Juvenile and adult hardhead thermal tolerances and preferences:

Temperature preference, critical thermal limits, active and resting metabolism, and blood-oxygen equilibria

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Abstract

California's populations of hardhead minnow, a fish species of special concern, have experienced population declines, possibly due to habitat perturbations, including dam construction with consequent temperature changes and the introduction of non-native species to California's mid- to low-elevation streams. Environmental temperature effects on this large (to 60 cm Standard Length) native species are poorly documented. To elucidate possible thermal preferences and tolerance in this species we designed a complementary behavioral and physiological study including: temperature preferences, resting and active metabolic rates, and thermal maxima and minima, all with fish acclimated to one of four environmental temperatures (11, 16, 21, or 25°C; 51.8, 60.8, 69.8, or 77°F). We also studied the blood-oxygen equilibria (including whole-blood oxygen affinity) of hardhead across a temperature range of 11-30°C. We found that hardhead can be kept in captivity for long periods of time between 11 and 25°C on commercially available diets, after a transition period including live food supplementation. Hardhead performed very well at moderate (ca. 16-21°C) temperatures in our experiments. Regardless of thermal acclimation history, hardhead preferred a mean water temperature of 19.4°C (66.9°F) and clearly avoided temperatures above ca. 26°C (78.8°F). Resting metabolic rates increased with increasing acclimation temperatures in both juveniles and adults, with low to moderate thermal sensitivity. Active metabolic rates ranged from 209-1,342 mg O_2 kg^{-1} h^{-1} for adult hardhead that swam in Brett-style respirometers at velocities from 30 to 90 cm s^{-1}, providing an estimate of the species' maximal continuous rate of oxygen consumption (i.e., aerobic capacity). Hardhead lost equilibrium (could no longer maintain themselves in an upright position) when rapidly exposed to water above 29.7°C (85.5°F) or below 7.4°C (45.3°F), not including acclimation acquired zones. These temperature thresholds are ecologically relevant because, in the wild, loss of equilibrium would expose a fish to potential death from predation. Finally, the blood-oxygen study indicated that hardhead had moderately sigmoidal blood-oxygen equilibria curves, high whole-blood oxygen affinities, high hematocrit, total hemoglobin concentration, and blood oxygen capacity, with relatively low nucleotide triphosphate values. These results suggest that this species is suited for some sustained aerobic activity over a range of dissolved oxygen concentrations and instream flow regimes, especially at temperatures <25°C. However, blood-oxygen affinities were lower at temperatures ≥25°C, indicating a somewhat decreased ability to bind oxygen at the gills at elevated stream temperatures, especially when combined with low dissolved oxygen levels (moderate environmental hypoxia).

Keywords: Public Interest Energy Research (PIER) Program, Pulsed flows, hardhead minnow, *Mylopharodon conocephalus*, temperature preference, critical swimming, critical thermal maxima and minima, active and resting metabolic rate, fish.

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EXECUTIVE SUMMARY

Introduction

Hardhead (*Mylopharodon conocephalus*) is a California fish Species of Special Concern, yet environmental limits for its management are largely unknown. Hardhead abundance may be declining throughout much of its range in California (Gard 2004; Moyle 2002). The decline is partially due to resource competition from non-native fish species, and also anthropogenic river alterations. These alterations may include temperature and flow changes due to hydroelectric-power-production operations. Knowledge of the temperature tolerances and preferences of hardhead, and the bioenergetic costs associated with survival and swimming across a range of water temperatures, would greatly assist management and conservation efforts. These data should assist in developing thermal targets for future and current Federal Energy Regulatory Commission (FERC) licensing of hydroelectrical projects in California. Maximum temperatures in California streams may exceed 28^{o}C in mid-summer, and may increase further in the future, due to climate change, enhancing the value of information on hardhead performance at higher temperatures.

There have been a small number of previous studies that examined hardhead's laboratory and field thermal performance and thermal preference (Baltz et al. 1987, Myrick and Cech 2000, Klimley et al. 2007), but these studies were limited by the use of adult fish (only) and a relatively narrow range of acclimation and maximum temperatures.

Project Objectives and Approach

The goal of this project was to conduct a comprehensive study of wild hardhead temperature tolerances and preferences, using both adult and juvenile fish, collected from the Feather, American, and Pit Rivers in California. We examined five key physiological and behavioral variables in hardhead, regarding temperature.

- Temperature preference of hardhead adults and juveniles, determined using large and small annular devices (doughnut-shaped water troughs; see Figures 4 and 6) at the Center for Aquatic Biology and Aquaculture (CABA) at UC Davis. We sought to determine the preferences of wild young-of-the-year and adult hardhead exposed to a thermal gradient ranging from 12 to 28°C (53.6 to 82.4 °F), when acclimated to different temperatures (11, 16, 21, 25°C).
- Resting metabolic (oxygen consumption) rates of adult and juvenile hardhead, determined using static respirometers (airtight aquarium chambers; see Figures 11, 12, and 13) at UC Davis, at environmentally relevant temperatures (11, 16, 21, 25°C), to measure energy turnover rates associated with survival.
- Active metabolic (oxygen consumption during exercise) rates of adult and juvenile hardhead, determined using three Brett-type (recirculating flow) respirometers (see Figures 14, 15, and 16) at UC Davis, at environmentally relevant temperatures (11, 16, 21, 25°C) and water velocities (up to 1.05 m s^{-1} for adults and 0.60 m s^{-1} for juveniles), to measure energy demands during exercise.

- Critical thermal maxima and minima (CTmax and CTmin, respectively) of adult and juvenile hardhead, via acute thermal challenge to a loss of equilibrium, using fish acclimated to environmentally relevant temperatures (11, 16, 21, 25°C).
- Hardhead blood-oxygen equilibria (including whole-blood oxygen affinity) assessed across a temperature range of 11-30°C (51.8-86°F), to understand the effects of temperature on hardhead oxygen uptake at the gills and transport to metabolizing tissue sites.

Project Outcomes

We quantified the thermal preferences of adult and juvenile hardhead minnows in unique annular preference chambers. Fish of both life stages were acclimated to 11, 16, 21, or 25°C for greater than 30 days. Fish were allowed to acclimate to the annular chamber for 30 minutes at their acclimation temperature before the thermal gradient was established (gradient temperatures ranged over ca. 12-28°C). Fish were exposed to the thermal gradient for a 4-hour period for preference testing. The thermal preference experiments showed that regardless of thermal acclimation history, adult hardhead minnows tended to prefer an overall mean (average) water temperature of 20.5°C (68.9°F) and juvenile hardhead preferred a mean water temperature of 19.5°C (67.1°F). Hardhead adults tended to be less active then juveniles. Adults would pass through all temperatures presented, but would spend large portions of the 4 hours near their mean preferred temperature. Juveniles were very active and would pace in the areas of the chamber near their mean selected temperature. Both adults and juveniles regularly avoided the warmest and coolest portions of the preference chamber.

We conducted a series of laboratory experiments to determine the resting metabolic rate (RMR) of both juvenile and adult hardhead acclimated to one of 4 acclimation temperatures (11, 16, 21 and 25°C). Resting metabolic rate (mg O_2 $kg^{-2/3}$ h^{-1}) generally increased with acclimation temperature, in juvenile and adult fish, as has been observed in other fishes. We calculated temperature quotients (as an index of thermal sensitivity in RMR, Q_{10}) for hardhead across temperatures. The Q_{10} values (range: 1.35-1.79) suggest that hardhead have a relatively low to moderate thermal sensitivity in resting metabolic rates.

In our tests of adult hardhead active swimming metabolism using our Brett-style swimming respirometers, hardhead easily swam at 75 cm s^{-1}, with many reaching water velocities of 90 cm s^{-1}. A few exceptional adult fish were able to swim continuously at 105 cm s^{-1} for 30-40 minutes. We found a strong positive correlation between tail beat frequency and metabolic rate with increasing water velocity. Mean metabolic (MO_2) rates ranged over 209-1,342 mg O_2 kg^{-1} h^{-1} for adult fish in the 660-L respirometer at velocities from 30 to 90 cm s^{-1} at temperatures between 11 and 25°C, revealing their maximal continuous rate of oxygen consumption (aerobic activity) A plateau in MO_2 was observed at 90 cm s^{-1} water velocity in adult fish, as fish became fatigued. In contrast, juvenile hardhead exhibited little swimming capability at 11-16°C, but between 21-25°C showed similar increases in oxygen consumption to those of adult hardhead, as water velocities increased from 10 to 50 cm s^{-1}.

We tested adult and juvenile hardhead's abilities to survive acute thermal challenges (quantified using standard Critical Thermal Methodology, CTM), by testing them in specially

prepared chambers (see Figure 19) capable of raising or lowering the water temperature by ca. 0.3°C min^{-1}. Both adult and juvenile hardhead showed a positive increase in CTMax and CTMin values in response to increasing acclimation temperatures. CTMax values ranged from 29.7 to 36.7°C in adult fish and 29.7-37.3°C in juvenile fish. CTMin values were ranged from 0.3 to 6.7°C in adult fish and 0.2-7.4°C in juvenile fish. Overall, adult and juvenile hardhead thermal tolerance limits were very consistent within a temperature-acclimation group across these two life stages, and these data predict lethality when hardhead are acutely exposed to water temperatures above 29.7°C and below 7.4°C. We did not measure CTMs for eggs or larvae of hardhead. These CTM results can be useful as an index, in comparing acute tolerance limits across species. However, such rapid temperature increases or decreases rarely happen in wild habitats. From studies on other species, realistic upper temperature tolerance limits are probably much lower, and realistic lower temperature limits are probably higher in hardhead, also.

We measured temperature effects on *in vitro* blood-oxygen affinity and equilibrium curve shape, key dynamics of a fish's oxygen-transport system, derived from blood collected from wild-caught, adult hardhead. Over an 11 - 30°C temperature range, the half-saturation value (P_{50}, an inverse measure of oxygen affinity) increased, with increasing temperature from 0.51 to 1.80 kPa for low-PCO_2 ("arterial") treatments and from 2.02 to 2.92 kPa for high-PCO_2 ("venous") treatments. The apparent heat of oxygenation (temperature effect, ΔH) for hardhead hemoglobin showed the greatest increase (in absolute value) between 19°C and 25°C compared with the other temperature intervals, i.e., 11°C-19°C and 25°C and 30°C. Therefore, hardhead's blood decreases its ability to bind oxygen at its gills at temperatures ≥ 25°C, compared to that at temperatures ≤ 19°C. The hardhead's Bohr factors, non-bicarbonate buffer values, nucleoside triphosphate (NTP) concentrations, and blood oxygen capacities showed no relationships with temperature. Overall, their blood-oxygen equilibria suggest that hardhead can tolerate moderate hypoxia and temperature variations in their environments and that they have some capacity for sustained, high-aerobic activity.

Conclusions and Recommendations

This study has provided new knowledge relevant to future field and laboratory studies, and to the management of hardhead habitat. Hardhead minnows can readily be captured via traps and hook and line fishing. Other methods may be suitable, but consideration of handling stress should be a high priority. For a thorough description of the disease and parasite burdens carried by hardhead and other cyprinids, see Alvarez (2008) and Haderlie (1953). Under conditions of chronic and/or acute stress, aggressive disease treatments are required to maintain hardhead for long periods of time in captivity. We were able to maintain hardhead in a healthy state once they had been treated for parasites and diseases that were present prior to capture. Hardhead can be kept in captivity for long periods of time in water temperatures of 11-25°C on commercially available diets, after a transition period involving supplementation with live foods.

Overall, our results show that hardhead, behaviorally and physiologically, perform well at moderate temperatures (i.e., above 16°C and below 25°C). This temperature range compares

well with that associated with the "squawfish-sucker-hardhead" zone described by Moyle and Nichols (1973) for Sierra Nevada foothill streams (Moyle 2002). In our thermal preference experiments, we showed that regardless of thermal acclimation history, adult hardhead minnows tended to prefer an overall mean water temperature of 20.5°C and juvenile hardhead preferred a mean water temperature of 19.5°C. Mean metabolic (MO_2) rates ranged from 209-1,342 mg O_2 kg^{-1} h^{-1} for adult fish in the 660-L swim chamber at velocities from 30 to 90 cm s^{-1} at temperatures between 11 and 25°C and revealed their maximal continuous rate of oxygen (aerobic activity) consumption. A plateau in MO_2 was observed at 90 cm s^{-1} water velocity in adult fish, as fish became fatigued. Whereas the juvenile hardhead exhibited little swimming activity in water temperatures from 11-16°C, between 21-25°C hardhead showed increases in oxygen consumption similar to those of adults as water velocities increased from 10 to 50 cm s^{-1}. Critical thermal limits studies revealed that, for both adult and juvenile hardhead, water above 29.7°C and below 7.4°C can be ecologically lethal, unless fish have experienced acclimation to cooler or warmer temperatures, and thus acquired broader temperature tolerance zones. The adult hardhead blood study showed that hardhead have a moderately sigmoidal blood-oxygen equilibria curves and relatively high whole-blood oxygen affinities and capacities, suggesting that hardhead are suited for some sustained aerobic activity over a range of dissolved oxygen concentrations and instream flow regimes, especially at temperatures <25°C.

We recommend that water managers simulate natural hydrographs as closely as possible. Hardhead have evolved to cope with seasonal shifts in water temperatures and flows. Altered flow conditions could force hardhead into smaller habitat areas (river sections), especially when river temperatures increase excessively. It is clear from our thermal preference experiments that hardhead avoid 12-14°C and 26-28°C water. The blood data suggest that hardhead are especially suited to water temperatures below 25°C, with temperatures above 25°C requiring higher dissolved oxygen concentrations (i.e., a higher partial pressure of oxygen for adequate oxygen binding to their hemoglobin).

Benefits to California

Lack of understanding of the temperature and related water flow requirements of native fishes may cause unnecessary curtailment of hydropower operations or unintended impacts on fishes if operation practices are incompatible with ecological and/or physiological species' requirements. Hardhead is a native minnow and a California fish Species of Special Concern, yet environmental limits for its management are largely unknown. Hardhead abundance may be declining throughout much of its range in California. Such a decline may be linked to resource competition from non-native fish species, and also anthropogenically induced river habitat alterations. These alterations may include temperature and flow changes due to hydroelectric operations. Overall, our results show that hardhead, behaviorally and physiologically, respond well to moderate temperatures (i.e., above 16°C and below 25°C). This temperature range compares well with those associated with the "squawfish-sucker-hardhead" zone of Moyle and Nichols (1973) in Sierra Nevada foothill streams (Moyle 2002).

This project provides comprehensive information on the temperature requirements of adult and juvenile hardhead. This research has also determined how the swimming ability of hardhead

varies with temperature and water velocity, advancing the understanding of how hardhead may respond to water management practices associated with hydropower production facilities that may alter flow and thermal regimes.

This research may allow more efficient operation of hydropower production facilities by avoiding unnecessary curtailment of operations due to excessive concerns about hardhead temperature and flow requirements. These data will assist in the development of thermal targets for current and future Federal Energy Regulatory Commission (FERC) licensing of hydro-electrical projects in California and research will be of critical value for management and conservation of hardhead. Furthermore, maximum temperatures in California streams often exceed 26°C in mid-summer, and may increase under the influence of climate change, enhancing the value of this information on hardhead performance at higher temperatures.

CHAPTER 1: Introduction

1.1 Hardhead Characteristics and Habitat

Hardhead minnow (HH), *Mylopharodon conocephalus*, is a large, California-native cyprinid listed as a species of special concern in California by the CA Department of Fish and Wildlife (Moyle et al. 1996) and as a sensitive species by the United States Forest Service (United States Forest Service 1998). Hardhead prefer clear, medium to large, mid to low-elevation streams with gravel, cobble, and boulder substrates typically with low water velocities (<25 cm s^{-1}) and cool to warm temperatures (Moyle and Baltz 1985; Moyle et. al. 1995; Moyle 2002). In summer they may be found in large pools where maximum temperatures reach 20-28°C (Knight 1985, Cech et. al. 1990). Hardhead are often found as part of the sucker-hardhead-pikeminnow guild (Moyle 2002), with the Sacramento pikeminnow as the hardhead's closet genetic relative in California (Avise and Ayala 1976).

Extensive construction of hydroelectric dams on nearly all medium to large streams and rivers in California, along with the introduction of non-native species, have fragmented and altered the native faunal hierarchy of historic hardhead habitat resulting in population declines and, in many cases, extirpation from heavily modified, downstream areas (Moyle et. al. 1995; Moyle 2002). In addition, hardhead populations are particularly vulnerable to predation and displacement by non-native species (Moyle et. al. 1995). The introduction of centrarchids (e.g., smallmouth bass, *Micropterus dolomieu*) into these modified aquatic ecosystems has been associated with declines and extirpation of hardhead from stream and river systems where this species was once historically abundant (Reeves 1964; Moyle and Nichols 1973; Brown and Moyle 1987; Herbold and Moyle 1986; Brown and Moyle 1993; Moyle 2002).

Hydroelectric dams have a near ubiquitous presence on streams and rivers containing hardhead populations and the timing and magnitude of water released from dams for electrical power generation, irrigation, and drinking water continue to modify natural, seasonal thermal and hydrological regimes that affect their life history parameters and bioenergetics. California leads the USA in the number (ca. 400) of hydroelectric generating facilities, located mainly in the Sierra Nevada Mountains (Hall and Reeves 2006). The daily operation of these run-of-river facilities is associated with once, or twice, daily power peaking flows supplying electricity when

demand is the greatest (Cushman 1985; Houck et. al. 1995). Peaking operations may cause reduced stream productivity, scouring of sediments, and changes in depth, width, velocity, water temperatures, and dissolved oxygen in areas immediately downstream of power-generating facilities (Cushman 1985, Young et al. 2011). The effects of hydroelectric power generation on stream flow, water temperature, turbidity, and oxygen content, and in turn, on fish and other aquatic species, raise concerns for the permitting, licensing, and operation of these facilities (Young et al. 2011).

Although modifications of habitat and hydrology, as well as the introduction of non-native species, have influenced current distributions and abundance of hardhead, there remains an incomplete understanding of how environmental variability may interact with hardhead physiology and influence its life history parameters. Temperature and oxygen levels in streams and rivers may limit the ability of hardhead to maintain their populations under changing conditions (e.g., modifications of hydrologic and thermal regimes, introduced species, and global climate change).

1.2 Temperature and hardhead

Temperature is a critical environmental variable for fish of all ages, because of its direct effect on metabolism, feeding, and growth (Brett 1971; Elliott 1981). Temperatures in low to mid-elevation California rivers and streams are influenced by both natural and anthropogenic factors. Water temperatures are controlled by input from tributary streams, incident radiation (which is influenced by riparian cover), and air temperatures as well as by human-controlled water releases from upstream reservoirs. Therefore, knowledge of thermal tolerances and preferences would help predict the success of hardhead in California stream habitats.

Hardhead are ectotherms, that is, they have body temperatures that closely reflect environmental temperatures, due to heat exchange across the gills, body wall, and fins (Stevens and Sutterlin 1976). It follows that, when presented with a temperature gradient, fishes often select temperatures that are optimal for their growth (Jobling 1981a). Optimal management of stream fish habitat should consider fish temperature preferences (e.g., the species' occupation frequency distributions over time, relative to temperature, as reviewed by Coutant 1987); along with physiological thermal limits and capacities in order to manage for both cold and warm seasons resulting from altered river hydrographs. There have been few studies on hardhead addressing laboratory and field thermal performance and preferences. In the Pit River (above Big Bend; Shasta County), hardhead with a total length of 7-37 cm have been observed in temperatures ranging from 16.6 to 20.2°C, swimming in mean water column velocities from 0.18 to 0.31 m/s (Baltz et al. 1987). These authors noted that more research was needed into seasonal microhabitat descriptions that reflect temperature-related shifts in habitats.

Knowledge of the temperature tolerance and preference of hardhead minnows, associated with swimming performance across a range of water velocities, is of critical value for management and conservation. Improved information should assist in developing thermal targets for future and current Federal Energy Regulatory Commission (FERC) licensing of hydro-electrical projects in California. Furthermore, maximum temperatures in California streams often exceed 28°C in mid-summer, and may increase under the influence of climate change. Conversely, cold

water (<8°C) may be stored in high elevation reservoirs longer into the summer, to compensate for low summer baseflows or to provide pulses of water for coldwater species such as spring-run Chinook salmon (Thompson et al. 2011) exposing juvenile hardhead to lower temperatures.

1.3 Project Objectives

The overall goal of this project was to conduct a comprehensive study of wild hardhead temperature preferences, tolerances, and effects on oxygen uptake and transport, using both adult and juvenile fish. We planned to examine several key physiological and behavioral variables of hardhead: temperature preference, resting metabolism, active swimming metabolism, critical thermal limits, and blood-oxygen equilibria. A previous study on hardhead thermal preference was conducted in our laboratory (Klimley et al. 2007), but was limited by the use of only adult fish, reuse of fish at each acclimation temperature, range of acclimation temperatures tested (12-18°C), and maximum temperature available to fish during preference testing (24°C). These limitations were due to a restricted capacity to chill and heat water at CABA, obstacles that were overcome in the current study. Myrick and Cech (2000) found that hardhead with a mean total length range of 22.5-28.5 cm that had been acclimated to a 10-20ºC temperature range were capable of critical swimming velocities (Ucrit) from 0.47 to 0.57 m s^{-1}. However, the maximum temperatures used in that study were lower than temperatures that may be encountered by fishes in California streams. Past studies investigated parasite loads of wild hardhead (Haderlie 1953; Alvarez 2008), finding relatively high natural loads of multiple parasite types. The authors suggested that warmer water temperature could have negative outcomes for hardheads carrying multiple parasites, potentially limiting hardhead distribution.

Our approach to study hardhead temperature tolerance and preference and potential habitat use was to use an eco-physiological approach, testing wild hardhead in a controlled laboratory setting. This involves testing responses of hardhead to thermal environmental conditions that fish may encounter in regulated rivers. To accomplish our objectives we aimed to expand on the work of Baltz et al. (1987), Myrick and Cech (2000), and Klimley et al. (2007) by testing hardhead over a broader range of temperatures representative of those found in the natural environment occupied by hardhead. Responding to the recommendations of Baltz et al. (1987) we designed a study of hardhead temperature preferences over a fine gradation of temperatures, using an annular temperature preference device (Klimley et al. 2007; Myrick et al. 2004). Furthermore, we designed detailed research into the metabolic costs of activity under environmentally appropriate temperature, as recommended by Cech et al. (1990). To expand on Knight's (1985), Castleberry and Cech's (1992), and Myrick and Cech's (2000) work on native California fishes' critical thermal tolerance we designed a study of both adult and juvenile hardhead critical thermal minima and maxima. We expanded on the above to included investigations of hardhead blood-oxygen equilibrium characteristics, because the dynamics of its oxygen-uptake and transport systems are useful in gaining insight into a fish's functional capacity and consequently its potential environmental limits (Powers 1932; Grigg 1974; Cech et al. 1994). The temperature sensitivity of a species' hemoglobin, quantified as the apparent heat of oxygenation, describes the effect of environmental temperature on blood oxygen affinities (Riggs 1970, Kaufman et. al. 2006).

Typically, active fishes inhabiting oxygen-rich water have a more sigmoidal-shaped blood-oxygen equilibrium curve and larger Bohr (CO_2 and pH-related) factors (Cameron 1971, Cech et al. 1984, Dobson et al. 1986), which help maintain oxygen delivery during activity when tissue pH declines and oxygen demand increases. Fishes inhabiting low oxygen environments typically have more hyperbolic-shaped curves and very low P50 (half-saturation) values (Cech et al. 1979a, Wood & Lenfant 1979), associated with high blood-oxygen affinities.

Our research objectives were to provide the following results:

- Temperature preference of hardhead adults and juveniles, determined using large and small annular devices at the Center for Aquatic Biology and Aquaculture (CABA) at UC Davis. We sought to determine the preferences of wild young-of-the-year and adult hardhead exposed to a thermal gradient ranging from ca. 12 to 28°C, when acclimated to different temperatures (11, 16, 21, 25°C).
- Resting metabolic rates (oxygen consumption) of adult hardhead and juvenile, determined using static respirometers at UC Davis' CABA facility, at environmentally relevant temperatures (11, 16, 21, 25°C), to measure energy turnover rates necessary for survival, in response to temperature.
- Active metabolic rates (oxygen consumption during exercise) of adult and juvenile hardhead, determined using three Brett-type (recirculating flow) respirometers at UC Davis' Eco-Physiology Lab, at environmentally relevant temperatures (11, 16, 21, 25°C) and water velocities for active metabolic rates (up to 1.05 m s^{-1} for adults and 0.60 m s^{-1} for juveniles), to understand energy demands during exercise.
- Critical thermal maximum and minimum (CTmax and CTmin, respectively) of adult and juvenile hardhead, acute thermal challenge followed by loss of equilibrium, using fish that have been acclimated at environmentally relevant temperatures (11, 16, 21, 25°C) at UC Davis' CABA facility.
- Hardhead blood-oxygen equilibria (including whole blood oxygen affinity) assessed across a temperature range of 11-30°C, to understand the effect of temperature on hardhead oxygen uptake and transport at UC Davis' Eco-Physiology Lab.

1.4 Report Organization

Chapter 2 describes the methods used in the study. Chapter 3 presents the results of the study. Chapter 4 discusses the conclusions and makes recommendations in context of hardhead's behavioral and physiological capabilities over a range of thermal conditions. Chapter 5 includes the References for all the chapters and the Executive Summary.

CHAPTER 2: Project Approach

2.1 Fish Collection and Care

2.1.1 Fish Capture Locations

The relatively accessible hardhead populations in the South Fork American River and North Fork Feather River, compared to those in the Pit and Yuba rivers, spread our limited collection impacts on the species (Table 1). Four hardhead were captured at Britton Reservoir (Pit River), but the low catch rate and larger distance from UC Davis precluded additional sampling at this location. We often noted that in clear water, with the substrate visible, hardhead collections were less successful, compared to those in waters where swimming hardhead could not be observed.

Table 1. Hardhead minnow adult collection rivers, total sites angled for fish, sites where hardhead were caught, and total number of adult fish captured for this study from each river. In Englebright Reservoir, on the Yuba River, hardhead were observed in small schools passing our vessel, but none were caught.

River	Total Sites Fished	Adult Hardhead Capture Sites	Adult Hardhead Captured
American, lower	1	0	0
Middle Fork American	3	0	0
South Fork American	5	4	54
Feather, lower	2	0	0
North Fork Feather	7	7	111
South Fork Feather	1	0	0
Pit	2	2	4
Rubicon	1	0	0
Yuba (above *Englebright*)	2	0	0
Middle Fork Yuba	1	0	0
South Fork Yuba	1	0	0
Totals	**25**	**13**	**169**

The South Fork American River has two reservoirs that hold hardhead in large numbers: Slab Creek Reservoir and Chili Bar Reservoir (Table 1). Upstream of Slab Creek Reservoir hardhead have access to several miles of river reach and tributaries, though cold water flowing from higher elevations may limit their winter and spring movements. Chili Bar Reservoir is a PG&E-operated hydro facility and is inaccessible to the general public due to safety concerns. We found a large hardhead populations there, perhaps due to lack of public angling pressure, and because hardhead have access to several miles of possible spawning habitat along the river between the reservoir and Slab Creek Dam. In this study, as in a previous study, we caught very small numbers of hardhead (<4) below Chili Bar Dam. Figure 1 shows all the sites sampled, including those where we found hardhead.

