THE EFFECTS OF CAPTURE BY TRAMMEL NETS ON NATIVE ARIZONA FISHES

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ABSTRACT

THE EFFECTS OF CAPTURE BY TRAMMEL NETS ON NATIVE FISHES

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Trammel nets are commonly used to sample fish; however, little is known about post-capture effects of this gear type on the fish captured. We conducted experiments to evaluate the effects of trammel net sampling on survival and cortisol levels of razorback sucker, *Xyrauchen texanus*, bonytail, *Gila elegans*, and roundtail chub, *Gila robusta*, at 15 °C, 20 °C, and 25 °C. Fish between 139 mm to 388 mm in total length (TL) were obtained from both captive hatchery stock and wild populations, quarantined for two weeks, and acclimated in two 18,000 L tanks for 13 days. Treatment fish were entangled in a trammel net for two hours and control fish were captured with a seine net. After capture, all fish were weighed, measured, PIT tagged. We extracted ~ 0.37 mL (between 0.1 mL and 0.5 mL) of blood from the caudal vasculature of 30-80% of fish in both the treatment and control groups. All fish were placed in a 36,000 L holding tank, where they were monitored for delayed mortality for 14 days.

Fish captured in the trammel net experienced up to 94% mortality within the first two weeks after capture, whereas seined fish experienced less than 24% mortality. There was little immediate net mortality for both control and treatment fish, which is consistent with field observations; however, up to two weeks after capture trammel netted fish were still dying. In warmer water temperatures, both bonytail and razorback suckers experienced significantly increased mortality rates ($p < 0.05$, Fisher's Exact Test); however, roundtail chub did not show this trend. Even higher levels of mortality may occur in nature, as any increased stress and/or

physical injury that fish incur during capture and handling may lead to a competitive disadvantage and impair foraging ability (Schreck et al. 1997, Wendelaar Bonga 1997). Post-capture mortality of wild fish may have gone undocumented thus far because of the time delay between capture and death. Our results suggest management agencies should re-examine the appropriateness of trammel net sampling for imperiled fish populations, especially when water temperatures are above 20 °C.

Although the magnitude of the cortisol response varied among species, cortisol levels were higher for fish captured by the trammel net than for fish captured by the seine net ($p < 0.05$). Fish captured at the highest temperature (25 °C) were more likely to have elevated cortisol levels than fish captured in the same way at lower temperatures ($p < 0.05$). Significantly more fish died after capture by trammel net ($p < 0.05$), and more fish died at the highest experimental temperatures: 20 °C and 25 °C ($p < 0.05$). For bonytail and razorback suckers, elevated cortisol levels were an effective predictor of mortality ($p < 0.05$); however, roundtail chub did not demonstrate a significant association between cortisol levels and subsequent mortality. These results suggest that cortisol could be used as a potential index of stress and post-capture mortality, at least for some species.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

Figure 2.4: Mean cortisol levels for razorback sucker at the three different temperatures (15 °C, 20 °C, and 25 °C) for fish entangled in a trammel net for two hours versus fish captured in a seine net. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

Figure 2.5: Mean cortisol levels for roundtail chub at the three different temperatures (15 °C, 20 °C, and 25 °C) for fish entangled in a trammel net for two hours versus fish captured in a seine net. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

Figure 2.6: Mean cortisol levels comparing the difference in mortality for treatment group (fish entangled in a trammel net for two hours), control group (fish captured in a seine net), and combined treatment and control. Asterisk denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

Figure 2.7: Logistic Regressions to compare cortisol levels from fish that lived to fish that died for each of the three species (bonytail (A), razorback suckers (B), and roundtail chub (C)). Cortisol appears to be a good marker for risk of mortality for bonytail (A) and razorback suckers (B), but not for roundtail chub (C).

CHAPTER 1

DLAYED MORTALITY RESULTS CAUSED BY CAPTURE OF NATIVE ARIZONA FISH IN TRAMMEL NETS

1. Introduction

Sampling and handling fish is critical for surveying fish populations, but sampling practices should, ideally, have minimal effects on the fish communities being studied. It is important because it allows biologists to obtain an accurate assessment of the population, without biasing the results or changing the population because of researcher investigation or error, Managers need to sample/monitor with little or no effect on the population being studied. Managers probably also observe that if the populations are rare and the monitoring program is killing the subjects being studied then the monitoring is actually doing harm and therefore should probably be discontinued (Rahel et al. 1999). Researchers often assume that fish handled during sampling survive and behave normally after they are released (Kelsch and Shields 1996), but non-lethal capture methods can cause injuries and even death (e.g., (Sharber and Carothers 1988); (Ruppert and Muth 1997); (Holliman et al. 2003); (Coggins et al. 2007) which may occur hours or even days after release because fish are unable to recover from physical injury or capture-induced stress (Barton 2002).

Repeated capture and handling (e.g., weighing, measuring, tagging) are necessary for many fish studies, such as mark-recapture estimates of population size, or studies of fish growth and movement. However, physiological responses of fish to sampling and handling vary with gear type, capture techniques and water temperature (Kelsch and Shields 1996). Many studies have examined the stress response in fish as it pertains to capture and handling (Stickney and

Kohler 1990, Kelsch and Shields 1996, Hosn et al. 2000). All aspects of sampling, including capture, handling, confinement, and time out of water, can cause reduced growth (Hosn et al. 2000, Barton et al. 1987), prevent spawning (Delahunty et al., 1979; Maule et al., 1996; Pickering et al., 1981), and reduce over all fitness. The effects of stress can be cumulative (Wedemeyer et al. 1990), and even standard handling procedures such as measuring and weighing fish may degrade condition and reduce growth (Paukert et al. 2001). Traumatized fish can exhibit abnormal physiological, behavioral, and ecological responses that defeat study purposes (Nickum 1988). Delayed mortality as a result of handling may occur hours, days, or even weeks after the handling occurred (Kelsch and Shields 1996).

