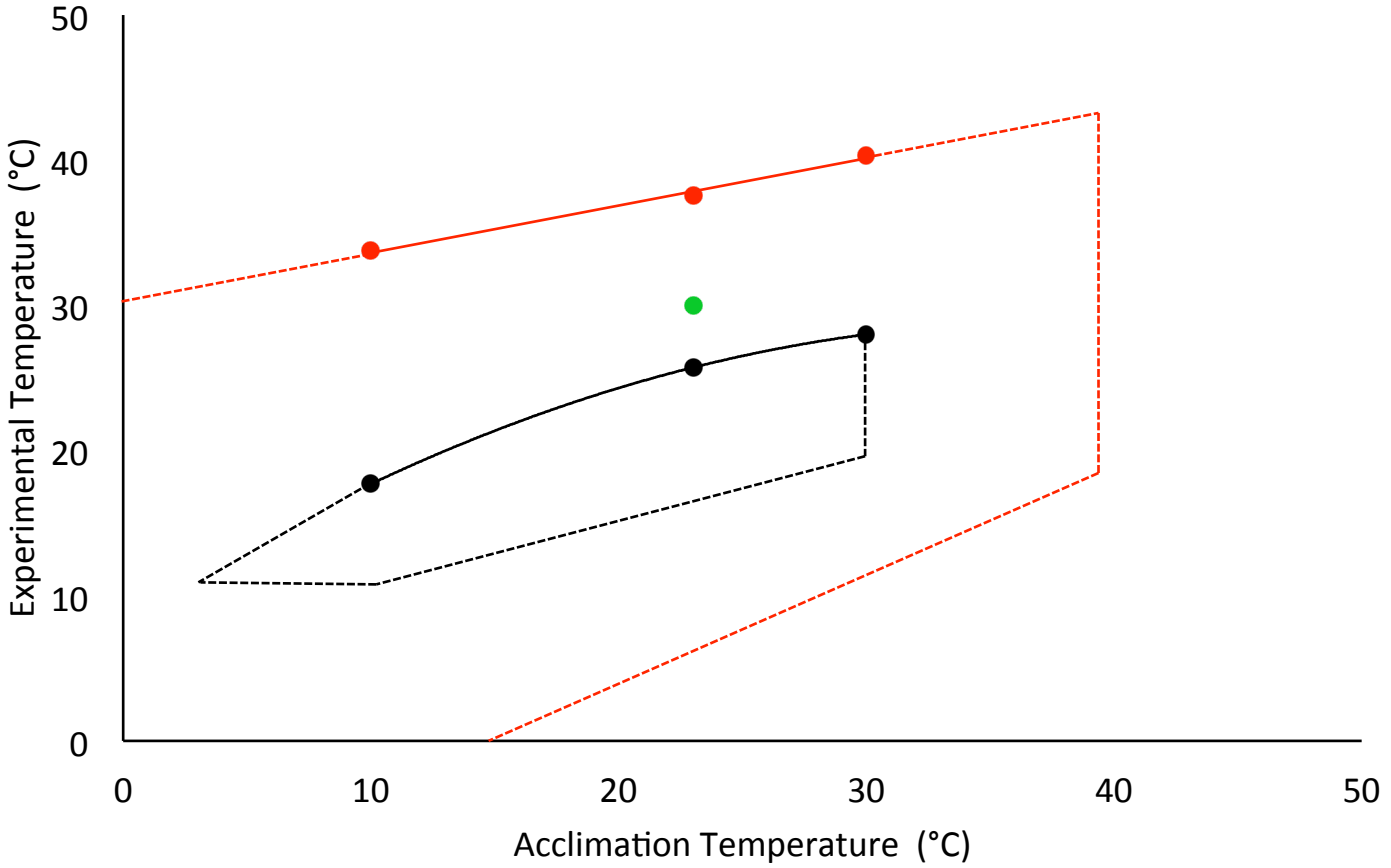

Figure 13: Hypothesized thermal tolerance polygon of combined Topeka Shiner thermal data showing the zones of growth based on ramped critical swimming speed tests (ramped U_{crit}) and feeding tests at 23°C. The zone framed in red lines represents the predicted lethal maximum. The zone framed in black lines represents a thermal zone promoting maximum growth. Points are calculated from swimming tests acclimated to 10°C, 23°C, and 30°C performed over a range of experimental temperatures. The green point is calculated from feeding tests for fish acclimated to 23°C. Solid lines are based on our thermal experiments while dashed lines are hypotheses based on laboratory observation during acclimation.

Appendix 1: For our hypoxia and thermal experiments, we assembled apparatus to measure physiological responses to our treatments. Parts and equipment are listed in each diagram.