The North Fork Feather River has a series of hydro power plants above Oroville Dam which support relatively large populations of hardhead. We sampled at Cresta Dam, Poe Reservoir, Rock Creek Dam, and Rock Creek Reservoir at Chipps Creek along Highway 70. Smallmouth bass (*Micropterus dolomieui*) were caught in these reservoirs, but only when temperatures were

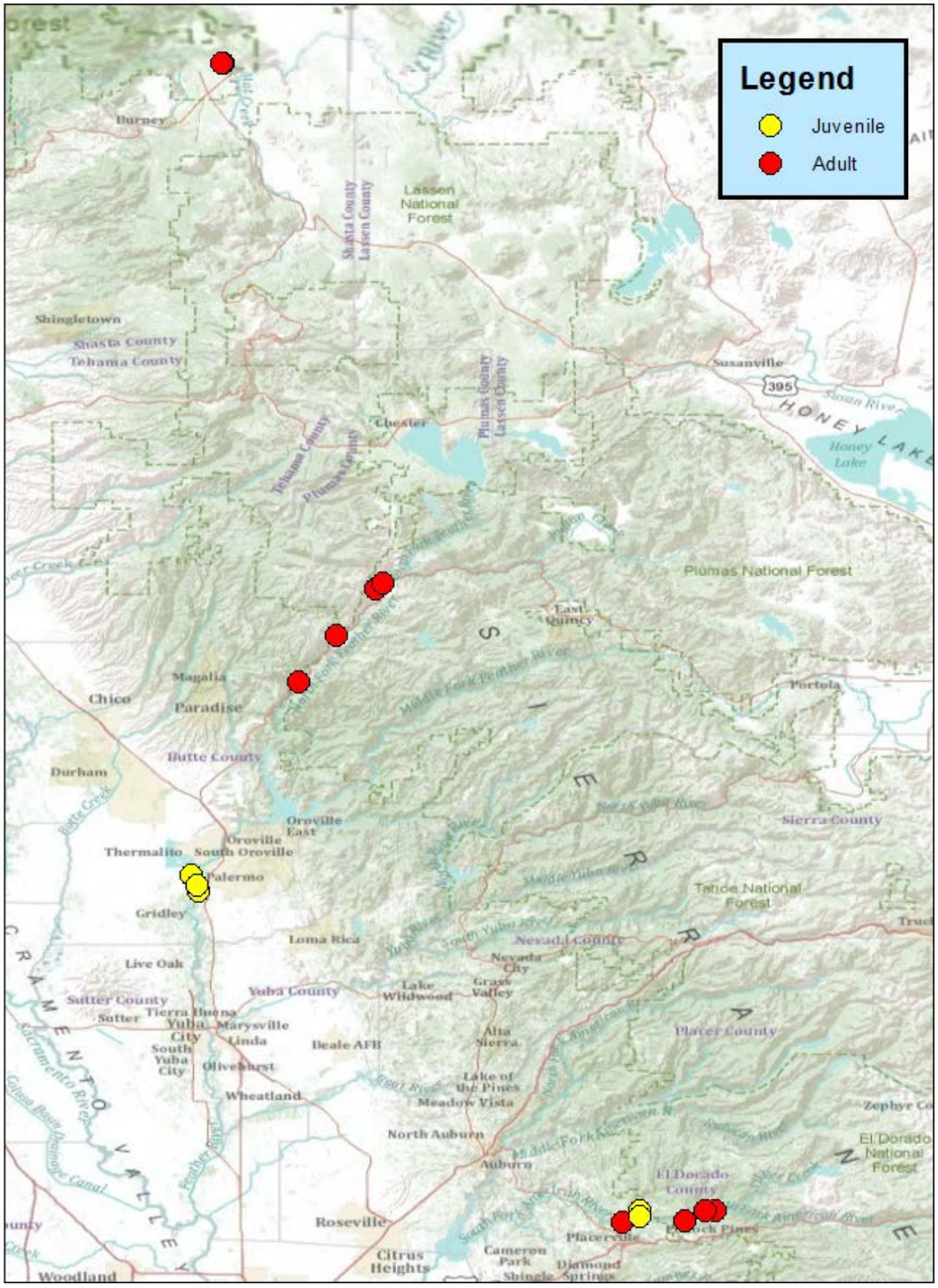

Figure 1. Map of collection sites for this study for both adult and juvenile hardhead minnow.

above 17.3°C. Interestingly, hardhead captures were lower when smallmouth bass were caught. At all the sites where hardhead were captured along the North Fork Feather and South Fork of the American River, Sacramento sucker (*Catostomus occidentalis*), Sacramento pikeminnow (*Ptychocheilus grandis*), rainbow trout (*Oncorhynchus mykiss),* brown trout (*Salmo trutta*), and sculpin (*Cottus sp.*) were frequently caught and released.

The main-stem Sacramento, Consumes, Mokelumne, Russian, and San Joaquin Rivers may support relatively large populations of hardhead, but our use of hook and line fishing on these river sections was limited by agency concerns over by-catch of protected Salmonidae species (e.g., winter run Chinook salmon). During April 26 – June 23, 2007, 390 hardhead were observed to move upstream in the Stanislaus River (Anderson et al. 2007).

2.1.2 Field Sampling Methods

Adult hardhead were captured by hook and line fishing from 3/24/2010 through 11/11/2010 (Table 2). Previous studies showed that this capture method works well to bring wild fish into the laboratory safely (Klimley et al. 2007). Our previous attempts with electro-fishing to capture cyprinids, including hardhead, resulted in poor physiological condition, including disease outbreaks, infections that were hard to control, and spinal deformities associated with electro-fishing (Kocovsky et al. 1997; Ruppert and Muth 1997). With hook and line fishing, we found only mild hook site infections (most likely *Columnaris*), which were treated with formalin baths (see 2.1.3). Salmon roe and halved earthworms were found to be the most productive baits compared to using spinning tackle or fly fishing. Our fishing technique involved watching for very small movements of the line as a cue to set the hook, thus only hooking the fish's lips and avoid hook ingestion. This was very successful and only a few fish were hooked in the tongue. Long surgical forceps were used to carefully and quickly remove the hook, typically in less than 5 seconds.

Table 2. Specific capture locations of adult hardhead minnows, by river and reservoir.

	Number of Hardhead Captured
North Fork Feather River	
Cresta Dam (2)	38
Poe Reservoir	12
Rock Creek Dam	24
Rock Creek Reservoir / Chipps Creek	37
South Fork American River	
Chili Bar Reservoir	27
Slab Creek Reservoir / Brush Creek	12
Slab Creek Reservoir / Dam	5
Slab Creek Reservoir at Forebay Rd.	10
Pit River	
Britton Reservoir	4

After capture, fish were held in transport coolers (142-l) of river water at up to six fish per cooler with up to three coolers used per fishing trip. The temperature and dissolved oxygen

levels of water in the coolers were monitored and maintained at river levels throughout the fishing day by adding fresh river water, including just prior to the ca. 2 to 3-h vehicle transport to the Center for Aquatic Biology and Aquaculture (CABA) at University of California Davis campus. During transport, each cooler was aerated via a large aquarium-grade air pump and individual air stones. Novaqua Plus™ was added as a prophylactic treatment to reduce transport stress and protect the fish's slime coat from netting. Upon arrival at CABA, fish were dip-netted from the transport cooler and immediately transferred to aerated 555-l tanks held within ±1.5°C of the capture location (often within ±0.5°C), with continuous flows of fresh well water, until experimental acclimations.

Juvenile fish were caught using standard minnow traps (Gee cylindrical trap, with two 2.54-cm diameter openings) placed near shore in riparian debris (fallen trees and submerged vegetation), often less than a meter deep. See Figure 1 for map of capture locations on the lower Feather River near Gridley. We used commercially available salmonid fish feed to bait the traps (Rangen, Inc. semi-moist and SilverCup®), suspended in the center of the traps in sandwich bags with small puncture holes. Traps were set overnight on 12/14/2010. Traps were set for only a short time (3-5 hours) on 12/20/2010 because fish were seen readily entering the traps immediately after they were set. By-catch consisted of sculpins, Sacramento suckers, Sacramento pikeminnow, centrarchids, blackfish (*Orthodon microlepidotus),* and tadpoles (Table 3).

Table 3. Juvenile hardhead capture, via minnow traps, and by-catch numbers for juvenile fish caught and released or used in this study. Fish were captured on the lower Feather River (Longitude 121:37:37.10W, Latitude 39:25:07.40N, Horizontal Datum WGS84) near Gridley.

Common Name	Scientific Name	Capture Date	Number	Disposition
Hardhead	*Mylopharodon conocephalus*	12/15/2010	73	Capture
Hardhead	*Mylopharodon conocephalus*	12/20/2010	143	Capture
Sculpin sp.	*Cottus sp.*	12/15/2010	38	Release
Sacramento sucker	*Catostomus occidentalis*	12/15/2010	37	Release
Sacramento sucker	*Catostomus occidentalis*	12/20/2010	68	Release
Centrarchid		12/15/2010	8	Release
Blackfish	*Orthodon microlepidotus*	12/20/2010	2	Release
Sacramento pikeminnow	*Ptychocheilus grandis*	12/15/2010	169	Release
Sacramento pikeminnow	*Ptychocheilus grandis*	12/20/2010	223	Release
Tadpoles sp.		12/15/2010	179	Release

Fish were sorted on the river's edge into separate containers for hardhead and by-catch. Identification was done with plexi-glass viewing chambers (Figure 2) to limit or prevent stressful air emersion of juvenile fish. After sorting, juvenile hardhead were loaded in 3 mm thick plastic bags (~50 fish per bag) containing ¾ full of river water (~9 l) and a cap full of NovAqua Plus™. The remaining head space in the bag was filled with pure oxygen and sealed for the ca. 2-h trip to CABA. By-catch was returned to suitable habitat near the capture location. At CABA, fish were placed into 140-l circular tanks, equipped with flow-through well water at 11.2°C (similar to the river at 11.5°C).

Figure 2. Capture location for juvenile hardhead used in this study. The picture shows Bethany DeCourten, an research assistant, using our acrylic viewing chamber, as well as the habitat type (large fallen trees, submerged vegetation, and swift main stem flows) in which large numbers of hardhead were found.

2.1.3 Fish Maintenance and Laboratory Acclimation

Adult fish were fed a 50:50 mix of SilverCup® 2 mm and 3 mm pellets amounting to 4.8 g of feed per fish per day. This was supplemented with rinsed and halved earth worms or red worms. Both foods were placed in a Pyrex dish that was gently lowered to the bottom of the tank. In general, it took hardhead several weeks to convert to commercial feed. Worms were re-offered to tanks that did not seem to be eating the pellet feed, and this was repeated as necessary through the acclimations and experimental treatments. Adults were kept at CABA in aerated, 555-l tanks held at one of four acclimation temperatures (11, 16, 21, or 25°C ± 0.5°C), with continuous flows of well water (conductivity 670-700 $\mu S\ cm^{-1}$, dissolved oxygen >6.5 $mg\ l^{-1}$, and pH 8.1), prior to experiments.

Because adult fish were from two main capture locations, we used Visible Implant Elastomer (VIE), a silicone-based material, from Northwest Marine Technology (Washington State) to mark hardhead by capture location. Feather River fish and American River fish were marked with green and orange VIE implants, respectively. We inserted the VIE marks into the interstitial spaces between the dorsal fin rays, after experimenting with a deceased hardhead and reviewing Hartman and Janney (2006). Elastomer was mixed per manufacture recommendations then loaded into the backof a syringe (B-D U-100). hardhead were lightly anesthetized with buffered Tricaine methanesulfonate (6 $g\ l^{-1}$ NaCl, 420 $mg\ l^{-1}$ $NaHCO_3$, and 150 $mg\ l^{-1}$ MS-222) and quickly (ca. <60 s) injected with the VIE mark, parallel to the rays, in the base soft connective tissue between the 1st and 2nd or the 2nd and 3rd dorsal fin rays (See Figure 3). This left a mark approximately 3-4 mm long. We noted that ectoparasites did poorly in the buffered MS-222 and were not observed on hardhead after tagging. After over a year in captivity, the VIE implants were still present in the dorsal fins and showed no loss in over 80 adult fish.

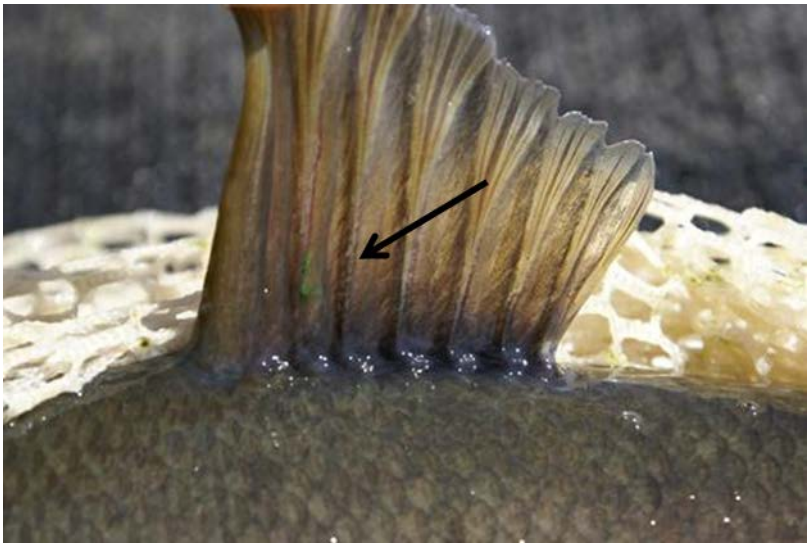

Figure 3. VIE tagging location on the dorsal fin of an adult hardhead minnow. Note the green marking near the base of the 3rd fin ray, indicated by the black arrow.

Immediately after tagging fish were randomly (coin flip) assigned to one of the four thermal acclimation groups (11, 16, 21, or 25°C). Thus, both Feather and American River fish were mixed together in a single tank for each acclimation group.

Juvenile fish were fed Rangen, Inc. starter diet (<0.6 mm) mixed with >0.6 mm Silvercup™ feed. This was supplemented with frozen baby and adult brine shrimp (San Francisco Bay Brand® Sally's Frozen Baby Brine Shrimp™) throughout the study. Juveniles were fed an excess ration where a minimal amount of uneaten food was present each morning from the previous day's feeding. Commercial diet was loaded into an automatic feeder set to add in food continually during the day. Because all the juveniles came from a single capture location no tagging was required.

Juvenile hardhead were kept in 140-l, circular tanks with a continuous flow of air-equilibrated well water (conductivity 670-700 μS cm^{-1}, dissolved oxygen >6.5 mg l^{-1}, and pH 8.1) under a natural photoperiod, until laboratory experiments.

During the first few weeks at CABA, all hardhead tanks were prophylactically treated with NovaAquaPlus™ (Kordon, LLC.). In adult hardhead, we noted flukes (monogenetic trematodes) and Blackspot disease. To treat for these conditions we selected a single treatment of 37% formaldehyde (1.5 h bath at 1.5 ml l^{-1}). During the treatment, aeration was significantly increased and fish were monitored closely for heavy respiration. Gyrodactylids (Monogeneans) and *Ichthyophthiriasis* ("Ich") were also observed on juvenile hardhead 2 months after capture. We first treated with 37% formaldehyde (formalin) for 6 days (1-h bath at 1.5 ml l^{-1} of water). This was successful in treating the condition, but caution is advised as hardhead are very sensitive to this harsh treatment regime (heavy mortality can be expected, especially if fish are in the later stages of an Ich infection). A potential explanation for mortality can be attributed to the factIch is known to attack delicate gill tissue (Ewing and Kocan, 1986) and formaldehyde

removes oxygen from the water (Noga 1996), both of which could impact oxygen uptake across the fish's gills.

A second outbreak of Ich occurred among juvenile hardhead two months later. The Ich was thought to have been induced by the added stress of a dissolved ammonia spike and a 5-6°C temperature increase, due to reduced chilled water flows to the holding tanks (overnight equipment failure). The reduced flow lasted over one night. To treat this outbreak Kordon, LLC.'s Rid-Ich™ was administered at 1.5 ml l^{-1} of water for 1 h in a water bath separate from the holding tank. At this point in our studies, fish were still held at their capture water temperature (11°C). Ich can survive >30 days in the cyst (trophont) stage at this low water temperature (Noga 1996), whereas in water above 25°C the Ich life cycle can be very short (4-5 days). Our recommendation for other researchers is to consider raising the water temperature to 18°C for Ich treatments of hardhead (under veterinary advice). We also treated the holding tank water with aquarium grade salt (NaCl), increasing tank salinity to 1-2 ppt daily during disease treatments, to break up Ich's life cycle (e.g., by causing cell lysing). Our holding tanks were equipped with flow through well water, restricting the salt's residence time to only a few hours per day. After these treatments, hardhead were held on untreated well water without disease outbreaks for the remainder of the study.

Tank temperatures were adjusted a rate of ±1°C/day and fish were kept a minimum of 30 days at their acclimation temperature before an experiment. Separate fish were collected for the blood-oxygen equilibria study, described in section 2.6 (below). If unexpected short-term temperature spikes were noted (< 12 h duration) during experimental periods, fish were held for a week at their acclimation temperature before restarting experiments. For both adults and juveniles, post-test tanks were used (maintained exactly the same as pre-test tanks) to prevent the added stress of trying to sort pre-test versus post-test fish each day. Due to limited opportunities to capture hardhead we could not use a unique fish for each type of physiological measure. Therefore, we decided to test fish in series with at least 30 days between experiment types. For adults the order of testing was: temperature preference, active metabolic rate, resting metabolic rate, and critical thermal minimum or maximum (deemed the most stressful procedure). For juveniles the order was slightly altered, with resting metabolic rate preceding active metabolic rate experiments.

2.1.4 Capture and Maintenance of Adult Fish for Blood–Oxygen Equilibria (Blood Gas Curves)

Adult hardhead (n = 45; TL 25.5-47.4 cm) used in the blood gas curve study were collected, via hook and line fishing, from the North Fork Feather River and the South Fork American River, California from early March to early November 2010. Captured fish were transported to CABA, in 150-l aerated insulated coolers. Fish were transferred into 950-l tanks supplied with a constant flow of aerated well water adjusted to match water temperatures at the time and place of capture. During transport and immediately after arrival hardhead were prophylactically treated with NovaAquaPlus™ (Kordon, LLC). The supply water for each tank was slowly adjusted (1°C d^{-1}) from conditions matching the time and date of capture (11-20°C) to ambient well water temperatures (i.e., 19°C). Fish were fed a daily ration of Silver Cup® commercial

trout pellets supplemented with live earthworms daily as previously described until they were used in blood-oxygen equilibria experiments (typically within only a few days after capture).

2.2 Temperature Preference Study

2.2.1 Adult

The adult temperature-preference chamber was first conceived in Klimley et al. (2007) as a scaled up version of the annular device used by Myrick et al. (2004), with only slight changes to its design for our current study. It was constructed of clear acrylic plastic and featured four concentric, circular walls, three were perforated inner walls separating the receiving/mixing chambers, the swimming chamber, and the final effluent chamber. The solid outer wall was 3-m in total diameter. Reflectix, Inc. insulation was placed around this wall to block the fish's view outside of the chamber and to insulate the mixing chambers from thermal influences in the room. The chamber's base was clear acrylic, on an acrylic sub frame (3.05 m x 3.05 m, 2 cm thick), mounted to a painted steel frame with leveling pads. For the current study opaque beige acrylic was attached underneath the base to create contrast for the video equipment.

The preference chamber was divided into 32 areal positions for tracking fish location and measuring water temperatures (Figure 4). Water was distributed to the mixing chambers via 16 constant-head 17-l reservoirs containing either chilled (12.0°C), ambient (18.0°C), or heated (28.5°C) water (Figure 5), mounted on a pulley system above the chamber. The 30-cm-wide swimming channel was kept at a constant 15.2-cm water depth. Temperatures were recorded using three calibrated AlphaMach, Inc. IBCod thermal loggers (Model 22L) mounted in the mixing chambers to verify that the gradient remained stable during each experiment.

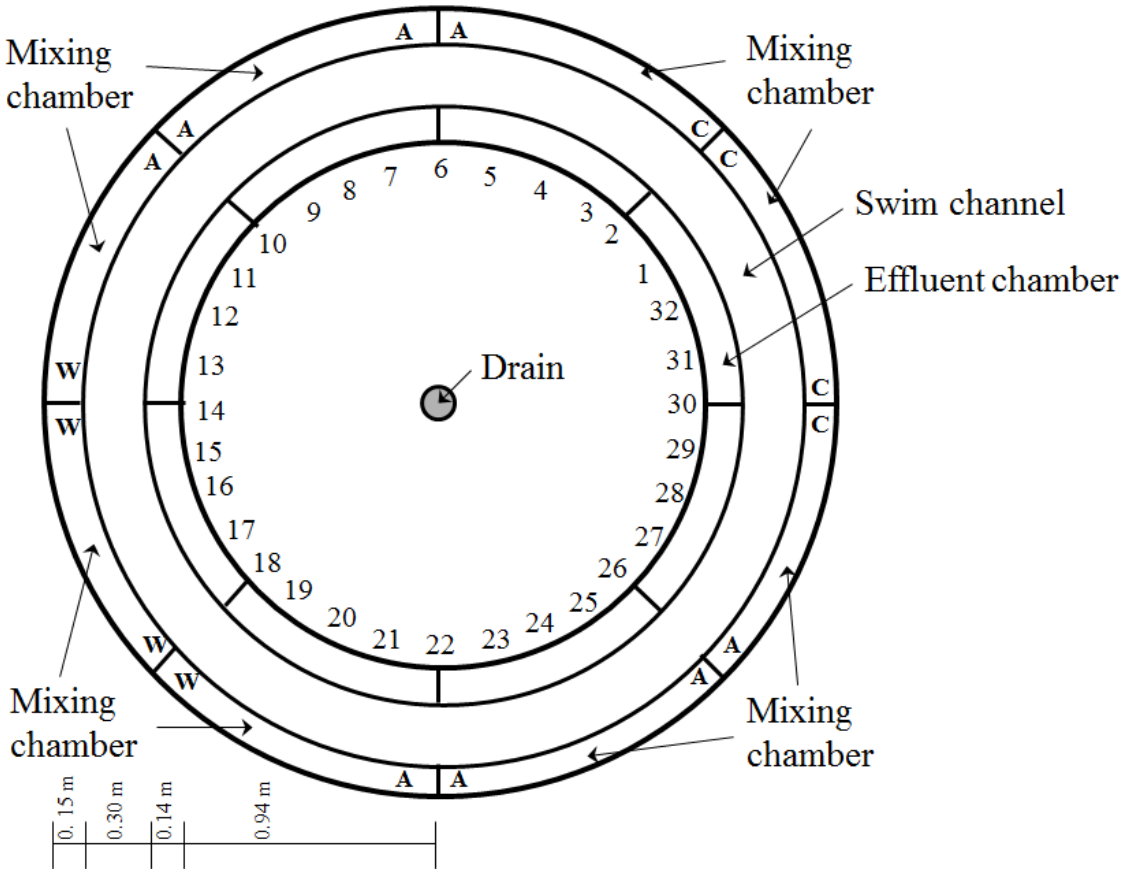

Figure 4. Top, diagrammatic view showing the temperature-preference apparatus' eight radial mixing and effluent chambers, swimming channel, and centered drain. The 32 areal locations are shown and the letters depict a typical inflowing water scheme (C=12°C, A=18°C, and H=28.5°C; SE are presented in the text). Note: positions 32 and 16 are the most extreme sides of the chamber and have no symmetric counter-part (e.g., Position 15 and 17, 14 and 18, 13 and 19, mirror identical thermal locations). Note shown are the constant-head 17-l reservoirs which are suspended above the mixing chambers of the apparatus.

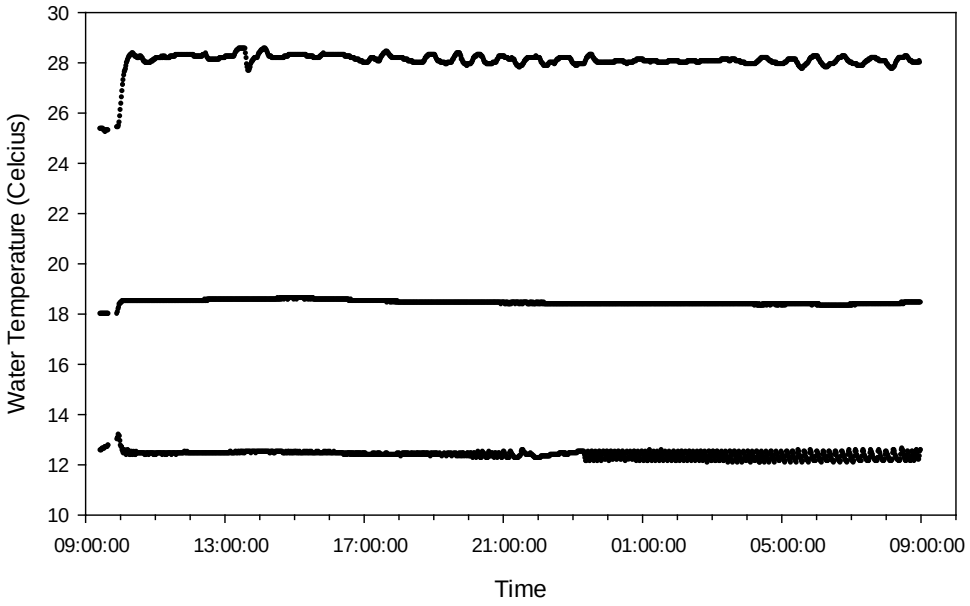

Figure 5. Typical incoming water temperatures delivered to the temperature-preference mixing chambers for the 25°C hardhead acclimation group. This verified that our gradient was stable for each experiment at 1 minute intervals. The early data points represent the adjustment of the chamber from the fish's acclimation temperature to the experimental gradient.

The chilling system consisted of two 15-horsepower heat exchangers (Heat Controllers, Inc.), retrofitted for cold ambient running conditions. This system supplied stable ~12.0°C water to four of the apparatus' overhead reservoirs. Three Mobius (model: T-M1 Takagi) on-demand, tankless gas boilers, plumbed in series, heated the ambient well water. Water from the heaters was first sent to two gas-equilibration columns (Aquatic Ecosystems Model No. AB12), that prevented nitrogen gas supersaturation, prior to its distribution into four of the overhead reservoirs. The other eight overhead reservoirs were supplied with air equilibrated well water. A single 0.5 horsepower Sweetwater® centrifugal pump (Model No. PS3SS) moved warm water from the gas-equilibration columns reservoir. This pump was oversized and required water bleeds back to the warm water reservoir.

Our warm and cold water supply lines leading to the apparatus were controlled by Belimo (model: LRB24-SR) and Honeywell (model: ML7984) mixing valves, respectively. The mixing valves were supplied with an 18°C (ambient) line and either a warm or cold supply line, depending on the system. Each valve was controlled by a separate Omrom (model: E5AK) digital controller. These systems are designed to fluctuate above and below the set temperature within a few tenths of a degree (see Figure 5). We used the mixing valves to set the acclimation temperature of the chamber to that of the fish's holding tank. The water distribution lines were plumbed to allow the cold and hot sides to alternate between experiments, with distribution decided randomly (coin flip) prior to each experiment. By changing the locations of the cold and warm areas in the temperature-preference apparatus, we increased the chances that hardhead

were responding to temperature cues, rather than other cues related to the apparatus' position in the laboratory.

To start each experiment the temperature of the entire chamber was stabilized at the fish's acclimation temperature, and then the fish was released into the apparatus at one of four possible randomly selected locations (Figure 6). Fish were allowed to acclimate to the chamber for 30 min at their acclimation temperature during which time the chamber had a minimum base flow to only 4 of the 16 overhead reservoirs in the adult system, and a single overhead reservoir in the juvenile chamber. After acclimation, all three source waters (chilled, ambient, and warm) were supplied to the relevant receiving/mixing chambers, producing the temperature gradient. All fish tested were easily able to swim around the entire annular ring in <15 s, minimizing possible space and time autocorrelations. During our temperature calibration tests (described in detail below), we found that the thermal gradient typically stabilized in less than 5 minutes. Each experiment lasted 4 hours. All temperature loggers and video data were downloaded to external USB hard-drives, and post-experiment fish were weighed, measured, and placed into a post-test holding tank.

Figure 6. Adult hardhead minnow in the large (left), and juvenile hardhead minnow in the small (right), temperature preference chamber's swimming channels.

2.2.2 Juvenile

The juvenile chamber was based on a design by Myrick et al. (2004), with some modifications. It consisted of an annular chamber, scaled down from the adult preference chamber, with a 122 cm by 122 cm footprint. We altered the original design by routing the water leaving the overhead reservoirs through a ½" PVC manifold, then to hose barbs (rather than directly to the hose barbs). The manifold was attached via a 2.54 cm bulkhead. The underside of the chamber was fitted with an opaque beige acrylic backing (2 mm thick) to enhance visual contrast of the fish for the video equipment. The hardhead's proclivity to jump out of the apparatus was prevented by the silicone attachment of 14 cm high and 1 mm thick acrylic sheets around both sides of the swimming channel rims. Similar to the adult chamber, there were 32 areal sections for analysis of fish position. The swimming channel was 9.5 cm wide and kept at a constant depth of 4.5 cm. The chamber was enclosed with landscape fabric to minimize disturbance of the fish, and natural light entered through the building's' translucent roof panels.