Timing of sampling may influence population-level responses of fish because of seasonal patterns associated with spawning condition and the physiological responses associated with water temperature. Sampling during the spawning season may adversely affect the ability of sampled fish to reproduce (Pickering and Pottinger 1987, Pankhurst and Sharples 1992, Haddy and Pankhurst 1999, Patiño and Redding 2000). Sampling during spawning migrations is common in river fishes because fish are typically concentrated in smaller areas increasing capture rates. For example, humpback chub monitoring in the Little Colorado River, Arizona, includes sampling in spring and fall when fish are concentrated in the river preparing to spawn (Van Haverbeke 2003). Sampling of razorback suckers in the upper Colorado River basin commonly corresponds with migration of the fish to spawning areas. Although sampling during these times may be necessary to determine adequate population estimates and model population trends, the effects of capture and handling on these fish are unclear.

Fish are susceptible to temperature stressors; water temperature is one of the most important environmental factors affecting the physiology and behavior in fish (Kikuchi et al.

1995, Hirayama et al. 2003). Warm water temperatures reduce oxygen content in the water and this increases metabolic rate thereby increasing oxygen demand of the fish. Cold temperatures typically slow physiological function and can ultimately reduce stress caused to the fish (Davis 2006). In this experiment, we considered a temperature effect associated with mortality, three different temperatures were chosen that are commonly used by managers in natural habitats (15 °C, 20 °C, and 25 °C).

Trammel nets are commonly used for sampling rare fish species. A trammel net consists of three layers of netting with small mesh inner netting (2 inch mesh size) between two layers of larger mesh (24 inch mesh size). Fish swim through the larger mesh and hit the smaller mesh where they become entangled by creating a temporary pocket that prevents the fish from leaving the net. This gear type is a passive sampling technique used to entangle the fish without causing damage to the gills. Little is known about the effects of this gear on individual fish or populations. Observations of injured humpback chub, captured with trammel nets in the Colorado River, Grand Canyon, provides some anecdotal evidence that unexplained high adult mortality may be linked to sampling (VanHaverbeke 2008). These fish are observed struggling to exhaustion in the net and developing severe skin lesions which have led researchers to question the use of trammel nets as non-lethal sampling gear. This experiment was designed to assess the delayed mortality that trammel nets may cause.

Three species of Colorado River fishes; razorback sucker *Xyrauchen texanus*, bonytail *Gila elegans*, and roundtail chub *Gila robusta*, were used in our study because these species are commonly captured with trammel nets in the wild. Two of the three species are on the endangered species list bonytail and razorback suckers. Roundtail chub is not on the endangered species list, but was chosen because the populations are not very stable and it is the humpback

chub's, *Gila cypha*, closest relative (Douglas and Douglas 2007). Most of the work done for endangered species in Grand Canyon primarily focuses on humpback chub, the few fish found outside wild populations are not considered to be 10j species, so sufficient supplies of fish were not feasible for this experiment. In this experiment we hypothesized that both fish captured in a seine net (control) and fish captured in a trammel net (treatment) would have similar survival rates for all three species at all three temperatures.

2. Materials and Methods

2.1. Experimental Design:

The experiment was designed as a three-way block method to determine the post-capture and handling mortality that occurs with trammel nets. We compared cortisol levels and mortality among three species (razorback sucker, bonytail, and roundtail chub) captured by two methods (a seine net and a trammel net) at three temperatures (15 °C, 20 °C, and 25 °C). Sample sizes for each treatment are provided in Table 1. The three different temperatures used to conduct the experiment were chosen because they represent a range of temperatures these species naturally experience in the wild: 15 °C, 20 °C, and 25 °C. We looked at three different species of fish: razorback sucker, bonytail, and roundtail chub, chosen because managers are interested in determining the best way to monitor these impearled fish with out causing detrimental affect on the population. Nine experimental trials were conducted (a trial was conducted for each species at each temperature) and a total of 550 fish were used in these experiments.

2.2. Animal care:

Experimental trails were conducted at the USFS Rocky Mountain Research Station in Flagstaff, Arizona from June 1, 2007 to March 10, 2008; the study was conducted under the

auspices of the Northern Arizona University Institutional Animal Care and Use Committee (Protocol 07-001). We obtained bonytail (n=177) and razorback suckers (n=154) from hatchery stocks (Bubbling Ponds Hatchery operated by the Arizona Game and Fish Department, Achii Hanyo Fish Facility (located on the Colorado River Indian Tribe land) operated by the Bureau of Reclamation, and Wahweap state Fish Hatchery operated by the Utah Division of Wildlife Resources. Roundtail chub (n=206) were captured from the wild (Fossil Creek, Arizona) using hoop nets. All fish were between 139 mm and 388 mm TL and weighed between 26 g and 574 g (Table 1.1 and Figure 1.6). After collection, fish were quarantined to remove diseases and parasites. The quarantine treatment consisted of ten days in water with elevated salinity (3 ppt), followed by a one hour ProForm C (200 ptt) bath.

Fish were assigned to one of two tanks with an attempt to distribute equal size classes and equal numbers in each tank. The two tanks were 18,000 L rectangular, above-ground swimming pools (5.8 m long x 1.4 m tall x 2 m wide). Each tank was plumbed with a 1/2 hp high-efficiency, centrifugal pump, an Aquadyne 2.2 bubble-bead filter, and a UV filter. Water circulated though the filtration system at 60 gallons per minute (227 L/min). To ensure oxygen remained at or near saturation, each tank was supplied with supplemental aeration from air pumps with air diffusers. Measurements of dissolved oxygen at 25 °C confirmed that O_2 levels were at saturation. The tanks were stocked with 30 razorback suckers for one month to ensure that the biological filters, which involve bacteria and other microorganisms to convert toxic ammonia and nitrites from fish waste into nontoxic nitrates, had become established prior to experimentation. Fish were fed *ad libitum* once daily with a sinking pelleted feed. They were fed two types of food purchased from Aquatic Eco-system. The first consisted of a sinking koi feed

that contains a minimum of 38% protein, minimum of 10% fat and a maximum of 4.5% crude fiber, the second was a dense culture food which contains 43% protein.

2.3. Experimental Protocol:

One of two 18,000 L tanks was arbitrarily designated as the treatment tank and the other as the control tank. Treatment and control tanks were switched half way through the experiment to distribute any potential tank effect among treatments. All fish were held in these tanks for 13 days before trials to minimize stress during treatment, and to assist in getting the fish accustomed to their new environment. The temperature in the tanks was generally about the same as the temperature each experiment was preformed at, although sometimes fluctuated no more then 5 degrees C.