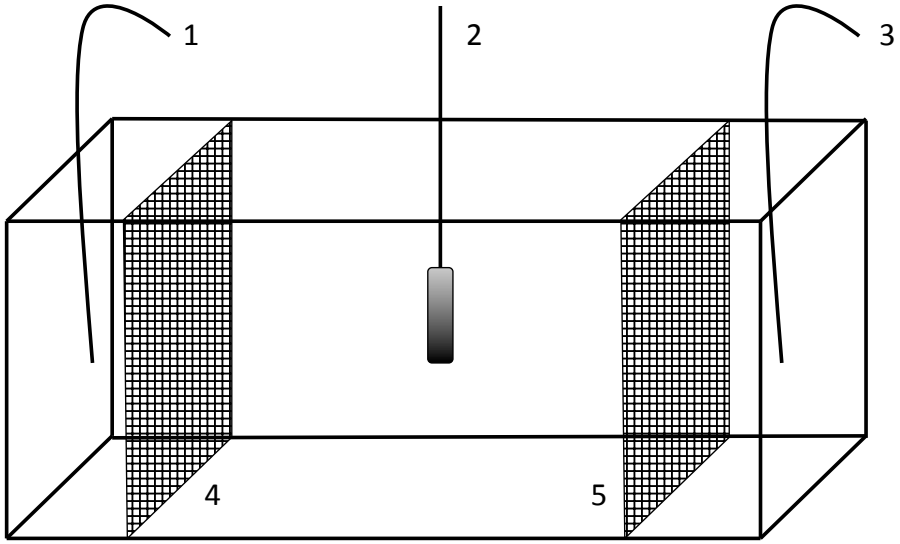

Figure A.1: Tank setup for hypoxia trials. Parts listed as follows: 1, 3 - nitrogen lines; 2 – YSI EcoSense ODO 200 DO probe; 4, 5 - meshed screen.

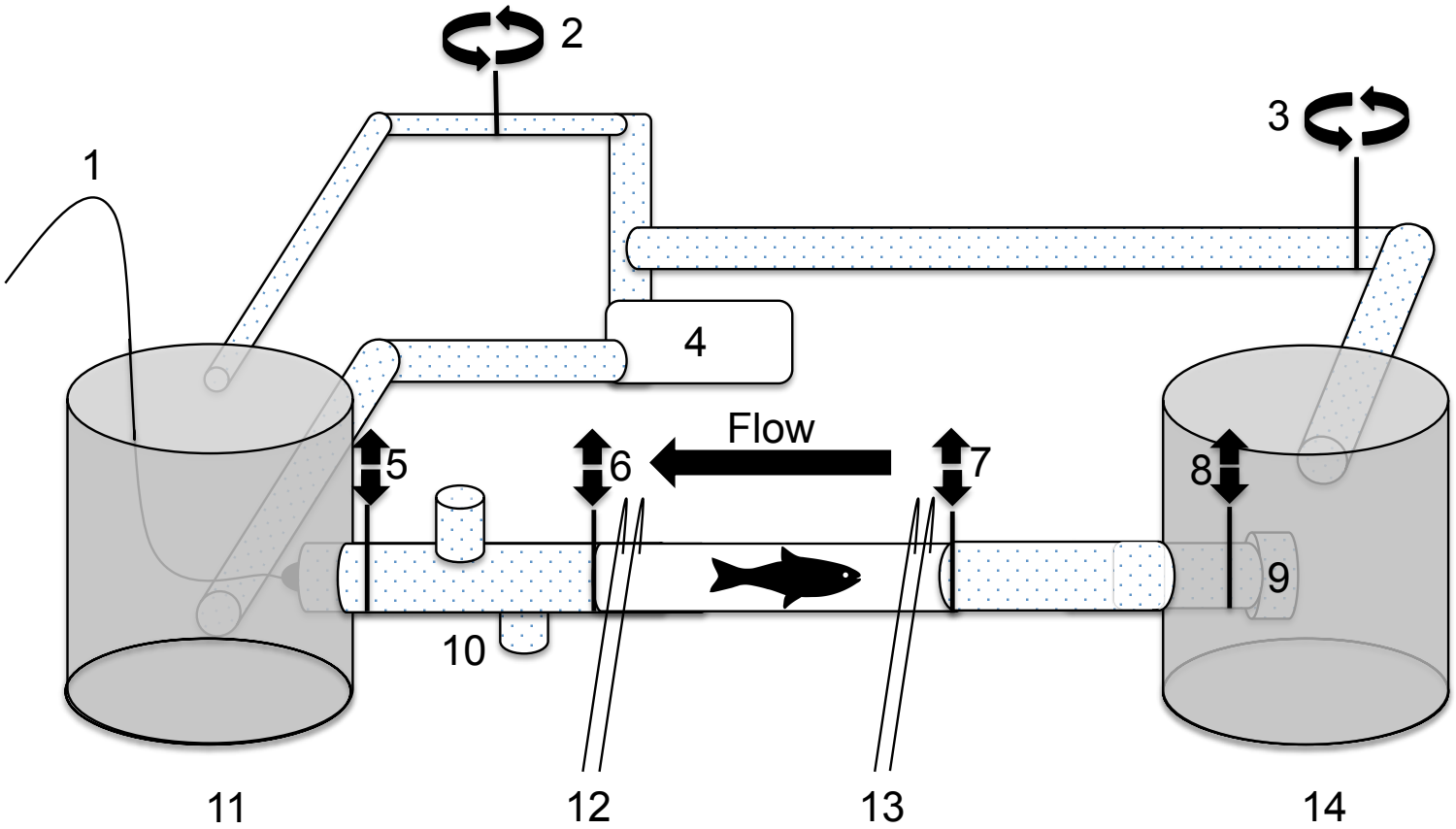

Figure A.2: A forward view schematic diagram of the swim tunnel used to determine ramped critical swimming speed. Parts are listed as follows: 1 - HACH FH950.0 flow meter; 2,3 - plastic screw valves; 4 - STA-RITE LT1/6L 1/6 horsepower centrifugal pump; 5,8 - solid gate valves; 6,7 - meshed gate valves; 9 - turbulence diffuser; 10 - 3'' sanitation tees with plugs; 11, 14 - 20 gal sumps; 12, 13 - electric barriers powered by Korad KA3005D-3S digital control DC power supply.