Inflowing water was supplied from the same system as for the adult preference chamber, supplying 28.5°C (~±0.3) on the warm side, 18.5°C (~±0.3) the ambient sides, and 12.0°C (~±0.3) on the cold side (Figure 7). Temperatures were recorded using three AlphaMach, Inc. IBCod thermal loggers (Model 22L) in the receiving/mix chambers to verify that the gradient remained stable during each experiment. Juvenile hardhead temperature-preference tests followed the same protocol described in 2.2.1 for adult fish.

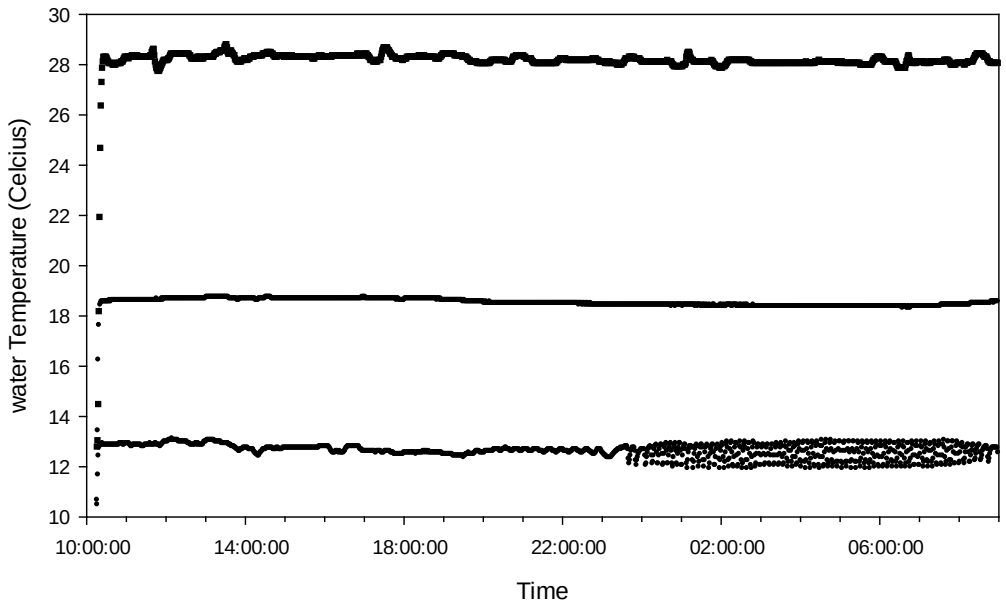

Figure 7. Typical incoming water temperatures delivered to the temperature preference mixing chambers for the 11°C juvenile hardhead acclimation group. This verified that our gradient was stable for each experiment at one minute intervals. The early data points represent the adjustment of the chamber from the fish's acclimation temperature to the experimental gradient.

2.2.3 Experimental Design, Video Data Collection, and Analysis

Temperature calibration tests, with no fish in the device, were performed in both annular preference chambers (n=2 large chamber and n=3 small chamber) to confirm the systems' ability to maintain and reproduce temperature gradients (Figures 8 and 9). Temperatures were measured using a custom mount that could hold six calibrated YSI 400 series probes at one time (see Figure 10 for layout) connected to a breakout box; we used a Cole-Parmer digital thermometer (Model No. 8402-00) to read temperatures. To record temperatures for the chamber calibration, the temperature probe mount was first positioned at areal position 1 (the observer took all six readings), then was manually moved to position 2 to repeat the process, then repeating the process until reaching position 32. The process was then repeated on additional calibration days.

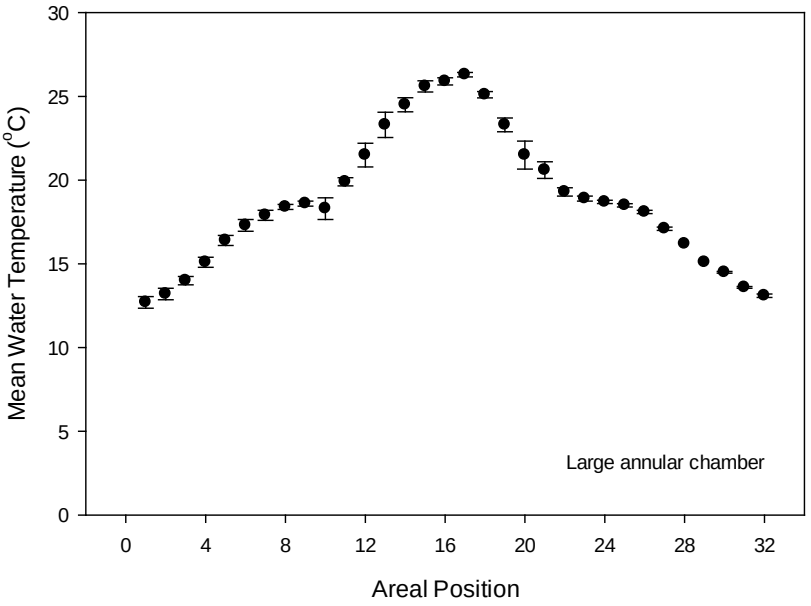

Figure 8. Temperature gradient calibration in the large annular preference chamber showing mean water temperature (±SE, ●, n=2). Note that positions 1 and 32 are next to each other in the chamber.

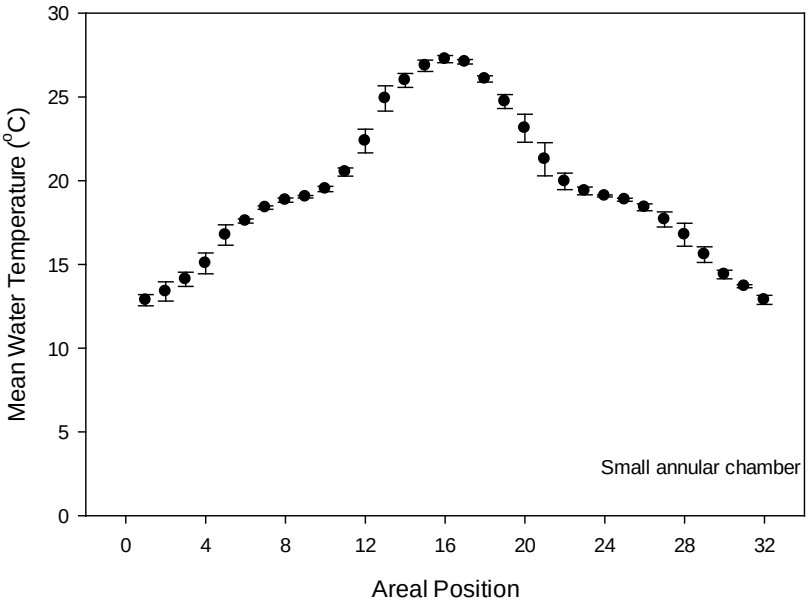

Figure 9. Temperature gradient calibration in the small annular preference chamber showing mean water temperature (±SE, ●, n=3). Note that positions 1 and 32 are next to each other in the chamber.

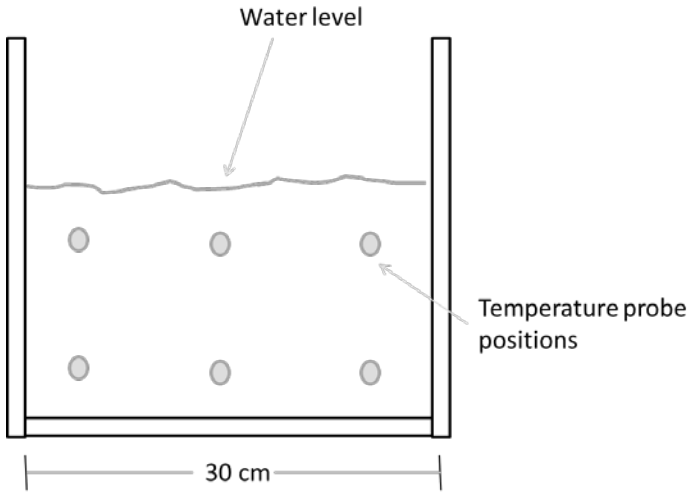

Figure 10. Locations of the calibrated YSI 400 series probes used to measure water temperatures in the swimming channel of the preference chambers. As shown in this cross-sectional diagram, in the adult chamber, the outer probes were positioned 2.5 cm from any acrylic edge and the water surface. The middle probes were positioned 15 cm from the vertical edges and either 2.5 cm from the water surface or the chamber bottom. In the juvenile chamber the mount was 9 cm wide and the outer probes had 0.5 cm gaps to the chamber and water surfaces.

Because of the long duration of each experiment we developed new methods to collect data that did not require the constant presence of a human observer during experiments. Both chambers were equipped with 520-line resolution video cameras (model No.QSC1352W with IR lighting), video data for both preference chambers was collected by programmable Q-See security recording systems (model No. QS408 for juveniles and QT528 for adults). The camera's IR lights were blacked-out in the juvenile chamber, and two separate LED infrared light modules (Q-See brand) were used instead. This change decreased the acrylic's direct reflection of infrared light back to the camera, causing a diamond-like sparkling effect. The adult preference chamber was large enough to mute the reflection of IR light back into the cameras. We also found that a camera that converts to a black and white image when the IR lights were activated provided images superior to those from a camera with software that adjusted to color feeds. Video data collection has several advantages over manual data collection: 1) it does not require constant real-time observation, 2) fish are less likely to be disturbed by a human observer, and 3) time can be taken post-test to determine the location of a fish that is very close to the wall of the chamber, whereas in a real-time situation the observer may not be able to locate the fish before the end of an observation interval.

Video analysis of the adult experiments was done by capturing images from the stored video files at 5-min intervals using Auto Screenshot Maker 3.0 (ASM), resulting in over 240 position observations per hardhead experiment. We used Q-See's SuperPlay software (version 1,2,0,723) to link all four camera angles together and the ASM to capture the active window. The images were viewed by the researcher and observations were entered manually into Excel. We opted not to employ video tracking software because of the parallax error associated with the large size of the chamber, and the limited area covered per camera. Instead, we were able to

determine the fish's position using the flooring joint seams (2.5 cm) to accurately track position, because these were directly under the fish at specific intervals.

Statistical comparisons of mean mass and fork length were made within each hardhead acclimation group (adults and juveniles) with a Kruskal-Wallis one-way ANOVA, because the data did not pass the normality test (Shapario-Wilk) and post hoc pairwise multiple comparisons were done using Dunn's method. The overall mean selected water temperatures of each acclimation group were compared with a Kruskal-Wallis one-way ANOVA (adults) and one-way ANOVA (juveniles). Post hoc pairwise multiple comparisons were done using Dunn's Method (adults) and Holm-Sidak's method (juveniles). Simple Linear Regression (SLR) of mean selected water temperature versus time were performed for each of the acclimation groups. Statistics were prepared with SigmaPlot™ Release 12 and alpha was considered significant below 0.05.

2.3 Resting Metabolism Study

2.3.1 Adult

Adult hardhead were acclimated to one of four temperature groups (11, 16, 21, or 25°C) for >30 days. The night before an experiment individual fish were placed into cylindrical acrylic chambers (64 cm long x 15 cm diameter area for the fish) with conical end caps and flow diffusing baffles to prevent a fish's tail or snout from clogging the inflow or outflow (similar to Cech et al. 1979, Figure 11). We calculated the two chambers' water volume three times (mean = 13.03 l) by comparing the weight of the chamber when full and empty. Overnight acclimation ensured that fish were in a post absorptive state (empty gut) for the follow morning's intermittent respirometry trials. Fish tended to struggle only a few seconds when initially entering the chamber before becoming very quiescent, exhibiting very shallow ventilatory beats during the acclimation and experimental periods. Two respirometers were placed, side by side, into an insulated, fiberglass 300-L (Minnow-O-Cool) tank and connected to the water supply manifold via a submersible pump (Danner Model No 2) with the bath water set to the fish's acclimation temperature (Figure 12). The water bath was heavily aerated, via air stones, and for the warmer water treatments a 17-l trickle column (wet-dry filter) was added to maintain dissolved oxygen >6 mg l^{-1} for all experiments. The fiberglass tank was then covered with heavy black plastic material to minimize disturbances to the fish.

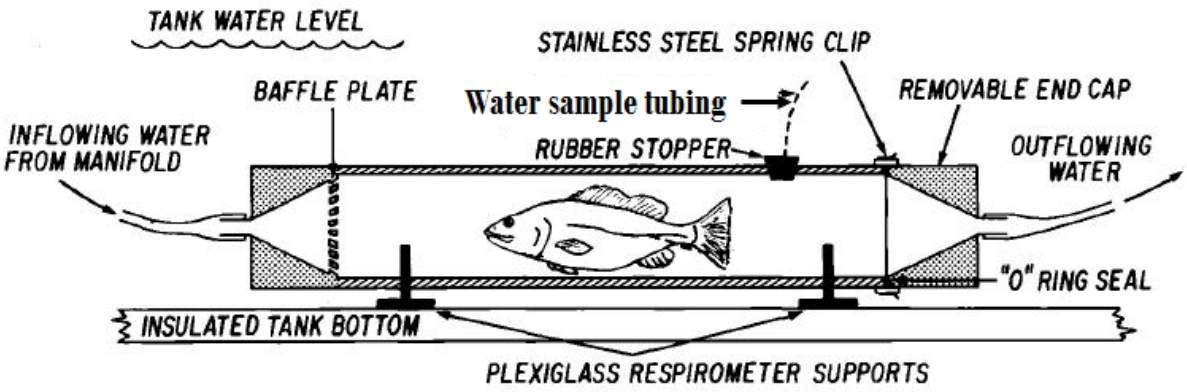

Figure 11. Chamber used for hardhead intermittent respirometry, outfitted with sampling port (cannulae tubing through a rubber stopper) for removal of water samples for oxygen measurements (the fish was not cannulated). Adapted from Cech et al. 1979.

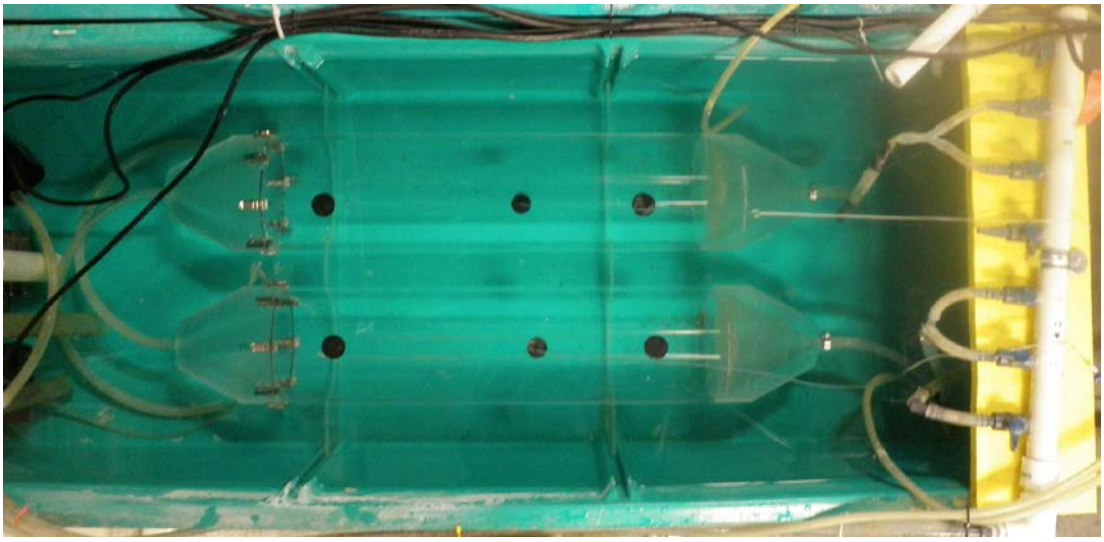

Figure 12. Two 13.01-l chambers used for our resting metabolic rate determinations of adult hardhead minnows.

We employed intermittent respirometry methods to quantify hardhead resting routine metabolism. For intermittent respirometry, water flow to the respirometry chamber alternates between flushing periods with continuous flow of well oxygenated water through the chamber and measurement periods where the chamber is sealed and the oxygen declines due to the aerobic respiration of the fish. To start the experiments, respirometry chambers were sealed at 0900-1000 each day, for both fish simultaneously. This measurement interval lasted ca. 45 minutes depending on acclimation temperature, allowing for a ca. 10 torr drop in the partial pressure of oxygen in the chamber water. The chambers were then flushed for 10 minutes and resealed. To determine the oxygen decline over time, the oxygen content of the water was sampled at the time at which the chamber was sealed and again just before it was unsealed. To sample the oxygen content of the respirometry water, an initial 2-ml sample was pulled through the cannula tubing to clear the line. Using a 1-ml syringe, a water sample was then drawn and quickly capped (Luer cap) before delivery into a water-jacketed temperature controlled

electrode cuvette sample port. These procedures were repeated until three measurement periods were completed for each fish. All chamber flushing and sealing occurred remotely through the valve manifold to avoid disturbing the fish. The oxygen content of the water was measured with a polarographic electrode (Model E101, Analytic Sensors, Inc.) connected to an AM Systems polarographic amplifier and converted to O_2 concentration (mg O_2 l^{-1}) using the nomogram of Green and Caritt (1967). Daily barometric pressure was determined using a Nova™ mercury barometer. Post-experiment fish were weighed, measured, and placed into a post-test holding tank. Resting routine oxygen consumption rates (MO_2,) were calculated by using the initial vs. final PO_2 difference, elapsed time in the respirometer, fish mass, and respirometer volume, following Cech (1990).

$$MO_2 = [(O_2(A) - O_2(B)) * V] / M^{2/3} / T$$

where MO_2 is O_2 consumption rate (mg O_2 $kg^{-2/3}$ h^{-1}), $O_2(A)$ is the concentration (mg O_2 l^{-1}) at the start of the interval in water, $O_2(B)$ is the concentration (mg O_2 l^{-1}) at the end of the interval in water, V is the chamber's volume (13.03-l, minus the fish volume assumed to be equal in milliliters to its mass in grams [Virani and Rees 2000]), M is the fish's mass (kg), and T is the elapsed time during the measurement (h).

To evaluate the influence of temperature on MO_2, we calculated the temperature coefficients. The Q_{10} is defined as a rate of change in a biological system over a 10°C change in temperature and has the following formula:

$$Q_{10} = (yMO_2/xMO_2)^{10/(Ty-Tx)}$$

Where $y$$MO_2$ was second rate, $x$$MO_2$ was first rate, Ty is the temperature (°C) at the second rate, and Tx is the temperature (°C) at the first rate. Q_{10} valves are unitless.

To test for differences in fish mass between acclimation groups we used a one-way ANOVA. To investigate if the mean metabolic rates differed between temperature acclimation groups we used a one-way ANOVA, for pairwise comparisons Holm-Sidak method was used. A simple linear regression was plotted to test for acclimation temperatures versus metabolic rate. All statistical analyses were conducted using SigmaPlot™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.3.2 Juvenile

Juvenile experiments were conducted with the same methods as for adults; except for the chamber size and type (glass), video equipment, and number of fish tested simultaneously. For juveniles we chose either a 225-ml or 470-ml glass respirometer, based on fish size (Cech 1990). All the 11°C and 16°C experiments were conducted in 225-ml vessels; all but one 21°C experiment was with the 225-ml respirometer, and all the 25°C treatment group were tested with 470-ml vessels (Figure 13). Preliminary trials suggested that juveniles were not as quiescent as the adults so a video camera (Speco Model No.627) equipped with IR lights was used to observe the hardhead remotely. Typically the juveniles showed active pectoral fin movements throughout the experiments and were observed to make repeated circles in the

chambers. Those intervals containing active circular movements were removed for that fish. We tested three juvenile fish per day, simultaneously.

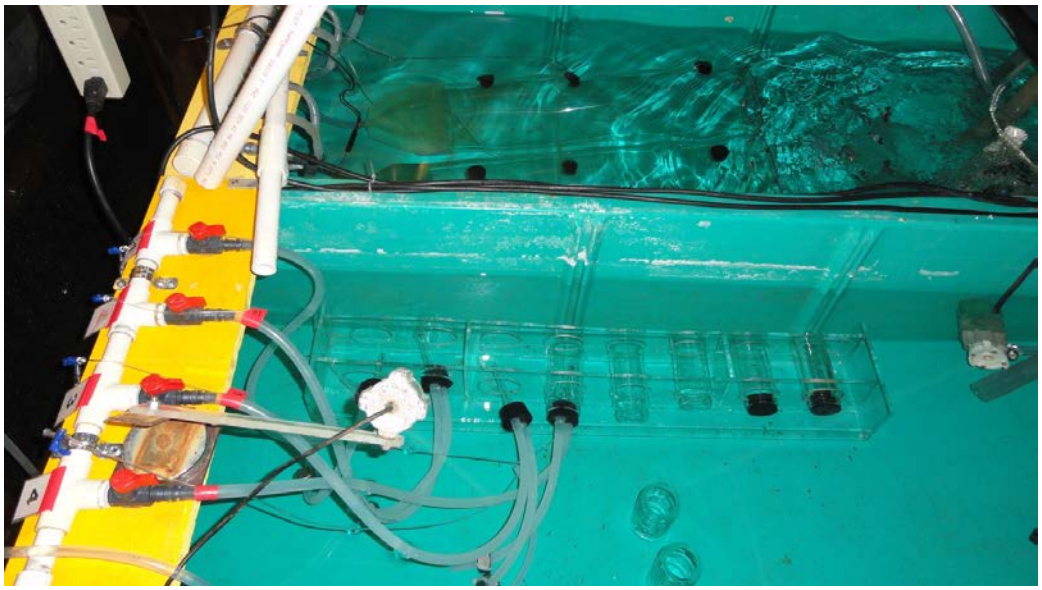

Figure 13. Chambers used for resting metabolic rate determinations for both juvenile (foreground, 225-ml respirometers) and adult hardhead minnows (in background). Video camera equipment was used for remote observations of the fish to assess activity.

MO_2 and Q_{10} were calculated in the same way as described for adults. To test for differences in fish masses between acclimation groups we used a one-way ANOVA, multiple pairwise comparisons were determined using the Holm-Sidak procedure. To test whether the mean metabolic rates differed between temperature acclimation groups we used a Kruskal-Wallis one-way ANOVA, for multiple pairwise comparisons we used Dunn's method. A simple linear regression was used to test the relationship between acclimation temperature and metabolic rate. All statistical analyses were conducted using SigmaPlot ™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.4 Active Swimming Metabolism Study

2.4.1 Adult

For the adult experiments we used two, velocity-calibrated Brett-style (Brett 1964) swim chambers; 150-L and 660-L (Cocherell et al. 2011; Figure 14). Fish that weighed more than 500 g were assessed in the larger chamber and hardhead weighing less than ca. 500 g were assessed in the 150-L chamber. Fish were placed inside the swim tunnel the previous night to allow for handling stress to subside. The chambers were maintained overnight at a water velocity of 5 cm s^{-1} and had constant exchange between the flow-through water bath (well water ca. 12 lpm flow rate through the bath) and the chamber with flushing water pumps (Danner Model No. 5). Combinations of chillers or heaters were used to hold water bath and swim chamber temperatures within ±0.5°C of the fish's acclimation temperature.

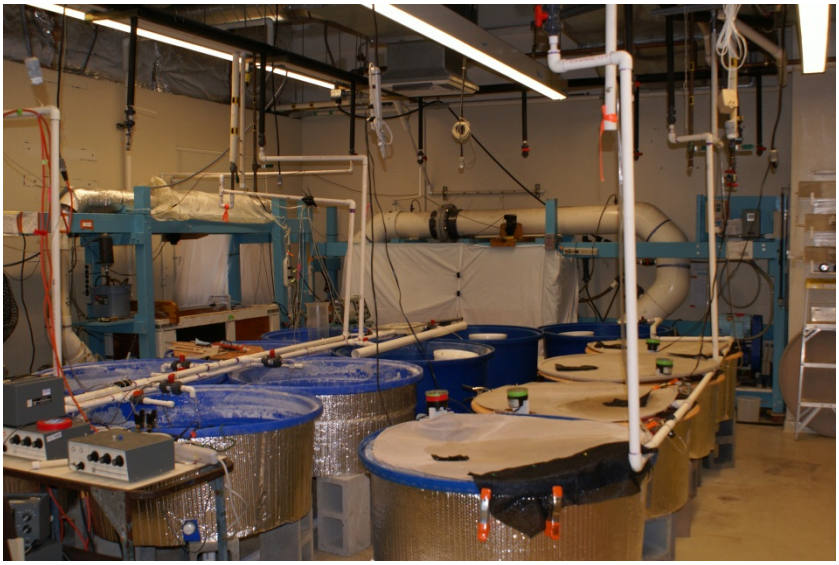

Figure14. Wet lab with the two Brett-style respirometers along the back and left lab walls. The tank systems in the foreground were under construction and not used for holding fish in these studies.

In the morning following acclimation, experiments were started between 0800-0900. First the chamber's water velocity was increased to 25 cm s^{-1} or 30 cm s^{-1} (velocities at which preliminary trials showed active swimming in adult fish) in the 150-l or 660-l chamber, respectively. Then flushing pumps were stopped,closed (depending on chamber), and the water bleed ball-valves were closed. Thus, the chamber was sealed from the water bath. An initial water sample (~3ml) was taken from the chamber and the partial pressure of oxygen was measured. After a waiting period (~1 h) another sample was taken to determine whether a ca. 10 torr decrease in oxygen partial pressure (PO_2) had occurred. If the drop was less than 10 torr the researcher waited a similar length of time before taking another sample (typically between 45-120 minutes, dependent on velocity step). After a 10 torr drop in PO_2 occurred, the flushing pumps were restarted and valves reopened to the chamber to flush the chamber with air-equilibrated water from the bath, ~30 min. The velocity was then increased by 10 cm s^{-1} (Jones et al. 1974) or 15 cm s^{-1}, 150-l or 660-l chamber respectively. Thus, both sets of fish (each chamber) received an approximately equal number of velocity step treatments until fatiguing. After the velocity stabilized (~5 min) the flushing system was closed and an initial PO_2 sample was taken. This procedure was repeated until the fish became fatigued twice at its highest swimming velocity, typically 10-12 hours. We allowed >30 min of recovery time before removing the fish to be weighed, measured, and placed in a post-test holding tank.

The oxygen content of the water was measured with a polarographic electrode (Model E101, Analytic Sensors, Inc.) connected to an AM Systems polarographic amplifier and converted to O_2 concentration (mg O_2 l^{-1}) using the nomogram of Green and Caritt (1967). Daily barometric pressure was determined using a Nova™ mercury barometer. If the dissolved oxygen level in the chamber fell below 70% of air saturation, the chamber was flushed with air-saturated water (Hammer 1995).

Swimming metabolic rate was calculated according to the equation:

$$MO_2 = [(O_2(A) - O_2(B)) * V] / M / T$$

where MO_2 is O_2 consumption rate (mg O_2 kg^{-1} h^{-1}), $O_2(A)$ is O_2 concentration in water (mg O_2 l^{-1}) at the start of the measurement period, $O_2(B)$ is O_2 concentration in water (mg O_2 l^{-1}) at the end of the measurement period, V is the volume of the respirometer (l), M is the fish's mass (kg), and T is the time elapsed (h) during the measurement period (Cech 1990).

During the active metabolism experiments the fish's tailbeat frequency (TBF) was noted by the observer every 20 min at each velocity step (Figure 15). Mean TBF was calculated for each fish at each velocity step, then the overall mean TBF for all fish at each velocity was calculated. Thus, a single fish could have had as few as one TBF determination or as many as eight, depending on how many velocity steps were successfully completed by that fish.

Figure 15. Overhead view of an adult hardhead minnow swimming in the 660-l Brett-style respirometer. Note the rubber stopper is connected to cannula tubing for remote water sample removal (not connected to the fish).

To compare the mean mass of fish in different acclimation groups we used One-Way ANOVA. We also used one-way ANOVA analyses to investigate differences in mean MO_2 values with water velocity within each temperature group. If significant differences were detected, we used post-hoc pairwise comparisons (Holm-Sidak Method) within the treatment group. The metabolic rate for hardhead acclimated to 11, 16, 21, and 25°C water temperatures, and that swam at 30, 45, 60, 75, and 90 cm s^{-1} water velocities, were analyzed using a two-way ANOVA with acclimation temperature and water velocity as factors. Post-hoc analyses were performed with Tukey tests.