After the acclimatizing period (13 days), capture experiments were conducted. In the treatment tank, a trammel net was stretched across the middle of the tank and gently moved from side to side to entangle the fish. The trammel net used in this experiment was 22 m long and 1.8 m deep and consisted of two outer walls of 60 cm multifilament netting and one inner wall of 5 cm multifilament netting. The fish remained entangled in the net for two hours, from 6:00 pm to 8:00 pm for every trial. This time period was chosen to replicate field conditions; two hours is the standard deployment time for humpback chub sampling in the Colorado River, Grand Canyon (Valdez and Ryel 1995; Van Haverbeke 2003; Paukert 2004). Fish in the control tank were captured at the end of the entanglement period using a seine net. The seine used in this experiment was lightweight, knotless nylon with 1 cm mesh and a lead line with 2.3 kg/100 m lead weights on the bottom and a foam filled float line on the top. The seine was used as the control because it does not hold the fish for longer then a minute and is a quick and effective way of capturing all the fish from the tank. All fish were captured from the control tank in less than

15 minutes. Two crews worked simultaneously so that the capture of the last fish in the seine net was timed to coincide with removal of the treatment fish from the trammel net.

All fish were removed from the nets and handled according to standardized fish handling procedures for the Colorado River in Grand Canyon (Ward 2002). Under these procedures, fish were carefully removed from the net and placed into a "live well" (a 76 L rectangular tote with aeration). Once all fish were in the live well, fish were weighed, measured (total length and fork length), and injected with a passive integrated transponder (PIT) tag that allows for identification of individual fish. The crews working up fish captured by the trammel net and crews working up the fish captured by the seine net were switched after handling five fish to ensure there was no systematic bias in how fish were treated by individual handlers.

To facilitate a concurrent study of fish stress hormones, blood samples were taken immediately after handling from approximately half the fish collected from the control tank and half the fish collected from the treatment tank (Table 1.1). Fish for blood extractions were randomly chosen while making sure equal numbers of blood samples were taken from the treatment and control tanks. Details of this methodology and experiment can be found in chapter 2 of this thesis. The total time it took for each fish to be removed from the live well, weighed, measured, implanted with a PIT tag, and have blood drawn (if performed) averaged 1 min and 15 sec (Table 1.1, Figure 1.6 A).

Immediately after handling, the fish were released into a 36,000 L recovery/holding tank. The recovery/holding tank was equipped and prepared in a similar manner to the control and treatment tanks, although it possessed a larger filtration and plumbing system to accommodate the larger volume of water (1 HP pump and Aquameties UV filter, 7.5 g/m).

Fish were held in the recovery/holding tank and monitored for 14 days to quantify post-experiment mortality. During this two-week period, all dead fish were removed and PIT tag numbers were recorded. At the termination of the study (15 days after the last experimental trial and 276 days after the first experimental trial), all fish in the recovery/holding tank were captured with a seine net, weighed, measured (TL and FL), and scanned to record the PIT tag.

2.4. Statistical Analyses:

The Fisher's Exact Test was used to evaluate a linear association of mortality between the seine and the trammel nets at the three different temperatures. This test was chosen because it is used to analyze categorical data with small sample sizes. This test examines the significance of the association between two variables in a 2 x 2 contingency table, and the p-value is computed as if the margins are fixed. The Mantel-Haenszel test was designed to test the association between two variables using information from several 2 x 2 tables. In this experiment, we used it to measure the strength of association by estimating the common odds ratio of the three different temperatures, seine or trammel nets, and lived or died. It is a K x R x C test. A Kaplan-Meier Survival curve was performed to analyze percent probability of survival for treatment and control groups over the 14 days after the experiment took place. This test is used to estimate the survival function from life-time data. There were two post-hoc one-way analysis of variance (ANOVA) used for determining if the fish sizes and time out of water were different for any of the trials. All statistical tests were preformed using JMPIN Version 4.0 (SAS Institute), and the level of significance (α) for all tests was 0.05.

3. Results

The results from this experiment indicate that the survival rate of razorback suckers and bonytail decreases as temperature increases, and significantly more treatment fish (captured with a trammel net) died than control fish (captured with a seine net) at both the 20 °C and 25 °C temperatures ($p < 0.05$ Fisher's Exact test, Figures 1.1 and 1.2). Roundtail chub did not show significant differences between treatment and control mortality at any temperature (Figure 1.3). Only one roundtail chub died during the capture and handling process; all other mortality was delayed. Mortality typically occurred two to seven days after capture (Figure 1.4 and Figure 1.5).

Fish in both the treatment and the control groups experienced a decrease in mass over the course of the study (6/5/07 to 2/25/08). To obtain this data only the fish that lived were weighed when they were placed into the holding tank and re-weighed when they were pulled out, so the amount of days between weightings could have been anywhere from 276 to 15 days. To control for minor variation in size among groups, we evaluated weight loss in terms of percent change: treatment fish experienced a 3.8% weight loss and control fish experienced a 1.8% weight loss, but these two values are not statistically significant (t-test, $p = 0.38$). We examined the possibility that drawing blood for the cortisol study presented another source of mortality; however, no correlations were found between blood taken and mortality ($p > 0.05$, ANOVA, data pooled for all species and temperatures). Treatment fish died, on average, approximately two days earlier then the control fish, after all fish were released into the recovery tank ($p < 0.05$, ANOVA Figure 1.4). For fish that died, treatment fish died on average four days after capture, whereas control fish died on average six days after the capture. Treatment fish had a lower probability of survival than control fish for the fish that died within the first week after the experiment ($p < 0.05$, Kaplan-Meier Survival curve Figure 1.5).

Roundtail chub demonstrated a different pattern relative to the other two species; mortality did not decline as temperature increased, so we performed an ANOVA to determine if there was variation in length of time out of the water for control versus treatment fish or among species. We found that control fish were out of the water longer than treatment fish (Table 1.1 and Figure 1.6), but all species were out of the water about the same amount of time (ANOVA, F-value = 0.6463, $p > 0.05$, Table 1.1 and Figure 1.6). We examined size of fish among trials because the size of the fish may influence the effects of trammel net entanglement. We found that there was no significant difference in size among the trials for roundtail chub (ANOVA, $p > 0.05$).