Appendix 2: As an additional analysis, we looked at boxplots for our treatments in all thermal and chemical ramped critical swimming speed tests for Topeka Shiners.

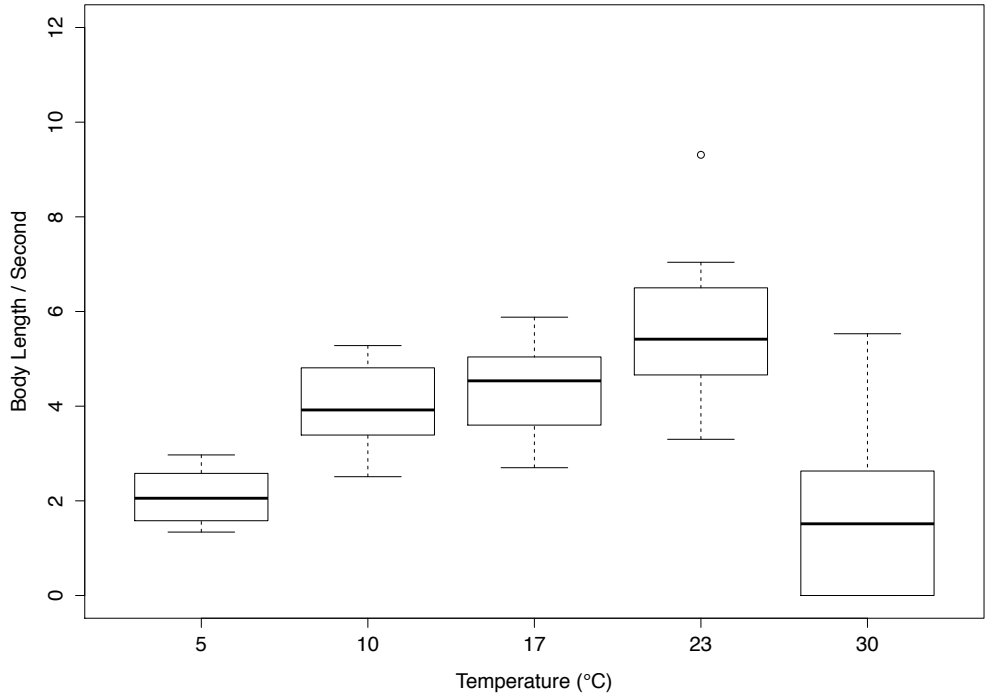

Figure A.1: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners acclimated to 10°C and tested over a ranged of experimental conditions. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

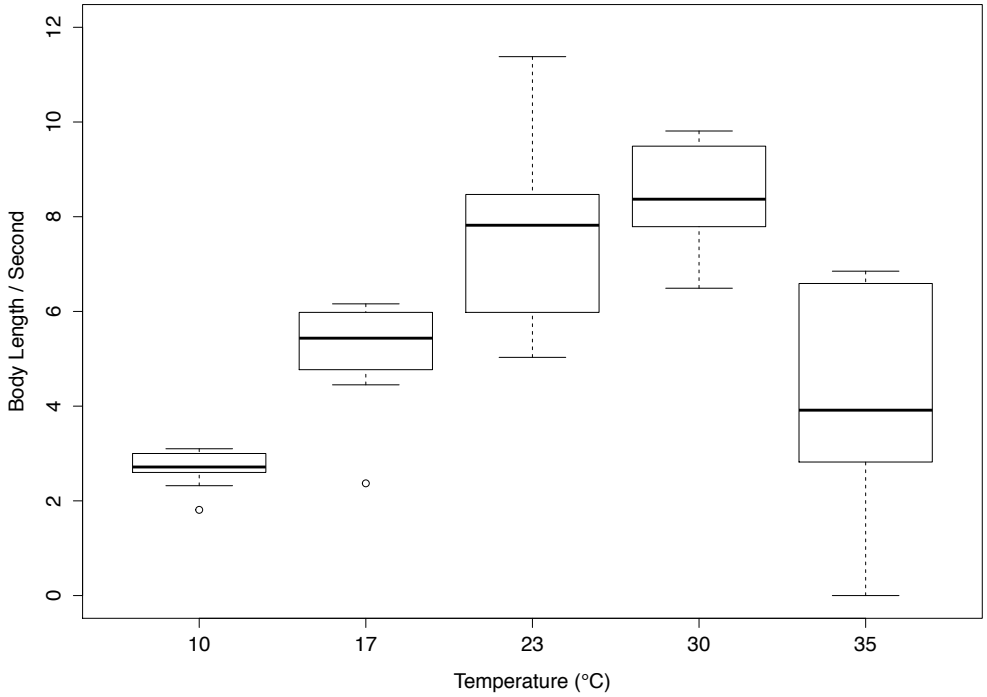

Figure A.2: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners acclimated to 23°C and tested over a ranged of experimental conditions. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