The sample size for TBF was calculated from the mean for each fish's velocity step, then the overall mean of all the fish. Thus, a single fish could have had as few as a single TBF determination or as many as eight. Fish that failed a velocity step for MO_2 still had TBF recorded. A one-way ANOVA analyses investigated differences in mean TBF values with water velocity within each temperature group. If significant differences were detected, we used post-hoc pairwise comparisons (Holm-Sidak Method) within the treatment group. To compare four acclimation groups and five water velocities fish were tested at we used a two-way ANOVA,

with acclimation group and water velocity as factors. Post-hoc multiple pairwise comparisons were performed with a Tukey test. All statistical analyses were conducted using SigmaPlot™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.4.2 Juvenile

Juvenile hardhead active metabolic experiments were conducted in a 5-l swimming respirometer (Loligo® Systems Model No. SW10050). This was coupled to a single-channel respirometry system with galvanic electrode (Loligo® Systems Model No. DAQ-PAC-G1) and Loligo's AutoResp™ software for continuious oxygen readings. The volume of our chamber (4.94-l) was calculated through UV/Vis spectroscopy (See Appendix for methods and results). Juvenile hardhead were given 30 min of acclimation in the chamber at 5 cm s^{-1}. Water velocity was calibrated using an electromagnetic flow meter (Marsh-McBirney Model No. 201D).

In preliminary trials we found that, after overnight acclimations at 11°C, the hardhead were unwilling to swim with water velocity increases (3 fish tested on 3 separate occasions). To encourage these juvenile fish to swim for the actual experiments, we decreased their acclimation time to 30 min to maintain the hardhead in a heightened activity state, versus the quiescent state that apparently resulted from overnight acclimation times. We also modified the square-section swim chamber by inserting a clear, acrylic tube (27.5 cm long x 7 cm diameter) in the testing area of the chamber (Figure 16), and found that when we replaced the gray-colored flow straightener with a white one, and shading the front 2-3 cm of the chamber, some hardhead were more willing to swim. The tube also prevented hardhead from utilizing any boundary layers near the vertical rectangular walls. The fish's view of the researchers was obscured by thick landscape fabric allowing filtered light to enter only from above the chamber. Hardhead were monitored remotely via video camera equipment.

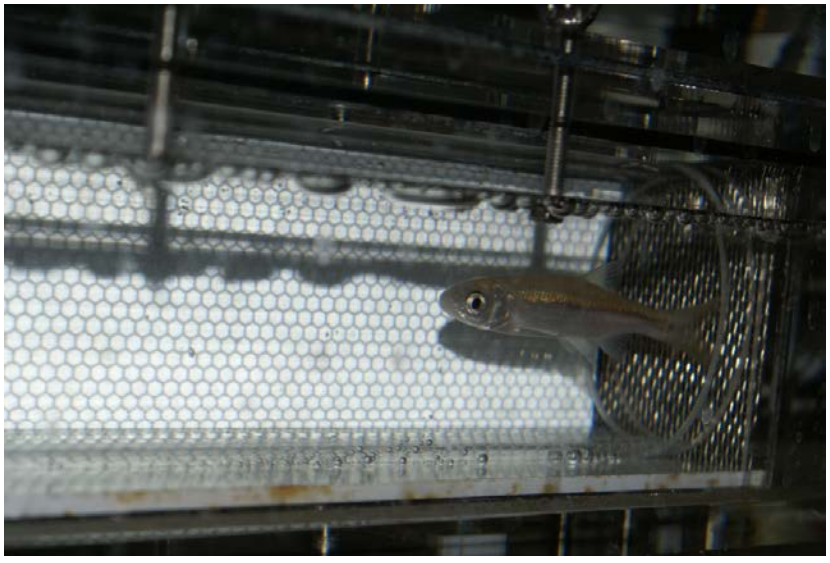

Figure 16. Side view of a juvenile hardhead swimming at 5 cm s^{-1} in the 5-l Loligo® swimming tunnel. Note the outline, near the back screen, of the cylinder added to the testing area. The bubbles shown in this picture were removed before starting the metabolism experiments.

For each experiment the swim chamber was set (±0.5°C) to the fish's acclimation temperature, using heaters or chillers, modifying the ambient temperature (~18°C) of the flow-through well water at CABA. After the 30-minute acclimation period, the water velocity was increased to 10 cm s^{-1} for the first velocity step. The chamber's flush pump was shut off and an electronic valve sealed the chamber. The partial pressure of oxygen was recorded every 10 min, until a 5-torr drop was noted. The chamber was then flushed for 10 min and the water velocity was increased by another 10 cm s^{-1}. Stepwise velocity increases, sealing and flushing of the chamber, and associated oxygen measurements were continued until the fish fatigued twice within a velocity interval (60 cm s^{-1} was the maximum velocity achieved in any experiment). Because of their small size it was very difficult to detect whether juvenile hardhead were "tail propping" (e.g., from fatigue) against the rear screen. Therefore, we decided to use one of two visible signs of fatigue before terminating each experiment: S-bending by the fish, or when fish had greater than 50% of its body in contact with the rear screen.

Post-test handling of juvenile fish was the same as for adults. To compare the mean masses of fish in different acclimation groups we used one-way ANOVA, and post hoc pairwise multiple comparisons were evaluated via the Holm-Sidak method. No statistical tests were performed for the 11 and 16°C acclimation groups due to very low successful sample sizes. To test for differences in mean metabolic rates over the tested velocities within the 21 and 25°C acclimation groups, we used a One-Way ANOVA followed by Holm-Sidak's method for pairwise multiple comparisons of the 21°C and Fisher LSD method for the 25°C acclimation group. The 21°C acclimation group's mean MO_2 was plotted versus water velocity and tested with a quadratic regression, whereas the 25°C acclimation group's mean MO_2 was plotted versus water velocity and tested with a simple linear regression. To compare the 21 and 25°C acclimation groups and the five tested water velocities, we used a two-way ANOVA with acclimation group and water velocity as factors. All statistical analyses were conducted using SigmaPlot™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.5 Thermal Limits Study

2.5.1 Adult

The critical thermal maxima or minima (CTM) apparatus for the adult hardhead consisted of a Minnow-O-Cool (150-l) flow-through water bath with two removable plastic CTM chambers (55 cm x 39 cm x 33 cm each). One hardhead was placed into each chamber and allowed to acclimate for 30 min prior to start of the experiment at their acclimation temperature. Water was pumped (Danner Model No. 3) from the bath into each chamber and allowed to spill out of a series of holes in the side of the chamber to maintain a water depth of 12.5 cm, and causing water from the bath and chambers to be exchanged. Air stones were placed in each chamber and in the water bath to ensure adequate mixing and to maintain dissolved oxygen levels (>6.0 mg l^{-1}) for all temperatures tested.

Acclimation temperature was maintained in the apparatus by mixing ambient (~18°C) and chilled (~11°C) water, for the 11 and 16°C temperature acclimation groups. Two Finnex titanium heaters (models TH300 and TH800) were placed in the bath and used in conjunction with a

proportional temperature controller (Yellow Springs Instruments [YSI] model 72), to maintain the water at the acclimation temperatures for the 21 and 26°C acclimation groups.

The water bath was heated (+0.33°C min^{-1} ±0.02 SE) during CTmax experiments using two 1800-watt immersion heaters (Clepco Smart Heater, QDMWS18 and Process Technology Heater, TA1-8117-PI) housed in two vertical 15 cm diameter, 60 cm deep insulated PVC tubes with capped bottoms. Each was controlled with a Clepco heat controller (QD 50-1). Bath water was circulated through the heating tubes with the same submersible pump used to circulate water through the adult chambers and allowed to overflow back into the bath. The tubes could be isolated from the system during CTmin experiments by closing a valve in line with the heating tubes.

The bath water was cooled (-0.24°C min^{-1} ±0.02 SE) during CTM experiments with three chillers (Frigid Units©), a 1-horsepower and two 1/2-horsepower units. All three chillers were mounted next to the bath with the heat exchange coils housed in three vertical 15 cm diameter, 60 cm deep, insulated PVC tubes capped on the bottom. Water was supplied to the chiller tubes with two submersible pumps (Danner E160713 Model 3 and 18), and allowed to overflow back into the water bath.

A submersible pump (Danner Model 2) was also attached to a PVC manifold to circulate water around the bath and ensure adequate mixing. Lids with small windows cut out (21 cm x 10 cm) for observations were placed over the chamber of each fish, due to their proclivity to jump. After the acclimation period a CTmax or CTmin experiment was initiated, using either heaters or chillers, respectively. Figure 17 depicts a model of critical thermal data and approximate laboratory and physiological death end points.

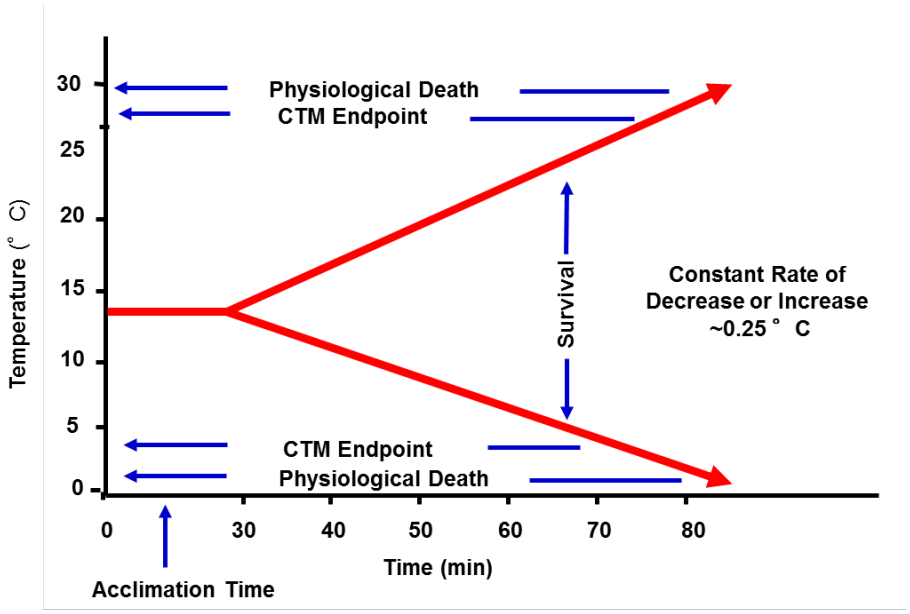

Figure 17. Model of fish's critical thermal minimum or maximum exposures to constantly increasing or decreasing temperatures until loss of equilibrium (CTM end point) is reached. Adapted from Beitinger et al (2000).

Temperature was monitored, using Fisherbrand thermometers (model No. 15-041-13A), and recorded at the beginning of acclimation and every five minutes during the experiment until the fish began to show signs of distress as it approached the CTM endpoint (loss of equilibrium), after which the temperature was observed continuously until the fish reached CTM. AlphaMach thermal loggers (Model No. 22L) were also used to verify the rate of temperature increase or decrease of the water bath (Figure 18).

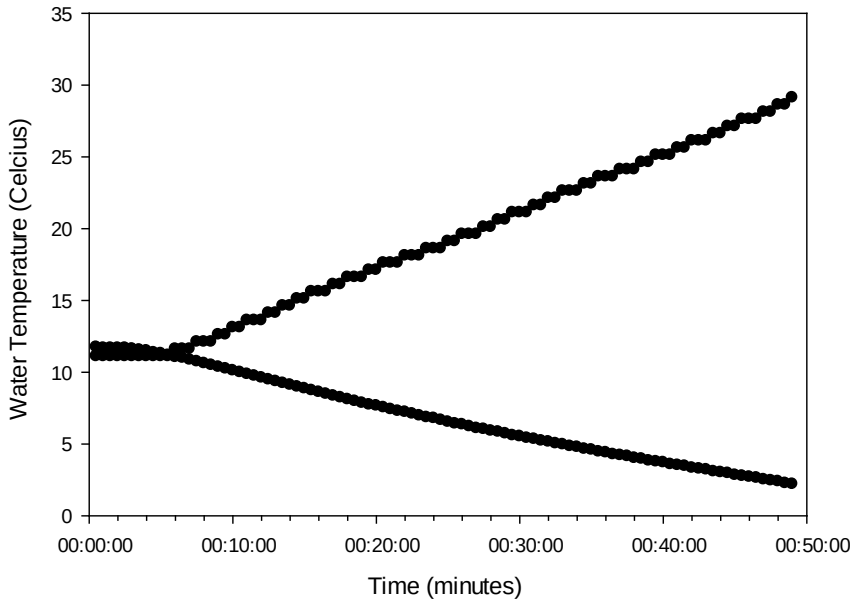

Figure 18. Example data logger water temperature readings, every 30 seconds, during an 11°C temperature acclimation group's critical thermal maxima and minima experiments.

A fish was considered to have reached CTM when it demonstrated loss of equilibrium (LOE) (Cox 1974; loss of righting response). At LOE, water temperature was measured and recorded, and each fish was immediately transferred to an isolated bath maintained at the fish's acclimation temperature for recovery. Fork lengths and wet mass were measured and recorded. Fish were then placed in a post-test tank at the appropriate acclimation temperature for 24-h observation. No adults died during or immediately after the 24-h observation period. Critical thermal maxima (CTmax) and minima (CTmin) were calculated as the arithmetic mean of the collective LOE endpoint temperatures of each group tested.

To test for difference in mean mass and fork length within a treatment (CTmin or CTmax) for each acclimation group we used a one-way ANOVA, and post hoc multiple pairwise comparisons were performed with the Holm-Sidak method. Thermal tolerance data sets were analyzed by analysis of covariance (ANCOVA) with fork length or mass as covariates. Corrected CTM values differed from actual values by no more than 0.1°C and were less than the standard error of unadjusted CTMs; therefore, thermal tolerance data sets were analyzed without statistical adjustment for body size. The mean CTmin or CTmax temperatures were evaluated with a one-way ANOVA, and post-hoc multiple pairwise comparisons were performed with the Holm-Sidak method. All statistical analyses were conducted using

SigmaPlot™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.5.2 Juvenile

Juvenile CTM experiments were conducted similarly to those of adults, with a few key differences. Prior to experiments three juvenile hardhead were netted from their acclimation tank and transported to the CTM apparatus via a small ice chest (4-l). Fish were placed into one of three 2-l Erlenmeyer flasks. The flasks each rested in a primary water bath; consisting of a 22-cm diameter, 18-cm deep, round plastic container. Water was then pumped from the secondary water bath (large Minnow-O-Cool tank) into the containers and allowed to spill out of a series of holes to create a 14.5 cm deep water jacket (Figure 19). This provided exceptional mixing and heat transfer at the flask's walls and obscured the fish's view of each other. Air-stones inside the flask facilitated mixing, but were regulated to prevent the formation of currents that would encourage the fish to swim or otherwise be active.

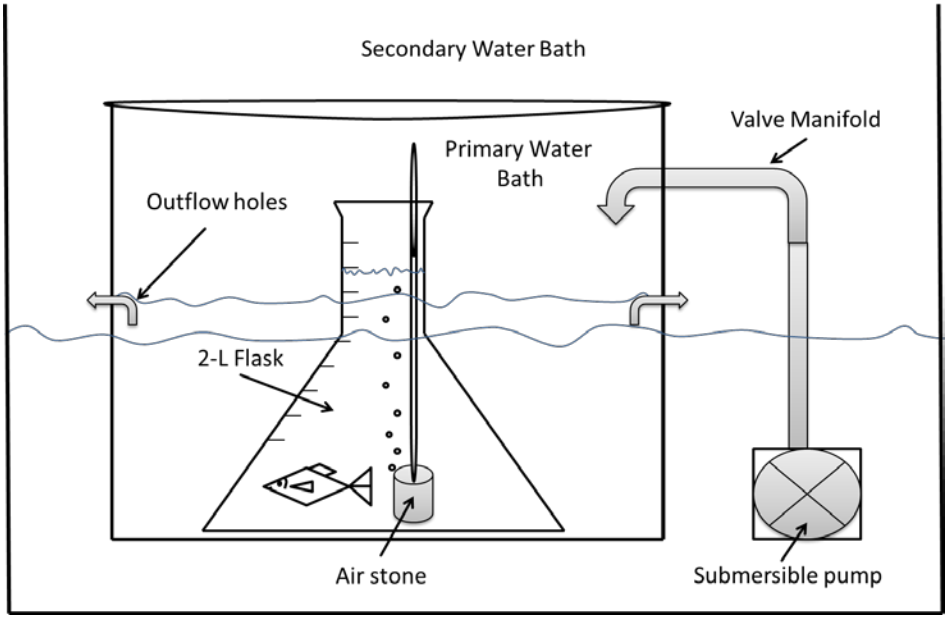

Figure 19. Plan-view of the juvenile hardhead minnow's critical thermal maximum and minimum experimental design. Up to three fish were tested at a time, in separate flasks, held in a larger secondary water bath for a given experiment.

To test for difference in mean mass and fork length within a treatment (CTmin or CTmax) for each acclimation group we used a one-way ANOVA. Thermal tolerance data sets were analyzed by analysis of covariance (ANCOVA) with fork length or mass as covariates. Corrected CTM values differed from actual values by no more than 0.1°C and were less than the standard error of unadjusted CTMs; therefore, thermal tolerance data sets were analyzed without statistical adjustment for body size. The mean CTmin or CTmax temperatures were evaluated with a one-way ANOVA, and post-hoc multiple pairwise comparisons were performed with the Holm-Sidak method. All statistical analyses were conducted using SigmaPlot™ (Release 12.0), SPSS Inc. statistical software. Alpha was considered significant below 0.05.

2.6 Adult Blood–Oxygen Equilibria Study

We studied the influence of temperature on the reversible oxygen-binding dynamics in hardhead blood by constructing hardhead blood-oxygen equilibria curves. The effect of CO_2 (simulating metabolic influence on blood-oxygen binding capacity) was also included to simulate arterial (low-CO_2) and venous (high-CO_2) blood dynamics, including Bohr and Root effects, via whole-blood tonometry techniques.

To obtain the required 22-24 ml of blood for each experimental replicate, all fish used in this study were over-anesthetized and bled via a dorsal-aortic cannula. Fish were quickly placed in a buffered anesthetic bath containing: 9 g l^{-1} NaCl, 420 mg l^{-1} $NaHCO_3$, 500 mg l^{-1} tricaine methanesulfonate (MS-222). Once fish reached stage-five anesthesia (3-5 min) they were transferred to a surgery table and placed in a dorsal recumbent position for cannulation of the dorsal aorta for blood collection (Summerfelt and Smith, 1990). The cannulation procedure was quickly accomplished (60-90 s) using heparinized PE 50 tubing fitted with an internal stainless steel stylet. The sharpened stylet facilitated the penetration through the roof of the buccal cavity and guided the PE 50 cannula tubing into the dorsal aorta, between the second and third gill arches. Once the stylet-cannula complex entered the dorsal aorta (1-2 cm), the stylet was withdrawn, and a three-way stopcock fitted with a 1-ml syringe containing 500 µl sodium heparin (500 IU) saline solution allowed rapid infusion of the heparin to prevent clot formation at the cannulation site prior to the collection of blood. Fish were bled using 3-ml syringes containing 300 IU heparin. Collected blood (e.g., from 3-6 fish) was placed into 50-ml Falcon tubes with air and placed horizontally on ice after gentle mixing. Immediately after blood collection hematocrit (packed cell volume) was measured (centrifugation @ 11,000 x g. for 3 min [Houston 1990]) and hemoglobin content was measured using a hemoglobin assay kit (Teco Diagnostics) and a UV-VIS Aquamate spectrophotometer.

Blood (ca. 6 ml) was placed (Figure 20) into each of two rotating glass tonometers (Hall 1960) for the construction of the first blood-oxygen equlibrium curve (low PCO_2) with the remaining blood held on ice for 60-80 min, with gentle mixing every 5 min, until loading into the second set of tonometers for the construction of the second curve (high PCO_2). Tonometer pairs were situated in a temperature-controlled water bath (11, 19, 25, or 30° C ± 0.3°C) and received either humidified air from an air pump, humidified nitrogen from a cylinder (blood with < 0.03 kPa PCO_2, for construction of 'low PCO_2' curves, estimating arterial conditions), or humidified gas mixtures (1% CO_2 with balance either air or nitrogen) from Wostoff gas mixing pumps, (1.01 kPa PCO_2, for 'high PCO_2' curves, estimating venous conditions). Blood was equilibrated with the gas mixtures for 40-60 min prior to data collection. Samples of oxygenated and deoxygenated blood were withdrawn from the tonometers (Figure 20) and mixed in a 1-ml polypropylene syringe with a mixing bead (Edwards & Martin 1966, Scheid & Meyer 1978). To reach target values of 0, 20, 35, 50, 65, 80, 95, and 100% oxygen saturation, proportional amounts of blood from both deoxy- and oxygenated tonometers were withdrawn and mixed as described above. The PO_2 (mm Hg) of each sample was determined using a Radiometer PHM71 blood gas apparatus with thermostatted E101 oxygen electrodes (Analytical Sensors, Inc.). Equilibration of blood, with the respective gas treatments, was defined to be complete after a measure of ≈ 0 kPa

(45 min) in the deoxygenated (nitrogen) tonometer's blood as outlined in Kaufman et al. (2006). The pH of each blood sample was determined using an Orion Dual Star pH meter equipped with thermostatted Analytical Sensors, Inc. E351 pH and RF-CM reference electrodes. Whole blood lactate (mg l^{-1}) determinations, using an YSI model 2700 Select analyzer, were made on blood from each tonometer after initial blood collection and at the conclusion of each experiment to assess the potential for lactic acidosis in tonometered blood. Following blood collections baseline lactate concentrations were 2.7 ± 0.5 mmol l^{-1} (mean ±SD, n=12). Lactate levels in pooled blood held on ice remained at baseline and showed no increases in lactate prior to use in tonometry experiments, which did not exceed 2 h. Results from the initial 30°C experiments were discarded due to increased (≥ 1 mmol l^{-1}) lactate concentrations over baseline concentrations (modified from methods described in Cech et al. [1994]). The 30°C experiments were repeated using decreased blood volumes (3 ml) in the tonometers, which shortened equilibration times, and decreased blood [lactate] increases to <1 mmol l^{-1} above baseline. Blood-oxygen equilibria data were converted from mm Hg to kPa, where 1 mm Hg = 0.13332 kPa and plotted, with curves fitted using nonlinear regression options with Sigma Plot 2000, SPSS, Inc.® software.

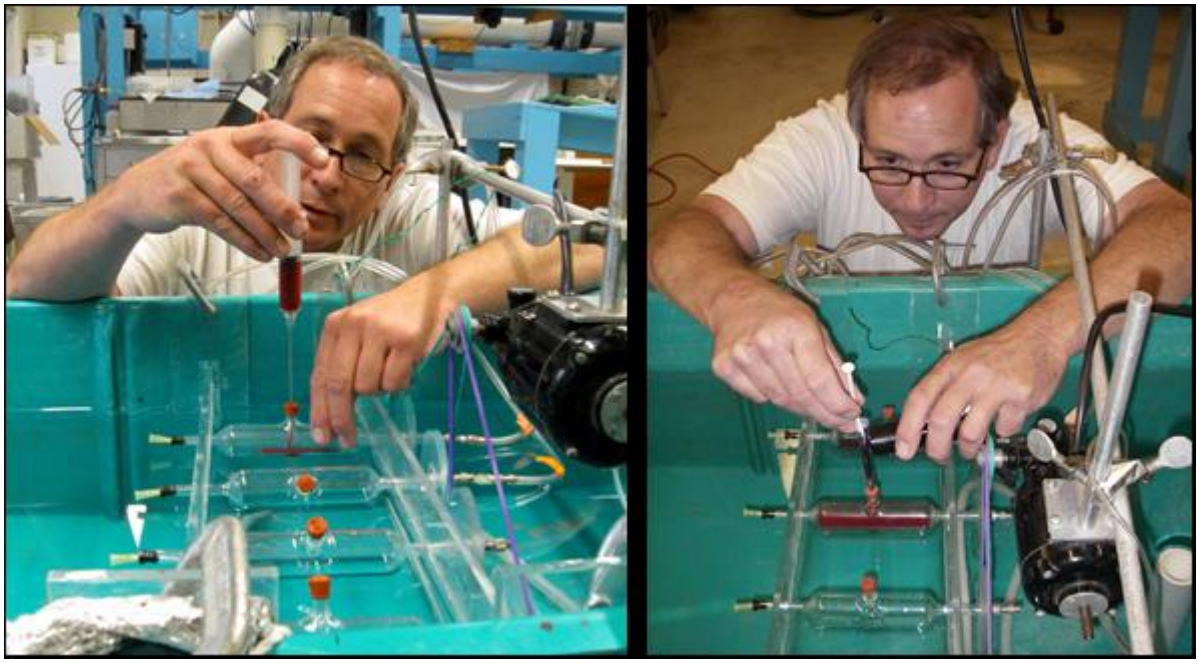

Figure 20. Dr. Robert Kaufman adds hardhead blood to a tonometer chamber (left) and uses a flashlight to check the volume of blood removed for blood-oxygen equilibria curve construction (right).

Bohr factors, temperature effects, and non-bicarbonate buffer concentrations were calculated from the collected data. Bohr factor (Φ), a measure of the blood-oxygen affinity's sensitivity to pH and CO_2, was calculated using.

$$\Phi = \Delta \log P_{50}/\Delta pH$$

where Δ log P_{50} and Δ pH are the changes in log PO_2 and the changes in pH respectively, in the 50% saturated sample. Temperature effect (ΔH, kiloJoules [kJ] mol O_2^{-1}, a measure of the blood-oxygen affinity's sensitivity to temperature), was calculated using a form of the Van't Hoff equation (Wood & Lenfant 1979) with kilocalories converted to kiloJoules:

$$\Delta H = 4.578(\Delta \log P_{50}/\Delta(1/T)\cdot 10^3)$$

where T is temperature in degrees Kelvin (Wyman 1964, Powers et al. 1979). Whole-blood, non-bicarbonate buffer capacity (β, in slykes), was calculated using:

$$\beta = \Delta[HCO_3^-]/\Delta pH$$

Bicarbonate ion concentration (HCO_3^-) was calculated using pH and PCO_2 data in the Henerson-Hasselbalch equation (Davenport 1974) and constants published in Boutilier et al. (1984).

Hemoglobin subunit cooperativity was estimated from slopes (n_{50}) of Hill plots:

$$\log (Y/100\text{-}Y) \text{ versus } \log (p)$$

where Y = percent saturations between 20 and 80% and $p = PO_2$ (Riggs 1970). Temperature and pH relationships were analyzed by least squares regression, and correlations between measured and calculated variables were tested for significance ($p < 0.05$) using Sigma Stat ™, SPSS Inc. statistical software.

The blood-oxygen capacity, (CBO_2, ml $O_2 \cdot dl^{-1}$ blood), of hardhead blood was determined using the methods and calculations outlined in Tucker (1967). Measurements from an acrylic Tucker cell, thermostatted to 37°C, were made with an E101 oxygen electrode (Analytical Sensors, Inc.) and an A-M Systems Polarographic Amplifier Model 1900.

Nucleoside triphosphates (NTPs) were extracted from whole-blood and stored at -80°C until analysis (Biovision Deproteinizing Sample Preparation Kit cat. #K808-200). Standards and samples were analyzed with modifications via Biovision ATP Colormetric/Fluorometric Assay Kit (cat. # K354-100). Modifications of the prescribed assay were as follows: a five-point standard curve was constructed (0, 6, 12, 18, and 24 nmols ATP in 200 ul standard solution) with standards and samples assayed using an Aquamate UV-VIS spectrophotometer, 1-mm path-length glass cuvette, and absorbance at 570 nm. All standard and sample volumes were increased threefold to provide sufficient volume for spectrophotometric analysis. NTP concentrations in prepared samples were calculated from the generated standard curve ($y=0.0335X-0.004$; $r^2=0.97$).

We used one-way ANOVA ($p < 0.05$) to investigate significant differences among treatment means. If significant differences were detected, we used post-hoc pairwise comparisons (Tukey test) to investigate differences between pairs of groups within and between treatments. All statistical analyses were conducted using Sigma Stat ™, SPSS Inc. statistical software.

CHAPTER 3: Project Outcomes

3.1 Fish Collection and Care

We did not observe any seasonal or diel patterns in our capture rate of adult hardhead. However, because of our travel schedule we typically did not begin angling until 9:30 am or later, so we were not able to observe capture rates of adults early in the morning. The majority of our captures of juvenile hardhead occurred in December 2010. It is not clear from our results whether this was due to young-of-year juveniles being larger later in the season, and therefore more likely to be caught in a trap after entering it, or whether we were simply fortunate to position traps in December in habitat more likely to contain hardhead.