4. Discussion

Our results indicate capture and handling of bonytail, razorback suckers, and roundtail chub under laboratory conditions causes delayed mortality days or even weeks after the sampling event. Capture of fish with trammel nets in water temperatures above 20 °C tended to increase mortality. The combined effects of capture in trammel nets and high temperatures led to very high mortality (for example, 94% for razorback suckers at 25 °C). Delayed mortality is difficult to measure in the field, but if it occurs at levels similar to what we obtained in the laboratory, this could represent a previously unrecognized threat to already diminished fish populations (Chopin et al. 1996). Furthermore, delayed effects of capture and handling may not result in mortality, but may result in whole-animal performance such as changes in growth, condition, metabolic scope for activity, and behavior (Wedemeyer and McLeay 1980; Wedemeyer et al. 1990). Handling stressors can interrupt gamete production by suppressing the reproductive hormones (Pickering and Pottinger 1987; Pickering 1993; Pankhurst and Dedualj 1994; Haddy and Pankhurst 1999).

These results present a major management consideration because many native species are sampled during spawning migrations when local densities of fish are highest.

It is possible that mortality in the field could exceed what we observed in the lab, as any physical damage and stress that fish incur during trammel net capture and handling may impair their foraging ability and overall resistance to diseases (Vander et al. 2004). Stress in fish often causes a reduction of food intake and reduced food intake could put fish at a competitive disadvantage (Schreck et al. 1997; Wendelaar and Bonga 1997). In addition to putting fish at a competitive disadvantage, reduced food intake and disease resistance could escalate to death in wild populations. The natural environment of these fishes may be more challenging than laboratory conditions.

However, the opposite could be true: the fishes may be more susceptible to fungal infection and mortality during the study. The densities of fish in the experimental tanks were relatively high compared to fish in the wild, although fish densities in our study were still much lower than rearing densities for these fish at hatcheries (Ward et al., 2007). Additionally, the controlled ambient temperature fluctuations were large which can increase stress to fish. It is possible that bacterial and fungal infections may have spread more quickly in our study than they would under natural conditions regardless of our UV filters. The fact that all fish lost weight during the study suggests that these fish were under quite a lot of stressed.

The cause of the delayed mortality seen in this study is not clear. We observed that if a fish died within the first two days, it was most likely do to trauma to the gills caused by the net. However, if it died 4 to 14 days after the trials occurred, fungal infection appeared to have been the cause of death.

Surprisingly, for roundtail chub, trammel netting did not increase mortality relative to seining as the temperature increased. This species did not show the same pattern as the other two species. Because all three species experienced the same amount of time out of the water, we conclude that experimental inequities in fish handling cannot account for this result (Figure 1.6 and Table 1.1). It is possible that roundtail chub have a different physiological response to temperature; their optimal temperature has a much larger range than bonytail and razorback suckers (Bulkley, Berry et al. 1981; Weitzel 2002). Because rountail chub are slightly different shaped, it is possible that they interacted with the trammel net in a manner that reduced physical damage and subsequent mortality at all temperatures. However, we note that this species was the only one of the three that was originally collected from the wild, and it is also possible that this had some, as yet undefined, effect on their reaction to the trammel net.

Undocumented mortality likely occurs after sampling in wild fish populations because of the time delay between capture and death. Yet, for imperiled fish populations, it is critical that sampling does not negatively impact already diminished populations. Based on our results, we suggest management agencies should re-examine the appropriateness of trammel net sampling for imperiled fish populations. They may try smaller inner mesh nets so damage is not incurred to the gills as easy, although it may then cause mortality in smaller fish. Managers should consider the potential impacts of the sampling when determining sampling frequencies, methods, gear types, seasons, and locations. We suggest that the seasonal timing and water temperature should be considered when planning sampling efforts for imperiled fish populations. For the southwest native fish examined in our study, sampling during the winter months would allow fish to be collected when water temperatures are cool, and would not interfere with spawning events.

CHAPTER 2

STRESS ASSOCIATED WITH CAPTURE OF ARIZONA NATIVE FISHES USING TRAMMELNETS AND ASSESSED BY CORTISOL

1. Introduction

Fish are often exposed to stressful situations in the wild as well as under culture conditions such as temperature fluctuation, water quality, capture and handling and many more. Capture and handling of fish for scientific studies is often necessary for measuring the size of populations, growth of individuals, and movement of fish. Biologists often assume that fish released after capture from the wild are minimally stressed and recover quickly and completely; however, mortality may occur immediately after release, days or even weeks after release because fish are unable to recover from physical injury or capture-induced stress (Barton 2002). The stress response from capture and handling depends on the duration and the magnitude of the sampling techniques (Barton 2002), and varies by fish species (Barton and Iwama 1991, Gamperl et al. 1994). The response of teleosts has been documented in a variety of species and is often measured using cortisol, the release of cortisol by the interrenal is a link between initial neuroendocrine perception of stress and the mobilization of energy (Vijayan et al., 1996, Barton, 2002). It is our aim in this paper to determine if cortisol can be used to indicate post capture mortality in bonytail, roundtail chub and razorback suckers after being captured with a trammel net and handled according to procedures commonly used in the Grand Canyon (Ward 2002).

Trammel nets are commonly used to capture endangered fish species because they are believed to be less harmful to fish than other capture gears. These nets are typically set anywhere from 1 to 24 hours, so fish can be entangled for relatively long periods of time; however, in the Grand Canyon net sets are limited to 2 hour net sets. Capture methods that involve physically

restraining fish for extended time periods, such as gill nets and trammel nets are known to cause stress to fish (Chopin et al. 1996); however, little information is available to quantify the post capture effects of trammel nets. Understanding these effects is critical when managing of fish populations, especially for rare species where mark-recapture estimators are used to quantify fish numbers. Capture methods that lead to delayed mortality or altered behavior that reduces capture probability can not only violate mark recapture assumptions and lead to significant bias in population estimates, but can further reduce population sizes in already threatened and endangered species.