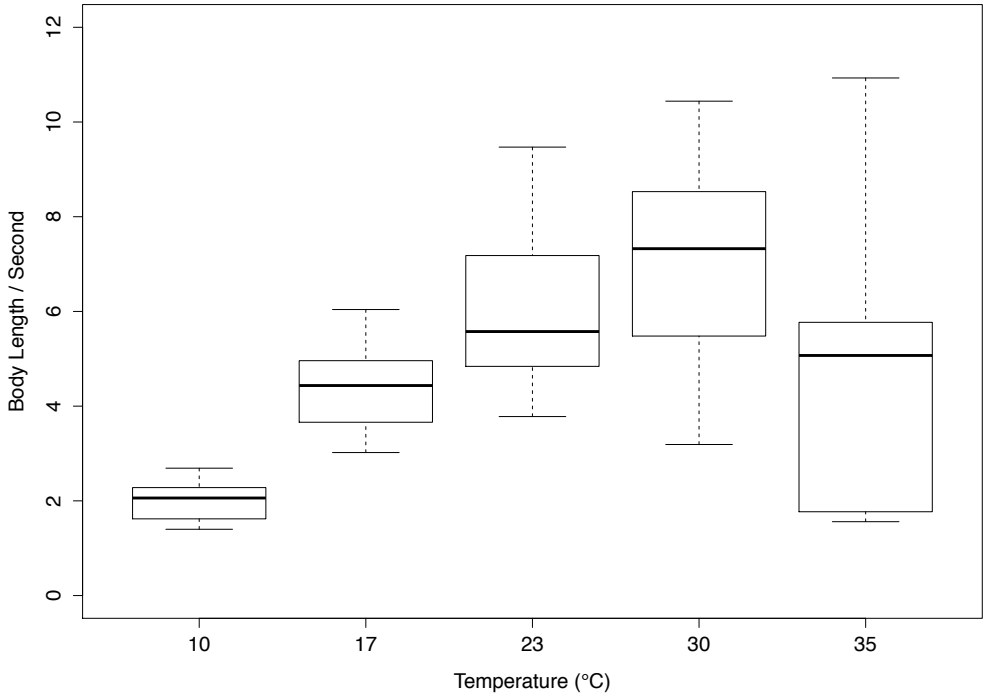

Figure A.3: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners acclimated to 30°C and tested over a ranged of experimental conditions. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

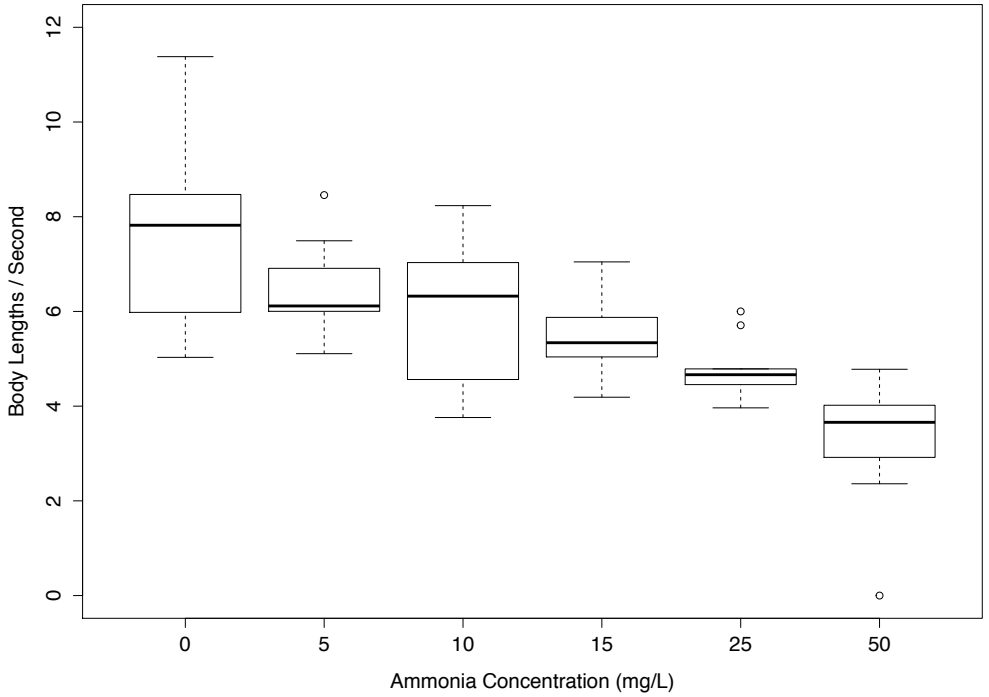

Figure A.4: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners exposed to a range of ammonia concentrations. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