Hardhead were readily maintained in a healthy state once they had been treated for parasites and diseases that were present prior to capture. Hardhead can be kept in captivity for long periods of time between 11-25°C on commercially available diets, after a transition period involving supplementation of live foods.

3.2 Temperature Preference Study

3.2.1 Adult temperature preference results;

Neither the adult hardhead's mass nor fork length were significantly different between the four acclimation groups (p=0.634 and p=0.987, respectively). Adult hardhead minnow's overall mean selected temperatures, calculated over the duration of the 4 h thermal preference trial, were between 18.4-21.6°C for the four acclimation groups (Table 4). Interestingly, the preferred temperature of the 11°C acclimation group was significantly lower than that of the 16°C and 25°C groups (p<0.001), but not the 21°C group. The 16°C acclimation group's mean selected temperature was significant higher than that of all other acclimation groups (p<0.0001). Overall, the range of selected temperatures was very small between the 11, 21, and 25°C groups. The hardhead groups utilized differing temperature ranges with the 16°C group selecting a warmer median, while the 21°C group utilized the largest range of temperature within the 75th percentile and the highest median temperature (Figure 21). All the acclimation groups had observations at all the 32 areal positions in the chamber.

Table 4. Mean (with ±SE) and median water temperatures selected during a 4 h experiment by adult hardhead acclimated to 11, 16, 21, or 25°C. Temperature acclimation group, sample size of acclimation group (N), number of total observations, mean fork length (FL), and mass are also presented. SE indicates standard error of the variable in the preceding column. Significant differences in mean preferred temperatures between acclimation groups are denoted by different superscript letters (p<0.05).

Acclimation Group	N	Number of Observations	Mean (°C)	SE	Median	FL (cm)	SE	Mass (kg)	SE
11°C	19	931	18.4^{A}	0.10	18.5	37.1	1.16	715.4	56.84
16°C	17	833	20.6^{B}	0.14	21.6	36.9	1.45	742.4	88.88
21°C	16	784	19.3^{AC}	0.16	17.9	37.9	0.89	753.5	96.29
25°C	16	784	19.5^{C}	0.14	18.0	38.9	1.14	730.9	58.43

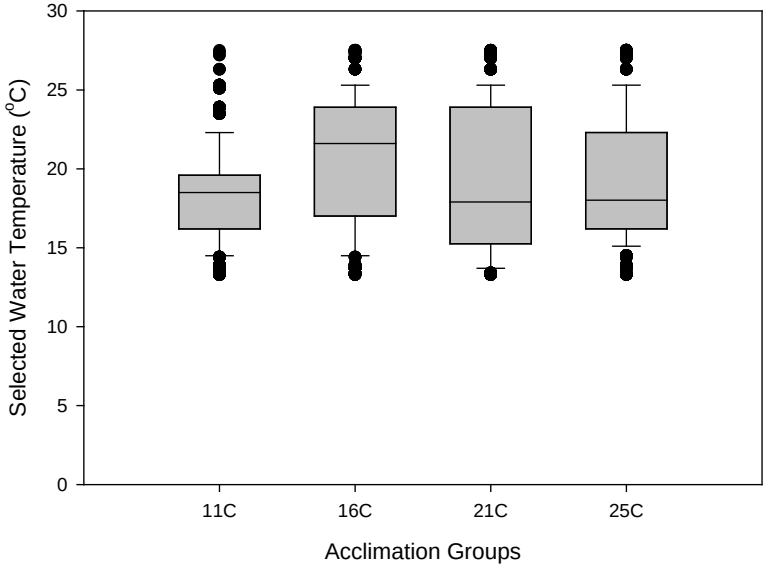

Figure 21. Box and whisker plot of the water temperatures selected by adult hardhead acclimated to 11, 16, 21, or 25°C (n=19, 17, 18, and 16 respectively) over 4 h in an annular 13-28°C gradient. All plots show the median (horizontal line within the box) and the 10th, 25th, 75th, 90th percentiles with outliers (●) for each acclimation group.

While the overall mean preferred temperatures of adult hardhead acclimation groups were similar, we also explored whether preferred temperatures changed over the 4 h experimental period. We plotted the mean selected temperature of each acclimation group at each 5 min interval over the 4 h experiment (Figure 22). Overall, acclimation temperature had a minor effect on preferred temperatures. Fish in the 11°C acclimation group quickly settled near their overall mean preferred temperature (18.4°C), whereas the 16°C acclimation group over the first 20 min selected a mean water temperature slightly cooler than that eventually chosen as their preferred temperature (20.5°C). The 21°C and 25°C acclimated fish both selected very similar water temperatures (~19°C) throughout the duration of the trial. The 11°C and 25°C acclimation groups showed a significant increase and decrease over time, respectively ($p<0.0001$, SLR).

In general adult hardhead frequented most temperatures presented by the apparatus with no particularly obvious preference for a narrow thermal range. All the groups tended to avoid the ~26-28°C area, but were frequently observed in the ~24-26°C range. The groups also tended to avoid the coldest section of the apparatus (~12-13°C), but were frequently observed at 14-16°C. Hardhead were most frequently observed in the 16-24°C zones of the apparatus. The 25°C group tended to avoid the cooler sections of the apparatus.

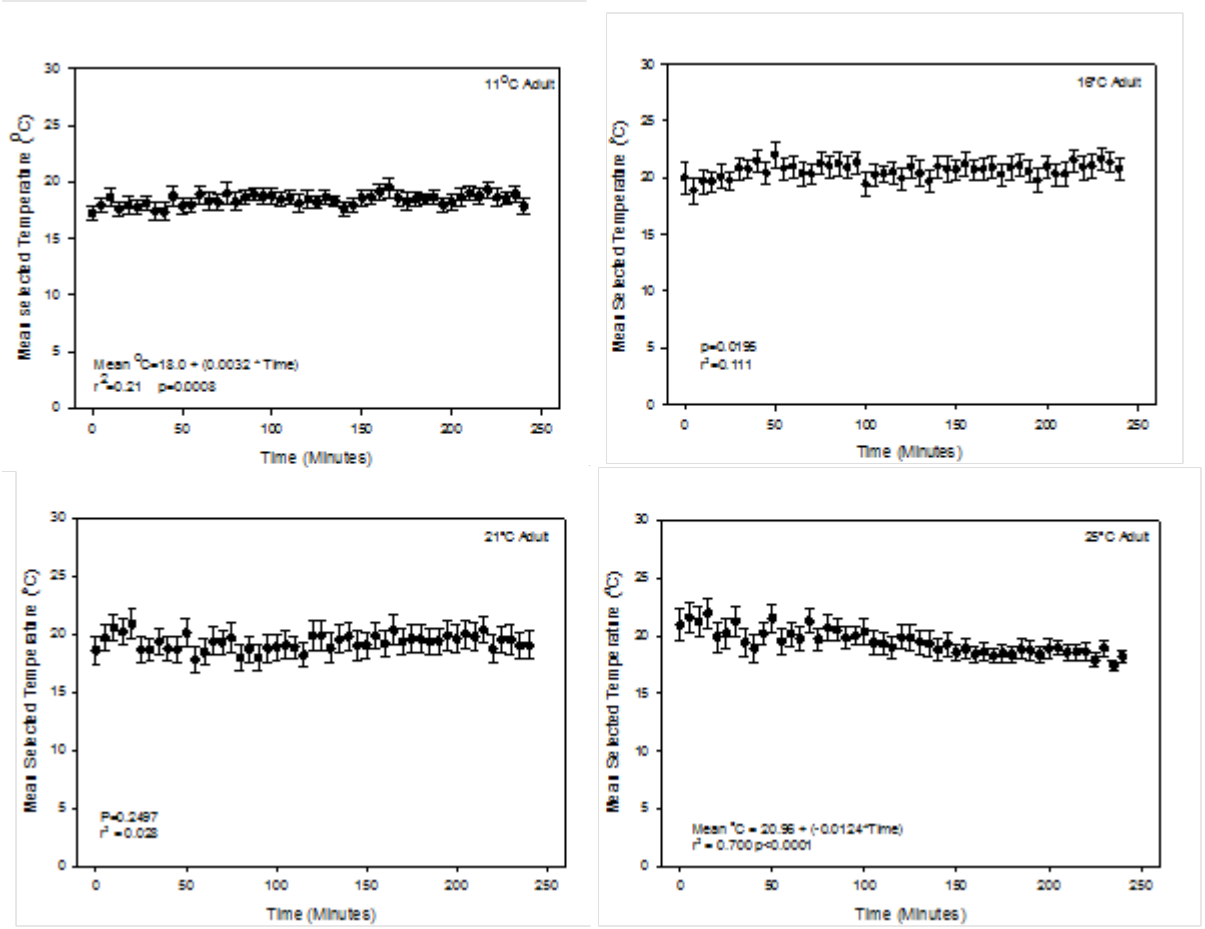

Figure 22. Mean (±SE) selected water temperature of adult hardhead in a ca. 12-28°C annular gradient apparatus at 5 min intervals over a 4 h period. Hardhead were acclimated to one of four temperature groups (11, 16, 21, 25°C). The R-square and p-value for the simple linear regression of each acclimation group are presented as well as the regression equation if significant.

3.2.2 Juvenile temperature preference results;

The mean masses of the four juvenile hardhead acclimation groups (11, 16, 21, and 25°C) differed significantly (p=0.001; Table 5). Juvenile hardhead's selected overall mean temperatures, calculated over the duration of the 4-h thermal preference trial, ranged from 17.2-21.9°C for the four acclimation groups. Interestingly, unlike the adults, there was a significant difference in the mean selected water temperature between each group (p>0.001), although the temperature range was very small for the 11 and 16°C groups and the 21 and 25°C groups. Juvenile hardhead utilized different temperature ranges, with the 25°C group selecting a warmer median, while the 21°C group utilized the largest range of temperature (similar to the adults) within the 75th percentile (Figure 23). All acclimation groups had observations at all 32 areal positions in the chamber.

Table 5. Mean (±SE) and median water temperatures selected during a 4 h experiment by juvenile hardhead acclimated to 11, 16, 21, or 25°C. Temperature acclimation group, sample size of acclimation group (N), number of total observations, mean fork length (FL), and mass are also presented. SE indicates standard error of the variable in the preceding column. Significant differences in mean preferred temperatures between acclimation groups are denoted by different superscript letters (p<0.05).

Acclimation Group	N	Number of Observations	Mean (°C)	SE	Median	Mass (g)	SE	FL (cm)	SE
11°C	21	1071	17.2[1]	0.10	16.8	1.9[a]	0.12	5.7	0.15
16°C	20	1020	17.9[2]	0.10	17.7	2.1[a]	0.13	5.6	0.12
21°C	19	969	21.1[3]	0.13	19.9	4.9[b]	0.39	7.3	0.19
25°C	15	765	21.9[4]	0.13	22.4	5.9[b]	0.56	8.0	0.23

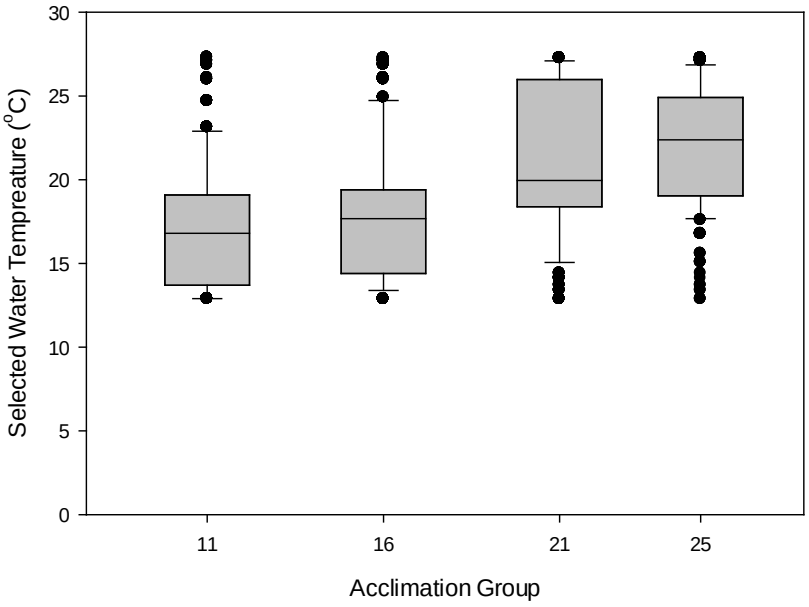

Figure 23. Box and whisker plot of the water temperatures selected by juvenile hardhead acclimated to 11, 16, 21, or 25°C (n=19, 17, 18, and 16 respectively) over 4 h in an annular 13-28°C gradient. All plots show the median (horizontal line within the box) and the 10th, 25th, 75th, 90th percentiles with outliers (●) for each acclimation group.

To determine whether preferred temperatures changed over the 4-h experimental period, we plotted the mean selected temperature of each acclimation group at each 5-min interval (Figure 24). Each acclimation group quickly settled near the overall mean preferred temperature (19.5°C). The 11 and 16°C and the 21 and 25°C acclimated fish both selected very similar water temperatures, ca. 17.5 and ca. 21.5°C, respectively, throughout the duration of the trial. The 16°C acclimation group showed a significant increase and decrease in preferred temperature over time, respectively (p=0.029, SLR).

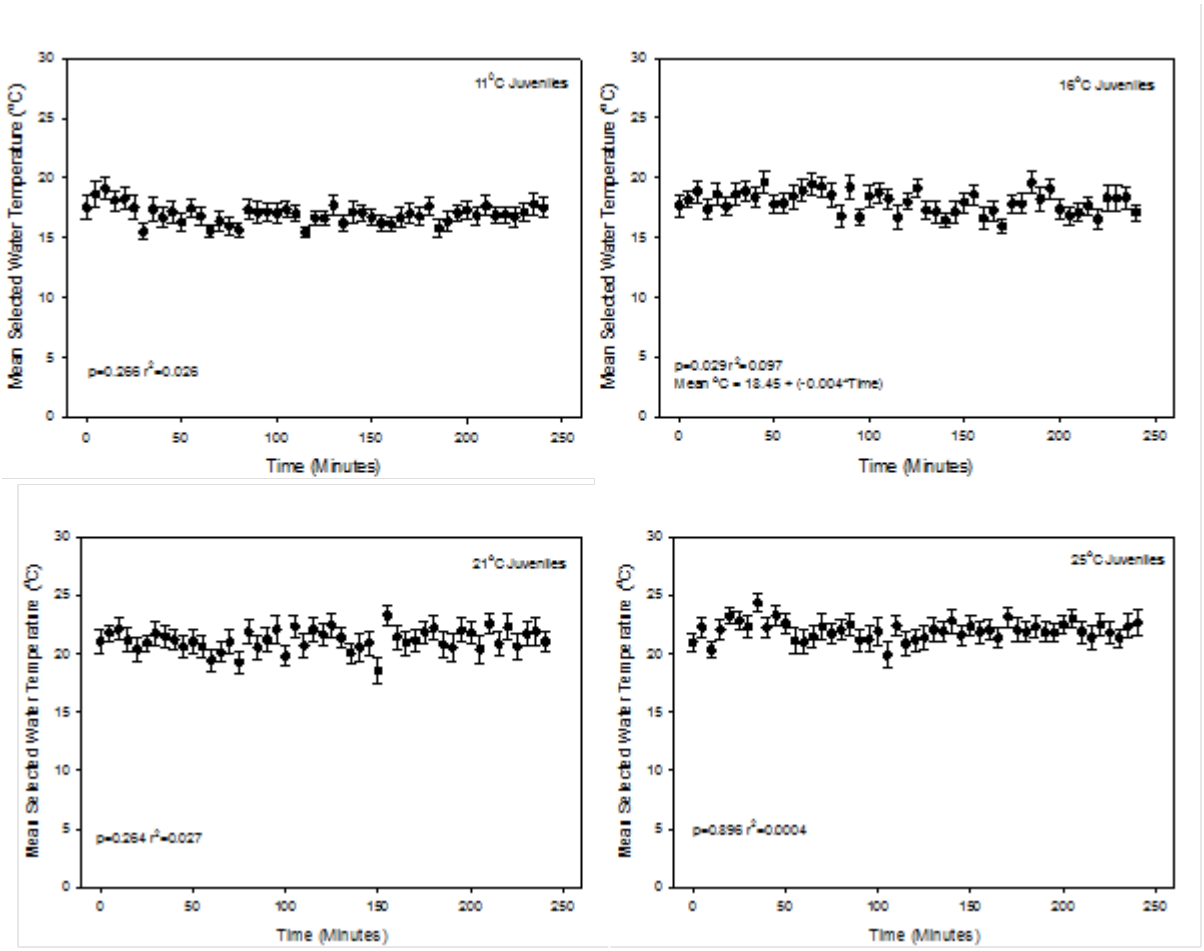

Figure 24. Mean (±SE) selected water temperature of juvenile hardhead in a 13-28°C annular gradient apparatus at 5 min intervals over a 4-h period. Hardhead were acclimated to one of four temperature groups (11, 16, 21, 25°C). The R-square and p-value for the simple linear regression of each acclimation group are presented as well as the regression equation if significant.

3.3 Resting Metabolism Study

The mean mass of adult hardhead did not differ significantly among acclimation groups (p=0.849). The mean mass of juvenile hardhead differed significantly among the thermal acclimation groups (p<0.05, Table 6) and tended to increase with increasing acclimation temperature while the mean masses of the 21°C and 25°C acclimation groups were indistinguishable (p=0.095). The resting metabolic rates (MO_2; mg O_2 $kg^{-2/3}$ h^{-1}) of adult and juvenile hardhead increased with increasing water temperatures (Figure 23 and Table 5). Simple linear regressions of MO_2 versus temperature were statistically significant for both adults (p=0.0274) and juveniles (p=0.0042). Comparisons between acclimation groups within a life stage, however, revealed that adult mean MO_2 values were significantly different only when separated by 9-10°C, and juvenile MO_2 values did not differ significantly between the 11°C and 16°C acclimation groups (Figure 25, p<0.001 for all significant comparisons).

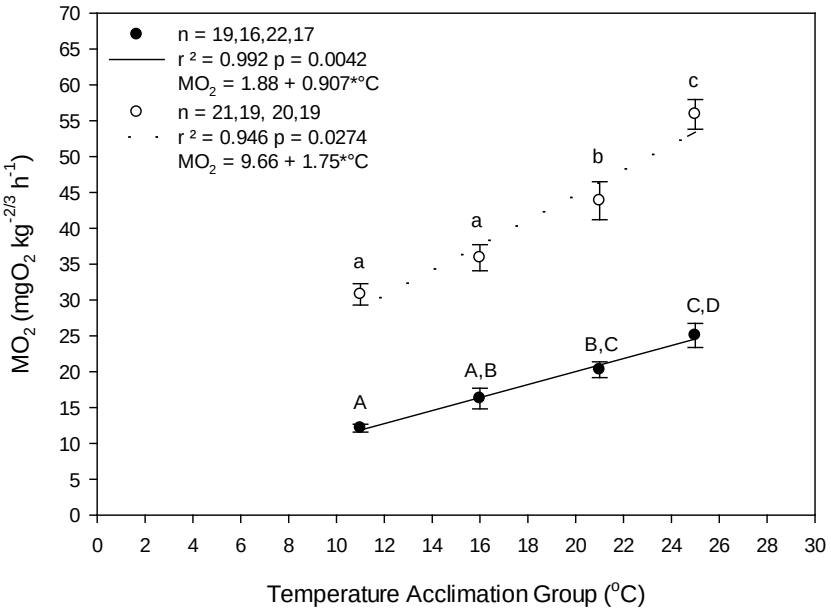

Figure 25. Mean (± SE) resting metabolic rate (mg O_2 $kg^{-2/3}h^{-1}$) of juvenile (●) and adult (○) hardhead, determined via intermittent static respirometry at acclimation temperatures of 11, 16, 21, and 25°C, data adjusted for mass-independence. Simple linear regression models are shown for adults and juveniles; and within a lifestage, different letters denote statistically significant differences between acclimation groups ($p<0.05$ for all significant comparisons). Sample size (n) for each acclimation group is given in order of increasing acclimation temperature.

Table 6. Resting metabolic rate (MO_2; mg O_2 $kg^{-2/3}$ h^{-1}, with ±SE) of juvenile and adult hardhead, determined by intermittent static respirometry. Temperature acclimation group, sample size, mean mass, and mean fork length (FL) are also presented. SE indicates standard error. Significant differences in mass between temperature groups and within life stage are denoted by different numbered superscripts, and significant differences in mean MO_2 are denoted by lettered superscripts (p<0.001).

			MO_2 (mgO_2 $kg^{-2/3}$ h^{-1})								
Life Stage	Temperature Group (°C)	N	Mean	SE	Min	Max	Median	Mass (kg)	SE	FL (cm)	SE
Juvenile	11	19	12.1^{A}	0.55	8.8	16.0	12.3	0.0028^{1}	0.18	5.7	0.15
Juvenile	16	16	16.3^{AB}	1.45	9.3	30.1	15.5	0.0043^{2}	0.21	6.6	0.15
Juvenile	21	22	20.3^{BC}	1.11	13.1	30.7	19.2	0.0055^{3}	0.36	7.7	0.20
Juvenile	26	17	25.1^{CD}	1.68	16.3	47.2	24.6	0.0062^{3,4}	0.45	7.9	0.25
Adult	11	21	30.8^{a}	1.50	21.2	51.9	30.2	705.0	53.20	37.0	0.99
Adult	16	19	35.9^{a}	1.82	22.3	51.1	36.1	743.9	71.38	36.8	1.06
Adult	21	20	43.9^{b}	2.64	23.6	68.8	43.7	736.7	49.41	38.8	0.82
Adult	26	19	55.9^{c}	2.07	37.9	68.0	56.9	680.8	47.72	38.8	0.91

We typically observed juvenile hardhead displaying light pectoral fin movements and occasional position shifting within the respirometers, and light to medium ventilatory beats. In contrast, the adult hardhead typically rested on the bottom of the respirometers and had very shallow opercular movements. The mass corrected resting MO_2 of juvenile hardhead was roughly 60% lower than that of adults at each acclimation temperature (Figure 25).

Q_{10} values calculated for both juvenile and adult hardhead resting MO_2 values (Table 7) were relatively low (ranging from 1.35 to 1.79), suggesting low to medium thermal sensitivity of resting metabolic rate between temperature acclimation groups and life stages.

Table 7. Calculated Q_{10} values for juvenile and adult hardhead resting metabolic rates (MO_2) across the range of acclimation temperatures used in this study.

Life stage	Acclimation Temperature (°C)	Q10
Juvenile	11-16	1.79
	16-21	1.55
	21-26	1.52
	11-25	1.62
Adult	11-16	1.35
	16-21	1.49
	21-26	1.62
	11-25	1.48

3.4.A Adult Active Swimming Metabolism Study

The mean mass of the fish did not differ between thermal acclimation groups (p=0.147). Adult hardhead active metabolic rate (mg O_2 kg^{-1} h^{-1}) and tailbeat frequency (TBF) increased with water velocity in the Brett-style temperature-controlled chambers in all four of our thermal acclimation groups. Overall, the adults performed well in the Brett-style swimming chambers. The fish acclimated to warmer temperatures tended to swim with less effort and willingly swam without attempting to tail prop against the back of the chamber, or to escape the chamber. We present only the successful velocity steps for which oxygen content measurements could be determined; variable sample sizes are noted for all steps. The fish that swam in the 150-l respirometer are presented only in the appendix, for reference. Below, we present our results from each thermal acclimation group separately, starting with the adult 11, 16, 21, and 25°C fish then move into comparisons between the four acclimation groups.

3.4.A.1 Swimming metabolism of adult hardhead acclimated and tested at 11°C

In the adult 11°C acclimation group 9 hardhead swam in the 150-l respirometer (see Appendix for data) and 16 hardhead swam in the 660-l chamber (Table 8). We were unable to collect oxygen content measures at some velocity steps because some hardhead refused to swim. At lower velocities, in this group, it was common for hardhead to tail prop on the downstream screen or rest along the chamber's bottom. At higher speeds some hardhead were observed to actively search for exit points or slower velocities; these behaviors increased the fish's overall activity. The mean metabolic rate of hardhead at 30 cm s^{-1} was significantly different than at 60

cm s^{-1} (p=0.006), 75 cm s^{-1} (p=0.029), and 90 cm s^{-1} (p=0.006), but not from the 45 cm s^{-1} velocity step (p=0.448). The differences in the mean TBFs were detect at several velocity steps (p<0.001), the 75 cm s^{-1} and 90 cm s^{-1} were not distinguishable (p=0.052).

For the 11°C acclimation group, tail beat frequency was plotted with active metabolic rate (MO_2, Figure 26) only for fish tested in the 660-l chamber because the hardhead sample size (n<5) for the 150-l chamber was too small to allow accurate estimates of performance. At 105 cm s^{-1} hardhead had a mean 105 TBF (±16.26 SE, n=3).

Table 8. Active metabolic rate (MO_2) of adult hardhead acclimated at 11°C and tested in the 660-l Brett-style swimming respirometer, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column.

						MO2 (mg O2 kg^{-1} h^{-1})				
N	Water Velocity (cm s^{-1})	Mean Mass (kg)	SE	Mean FL (cm)	SE	Mean	SE	Min	Max	Median
12	30	0.809	0.052	39.4	0.858	209.5	26.45	84.2	377.9	184.1
15	45	0.785	0.051	39.1	0.800	338.9	54.33	136.7	790.5	252.0
15	60	0.807	0.042	39.3	0.687	501.1	76.00	218.4	1160.5	395.2
15	75	0.807	0.042	39.3	0.687	452.0	45.72	215.6	967.1	415.6
7	90	0.796	0.066	39.2	1.040	565.8	62.62	289.4	773.7	565.0

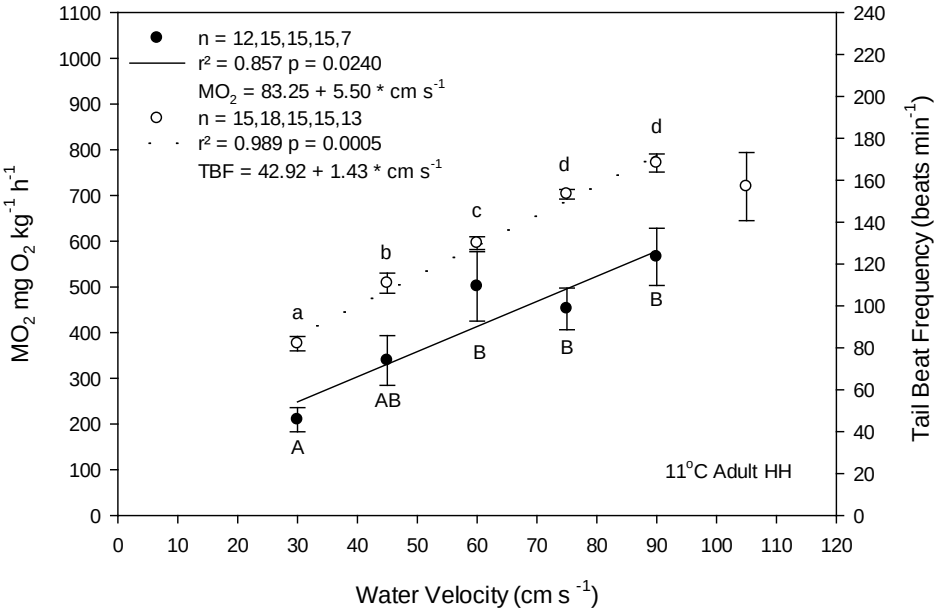

Figure 26. Mean (± SE) active metabolic rate (●) and tail beat frequency (○) of adult hardhead acclimated to 11°C, that swam in a 660-l Brett-style chamber. The 105 cm s^{-1} TBF was excluded from the simple linear regression, due to the low sample size (n=3) at that velocity. Letters denote statistically significant differences at the velocity steps (p<0.05 for all significant comparisons). Sample sizes (n) for each acclimation group are given in order of increasing acclimation temperature.

3.4.A.2 Swimming metabolism of adult hardhead acclimated and tested at 16°C

In the adult hardhead 16°C acclimation group 5 hardhead swam in the 150 L chamber (Appendix) and 17 hardhead swam in the 660-l chamber (Table 9). As occurred for the 11°C group, at lower velocities it was common for hardhead to tail prop on the downstream screens or rest along the chamber bottom. At higher speeds some hardhead were observed to search actively for exit points or slower velocities, and these behaviors increased the fish's overall activity level.