Temperature appears to have a significant influence on the ability of a fish to withstand traumatic events, such as capture and handling (Strange et al., 1977). Additionally, water temperature is one of the most important environmental factors that affect the physiology and behavior in fish (Kikuchi et al. 1995, Hirayama et al. 2003). Acclimatization to changing water temperature requires significant physiological responses ranging from dormancy (in cold temperatures), to increased energy consumption, as well as direct effects of temperature on ligand binding, diffusion, and enzyme catalysis (Cossins and Bowler 1987). High temperatures are also detrimental to a fish's ability to withstand infections (Sha et al. 2008). Warm water temperatures reduce the ability of the water to hold oxygen while simultaneously increasing the metabolic rate and oxygen demand of the fish. In contrast, cold temperatures slow the activity of many physiological functions, which ultimately reduces the response of the stressor (Davis 2006).

The fishes physiological responses to stressors, such as capture and handling or environmental stressors such as temperature, have been broadly grouped into the primary, secondary, and tertiary response (Gamperl et al. 1994, Iwama 2004). The primary response for

fish involves the initial neuroendocrine responses, the production and release of stress hormones such as cortisol and catecholamines. The secondary response comprises the various biochemical and physiological effects associated with stress, which are mostly mediated by the stress hormones involved in the primary response. The biochemical and physiological changes are things like metabolic changes, osmoregulatory disturbances, changes in hematological features, and changes in the immune functions. The tertiary responses are also mediated by the previous two responses; it refers to aspects of whole-animal performance. Some examples categorized in this group are changes in growth, swimming capacity, disease resistance, and behavioral changes in feeding and aggression. These three response are considered to be an adaptive mechanism that allows fish to cope with real or perceived stressors in order to maintain its normal or homeostatic state (Randall and Perry 1992). Stress is not always detrimental, but if the intensity and duration is severe, enough adaptive responses may be compromised causing deterioration in the overall health of the fish and mortality (Barton 2002).

Cortisol is commonly used as an index of stress (Donaldson and Pickering 1981) or to track recovery from stressful events (Pickering 1993) because it is the most active and abundant stress hormone in fish blood plasma (Van Der Boon et al. 1991) and has a short lag time of only a few minutes, making it a good indicator of stress (Wedemeyer et al. 1990, Gamperl et al. 1994). This delay or lag time, for cortisol to be detected in the plasma of fish, is caused by the pathway in which cortisol is formed. This pathway begins in the HPI axis with the release of corticotrophin-releasing hormone (CRH), or factor (CRF), chiefly from the hypothalamus in the brain. This stimulates the corticotropic cells of the anterior pituitary to secrete adrenocorticotropin (ACTH) into the blood. This hormone stimulates the interrenal cells, which are imbedded in the head of the kidney to synthesize and release cortisol. Cortisol circulates in

the blood to target tissues which in turn cause secondary and tertiary responses to the perceived stressor. The purpose of this study was to determine plasma cortisol levels to monitor physiological changes caused by stress associated with capture in a trammel net and see if elevated levels of cortisol can be an indicator of mortality. We quantified blood cortisol levels and subsequent mortality of razorback sucker, *Xyrauchen texanus*, bonytail, *Gila elegans*, and roundtail chub, *Gila robusta* in the laboratory at 15 °C, 20 °C, and 25 °C. These water temperatures are similar to those normally experienced by these species during field sampling. All three of these species are commonly captured with trammel nets and are either endangered or candidates for listing.

2. Materials and Methods

2.1 Experimental Design:

The experiment was designed as a three-way block method to determine cortisol levels found in the plasma of fish after they have been captured in a trammel net or a seine net. We compared cortisol levels and mortality among 3 species (razorback sucker, *Xyrauchen texanus*, bonytail, *Gila elegans*, and roundtail chub, *Gila robusta*) captured by two methods (trammel nets and seine nets) at three temperatures (15 °C, 20 °C, and 25 °C). Samples sizes for each treatment are provided in Table 2.1. The three different temperatures, 15 °C, 20 °C, and 25 °C, used for this experiment were chosen because they represent a range of temperatures these species naturally experience in the wild. We looked at three different species of fish: razorback sucker, *Xyrauchen texanus*, bonytail, *Gila elegans*, and roundtail chub, *Gila robust* chosen because managers are interested in determining the best way to monitor these impearled fish with out causing detrimental affect on the population. Nine experimental trials were conducted (a trial

was conducted for each species at each temperature) and a total of 550 fish were used in these experiments.

2.2. Animal care:

Experimental trails were conducted at the USFS Rocky Mountain Research Station in Flagstaff, Arizona from June 1, 2007 to March 10, 2008; the study was conducted under the auspices of the Northern Arizona University Institutional Animal Care and Use Committee (Protocol 07-001). We obtained bonytail (n=177) and razorback suckers (n=154) from hatchery stocks (Bubbling Ponds Hatchery operated by the Arizona Game and Fish Department, Achii Hanyo Fish Facility (located on the Colorado River Indian Tribe land) operated by the Bureau of Reclamation, and Wahweap state Fish Hatchery operated by the Utah Division of Wildlife). Roundtail chub (n=206) were captured from the wild (Fossil Creek, Arizona) using hoop nets. All fish were between 139 mm and 388 mm TL and weighed between 26 g and 574 g (Table 1.1 and Figure 1.6). After collection, fish were quarantined to remove diseases and parasites. The quarantine treatment consisted of ten days in water with elevated salinity (3 ppt), followed by a one hour formalin bath (100 mg/L). Treatment began 13 days following quarantine.

Fish were assigned to one of two tanks with an attempt to distribute equal size classes and equal numbers in each tank. The two tanks were 18,000 L rectangular, above-ground swimming pools (5.8 m long x 1.4 m tall x 2 m wide). Each tank was plumbed with a 1/2 hp high-efficiency, centrifugal pump, an Aquadyne 2.2 bubble-bead filter, and a UV filter. Water circulated though the filtration system at 60 gallons per minute (227 L/min). To ensure oxygen remained at or near saturation, each tank was supplied with supplemental aeration from air pumps with air diffusers. Measurements of dissolved oxygen at 25°C confirmed that O_2 levels were at saturation. The tanks were stocked with 30 razorback suckers for one month to ensure

that the biological filters, which involve bacteria and other microorganisms to convert toxic ammonia and nitrites from fish waste into nontoxic nitrates, had become established prior to experimentation. Fish were fed *ad libitum* once daily with a sinking pelleted feed. They were fed two types of food purchased from Aquatic Eco-system. The first consisted of a sinking koi feed that contains a minimum of 38% protein, minimum of 10% fat and a maximum of 4.5% crude fiber, the second was a dense culture food which contains 43% protein.