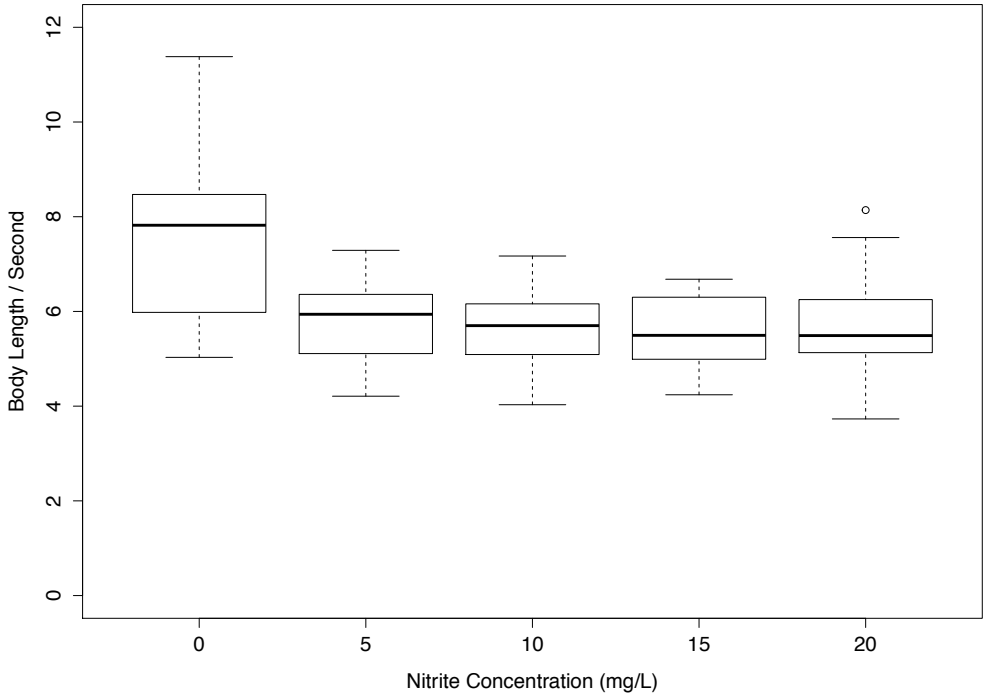

Figure A.5: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners exposed to a range of nitrite concentrations. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

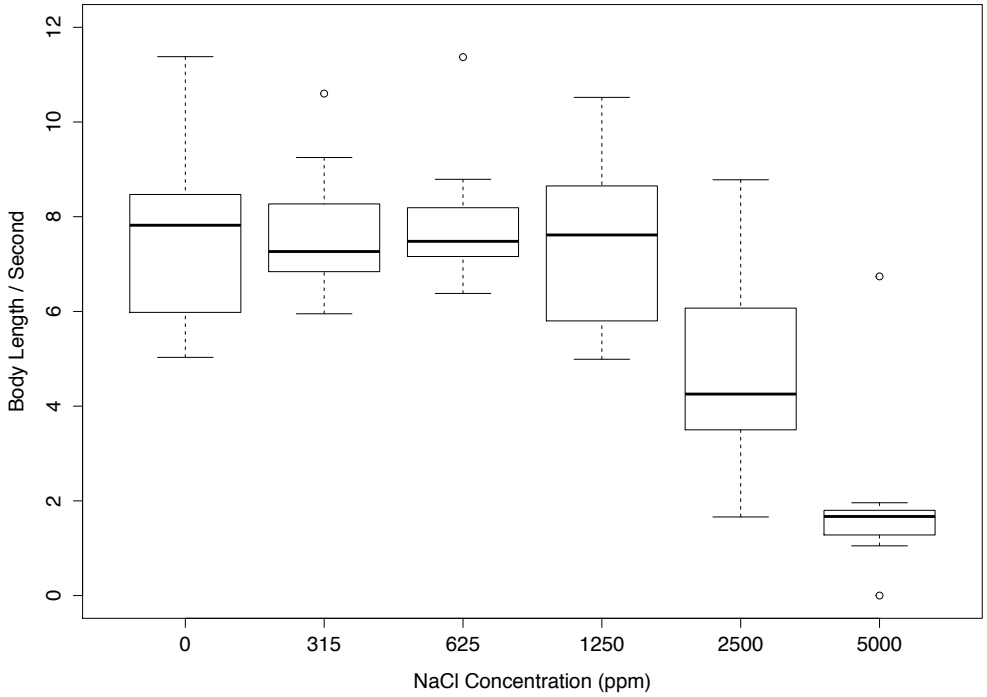

Figure A.6: Boxplot of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Topeka Shiners exposed to a range of sodium chloride concentrations. Each box represent a test temperature of n = 10 fish with the outside bars indicating max and minimum, the upper and lower box edges representing first and third quartile, and the center line showing median.

Appendix 3. As part of this study, we used Red Shiners (*Cyprinella lutrensis*) to test and refine our methodology prior to experimentation on the federally endangered *N. topeka*. Our pilot work allowed us to obtain information on this species similar to what is contained in this thesis, summarized in tables and figures below.