Mean metabolic rates of hardhead at 30, 45, and 60 cm s^{-1} were not significantly different ($p=0.475$, 0.303, 0.125, respectively). The metabolic rate at 75 cm s^{-1} was higher than at 30 cm s^{-1} ($p=0.001$) and 45 cm s^{-1} ($p=0.012$) but not different than that at 60 cm s^{-1} ($p=0.082$). The metabolic rate of hardhead at 90 cm s^{-1} was significantly higher than at all of the other velocity steps ($p<0.05$). The metabolic rates at 60 and 75 cm s^{-1} were statistically indistinguishable ($p=0.082$). The mean TBFs were different at each velocity ($p<0.001$), except the 30 cm s^{-1} and 45 cm s^{-1} means were not statistically distinguishable ($p=0.303$). For the 16°C acclimation group TBF was plotted with the active metabolic rate (MO_2, Figure 27) for fish tested in the 660-l chamber only, because the hardhead sample size ($n<4$) for the 150-l chamber was too small to allow accurate estimates of performance (appendix).

Table 9. Active metabolic rate (MO_2) of adult hardhead acclimated at 16°C and tested in a 660-l Brett-style swimming respirometer, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column.

						MO2 (mg O2 kg^{-1} h^{-1})				
N	Water Velocity (cm s^{-1})	Mean Mass (kg)	SE	Mean FL (cm)	SE	Mean	SE	Min	Max	Median
8	30	0.759	0.101	37.8	1.464	239.3	22.69	160.4	343.7	221.8
12	45	0.803	0.077	38.7	1.048	351.9	48.06	153.8	621.5	340.2
15	60	0.808	0.067	38.9	0.917	442.1	48.27	103.1	740.1	467.9
14	75	0.806	0.072	38.9	0.983	639.8	77.03	289.7	1412.2	597.6
6	90	0.601	0.041	36.9	0.700	934.6	141.34	563.1	1461.5	818.6
1	105	0.580	-	36.0	-	642.0	-	-	-	-

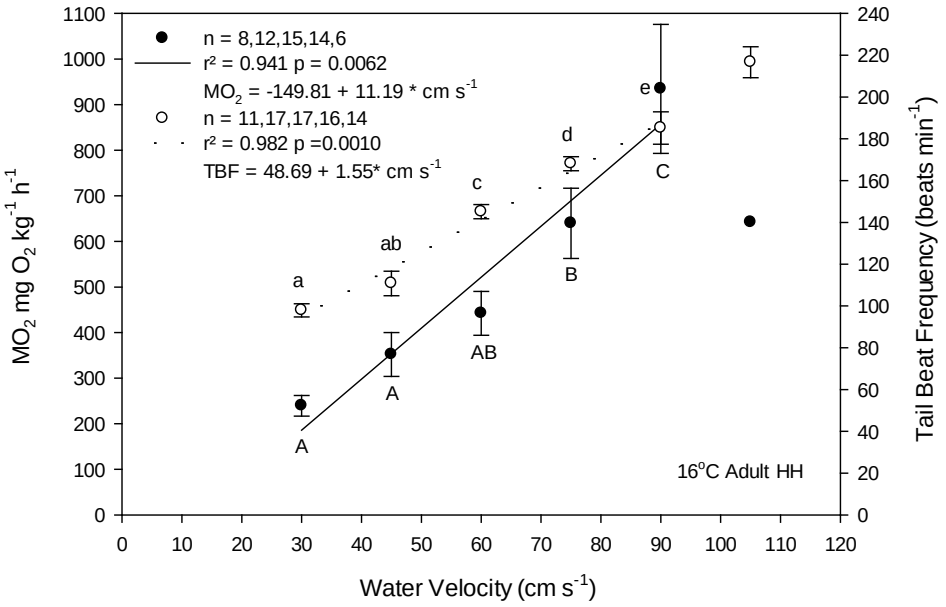

Figure 27. Mean (± SE) active metabolic rate (●) and tail beat frequency (○) of adult hardhead acclimated to 16°C, that swam in a 660-L Brett-style chamber. The 105 cm s^{-1} TBF was excluded from the simple linear regression, due to the low sample size (n=3) at that velocity, as was the MO_2 (n=1). Different letters denote statistically significant differences among the velocity steps (p<0.05). Sample sizes (n) for each acclimation group are given in order of increasing acclimation temperature.

3.4.A.3 Swimming metabolism of adult hardhead acclimated and tested at 21°C

In the adult hardhead 21°C acclimation group a total of 2 hardhead swam in the 150 l chamber (Appendix) and 18 hardhead swam in the 660-l chamber (Table 10). The mean metabolic rates of hardhead at 30 and 45 cm s^{-1} were not significantly different (p=0.429). However, at 60 cm s^{-1} the MO_2 was higher than that at 30 cm s^{-1} (p=0.016), but not higher than at either 45 cm s^{-1} or 75 cm s^{-1} (p=0.214 and p=0.397, respectively). Metabolic rate at the 90 cm s^{-1} step was significantly higher than at the 30 and 45 cm s^{-1} velocity steps (p<0.05), but not higher than at the 60 and 75 cm s^{-1} steps (p=0.253 and p=0.443, respectively). For the 21°C acclimation group, TBF was plotted with active metabolic rate (MO_2, Figure 28) only for fish tested in the 660-l chamber. The sample size for the 150-l chamber size (n=2) was too small to allow accurate estimates of performance. The mean TBFs were different at each velocity (p<0.001). TBF for the single fish that made it to the 105 cm s^{-1} velocity step was 200 beats/min.

Table 10. Active metabolic rate (MO_2) of adult hardhead acclimated at 21°C and tested in a 660-L Brett-style swimming respirometer, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column.

						MO2 (mg O2 kg^{-1} h^{-1})				
N	Water Velocity (cm s^{-1})	Mean Mass (kg)	SE	Mean FL (cm)	SE	Mean	SE	Min	Max	Median
13	30	0.782	0.053	39.9	0.812	239.0	21.23	95.4	386.8	247.9
17	45	0.728	0.048	38.9	0.753	336.3	35.01	117.9	666.1	319.2
17	60	0.751	0.047	39.2	0.737	462.1	70.40	173.0	1377	406.1
14	75	0.772	0.055	39.3	0.898	520.7	37.62	307.7	712.9	560.7
9	90	0.719	0.079	38.5	1.269	614.4	69.58	303.6	924.2	604.9
1	105	0.620	-	36.5	-	1342	-	1342	1342	1342

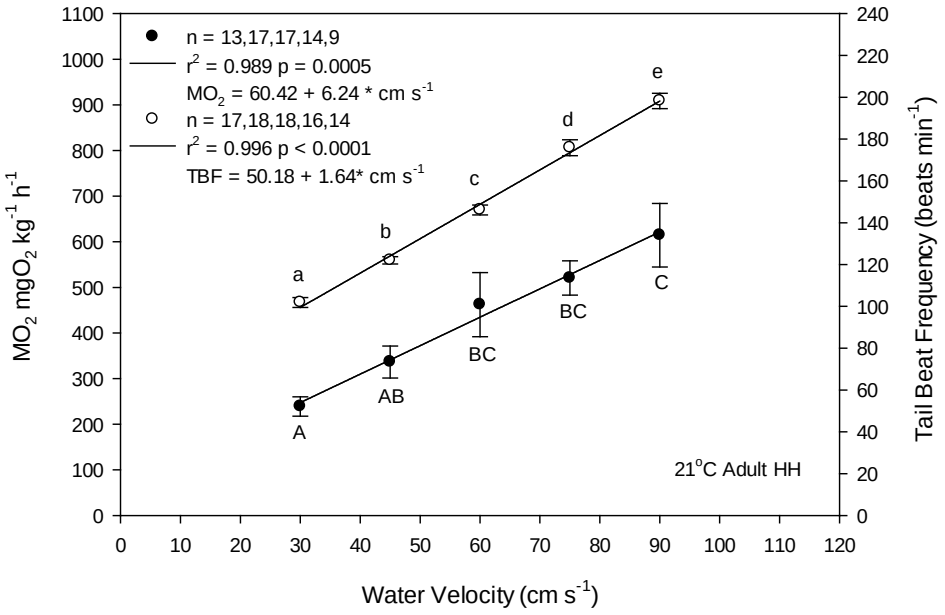

Figure 28. Mean (± SE) active metabolic rate (●) and tail beat frequency (○) of adult hardhead acclimated to 21°C, that swam in a 660-l Brett-style chamber. The 105 cm s^{-1} TBF was excluded from the simple linear regression and plotting, due to the low sample size (TBF = 200; n=1) at that velocity, as was the MO_2 (1342; n=1). Different letters denote statistically significant differences at the velocity steps (p<0.05). Samples sizes (n) for each acclimation group are given in order of increasing acclimation temperature.

3.4.A.4 Swimming metabolism of adult hardhead acclimated and tested at 25°C

In the adult hardhead 25°C acclimation group 20 fish swam in the 660-l chamber (Table 11). The mean metabolic rates of hardhead at 30, 45, and 60 cm s^{-1} were statistically indistinguishable (p>0.05), and metabolic rate at 60 cm s^{-1} was not different than that at 90 cm s^{-1} (p=0.066). The MO_2 rate at the 75 cm s^{-1} step was significantly higher than at the 30, 45, and 60 cm s^{-1} velocity steps (all p<0.001), but was not statistically distinguishable from that at the 90 cm s^{-1} step (p=832). The mean tail beat frequencies were different at each velocity step (p<0.001). For the

single fish that swam at 105 cm s^{-1} the TBF averaged 195 beats/min (±43 SE, n=2). No fish from the 25°C acclimation group swam in the 150-l chamber. For the 25°C acclimation group TBF was plotted with active metabolic rate (Figure 29) for fish that swam in the 660-l chamber. No fish in this temperature group were smaller than the ~500 g cut off to be swam in the 150 l chamber.

Table 11. Mean active metabolic rate (MO2) of adult hardhead acclimated at 25°C and tested in a 660-L Brett-style swimming respirometer, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column.

						MO2 (mg O2 kg^{-1} h^{-1})				
N	Water Velocity (cm s^{-1})	Mean Mass (kg)	SE	Mean FL (cm)	SE	Mean	SE	Min	Max	Median
17	30	0.731	0.051	39.2	0.759	234.7	30.06	81.9	482.7	182.8
19	45	0.749	0.048	39.4	0.709	234.0	13.63	152.1	389.5	223.0
18	60	0.749	0.048	39.4	0.709	295.6	13.28	201.4	416.7	289.3
18	75	0.753	0.051	39.3	0.744	439.9	37.35	214.3	920.5	397.7
7	90	0.649	0.083	37.9	1.409	414.5	28.24	324.2	499.7	417.6

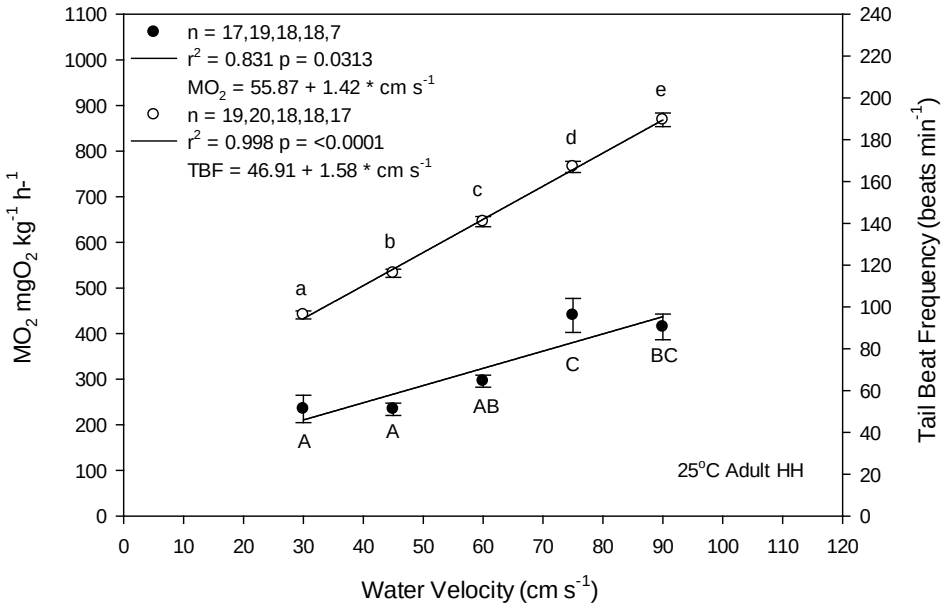

Figure 29. Mean (± SE) active metabolic rate (●) and tail beat frequency (○) of adult hardhead acclimated to 25°C, that swam in a 660-L Brett-style chamber. The 105 cm s^{-1} TBF was excluded from the simple linear regression and plotting, due to the low sample size (TBF = 195; n=2), and no MO_2 values were recorded at this velocity step. Different letters denote statistically significant differences at the velocity steps (p<0.05). Sample sizes (n) for each acclimation group are given in order of increasing acclimation temperature.

3.4.A.5 Active MO_2 comparison of adult hardhead acclimated to 11, 16, 21, and 25°C

There were significant effects of acclimation temperature and water velocity on metabolic rate (two-way ANOVA, $p<0.001$ for both comparisons), with no significant interaction between the effects of acclimation temperature and water velocity on hardhead active MO_2 values ($p=0.273$; Figure 30). Post hoc tests revealed that, at all acclimation temperatures the 25°C had significantly lower MO_2 ($p<0.008$). Water velocities steps of 30 and 45 cm s^{-1} ($p=0.279$), 60 and 75 cm s^{-1} ($p=0.083$), and 75 and 90cm s^{-1} ($p=0.893$) were not distinguishable from each other. The 60 and 75 cm s^{-1} MO_2 rates were higher than the previous velocity steps ($p<0.004$ for all comparisons). The 90 cm s^{-1} water velocity step's MO_2 was significantly higher than the first three steps ($p<0.028$ for all comparisons). The mean TBF of hardhead was significantly higher at each velocity step tested ($p<0.001$; Figure 31). The 11°C acclimation group had the lowest mean TBF and the 21°C acclimation group had the highest mean TBF ($p<0.001$). The 16 and 25C acclimation were not distinguishable ($p=0.521$) and the 21 and 25°C were not distinguishable for TBF ($p=0.065$; Figure 29).

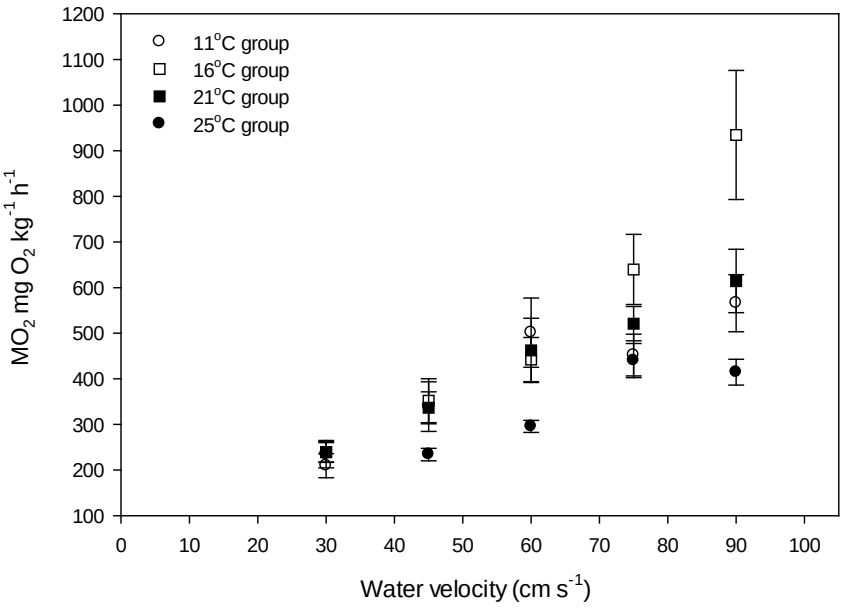

Figure 30. Metabolic rate (MO_2 ± SE), normalized by fish mass, of hardhead that swam in the 660-l Brett-style chamber after being acclimated to one of four treatment temperatures (11, 16, 21, or 25°C). Note that the MO_2 data at the 105 cm s^{-1} velocity were not included in the analysis because of small sample sizes.

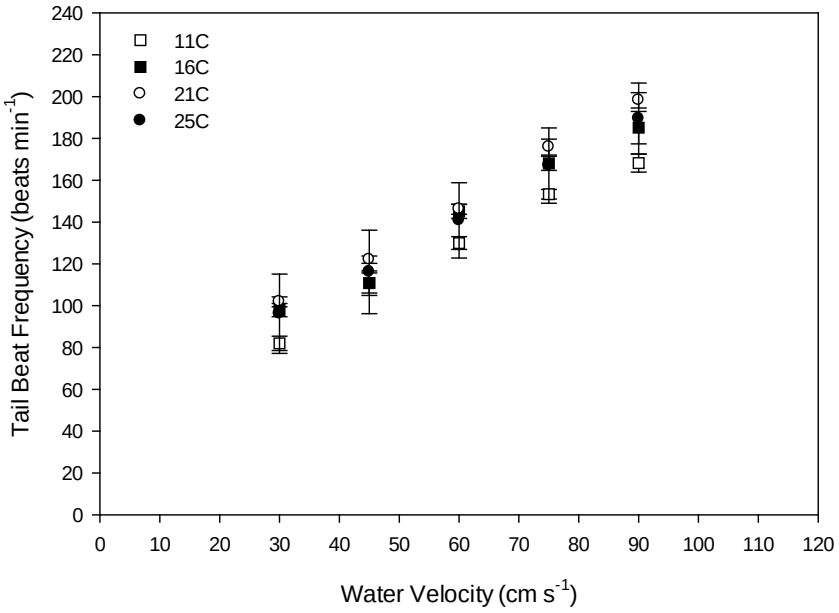

Figure 31. Tail beat frequency (±SE) for 11, 16, 21, and 25°C acclimated adult hardhead that swam in the 660-L Brett-style chamber. The linear regressions for each can be found in previous figures, but the 105 cm s^{-1} data were omitted from this figure due to low sample sizes.

3.4B Juvenile Active Swimming Metabolism Study

Similar to the pattern seen for the adults, juvenile hardhead active metabolic rate (MO_2; mg O_2 kg^{-1} h^{-1}) increased with increased water velocities. However, many juvenile hardhead were reluctant to swim in the respirometer. The 21 and 25°C acclimation group fish performed well, but the 11 and 16°C acclimation groups performed very poorly, despite repeated attempts to stimulate swimming behavior. The mean mass of the 25°C (9.9 g ± 0.72 SE) acclimated fish was significantly larger than those of the other acclimation groups ($p \leq 0.001$), due to our design of sequentially swimming the fish to match the acclimation group temperature to the appropriate seasonal temperature (e.g., 11°C in the winter months and 25°C in summer). Size, though, is not believed to be the source of the difference in swimming performance (see results below). Below, we present our results from each thermal acclimation group separately followed by comparisons between all four groups. Due to the hardhead's small size and very high tail beat frequency, we were unable to collect accurate TBF data in real time.

3.4B.1 Swimming metabolism of juvenile hardhead acclimated and tested at 11°C

The active metabolic rate of juvenile hardhead acclimated to 11°C are presented in Table 1. Of the 20 fish we attempted to swim most completely refused to swim longer than a few seconds per attempt, typically only **burst swimming.** We attempted to increase water velocity to the next velocity step to encourage swimming. We also attempted to add fish into the chamber and directly ramp the water velocity (15-20 cm s-1), instead of giving the fish an acclimation period,

to get the hardhead to orient **upstream** and begin directed swimming. This seemed promising, but continuous swimming longer than 10-15 s was rare.

We also tested eight juvenile hardhead (mean mass = 4.6 g) in a preliminary study, and found that at 11°C only two of the eight fish swam, whereas at 25°C seven of the eight hardhead were willing to swim. These fish were captured from the Pit River along with our adult fish collections, so we believe this observation of indifference to active swimming at lower temperatures occurs in multiple populations of this species.

Table 12. Active metabolic rate (MO_2) of juvenile hardhead held at the 11°C acclimation temperature, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column .Note 20 fish were tested but the data presented represent only the successful velocity steps (>5 torr decrease in oxygen partial pressure).

						MO_2 (mg O_2 kg^{-1} h^{-1})				
Velocity (cm s^{-1})	N	FL (cm)	SE	Mass (g)	SE	Mean	SE	Max	Min	Median
10	2	6.2	0.2	3.34	0.1	236.6	17.82	254.5	218.8	236.6
20	1	6.4	--	3.44	--	303.6	--	--	--	--
30	1	6.4	--	3.44	--	387.6	--	--	--	--

3.4B.2 Swimming metabolism of juvenile hardhead acclimated and tested at 16°C

The active metabolic rate of juvenile hardhead acclimated to 16°C are presented in Table 13. The 16°C group performed better than the 11°C acclimation group, but only marginally (five fish completed the first velocity step versus two for the 11°C group). Again our attempts to promote swimming in the hardhead were unsuccessful.

Table 13. Active metabolic rate (MO_2) of juvenile hardhead acclimated at 16°C, including the sample size (N), water velocity, mass), and fork length (FL). SE indicates the standard error (±) of the variable in the previous column. Note 16 fish were tested but the data presented represent only the successful velocity steps (>5 torr decrease in oxygen partial pressure).

						MO_2 (mg O_2 kg^{-1} h^{-1})				
Velocity (cm s^{-1})	N	FL (cm)	SE	Mass (g)	SE	Mean	SE	Max	Min	Median
10	5	7.2	0.198	5.06	0.505	493.7	62.55	717.6	357.4	437.6
20	4	7.1	0.204	4.87	0.6	483	45.5	606.4	392.2	466.8
30	3	7	0.273	4.92	0.844	617.3	126.31	841.9	404.8	605.2
40	1	7.4	--	5.18	--	719.1	--	--	--	--

3.4B.2 Swimming metabolism of juvenile hardhead acclimated and tested at 21°C

The active metabolic rate of juvenile hardhead acclimated to 21°C are presented in Table 14. This acclimation group had seven fish make it to the 50 cm s^{-1} velocity step successfully and all the fish tested completed the 10 and 20 cm s^{-1} velocity steps. This group was characterized by

fish that swam with fewer of the impingements and "tail-propping" events that were common among the groups acclimated at lower temperatures. While the mean metabolic rates of the 21°C group at 10 and 20 cm s^{-1} were identical (p=1), the rate at 30 cm s^{-1} was higher than those at the previous two steps (p<0.05 for both comparisons). Finally, the rates at the 40 and 50 cm s^{-1} steps were significantly higher than those at the preceding steps (p<0.001 for both individually; Figure 30).

Table 14. Active metabolic rate (MO_2) of juvenile hardhead acclimated at 21°C, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column. Note that 19 fish swam but the results represent only the successful velocity steps (>5 torr decrease in partial pressure). Superscripts represent mean MO_2 rates that are significantly different than each other (P<0.05 for all).

						MO_2 (mg O_2 kg^{-1} h^{-1})				
Velocity (cm s^{-1})	N	FL (cm)	SE	Mass (g)	SE	Mean	SE	Max	Min	Median
10	19	8.1	0.239	6.61	0.364	393.1^{A}	20.68	608.5	252.2	369.2
20	19	8.1	0.239	6.61	0.364	$393.1^{A,B}$	18.66	545.8	237.8	382.5
30	17	8	0.265	6.52	0.402	471.9^{B}	28.54	754.2	331.5	457.4
40	16	8	0.282	6.47	0.424	571.7^{C}	27.3	719.6	387.2	579.6
50	7	8	0.534	6.82	0.553	769.8^{D}	50.51	901.8	519.8	763.9
60	1	9	--	8.5	--	725.4	--	--	--	--

3.4B.2 Swimming metabolism of juvenile hardhead acclimated and tested at 25°C

The active metabolic rate of juvenile hardhead acclimated to 25°C are presented in Table 15. The mean metabolic rates at 10, 20, 30 cm s^{-1} were statistically indistinguishable (p=0.099-0.988). The rates at 40 and 50 cm s^{-1} were significantly higher than those at the 10 and 20 cm s^{-1} steps (p<0.01 for all comparisons), but not the (intermediate-level) MO_2 at 30 cm s^{-1} (p=0.007, Figure 30). For both the 21 and 25°C groups the 50 cm s^{-1} velocity step metabolic rates were highly variable, this water velocity may approach the juvenile's maximum sustained swimming velocity (aerobic scope). Unsteady swimming is often observed near a fish's maximum speed and this would cause significant deviations in metabolic rates. This is supported by the fact that of fish that successfully completed the 40 cm s^{-1} velocity step, less than 50% completed the 50 cm s^{-1} velocity step.

Table 15. Active metabolic rate (MO_2) of juvenile hardhead acclimated at 25°C, including the sample size (N), water velocity, mass, and fork length (FL). SE indicates the standard error (±) of the variable in the previous column. Note that 17 fish swam and these data only represent the successful velocity steps (>5 torr oxygen water content decreased). Superscripts represent mean MO_2 rates that are significantly different than each other (P<0.05 for all).

						MO_2 (mg O_2 kg^{-1} h^{-1})				
Velocity (cm s^{-1})	N	FL (cm)	SE	Mass (g)	SE	Mean	SE	Max	Min	Median
10	16	9.8	0.28	10.21	0.72	445.1^{A}	22.96	645.3	276.8	433.2
20	15	9.8	0.3	10.36	0.75	445.7^{A}	22.9	600.9	271.1	444.3
30	13	9.8	0.34	10.53	0.86	$515.5^{A,B}$	36.28	754.2	292.2	497.5
40	12	9.9	0.36	10.83	0.9	565.5^{B}	33.86	848.5	413.4	535.8
50	6	10.1	0.53	11.09	1.28	592^{B}	64.88	856.7	434	557.3
60	1	10	--	10.8	--	891.9	--	--	--	--

3.4B.2 Active MO_2 comparison of juvenile hardhead acclimated to 21°C and 25°C

The metabolic rates for juvenile hardhead acclimated to 21 and 25°C had no significant interactions with water velocity and acclimation temperature (p=0.072 and p=0.879, respectively; Figure 32). Although the 25°C acclimation group performed similarly to the 21°C acclimation group at the 40 cm s^{-1} step, the mean metabolic rates at the first 3 velocity steps were higher for the 25°C group. Interestingly, the 21°C acclimation group's MO_2 rate increased above that of the 25°C acclimation group at 50 cm s^{-1}.

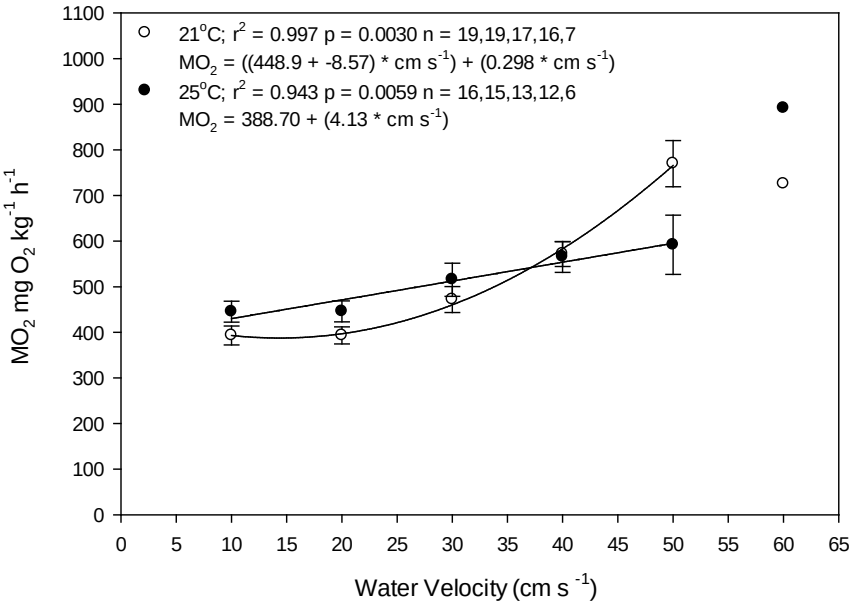

Figure 32. Mean active swimming metabolism (± SE) of juvenile hardhead that swam in a 5-l swimming respirometer after acclimation to 21°C (○) or 25°C (●). Note that the 60 cm s^{-1} data point is not included in the polynomial (21°C acclimation group) or common linear regressions (25°C acclimation group) due to the low sample sizes (n=1 both points). Sample sizes (n) for each acclimation group are given in order of increasing acclimation temperature.