2.3. Experimental Protocol:

One of two 18,000 L tanks was arbitrarily designated as the treatment tank and the other as the control tank. Treatment and control tanks were switched half way through the entire experiment to distribute any potential tank effect among treatments. All fish were held in these tanks for 13 days before trials to minimize stress during treatment, and to assist in getting the fish accustomed to their new environment. The temperature in these two tanks were generally about the same as the temperature of each experiment at the time it was preformed, although sometimes fluctuated no more then 5°C.

After the acclimatizing period (13 days), capture experiments were conducted. In the treatment tank, a trammel net was stretched across the middle of the tank and gently moved from side to side to entangle the fish. The trammel net used in this experiment was 22 m long and 1.8 m deep and consisted of two outer walls of 60 cm multifilament netting and one inner wall of 5 cm multifilament netting. The fish remained entangled in the net for two hours, from 6:00 pm to 8:00 pm for every trial. This time period was chosen to replicate field conditions; two hours is the standard deployment time for humpback chub sampling in the Colorado River, Grand Canyon (Valdez and Ryel 1995; Van Haverbeke 2003; Paukert 2004). Fish in the control tank were captured at the end of the entanglement period using a seine net. The seine used in this

experiment was lightweight, knotless nylon with 1 cm mesh and a lead line with 2.3 kg/100 m lead weights on the bottom and a foam filled float line on the top. The seine was used as the control because it does not hold the fish for longer then a minute and is a quick and effective way of capturing all the fish from the tank. All fish were captured from the control tank in less than 15 minutes. Two crews worked simultaneously so that the capture of the last fish in the seine net was timed to coincide with removal of the treatment fish from the trammel net.

All fish were removed from the nets and handled according to standardized fish handling procedures for the Colorado River in Grand Canyon (Ward 2002). Under these procedures, fish were carefully removed from the net and placed into a "live well" (a 76 L rectangular tote with aeration). Once all fish were in the live well, fish were weighed, measured (total length and fork length), and injected with a passive integrated transponder (PIT) tag that allows for identification of individual fish. "Treatment" and "control" crews switched after handling five fish to ensure there was no systematic bias in how fish were treated by individual handlers.

For the purpose of this study, immediately after handling, blood samples were taken from approximately half the fish collected from the control tank and half the fish collected from the treatment tank. The total time it took for each fish to be removed from the live well, weighed, measured, implanted with a PIT tag, and have blood drawn (if performed) averaged 1 min and 15 sec (Table 2.1). Fish for blood extractions were randomly chosen while making sure equal numbers of blood samples were taken from the treatment and control tanks. Blood samples were collected from the caudal vasculature into a heparinized 1 mL syringe (25 gauge, 5/8" needle) and placed into a 1.5 mL centrifuge tube. The centrifuge tube was spun at 10,000 rpm for ten minutes to separate the plasma from the red blood cells. The supernate (or plasma) was removed off the top with a pipette and stored in a labeled vial at -70 °C.

Immediately after handling, the fish were released into a 36,000 L recovery/holding tank. The recovery/holding tank was equipped and prepared in a similar manner to the control and treatment tanks, although it possessed a larger filtration and plumbing system to accommodate the larger volume of water (1 HP pump and Aquameties UV filter, 7.5 g/m).

Fish were held in the recovery/holding tank and monitored for 14 days to quantify post-experiment mortality. During this two-week period, all dead fish were removed and PIT tag numbers were recorded so that treatment group could be assigned. At the termination of the study (15 days after the last experimental trial and 276 days after the first experimental trial), all fish in the recovery/holding tank were captured with a seine net, weighed, and measured (TL and FL), and scanned to record the PIT tag.

2.4. Assay:

Plasma cortisol was measured using a 96 well enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, Michigan). Serial dilution of plasma ran parallel to the standard curve. Samples were diluted 1:200 with the provided assay buffer. This dilution allowed the plasma cortisol samples to be within the detection limits of the assay. All samples were measured in duplicate and every plate had 8 pooled samples; 4 wells randomly placed through the plate were from one treatment sample and 4 wells randomly placed through the plate were from one control sample. Intraassay coefficient of variation was 0.124, and the interassay coefficient of variation was 0.054. This assay was conducted according to the instructions provided with the kit and besides dilution, the plasma was unmodified. No purification processes were preformed. Cortisol was measured using a microplate reader with a wavelength filter of 405 nm.

2.5. Statistical Analysis:

The data was statistically analyzed using JMPin 4 to perform a three-way analysis of variance (ANOVA) in which the main effects were species, temperature, and net type. Species (bonytail, razorback suckers, and roundtail chub), temperature (15 °C, 20 °C, and 25 °C), and the net type were always treated as if they were fixed factors, whereas cortisol levels were treated as random factors. We preformed a three-way ANOVA following the guidelines presented in Sokal and Rohlf, the F-test for individual effects was computed as the mean square (Mommsen et al.) cortisol divided by MS residual. Both species x treatment, control and treatment, and control temperature two-way interaction terms were divided by the MS residual in order to determine the F-value. The two-way interaction terms were divided by the MS residual in order to determine the F-value. The p-value was set at < 0.05 for the purpose of this experiment. We used the Shapiro-Wilkes test to determine if the data was normal and the Levene's test to determine the homogeneity of variance. When necessary, the data was transformed using log transformations to meet the assumptions. Logistic regression was also used to determine if cortisol could be used as a predictor of mortality.