Table A.1: Regression analysis used for Red Shiner thermal test using ramped critical swimming speed performed under different acclimation temperatures and ambient test temperatures.

Acclimation Temperature (°C)	Regression	Equation	P Value	Multiple R Squared	Adjusted R Squared
10	2nd poly	$y = -2.496e\text{-}2(x^2) + 8.881e\text{-}1(x) - 1.922$	< 0.0001	0.447	0.423
23	3rd poly	$y = -1.502e\text{-}3(x^3) + 6.829e\text{-}2(x^2) - 5.181e\text{-}1(x) + 1.628$	< 0.0001	0.648	0.625
30	3rd poly	$y = -7.554e\text{-}4(x^3) + 3.224e\text{-}2(x^2) + 4.421e\text{-}2(x) + -1.883$	< 0.0001	0.779	0.760

Table A.2. Effects table for Red Shiner thermal tests using ramped critical swimming speed. Shown are predicted optimum and predicted 100% mortality from each acclimation temperature with different ambient test temperatures.

Acclimation Temperature (°C)	P Value	Predicted Optimum (°C)	Predicted 100% Mortality (°C)
10	< 0.0001	17.79	33.26
23	< 0.0001	25.86	36.92
30	< 0.0001	29.12	42.68

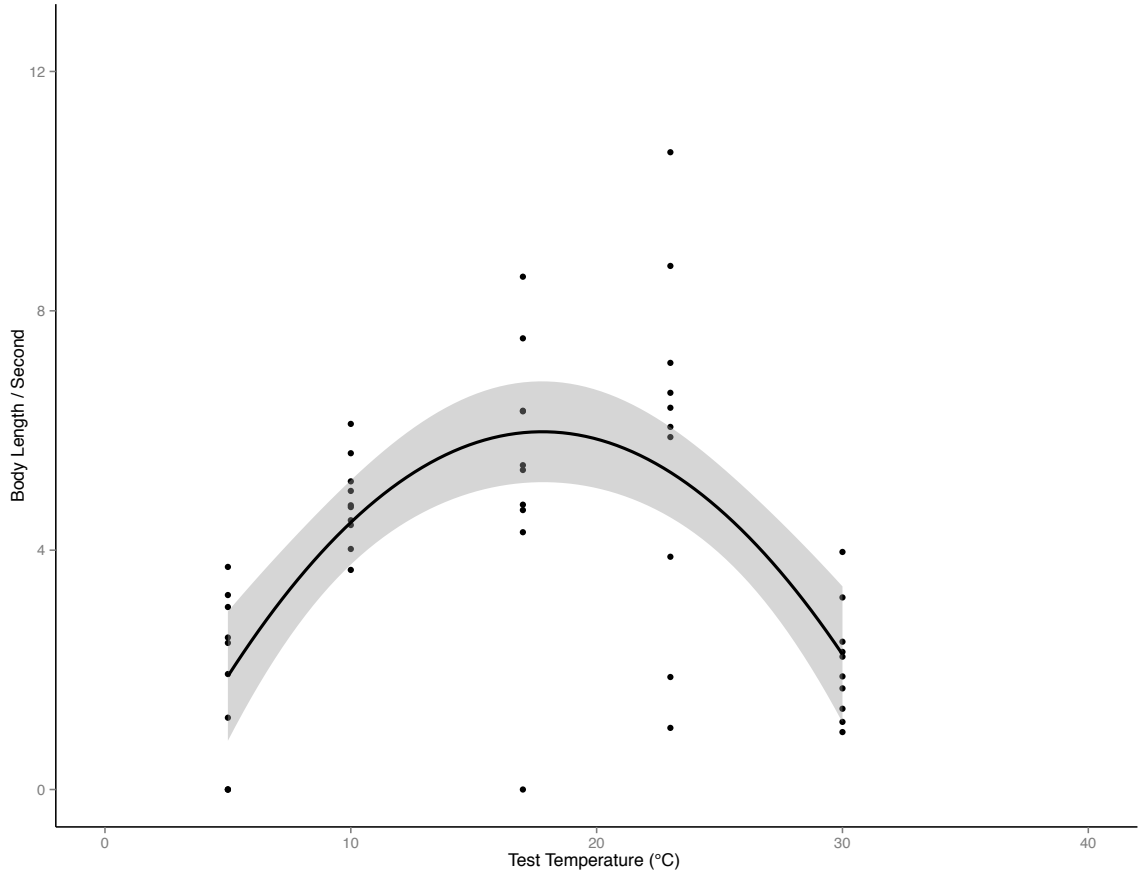

Figure A.1: Polynomial regression of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Red Shiners acclimated to 10°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. One mortality was observed at 5°C.