3.5 Adult and juvenile thermal tolerance study

The mean mass and fork length (FL) for both the critical thermal minimum and maximum are presented in Table 16. In the CTmin acclimation groups, mean adult hardhead FL were indistinguishable (p=0.500) while, significant differences were noted between the CTmax groups (p=0.016). Fish in the 25°C acclimation group were longer than in the 11 and 16°C acclimation groups (p=0.033 and p=0.032, respectively). The mean mass of the four acclimation groups for the CTmin trials were all different (p<0.001 for all comparisons), except that the 11 and 16°C acclimation group were not distinguishable (p=0.211). For fish used in CTmax determinations, the mean mass of fish in the 21°C acclimation group was significantly lower than in the 16 and 25°C groups (p=0.001 and p=0.004, respectively).

There was a significant effect of thermal acclimation on CTmin values (p value of the ANOVA), and pairwise tests revealed that significant differences at each acclimation temperature (p<0.028 for all comparisons), except the 11 and 16°C were indistinguishable (p=0.213). For CTmax, there were no significant differences between the 11 and 16°C (p=0.089) and 16 and 21°C (p=0.056) groups, while all other pairwise comparisons differed (p<0.05; Table 16).

Table 16. Upper and lower critical thermal limits of adult hardhead, for groups of fish held at one of four target acclimation temperatures, including critical thermal maximum (CTmax) or minimum (CTmin), sample size (N), mass, and fork length. Within a treatment type, different superscripts represent significantly different comparisons (p<0.05).

Acclimation Group (°C)	Treatment type	N	Mass (g) ± SE	Fork length (cm) ± SE	CTM (°C) ± SE
11	CTmin	11	734.9 ± 15.2^{a}	37.0 ± 1.4	0.3 ± 0.6^{1}
16	CTmin	10	762.2 ± 18.8^{a}	37.4 ± 1.7	1.5 ± 0.8^{1}
21	CTmin	10	882.0 ± 13.2^{b}	39.7 ± 0.9	4.2 ± 0.7^{2}
25	CTmin	10	628.4 ± 13.4^{c}	37.7 ± 1.2	6.7 ± 0.6^{3}
11	CTmax	10	$736.2 \pm 18.0^{A,B}$	34.0 ± 1.4^{X}	29.7 ± 0.7^{1}
16	CTmax	10	780.7 ± 18.2^{A}	34.1 ± 1.7^{X}	$31.9 \pm 0.7^{1,2}$
21	CTmax	10	681.2 ± 15.8^{B}	$36.9 \pm 1.3^{X,Y}$	34.0 ± 0.8^{2}
25	CTmax	10	770.7 ± 15.8^{A}	39.8 ± 1.1^{Y}	36.7 ± 0.8^{3}

There were no significant differences in juvenile hardhead masses between CTmin acclimation groups (p=0.213) nor between CTmax acclimation groups (p=0.340, respectively, Table 17). Similarly, there were no significant differences in juvenile hardhead mean FL for CTmin and CTmax groups (p=0.511 and p=0.770, respectively). There was a significant effect of temperature acclimation on juvenile hardhead CTmin values (p value of ANOVA), and post-hoc tests revealed that the CTmin of juvenile hardhead acclimation groups differed significantly (p<0.001 for all comparisons), except for the 11 and 16°C acclimation groups CTmin which were indistinguishable from one another (p=0.057). The mean CTmax of juvenile hardhead 21 and 25°C acclimation groups were significantly different than each other and as well as from the lowest acclimation temperature group (p<0.05). The 11 and 16°C acclimation groups CTmax (p=0.078) did not differ from one another.

Table 17. Upper and lower critical thermal limits of juvenile hardhead, for groups of fish held at one of four target acclimation temperatures, including critical thermal maximum (CTmax) or minimum (CTmin), sample size (N), mass, and fork length. Different superscripts represent significantly different comparisons (p<0.05).

Acclimation Group	Treatment type	N	Mass (g) ± SE	Standard length (cm) ± SE	CTM (°C) ± SE
11	CTmin	9	3.59 ± 1.08	5.35 ± 0.81	0.2 ± 0.4^{A}
16	CTmin	8	5.55 ± 0.94	6.45 ± 0.68	1.7 ± 0.5^{A}
21	CTmin	10	7.34 ± 1.44	7.42 ± 0.87	4.6 ± 0.4^{B}
25	CTmin	8	11.52 ± 1.89	8.64 ± 1.05	7.4 ± 0.8^{C}
11	CTmax	9	3.04 ± 0.94	4.98 ± 0.80	29.7 ± 0.8^{a}
16	CTmax	8	5.29 ± 0.94	6.20 ± 0.81	$32.2 \pm 0.9^{a,b}$
21	CTmax	10	8.67 ± 1.69	7.85 ± 0.92	34.4 ± 0.8^{b}
25	CTmax	9	10.38 ± 1.81	8.54 ± 0.95	37.3 ± 0.7^{c}

We constructed thermal tolerance polygons by plotting CTmax and CTmin versus acclimation temperature for adults (Figure 33) and juveniles (Figure 34). The polygons were expressed quantitatively using the area $°C^2$ (areal units). The polygonal area is further divided into an intrinsic tolerance zone (i.e., thermal tolerance independent of previous thermal acclimation) as well as upper and lower acquired tolerance zones (i.e., thermal tolerance gained through thermal acclimation) by dividing the polygon with horizontal lines originating at the inter-section of the CTmin and CTmax regressions at their respective upper and lower thermal acclimation limits. We found that of the total polygonal area the intrinsic zone accounted for 77.4% and 75.0% of the total area in adults (Total area = 418.5$°C^2$) and juveniles (Total area = 406.2$°C^2$), respectively.

Both adult and juvenile hardhead displayed increased heat tolerance (CTmax) with increasing acclimation temperature, and a greater tolerance of colder temperature (CTmin) with decreasing acclimation temperature (Figures 31, 32). For every 1°C in acclimation temperature adult hardhead acquired 0.46°C and 0.49°C increases in critical thermal minimum and maximum, respectively. For every 1°C in acclimation temperature juvenile hardhead acquired 0.52°C and 0.53°C increases in critical thermal minimum and maximum, respectively.

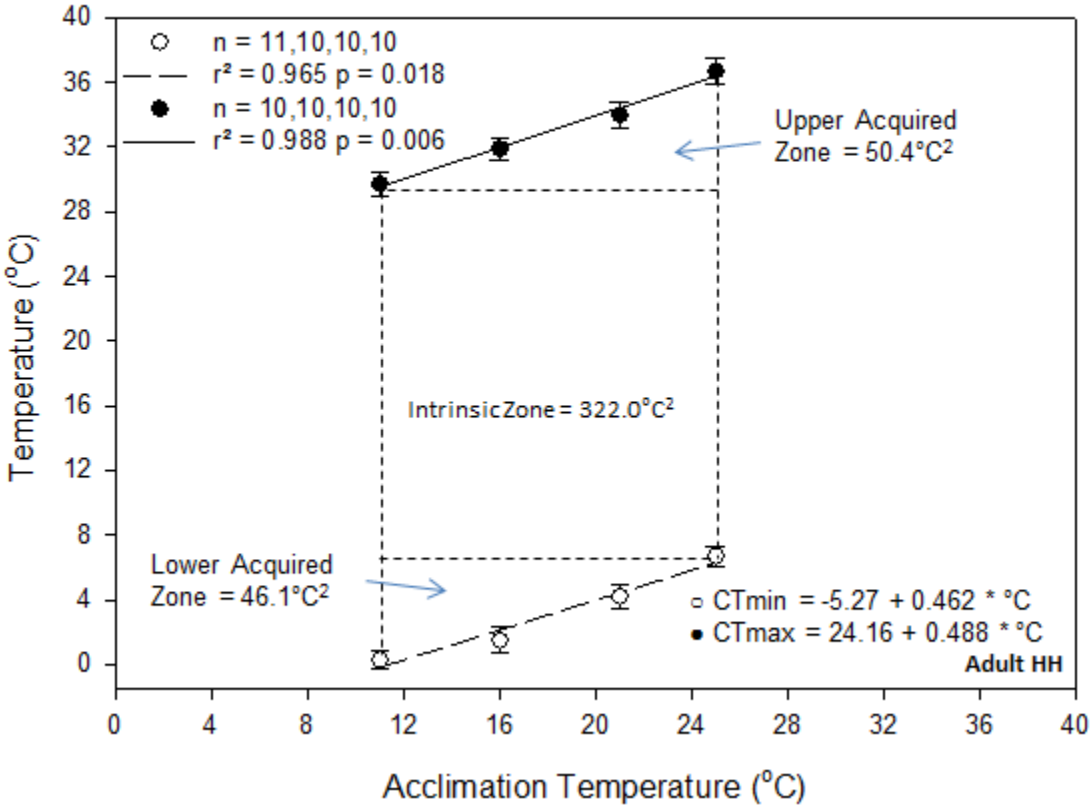

Figure 33. Mean (± SE) adult hardhead minnow critical thermal maxima and minima across temperature acclimation groups (11, 16, 21, and 25ºC). Maxima (●) and minima (○) are bracketed by an environmental thermal tolerance constructed polygon including upper and lower acquired zones and the zone of intrinsic tolerance. Line equations were determined by simple linear regressions.

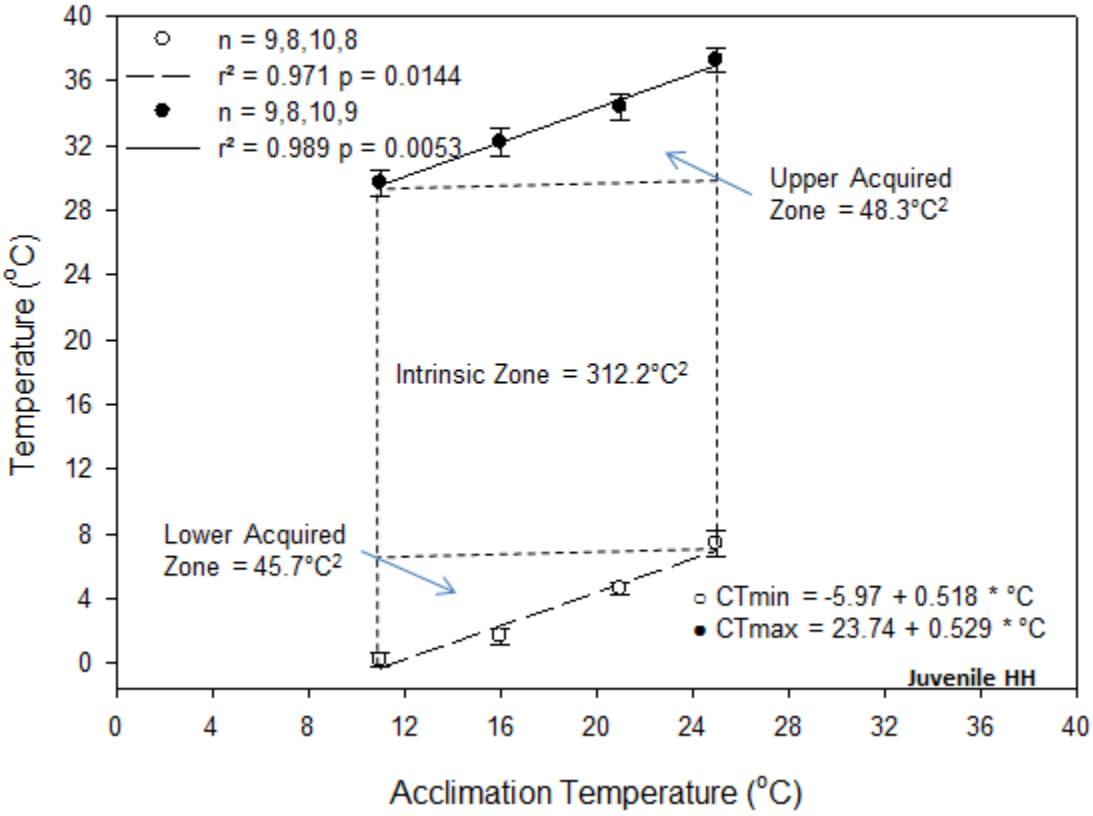

Figure 42. Mean (± SE) juvenile hardhead critical thermal maxima and minima across temperature acclimation groups (11, 16, 21, and 25ºC). Maxima (●) and minima (○) are bracketed by an environmental thermal tolerance constructed polygon including upper and lower acquired zones and the zone of intrinsic tolerance. Line equations were determined by simple linear regressions.

3.6 Adult Blood-Oxygen Equilibria Study

Hardhead blood-oxygen affinity showed low to moderate decreases (i.e., increased P_{50}) in response to increased temperature. In the 11°C and 19°C treatments both the low- and high-PCO_2 equilibrated blood P_{50}s were statistically indistinguishable ($p>0.05$) between the two temperatures (Table 18). Although the 25°C and 30°C low- and high-PCO_2 blood-oxygen affinities were statistically indistinguishable from each other, they were both lower ($p<0.05$) when compared to the 11°C and 19°C treatments (Table 18). Therefore, the higher temperature intervals (i.e., between 19°C and 25°C and between 25°C and 30°C) showed the greater temperature sensitivity (ΔHs, absolute values) of hemoglobin oxygen loading andunloading in both low- and high-PCO_2 treatments, compared with that between 11 and 19°C, in both low- and high-PCO_2 treatments (Table 19). Least-squares regression analysis of P_{50} versus temperature conditions yielded positive slopes (0.047 kPa $°C^{-1}$ [r^2=0.90]) for the low-PCO_2 treatment and (0.052 kPa $°C^{-1}$ [r^2 = 0.84]) for the high low PCO_2 treatment. Blood pH tended to

decrease with increased temperature in both the low-PCO_2 (-0.0281 pH units $°C^{-1}$ [r^2= 0.91]) and high-PCO_2 (-0.0039 pH units $°C^{-1}$ [r^2= 0.42]) treatments.

The CBO_2 measurements showed no statistically significant differences nor were there any apparent trends in relation to the temperature treatments (Table 18). Mean Bohr factors, Hill-plot slopes (n_{50}), and β showed no statistically significant relationship across the temperature treatments.

Predictably, high-PCO_2 treatments decreased oxygen affinities (Bohr effect) when compared to their low-PCO_2 paired treatment within a temperature regime. The observed decrease in oxygen affinity as a result of exposure to CO_2 shifted all high-PCO_2 curves to the right (Figure 35 A-D). Increased CO_2 exposure (from <0.03 kPa to 1.01 kPa) produced statistically ($P<0.001$) lower pH values when compared to corresponding treatment low-PCO_2 P_{50} pH values (mean pH ± SE values for low-PCO_2: 7.971 ±0.120, for high-PCO_2: 7.424 ± 0.038). No significant relationships were observed between CBO_2 and CO_2 treatments, indicating no Root effect (Root 1931). We observed a small, but statistically significant, decrease in HCT values at 19°C, compared with the other treatments (a possible artifact from dilution with the anticoagulant solution), with no statistical difference noted in [Hb] across temperature treatments. There were no statistical differences in whole-blood NTP concentrations or NTP:Hb ratios (μmol:μmol) (Table 19) across temperatures.

Table 18. Mean (with ±SE), pH, P_{50} (kPa), total hemoglobin concentration ([Hb], in $g \cdot dl^{-1}$), blood oxygen capacity (CBO_2, ml $O_2 \cdot dl^{-1}$ blood), hematocrit (HCT in %), and n (sample size) in low- and high-PCO_2 treatments over the experimental range of temperatures (11, 19, 25, and 30°C) in blood from wild-caught hardhead.

Temp.	PCO_2	pH	P_{50}	[Hb]	CBO_2	HCT	n
11	≤0.03	8.197 (0.068)	0.51 (0.08)	-	17.5 (1.0)	-	3
				10.9 (0.6)		29 (0.3)	
11	1.01	7.430 (0.047)	2.02 (0.02)	-	14.6 (0.6)	--	3
19	≤0.03	8.132 (0.087)	0.67 (0.12)	-	16.5 (1.7)	-	3
				10.5 (0.6)		27 (0.4)	
19	1.01	7.528 (0.058)	2.16 (0.06)	-	16.1 (1.5)	-	3
25	≤0.03	7.881 (0.157)	1.07 (0.15)	-	14.7 (0.8)	-	3
				8.7 (0.3)		30 (1.6)	
25	1.01	7.359 (0.120)	2.62 (0.16)	-	15.1 (0.5)	-	3
30	≤0.03	7.675 (0.02)	1.80 (0.13)	-	17.2 (0.3)	-	5
				10.5 (1.3)		31 (1.3)	
30	7.6	7.380 (0.03)	2.92 (0.12)	-	14.2 (2.3)	-	5

Table 19. Hardhead hematological parameters derived from tonometered blood used in blood-oxygen equilibria experiments. Temperature in °C, PCO_2 in kPa, n_{50} = hemoglobin subunit cooperativity, β = whole-blood nonbicarbonate buffer value (slykes), Φ = Bohr factor, ΔH = temperature effect in low- and high-PCO_2 treatments (kJ mol O_2^{-1}, NTP (NTP µmol l^{-1}), NTP:Hb (µmol NTP:µmol Hb), and n = number of experimental replicates. Measurements are presented as means (with ±SE).

					ΔH				
Temp.	**PCO_2**	**n_{50}**	**β**	**Φ**	**Low-PCO_2**	**High-PCO_2**	**[NTP]**	**NTP:Hb**	**n**
11	≤ 0.03	1.41 (0.2)	-	-	-	-	-	-	3
11	-	-	-2.6 (0.1)	-0.794 (0.09)	-	-	454.6 (48.6)	0.271	-
11	1.01	1.43 (0.06)	-	-	-	-	-	-	3
11-19	-	-	-	-	-5.48	-1.37	-	-	
19	≤ 0.03	1.17 (0.07)	-	-	-	-	-	-	3
19	-	-	-8.5 (4.4)	-0.916 (0.14)	-	-	355.3 (40.6)	0.22	-
19	1.01	1.38 (0.6)	-	-	-	-	-	-	3
19-25	-	-	-	-	-13.56	-5.66	-	-	-
25	≤ 0.03	1.29 (0.04)	-	-	-	-	-	-	3
25	-	-	-6.7 (2.2)	-0.770 (0.05)	-	-	na*	na*	-
25	1.01	1.49 (0.03)	-	-	-	-	-	-	3
25-30	-	-	-	-	-18.81	-.3.87	-	-	-
30	≤ 0.03	1.32 (0.05)	-	-	-	-	-	-	5
30	-	-	-16.5 (4.1)	-0.819 (0.11)	-	-	383.7 (26.6)	0.238	-
30	1.01	1.52 (0.03)	-	-	-	-	-	-	5

*na = missing data

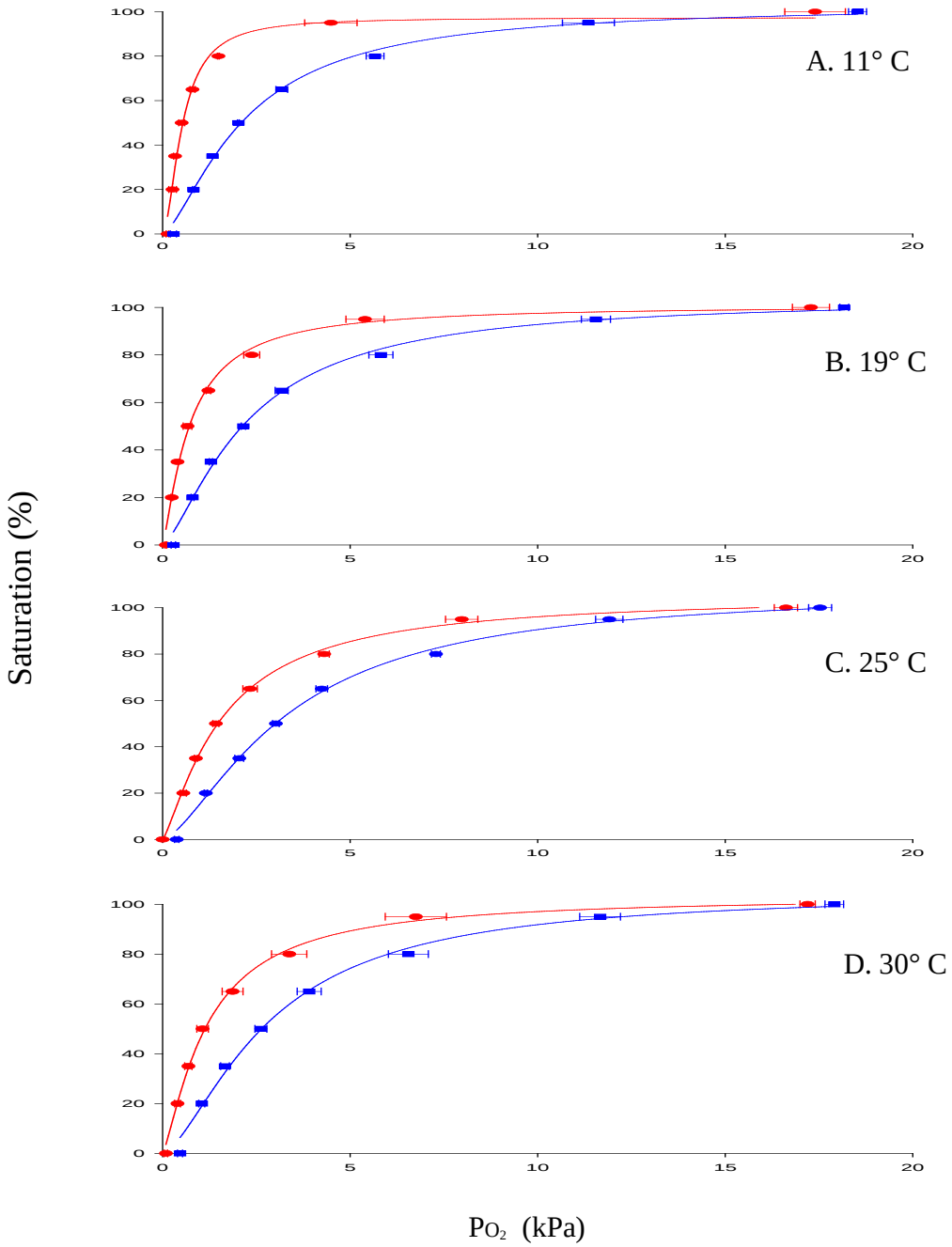

Figure 35. Hardhead blood-oxygen equilibria at low-PCO_2 (solid circles, PCO_2 = 0.2 mm Hg) and high- PCO_2 (solid squares, PCO_2 = 7.6 mm Hg) conditions and temperatures:(A) 11°C, (B) 19°C, (C) 25°C, and (D) 30°C. All curves were fitted to data means (±SE) with nonlinear regressions, and R^2 values ranged from 0.998 to 1.000.

CHAPTER 4: Conclusions and Recommendations

4.1 Fish Collection and Care

Hardhead minnows are readily captured via traps and hook and line fishing. Other methods may be suitable, but consideration of handling stress should be a high priority. Our experiences in other studies suggest that electro-shocking and gill-netting are very stressful for cyprinids and can compromise the fish's ability to fight infections and disease. For a thorough description of the disease and parasite burdens carried by hardhead and other cyprinids, see Alvarez (2008) and also Haderlie (1953). Under conditions of chronic and/or acute stress, aggressive disease treatments are required to maintain hardhead for long periods of time in captivity. We used only flow-through, freshwater fish-holding systems and have no recommendations regarding typical constituents in recirculating systems (e.g., dissolved ammonia, nitrite, and nitrate concentrations), which were maintained near zero in our systems.

We were able to maintain hardhead in a healthy state once they had been treated for parasites and diseases that were present prior to capture. Hardhead can be kept in captivity for long periods of time at temperatures between 11 and 25°C and fed commercially available diets, after a transition period involving supplementation with live foods. We noted no evidence of spawning in our "featureless" holding tanks, but post-test adult hardhead that were sampled via ultrasound showed gonadal tissue, suggesting that hardhead could be spawned in captivity if appropriate endogenous or exogenous cues were provided.

4.2 Temperature Preference, Performance, and Tolerance of Hardhead

Our hardhead performed very well at moderate temperatures (ca. 16-21°C) within our experiments. Regardless of thermal acclimation history, adult hardead minnows preferred a mean water temperature of 20.5°C and clearly avoided temperatures above ca. 26°C, whereas juvenile hardhead preferred 19.5°C water. Resting metabolic rates of hardhead increased with increasing acclimation temperatures in both adults and juveniles and have a low to moderate thermal sensitivity. Active metabolic rates ranged over 209-1342 mg O_2 kg^{-1} h^{-1} for adult fish that swam in the 660-l respirometer at velocities from 30 to 90 cm s^{-1} estimating their maximal continuous rate of oxygen consumption (aerobic activity). In contrast, juvenile hardhead exhibited poor ability and little willingness to swim at temperatures from 11-16°C. However, between 21-25°C they showed an increase in oxygen consumption as water velocities increased from 10 to 50 cm s^{-1}. Both adult and juvenile hardhead had nearly identical thermal tolerance limits, and we showed that water temperatures above 29.7°C and below 7.4°C likely approach ecologically lethal limits for these fish depending on their thermal acclimation history. These CTM results can be useful as an index, in comparing acute tolerance limits across species. However, such rapid temperature increases or decreases rarely happen in wild habitats. From studies on other species, realistic upper temperature tolerance limits are probably much lower, and realistic lower temperature limits are probably higher in hardhead, also.

Hardhead have moderately sigmoidal blood-oxygen equilibria curves, high whole-blood oxygen affinities and capacities (Table 18) and seem suited for some sustained aerobic activity over a range of dissolved oxygen concentrations and instream flow regimes, especially at

temperatures <25°C. Their decreased blood-oxygen affinities at temperatures ≥25°C may decrease oxygen binding at the gills, which may be unfavorable to hardhead's toleration of elevated stream temperatures, especially when combined with moderate environmental hypoxia.

We determined the thermal preference of adult and juvenile hardhead minnows in unique, annular preference chambers. Fish were acclimated to 11, 16, 21, or 25°C for greater than 30 days, and tested over a 4 hour period. When adult hardhead were presented with a temperature range of ca. 13-28°C they preferred 19.1-21.3°C, and juveniles preferred a range of 17.2-21.9°C. Interestingly both adult and juvenile hardhead acclimated well to 25°C but immediately preferred a cooler temperature in the range of temperatures preferred by hardhead in general. The inverse was true for the 11°C acclimation groups, where they quickly preferred a warmer temperature relative to their acclimation history and tended not to use temperatures above 25°C. Finally, the 21°C acclimated adults and juveniles, the group likely held at the most suitable and preferred temperature, demonstrated the most variability in their selected temperatures, with some at ca.13-15°C, some at 17-19°C, and others at ca. 24-26°C.

We measured resting metabolic rates at several environmentally relevant temperatures because these rates are a basic property in fish, which could be used to make direct comparisons to other species, under similar conditions. We did not test hardhead at extreme temperatures (high or low, e.g., that would have caused metabolic depression or death based on CTM data), and we were able to reuse the specimens in subsequent experiments. For example, we did not test fish at 30°C, due to their general avoidance of the 28°C zone in our temperature-preference apparatus, or below 11°C because we rarely caught hardhead when river water temperatures were below 10°C. Instead, we measured hardhead's critical thermal min/max to elucidate its very-short-term thermal limits.

Hardhead resting metabolic rates generally increased at increased acclimation temperatures in both juveniles and adults, as has been observed in other fish species (Jobling 1981). Pang et al. (2011) investigated the resting metabolic rates for three juvenile cyprinid fishes at both 15 and 25°C while the fish were not digesting food. These authors noted significantly higher MO_2 values while the fish were digesting food, but metabolic rates decreased to pre-fed values between 7.6 and 15.6 hours after feeding, suggesting that our fish were potentially in a post-absorptive state after their long (ca. 12 hour) chamber acclimations. Overall we found similar results to those of Cech et al. (1990), despite the use of somewhat different respirometric techniques, that hardhead had an intermediate response to temperature corresponding to their intermediate river distributions (low to mid elevations, Moyle 2002). Cech et al. (1990) found hardhead resting MO_2 rates only increased ~30% from 10°C to 20°C, considerably less than the 2 to 3-fold increase generally seen in poikilotherms (Schmidt-Nielsen 1990). We noted a 40% (juveniles) and 30% (adult) increase from 11°C to 21°C and only a slightly higher 52% (juvenile) and 45% (adult) resting metabolic rate increase from 11°C to 25°C.