3. Results

To clarify the extent of differences in amplitudes of cortisol within species, temperature, and treatment group, we preformed a three-way ANOVA which revealed that cortisol in all three variable groups varied significantly (Table 2.2). The strongest effect was net effect; trammel netted fish showed much higher levels of cortisol than the seine netted fish (Table 2.2, Figure 2.1). There was also a significant effect among the three temperatures (15 °C, 20 °C, and 25 °C); as temperature increased so did cortisol. There was an effect among species, as well; roundtail

chub had the highest amount of cortisol (283.5 ± 14.4), then razorback suckers (227.9 ± 16.0), closely followed by bonytail (227.9 ± 16.1). Temperature also influenced species reaction to cortisol, but species did not influence treatment (Figure 2.2). The three-way interaction effect indicated that temperature, treatment, and species all influenced one another (Table 2.2).

Plasma cortisol levels for these three fish species ranged from 40.7ng/mL to 1207.6ng/mL. The mean cortisol levels for the trammel netted fish were 295.9ng/mL (± 16.7), while the mean cortisol levels for the fish captured with a seine net were 215.8ng/mL (± 10.1). Trammel netted fish had significantly higher cortisol levels than seine netted fish (Figure 2.1 and Table 2.3). The fish captured by the trammel net at 25°C had significantly higher cortisol levels than those captured in the treatment tank at 20°C and 15°C combined ($p < 0.05$, ANOVA). In fact, when trammel netted fish and seined netted fish were combined, fish captured at 25°C still had significantly higher cortisol levels than fish captured at 15 °C and 20 °C combined ($p < 0.05$, ANOVA).

Temperature seems to have an important role when it comes to capturing these three species in a trammel net. The cortisol levels for these three species were broken out and analyzed at the three different temperatures, 15 °C, 20 °C, and 25 °C. Bonytail (Figure 2.3) and razorback sucker (Figure 2.4) showed similar results; at the highest temperature cortisol levels were higher in the trammel netted fish than in the seined fish ($p < 0.05$, ANOVA). There was no statistical difference in cortisol levels between treatment fish and control fish at 20 °C ($p > 0.05$, ANOVA) for any of the three species. Roundtail chub, however, captured at 15 °C and 25 °C had significantly higher cortisol levels than fish captured at 20 °C. The only statistically significant difference in treatment and control for roundtail chub was also found at 15 °C.

The mortality data indicates that fish captured in the trammel net experienced up to 94% mortality within the first two weeks after capture, whereas seined fish experienced less than 24% mortality. There was little immediate net mortality for both control and treatment fish, which is consistent with field observations; however, up to two weeks after capture trammel netted fish were still dying. In warmer water temperatures, both bonytail and razorback suckers experienced significantly increased mortality rates ($p < 0.05$, Fisher's Exact Test); however, much like the cortisol results roundtail chub did not show the same trend when looking at the mortality results (Chapter 1 of this thesis). Only 20% of the control fish died, resulting in minimal data to detect the difference in cortisol levels of fish that lived versus fish that died in the control group.

When all fish were pooled, those that lived for the entire recovery period had lower cortisol levels than those that died. Further evaluation demonstrates that this result is due to the trammel netted fish with the highest cortisol levels having a higher mortality compared to those with lower cortisol levels. The seine netted fish showed no association between cortisol and mortality.

Cortisol appears to be a good marker for risk of mortality for bonytail and razorback suckers when all temperatures are pooled, but not for roundtail chub ($p < 0.05$, ANOVA and Logistic Regression, Figure 2.7). It also appears that cortisol is a good marker for risk of mortality at 25 °C, but not at 15 °C or 20 °C ($p < 0.05$, ANOVA and Logistic Regression). The fish that died within the first two days after handling contained statistically higher cortisol levels than the fish that lived beyond these two days ($p < 0.05$, ANOVA). The percent mortality occurring at the three different temperatures in both the treatment and the control groups were published separately and percent survivability decreased as temperature increased. We also examined the possibility that drawing blood for the cortisol study presented another source of

mortality; however, there was no correlation found between blood taken and mortality ($p > 0.05$, ANOVA).

4. Discussion

Our results indicate that there is a significantly higher primary physiological response of cortisol levels for fish captured in trammel nets than for fish captured in seine nets. In this experiment, roundtail chub exhibited higher amounts of cortisol than the other two species; however, cortisol levels vary widely among fish species (Barton and Iwama 1991, Gamperl et al. 1994, Barton 2002). Overall, razorback suckers, bonytail and roundtail chub exhibit relatively high amounts of cortisol compared to published studies on other fish species (Delahunty et al. 1980, Redding et al. 1984, Barton 2002) with the exception of striped bass, *Morone saxatilis*, (Mazik 1991).

Water temperature is one of the most important environmental factors that affect the physiology and behavior in fish (Kikuchi et al. 1995, Hirayama et al. 2003). Water temperature is known to affect the metabolic rate of fish as well as oxygen availability and demand. Optimum water temperature for a given species is also associated with a fish's ability to withstand infections (Sha et al. 2008). Thus temperature effects must be taken into account when considering the stress caused by a particular sampling and handing methodology.

We measured cortisol levels at three different water temperatures (15 °C, 20 °C, and 25 °C). Differences in cortisol levels between the seine netted and the trammel netted fish were seen at both the lowest and highest water temperatures tested, but no differences were observed between the seine and trammel net for any fish species at 20 °C. There is little information on the critical thermal maximum and minimum temperatures for these species; however, all three

species seem to live in similar temperature regimes ranging from 16 °C to 28 °C. (Bulkley et al. 1981, Weitzel 2002). Our results indicate that at or near the thermal optimum for these species (20 °C), the effects of trammel netting induce a lower cortisol response than at higher or lower temperatures.

Increased stress influences some tertiary stress responses, such as changes in growth, condition, and overall resistance to disease (Wedemeyer and McLeay 1980, Wedemeyer et al. 1990). All fish lost weight in this experiment; however fish captured in trammel nets lost twice as much weight as fish captured in the seine net. It also appeared that four to 14 days after the experiment almost all the mortality was caused by fungal infections (Chapter 1).