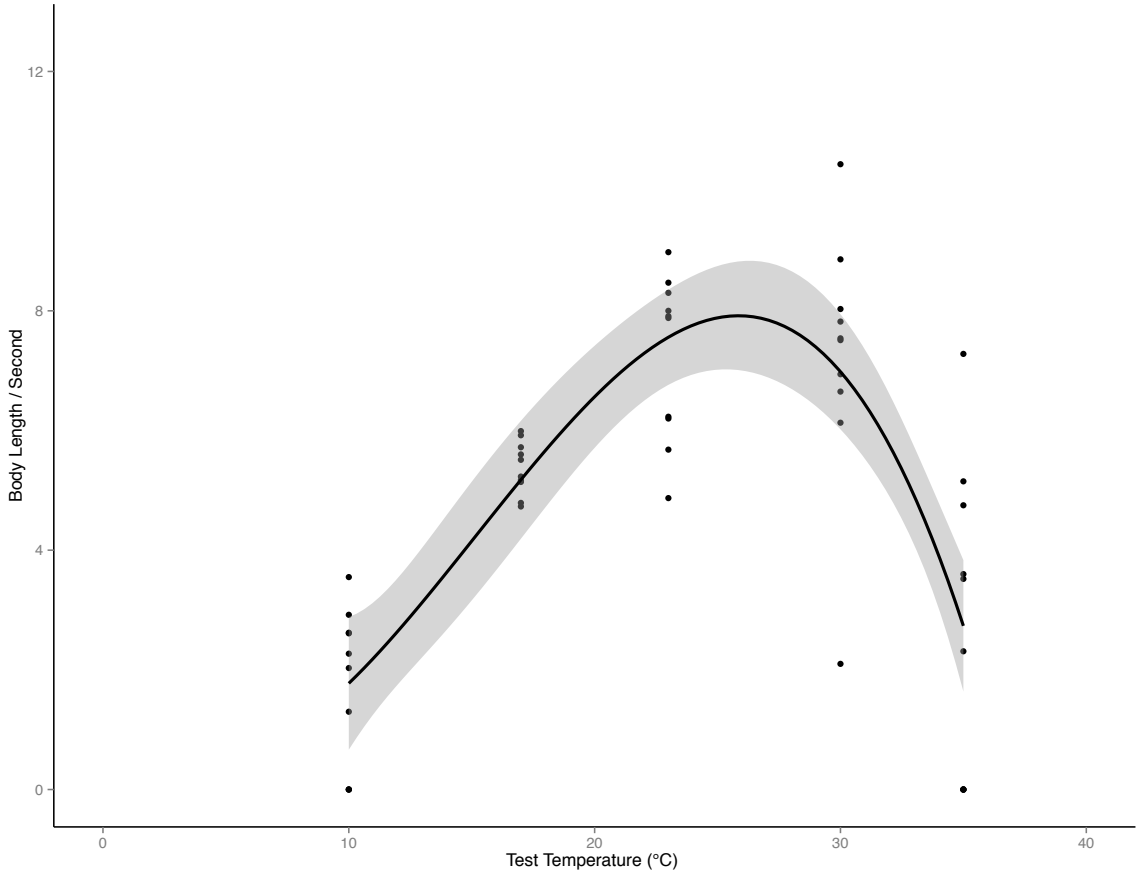

Figure A.2: Polynomial regression of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Red Shiners acclimated to 23°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. One mortality was observed at 10°C.