Our Q_{10} data (range 1.25-1.79) suggest hardhead have a relatively low to moderate thermal sensitivity, regarding resting metabolic rates. Clarke and Johnston (1999) reviewed 14 papers and found a median Q_{10} = 2.40, suggesting that our data show a degree of evolutionary

adjustment by hardhead. For example, Novinger and Coon (2000) found, in redside dace (*Clinostomus elongates*, Cyprinidae), metabolic rate increases with acclimation temperature increases (over 6-20°C, Q_{10} = 2.3), suggesting that hardhead have evolved a biochemical moderation of whole-body metabolism across diel or seasonal temperature ranges that they might experience in California's rivers and streams. Both juveniles and adults showed similar trends between acclimation groups with a small changes over 11-26°C, but our juvenile showed a marked increase from 11 to 16°C. Interestingly, Cech et al. (1994) found northern pikeminnow (a closely related species) had a low Q_{10} (1.80) from 18-21°C, but a very high Q_{10} (3.23) over 9-15°C. See Cech's et al. (1994) discussion for a comparison of other cyprinid's and California native fishes' Q_{10}'s, and also Cech's et al. (1990) discussion regarding the hardhead's sensitivity to hypoxia after overnight 5°C temperature increases.

Overall, adult hardhead performed well in our Brett-style swimming respirometer from our four acclimation temperatures. Fish were easily capable of reaching 75 cm s^{-1}, with many reaching 90 cm s^{-1} water velocities. A few exceptional fish were able to swimming continuously at 105 cm s^{-1} for 30-40 minutes. At lower velocities and temperatures hardhead spent much of their time refusing to swim and apparently trying to avoid activity (e.g., clinging to the bottom, trying to escape, "tail propping" on the rear screen). This was especially true at the lower water temperatures where intermittent swimming was used until moderate velocities were achieved. In contrast, fish tended to swim well at lower velocities at the higher temperatures and spent less time avoiding activity. Within a species' limits, muscles are usually more efficient at warmer temperatures. Also, the viscosity of water decreases, somewhat, as water warms, facilitating swimming. Rome et al. (1984) observed that the water velocity threshold for recruitment of fast motor units in a cyrinid correlated with decreased water temperatures. Thus, non-adapted fish would need to use more white (anaerobic) musculature at lower swimming speeds, potentially resulting in a reduced maximum sustained swimming speed.

Our hardhead data compare with other cyprinid data from Zhang (2012) featuring MO_2 rates starting ca.200 mg O_2 kg^{-1} h^{-1} and exceeding 900 mg O_2 kg^{-1} h^{-1} at higher velocities. Mean MO_2 rates ranged from 209-1342 mg O_2 kg^{-1} h^{-1} for our adult hardhead that swam in our 660-L respirometer at velocities from 30 to 90 cm s^{-1}, estimating the maximal continuous rate of oxygen consumption (aerobic activity) in hardhead. A plateau was observed at 90 cm s^{-1} in adults, possibly due to fish nearing fatigue (Fry 1971). Fry (1971) noted that locomotor activity changes associated with temperature are responsible for plateau effects when looking at non-linear relationships of metabolic rate and temperature. The adult data at 105 cm s^{-1} may represent unsustainable activity under extreme conditions. This velocity was significantly higher than the critical swimming velocities (U_{crit}) reported in Myrick and Cech (2000, U_{crit} mean: 0.52 m s^{-1}).

Our study was not designed to determine endurance velocities of hardhead. Our smaller fish tended to have higher tail beat frequencies and lower metabolic rates (mg O_2 h^{-1}) than the larger adults.

The juvenile fish in our study did not perform as well as the adults in a similar type of swimming respirometer, especially at lower temperatures. Most of the juveniles refused to

swim at 11°C and 16°C with a total of seven of 36 individuals actually swam in any velocity step allowing the collection of metabolic data. From the 11°C group only one fish progressed to 30 cm s^{-1} and in the 16°C group, only one fish progressed to 40 cm s^{-1}. The 21°C and 25°C hardhead groups fared better with several fish progressing to the 50 cm s^{-1} velocity step, comparable to Myrick and Cech's (2000) results for hardhead weighing 102-194 grams. We did not test juvenile hardhead at night, although some evidence suggests that cyprinids can shift to being nocturnal at lowered water temperatures, which could improve their chances of swimming in an artificial swimming chamber (Greenwood and Metcalfe 1998). Nor did we employ a potentially stressful electric (typically 3v) grid as our downstream screen to induce swimming and discourage "tail propping."

The thermal scope (range of survivable temperatures), regarding critical thermal maxima and minima, for hardhead adults was 36.4°C and for juveniles was 37.1°C (including acquired acclimation zones). Without considering the acclimation acquired zones, the thermal scopes for adults and juveniles were equal (22.3°C). When comparing our thermal tolerance polygons, care should be exercised as we did not include chronic temperatures (CTMs are acute tests). Chronic temperatures bracket the x-axis of Figures 21 and 22 for extrapolating out the intrinsic zone. See Fangue and Bennett (2003) for discussion of comparative ecological thermal tolerance polygons.

Hardhead critical thermal maxima and minima increased in response to increased acclimation temperatures. Several studies have tested CTmin in North American fish (see review in Beitinger et al. 2000), but only rainbow trout (acclimated to 10 and 15°C) reached temperatures as low (0.0-2.0°C) as did our 11°C and 16°C acclimated hardhead (0.2-1.7°C). Several studies that included California species either sympatric or that are related to hardhead showed similar CTmax values (Myrick and Cech 2000, Castleberry and Cech 1992, Knight 1985, and Young and Cech 1996). For temperature criteria purposes one should consider hardhead to be physiologically capable of surviving water temperatures ranging from 8°C to 29°C (rounded CTM data excluding the intrinsic zones). Factors such as dissolved oxygen, food availability, disease, competition, and predation should be carefullyconsidered when evaluating these results or when applying them to resource-management decisions.

Hardhead whole-blood has the ability to bind oxygen (at the gills) at low environmental oxygen partial pressures (i.e., low P_{50}s = high O_2 affinity) as well as the capacity to deliver oxygen efficiently to metabolically active tissue sites (i.e., relatively high Bohr factors). Moderately high non-bicarbonate buffer values (β), moderate n_{50}'s, combined with the observed high oxygen affinities suggest that this species may tolerate environments which may, on occasion, become hypoxic and/or hypercapnic (Kaufman et al. 2006).The relatively high CBO_2 and Bohr factor values indicate that this species may have a high capacity for aerobic activity across the range of environmental temperatures examined in this study. These results compare well with the active metabolism experimental results showing good swimming to the higher velocity steps in adult hardhead.

Generally, increases in temperature result in decreased blood-oxygen affinity (Wood and Lenfant 1979; Powers 1980, 1983). The apparent heat of oxygenation (ΔH) reflects the combined effects of the exothermic binding of oxygen and the endothermic release of allosteric modifiers,

i.e., NTPs and protons, from hemoglobin and the ΔH calculation is a quantitative measure of the temperature sensitivity of the Hb-oxygen complex (Wood and Lenfant 1979; Jensen et. al. 1993). Hardhead, with their moderately sigmoidal blood-oxygen equilibria curves, have high whole-blood oxygen affinity (low P_{50}s) which align them with other cyprinids, such as the Sacramento blackfish (*Orthodon microlepidotus Ayres*), northern pikeminnow (*Ptychocheilus oregonensis*), carp (*Cyprinus carpio*), and tench (*Tinca tinca*), species capable of tolerating hypoxic environments (Eddy 1973; Cech et. al. 1979, 1994). Sacramento blackfish and northern pikeminnow have high whole-blood oxygen affinities with hyperbolic blood-oxygen equilibria curves, low P_{50} values, moderately large Bohr factors, moderate ΔHs, and moderate CBO_2 values (Cech et. al. 1979; Cech et. al. 1994). In contrast, hardhead have moderately sigmoid blood-oxygen equlibria curves (mean n_{50} = 1.38), with mean P_{50} values (low-PCO_2) slightly higher than those of Sacramento blackfish but analogous to those found in the northern pikeminnow. Additionally, high hardhead ΔH (absolute values) above 19°C suggest that exposure to 25°C and 30°C is unfavorable for hardhead, regarding Hb-oxygen binding. While an increased temperature effect facilitates oxygen unloading from hardhead hemoglobin at temperatures ≥ 25°C, the temperature-induced increase in P_{50} may limit oxygen binding at the gills, especially in hypoxic environments. Adult hardhead volitionally choose 19-21°C temperatures, in both field and laboratory studies, suggesting that hardhead behavior is an important factor in regulating metabolic rate as well as Hb-oxygen affinity in this species (Knight 1985; Klimley et. al. 2011).

Fish respond to changes in temperature and oxygen content, e.g., hypoxia, with a combination of mechanisms directed at increasing oxygen carrying capacity or changing Hb-O_2 affinity. The most commonly found mechanisms are the expression of multiple iso-Hb forms, with differential properties of oxygen binding and affinity, as well as the modification of Hb-O_2 affinity via allosteric modifiers, i.e., ATP and GTP (Greaney et. al. 1980; Albers, et. al. 1983; Rutjes et. al. 2007). The hardhead's toleration of hypoxia may be a function of the rapidity of environmental changes, particularly temperature, and how this species responds by modulating their blood-oxygen affinities via acute (e.g., [NTP]) or chronic (e.g., expression of Hb isoforms) adjustments. Because hardhead have higher HCT, [Hb], and correspondingly larger CBO_2 values (Table 19) than the Sacramento blackfish or northern pikeminnow, hardhead presumably benefit from a greater aerobic capacity (Cech et. al. 1979; 1994).

High aerobic capacity, which is linked to several factors (e.g., Φ, CBO_2s, [Hb], HCT), should increase the ability of hardhead to forage, avoid predators, access suitable spawning habitat, and persist during low- and high-flow during periods,. Hardhead are not recognized to be a highly migratory species but, like most other California-native stream fishes, are highly dispersive and rapidly re-colonize areas after periods of drought or after displacement from a flushing event (Moyle 2002). Hardhead inhabiting these mid- to low-gradient streams/rivers typically remain within a kilometer of their home range(s) although some extended (30-75 km) spawning migrations from reservoirs have been reported (Grant and Maslin 1997; Moyle 2002).

Relatively high (absolute value) Bohr factors are characteristic of active fishes, e.g., rainbow trout, *Oncorhynchus mykiss* (-0.57, Cameron 1971; -0.49, Tetens and Christensen 1987), tunas (skipjack, *Katsumonus pelammis*, -0.98, Bushnell and Brill 1991; yellowfin, *Thunnus albacares*, -

0.90, Bushnell and Brill (1991); kawakawa, *Euthynnus affinis*, -0.83, Jones et. al. 1986; albacore, *Thunnus alalunga*, -1.17, Cech et. al. 1984) and assist in unloading oxygen from the Hb at metabolically active tissue sites. Interestingly, hardhead Bohr factors were higher (mean: -0.825) than those found in rainbow trout and in the closely related northern pikeminnow (-0.70:18°C to -0.46:21°C, Cech et. al. 1994) suggesting that hardhead can sustain high aerobic activity. The hardhead CBO_2s (mean: 15.7 ml dl^{-1}) also exceeded those found in rainbow trout (8.9-9.8 ml dl^{-1}, Cameron 1971) and in the northern pikeminnow (10.7-13.0 ml dl^{-1}, Cech et. al 1994), although they were less than those of the very active albacore (21.8 ml dl^{-1}, Cech et. al. 1994). These Bohr factor and CBO_2 data, combined with hardhead's relative insensitivity to temperatures less than 25°C suggest that this species is suited for sustained aerobic activity over a range of environmental temperatures, dissolved oxygen concentrations, and instream flow regimes, especially at temperatures < 25°C. This is consistent with Myrick and Cech's (2000) findings that hardhead critical swimming velocity, Ucrit (mean: 0.52 m s^{-1}), did not vary significantly over a range of cool temperatures (10-20°C).

Fish respond to environmental hypoxia and hypercapnia with a number of behavioral and physiological mechanisms once low oxygen levels are detected. They may migrate to more favorable habitats, increase ventilation rate and volume, increase heart rate, increase erythrocyte density (e.g., via splenic contraction) resulting in a increase in HCT and Hb concentration as mechanisms to increase the oxygen carrying capacity of the blood (Weber and Jensen 1988; Perez et. al. 1995). Additionally, fish may modify the oxygen affinity of their Hb using allosteric modifiers (e.g., binding NTPs decreases oxygen affinity) or through the expression of hemoglobins which are temperature or pH insensitive (Perez et. al. 1995). Hardhead have high HCT, [Hb], and CBO_2s with relatively low NTP values (Table 20) suggesting that they could be classified as an "active" fish (Cameron and Davis, 1970; Weber and Wells, 1989; Perez et. al. 1995). Decreased NTP:Hb ratios may act to safeguard oxygen binding at the gills at elevated temperatures and/or during hypoxia. This mechanism may provide a benefit to a species, such as hardhead, in habitats that may be substantially modified (e.g., regarding oxygen content or temperature) on a seasonal or daily basis due to stream water retention or releases for future or current power generation (PG&E 1985, Brown and Moyle 1987, Brown and Moyle 1993, Frey et. al., 1998, Moyle 2002). Temperature acclimation in fish characteristically found inhabiting hypoxic waters show a similar downward adjustment to the NTP:Hb ratio as a mechanism to increase oxygen carrying capacity and delivery (Weber 1996 cited in Frey et al. 1998). Frey et al. (1998) found that mudfish (*Labeo capensis*) acclimated to hypoxia and elevated temperatures had lower NTP:Hb ratios that were not attributable to a modification of intrinsic NTP concentrations but rather due to an increased [Hb].

Hardhead display *in vitro* blood-oxygen equilibrium characteristics (e.g., mildly sigmoidal curve, high oxygen affinities, low bicarbonate buffer values, low to moderate temperature sensitivity) which seem to fit a species that evolved in California's mid- to low-elevation streams and rivers of moderate temperature variation and occasional hypoxic events. Our data suggest that at temperatures <25°C hardhead are capable of increased aerobic activity but additional research is needed to further examine the complex nature of hardhead physiology and behavior in response to environmental stimuli. Certainly, additional research is essential to

more firmly establish its physiological and behavioral responses to environmental stimuli. Until additional information is revealed, fisheries and water managers should exercise informed judgment regarding the timing and duration of water releases from instream reservoirs (e.g., as associated with hydro-electric power production). Such informed judgment should ensure that associated habitat changes will not adversely affect current hardhead populations by exceeding tolerable temperatures, dissolved oxygen levels, and water velocities for this species, or inadvertently enhance the survival, reproduction, and maintenance of introduced species (e.g., smallmouth bass, *Micropterus dolomieu*) as potential competitors or predators (Moyle 2002).

4.3 Conclusions

We found that hardhead can be kept in captivity for long periods of time between 11-25°C on commercially available diets, after a transition period involving supplementation with live foods. We were able to maintain hardhead in a healthy state once they had been treated for parasites and diseases that were present prior to capture. Overall, our data on hardhead indicate that they performed very well at moderate temperatures (ca. 16-21°C) in our experiments. In our thermal preference experiments, regardless of their thermal acclimation history, hardhead tended to prefer a mean water temperature of 19.4°C and clearly avoided temperatures above ca. 26°C. Hardhead's resting metabolic rates increased with increasing acclimation temperatures in both juveniles and adults and had a low to moderate thermal sensitivity. Adult hardhead's active metabolic rates ranged over 209-1342 mg O_2 kg^{-1} h^{-1} while swimming in the 660-l respirometer at velocities from 30 to 90 cm s^{-1}, providing an estimate of the species' maximal continuous rate of oxygen consumption (aerobic activity). In the critical thermal limits study hardhead had ecologically lethal responses in water above 29.7°C and below 7.4°C, not including acclimation acquired zones. Finally, adult hardhead had moderately sigmoidal blood-oxygen equilibria curves, high whole-blood oxygen affinities, high HCT, [Hb], and CBO_2 with relatively low NTP values (Table 18). This suggests that this species is suited for some sustained aerobic activity over a range of dissolved oxygen concentrations and instream flow regimes, especially at temperatures <25°C. However, high ΔH (absolute values) between 19°C and 25°C indicate an increased partial pressure of oxygen requirement in binding oxygen at the gills at temperatures >19°C, which may be unfavorable to hardhead's toleration of elevated instream temperatures, especially when combined with moderate environmental hypoxia. We emphasize caution interpreting these results for eggs or larvae of hardhead.

4.4 Recommendations

Future research, including a suite of physiological tests, would help enlighten scientists and environmental resources managers regarding hardhead's unique cold-water temperature responses. Chronic thermal limits studies would help bracket thermal polygons. Measuring the length of time for temperature acclimation (gained or lost) and determining the different proportions of hardhead muscle types, compared to sympatric species, would be useful. Swimming performance studies coupled with biochemical investigations would improve our understanding of the glycolytic capacity of juvenile versus adult hardhead. Finally, growth,

metabolic, and food consumption rate measurements at relevant temperatures would be critical to develop a bioenergetics model for hardhead and, possibly, other stream fishes.

Field studies of hardhead spawning and rearing habitat preferences and requirements would allow managers to target stream conditions and water management to accommodate and conserve hardhead, while minimizing unnecessary curtailment of other water uses. Cross-watershed, and longitudinal (elevation-based) studies of the current and potential distributions of hardhead, as affected by passage barriers, temperature, dissolved oxygen, disease, and interactions with non-native species (including smallmouth bass) would also assist conservation managers in making informed decisions about hardhead and their habitats.

4.5 Synthesis

The temperature preferences and tolerances of hardhead are moderate for California waters, and not unexpected given the common appearance of hardhead in mid-elevation streams and reservoirs in the Sierra Nevada, where their distributions overlap with trout species upstream and other minnow species downstream. Hardhead is a relatively thermally tolerant fish species, when compared to native trout species, but hardhead prefer cooler water temperatures than do Sacramento pikeminnow, blackfish, and splittail. Hardhead swimming capabilities are also more trout-like than those of other California native minnows that have been studied. Management of hardhead habitat should take into account the species' preferred temperature range, age distribution, acclimation state, and dissolved oxygen requirements. Furthermore, water managers should seek to minimize unseasonal flow and temperature fluctuations that may have particularly adverse effects on the swimming capabilities of juvenile hardhead.

CHAPTER 5: References

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6.0. Glossary

ANOVA analysis of variance

CABA Center for Aquatic Biology and Aquaculture

Ca. circa, or about

FL fork length

GPS global positioning system

Hb hemoglobin

HH hardhead minnow

LOE loss of equilibrium

MO_2 metabolic rate

N sample size

NTP nucleotide triphosphate

PIER Public Interest Energy Research

SE standard error

SL standard length

SMUD Sacramento Municipal Utility District

TBF tail beat frequency

TL total length

UC University of California

VIE visual implantable elastomer

APPENDIX A: Standard Operating Procedures for Hardhead Handling

Estimation of swim chamber volume via UV/Vis spectroscopy

The volume of our 5-L Loligo Systems swim chamber was estimated to be 4.95 L ± 0.01 SE, by diluting known volumes of a 3.13×10^{-5} M methylene blue solution into the chamber. The absorption spectra of the resulting solution was measured and compared to the best fit regression of the concentration versus absorption of a serial dilution.

The stock methylene blue solution (1.56×10^{-2} M) used throughout this experiment was prepared by dissolving 0.5006g of methylene blue powder (Sigma Chemical Co., Lot # 25F-35241) in a 100 mL ± 0.08 SE volumetric flask. 200 µL of the stock solution was diluted in a 100 mL ± 0.08 SE volumetric flask using a 200 µL Fisher Scientific pipetman. The resulting 3.13×10^{-5} M solution was used to generate a standard curve by plotting the absorption spectra of a serial dilution, measured using an Aquamate Spectrophotometer (Thermo-Electra Corporation), against the concentration of the dilution series. A best fit regression of concentration versus absorption spectra was plotted in Microsoft Excel™.

A 2000 µL Fisher Scientific pipetman was used to transfer 2000 µLs of the 3.13×10^{-5} M solution into the swim chamber via the oxygen probe port. The solution was mixed by running the swim chamber at 5 cm s^{-1} for approximately 2 min. A dummy oxygen probe was inserted to account for the volume that the probe would displace. A 2-3 mL sample was taken from the chamber using a disposable transfer pipette and analyzed using the spectrophotometer. 500 µLs of the 3.13×10^{-5} M solution was added and mixed as described above. This process was repeated until a total of 3500 µLs of 3.13×10^{-5} M solution had been added to the chamber (N = 4). The swim chamber was allowed to flush for > 1 week before repeating the measurements. A new serial dilution and standard curve were created using the 1.56×10^{-2} M stock solution for the second set of measurements.

We estimated to be 4.95 L ± 0.01 SE, by diluting known volumes of a 3.13×10^{-5} M methylene blue solution into the chamber, n=8 measurements (Table A1). Both linear regressions R-values were greater than 0.996 for data sets 1 and 2 (Figures A1 and A2).

Table A1. Results of the chamber volume determination via UV/Vis spectroscopy.

Set Number	Vol. 3.13×10^{-5} M solution added (L)	% ABS	Estimated Volume (L)

1	2.000×10^{-6}	0.446	4.99
1	2.500×10^{-6}	0.542	4.87
1	3.000×10^{-6}	0.62	4.96
1	3.500×10^{-6}	0.701	5
2	2.000×10^{-6}	0.445	4.98
2	2.500×10^{-6}	0.535	4.91
2	3.000×10^{-6}	0.627	4.86
2	3.500×10^{-6}	0.696	5.01

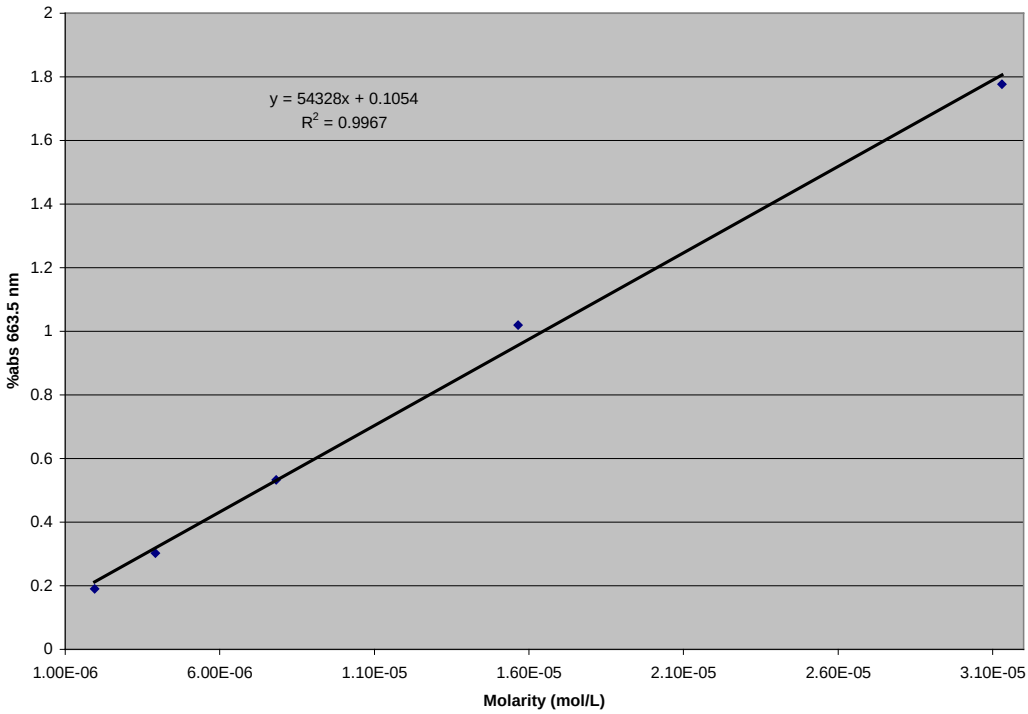

Figure A1: Standard curve for data set 1.

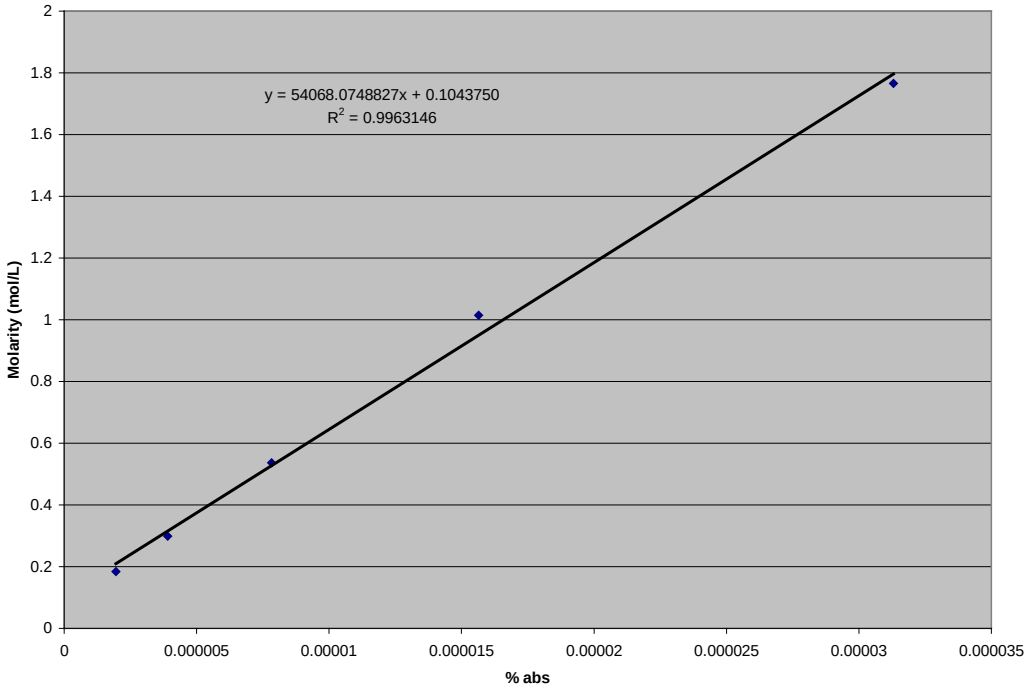

Figure A2: Standard curve for data set 2.

Results; Active metabolic rate of adult hardhead minnows acclimated to 11, 16, and 21°C and that swam in the 150-l (Table A2). The small sample size precluded statistical analysis and are present as a reference of how smaller adults or sub-adult perform compared to larger adult hardheads.

Table A2. Mean ±SE active metabolic rate (MO_2) of adult hardhead, for fish held at the 11, 16, and 21°C acclimation temperatures and tested in a 150-l Brett-style swimming respirometer, including the sample size (N), water velocity, mean mass (±SE), and mean fork length (FL ±SE).

							MO2 (mg O2 kg^{-1} h^{-1})				
Acclimation group	**N**	**Water Velocity (cm s^{-1})**	**Mass (kg)**	**SE**	**FL (cm)**	**SE**	**Mean**	**SE**	**Min**	**Max**	**Median**
11°C	1	25	0.211	-	27	-	193.2	-	-	-	-
11°C	1	35	0.211	-	27	-	138	-	-	-	-
11°C	2	45	0.221	0.01	26.5	0.50	342.1	148.86	193.2	490.9	342.1
11°C	3	55	0.322	0.006	29.1	0.33	305.5	114.92	155.6	531.4	229.5
11°C	5	65	0.402	0.073	31.5	2.19	310.1	48.65	186.5	483.1	293.9
11°C	3	75	0.425	0.009	32.2	3.00	310.6	28.32	259.3	357	315.5
11°C	1	85	0.533	-	35.5	-	206.5	-	-	-	-
16°C	3	25	0.267	0.032	25.8	1.17	193.4	43.19	125.5	273.6	181.1
16°C	4	35	0.318	0.056	27.9	2.20	134.5	19.13	93	183.6	130.7
16°C	4	45	0.318	0.056	27.9	2.20	188.8	36.23	101.8	252.5	200.5
16°C	3	55	0.335	0.075	29.2	2.52	288.7	18.26	252.5	311	302.5
16°C	1	65	0.212	-	25.5	-	458.4	-	-	-	-
21°C	2	25	0.367	0.01	31.5	0.50	193.3	5.9	187.4	199.2	193.3
21°C	2	35	0.367	0.01	31.5	0.50	169.6	88.2	81.4	257.8	169.6
21°C	1	45	0.377	-	31	-	258.3	-	-	-	-
21°C	1	55	0.377	-	31	-	425.4	-	-	-	-