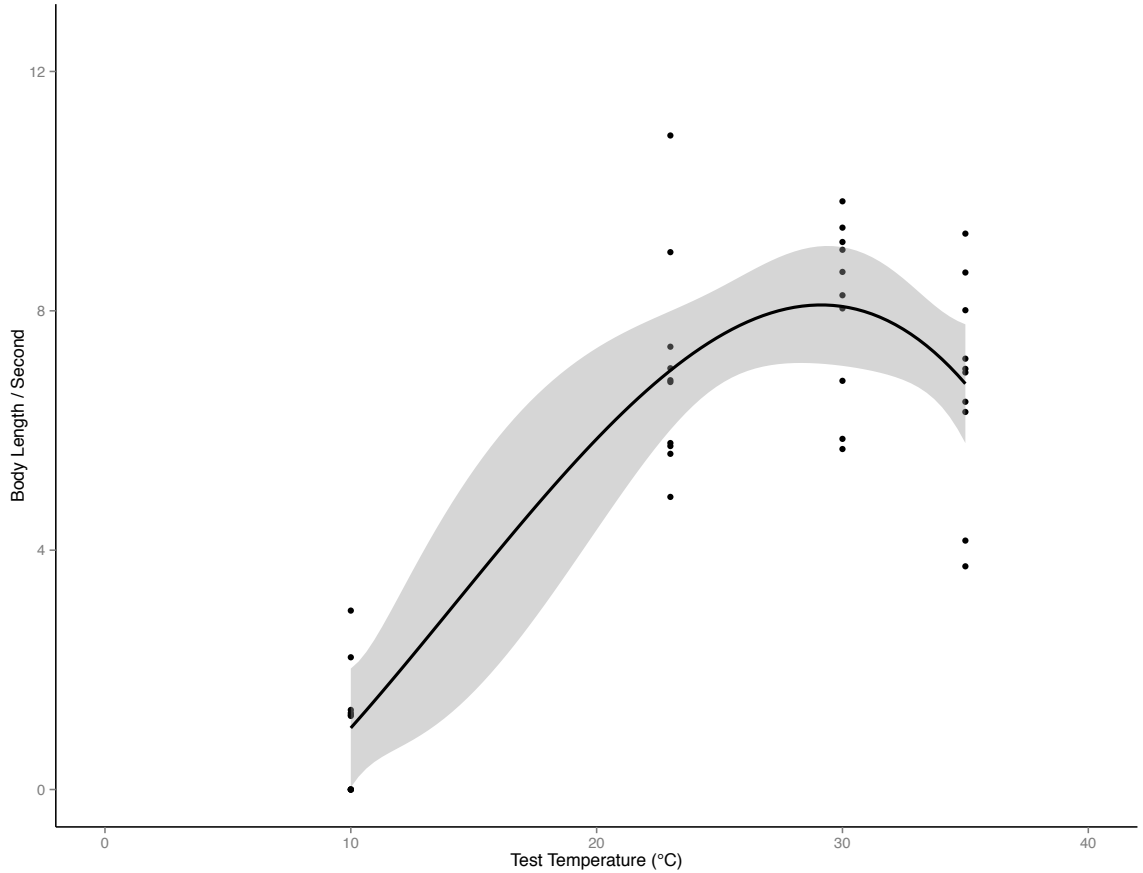

Figure A.3: Polynomial regression of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Red Shiners acclimated to 30°C and tested over a ranged of experimental conditions. The shaded zone represents the 95% confidence interval around this line. No mortalities took place during experimentation.

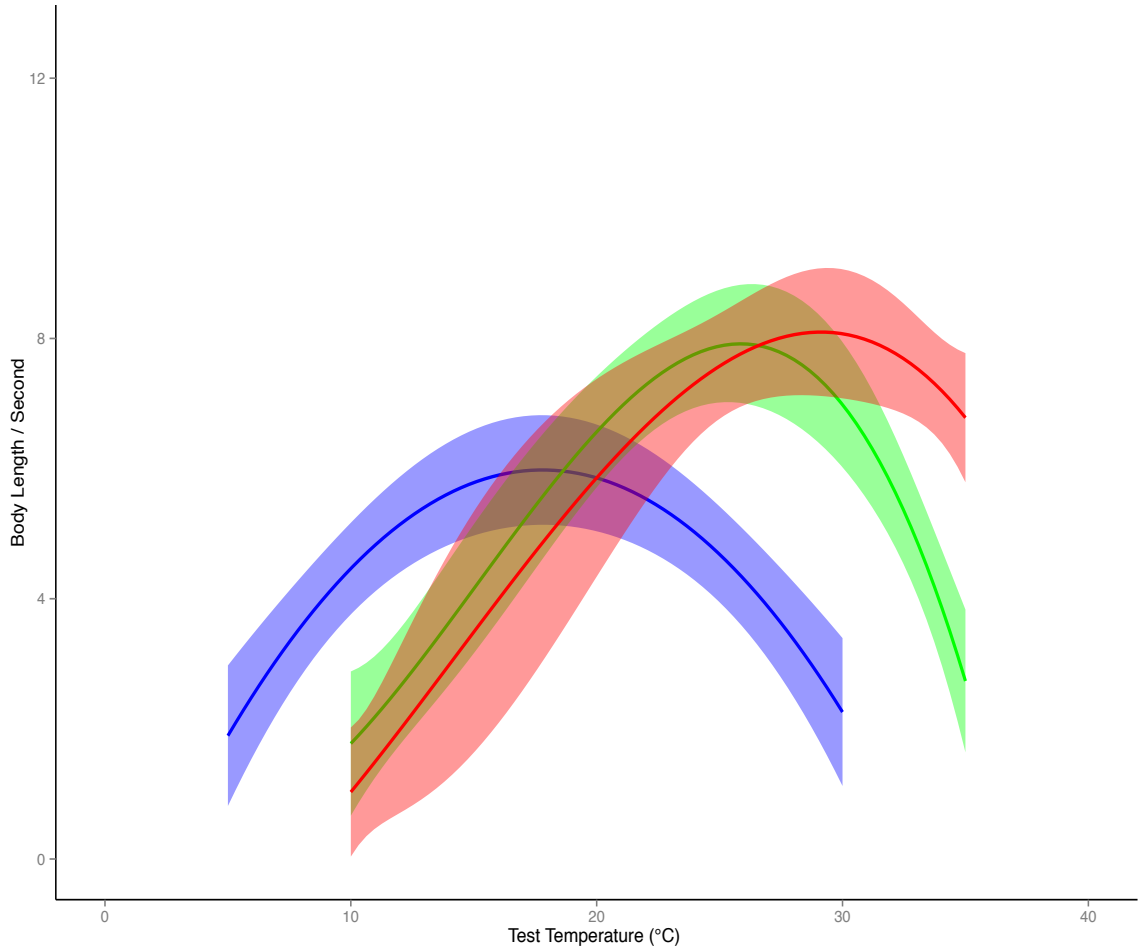

Figure A4: Polynomial regression of ramped critical swimming speed (ramped U_{crit}) standardized by body length of Red Shiners acclimated to 10°C (blue), 23°C (green), and 30°C (red) and tested over a ranged of experimental conditions. The shaded color zone represents the 95% confidence interval around the similar color line.