Cortisol levels of fish that died not only depended on treatment group and water temperature, but also among individual fish; some fish that had relatively high cortisol levels lived while other fish that had relatively low cortisol levels died. Cortisol appears to be an indicator of mortality for all the fish pooled in the treatment group (Figure 2.6), but does not seem to be an indicator of mortality for fish captured in the control group (Figure 2.6). Additionally, we observed that the treatment fish died sooner than the control fish which may indicate that the control fish deaths were caused by handling or fungal infections (Hunt et al. 2008 (in prep)). There is a clear relationship between cortisol and mortality for some species (Figure 2.7). Roundtail chub is also closely related to humpback chub, *Gila cypha*, (Douglas 2007) which are sampled in the Grand Canyon with trammel nets. Humpback chub could not be used for this experiment because sufficient supplies are not available from hatchery stocks; however, roundtails were collected from wild populations. This could be one of the reasons that roundtail chub did not show the same results as razorback suckers and bonytail.

Tables

Table 1.1 and 2.1: Number of fish for each experiment(# of fish), number of fish in each experiment that blood was drawn from (n = blood), the fraction of the total study group that died in each experimental treatment (% mortality), mean (± SE) handling time, mean (± SE) total length in millimeters (mm), mean (± SE) weight in grams (g), and total number of fish from each species.

Species	Temp	Tank	# of fish	n = blood	% mortality	mean seconds handling time	TL mm Mean	Mean weight g
Bonytail	15	Trammel net	24	11	8%	77 ± 7.2	235 ± 6.2	84 ± 11.3
		Seine net	24	11	9%	53 ± 7.3	246 ± 5.8	114 ± 11.6
	20	Trammel net	37	21	68%	78 ± 6.5	183 ± 5.1	134 ± 10.3
		Seine net	29	17	10%	59 ± 6.1	266 ± 6.3	144 ± 9.7
	25	Trammel net	26	11	88%	78 ± 6.0	254 ± 5.7	81 ± 9.7
		Seine net	37	14	21%	71 ± 5.9	224 ± 5.4	95 ± 9.3
Razorback	15	Trammel net	30	19	0%	84 ± 6.7	233 ± 5.7	233 ± 10.5
		Seine net	29	16	3%	50 ± 6.4	239 ± 5.8	240 ± 10.1
	20	Trammel net	19	6	26%	49 ± 6.7	179 ± 5	119 ± 10.5
		Seine net	30	15	3%	75 ± 6.3	240 ± 5.4	112 ± 10
	25	Trammel net	30	21	94%	77 ± 7.1	234 ± 5.6	142 ± 11.1
		Seine net	29	25	24%	97 ± 6.8	201 ± 5	145 ± 10.7
Roundtail	15	Trammel net	35	18	11%	80 ± 5.7	229 ± 5.4	61 ± 9
		Seine net	38	21	8%	59 ± 6.1	232 ± 6.1	122 ± 9.7
	20	Trammel net	32	15	44%	83 ± 5.6	174 ± 5.5	70 ± 8.8
		Seine net	44	14	8%	50 ± 5.5	238 ± 5.3	103 ± 8.7
	25	Trammel net	24	15	25%	76 ± 6.1	235 ± 5.9	65 ± 9.7
		Seine net	33	20	10%	65 ± 7.8	199 ± 6.5	116 ± 12.4

Table 2.2: Summary of F-tests for significance of effects in three-way ANOVA looking at the interactions of the treatment, temperature, and species. Asterisks denote significant differences.

	Effect						
Variable	Treatment df=1	Temp df=2	Species df=2	Treatment x Temp df=2	Treatment x Species df=2	temperature x Species df=4	Treatment x Temp x Species df=4
Cortisol	17.9641***	8.5134***	4.607*	4.8776**	0.875	10.4899***	2.4156*

*p<0.05; **p<0.01; ***p<0.001

Table 2.3: The mean (± SE) cortisol levels for the three species at the three different temperatures for both treatment and control groups. The bold values indicate statistical significance between the treatment and control ($p < 0.05$).

	15		20		25	
	Control	Treatment	Control	Treatment	Control	Treatment
Bonytail	**108.661 ± 43.441**	**108.204 ± 43.441**	265.229 ± 34.944	333.515 ± 31.440	**145.416 ± 38.506**	**334.383 ± 43.441**
Razorback	212.495 ± 36.019	182.776 ± 34.944	212.375 ± 37.200	224.584 ± 58.819	**179.683 ± 28.815**	**355.588 ± 31.440**
Roundtail	**232.240 ± 31.440**	**379.967 ± 33.959**	180.061 ± 38.506	145.401 ± 37.200	340.241 ± 32.216	422.938 ± 37.200

Figures

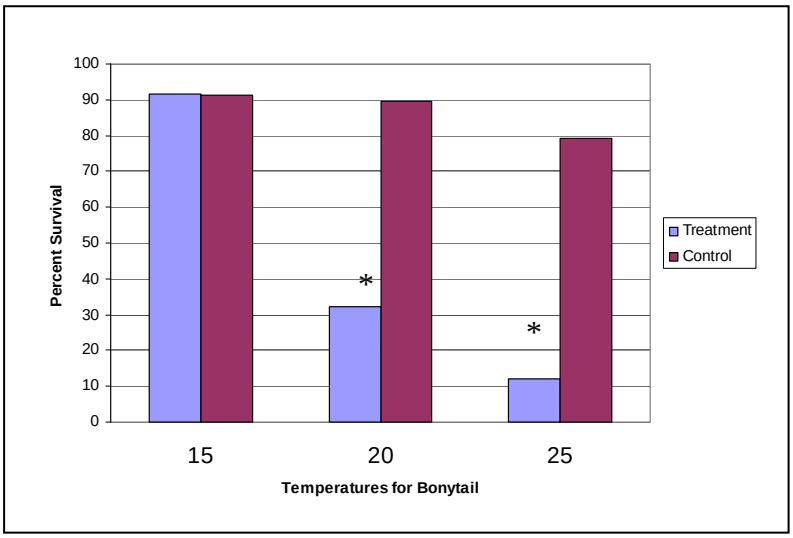


Figure 1.1: Percent survival of bonytail at 15 °C, 20 °C, and 25 °C for fish entangled in a trammel net for two hours (treatment) versus fish captured with a seine net (control). Asterisks denote significant differences ($p < 0.05$, Fisher's Exact Test).

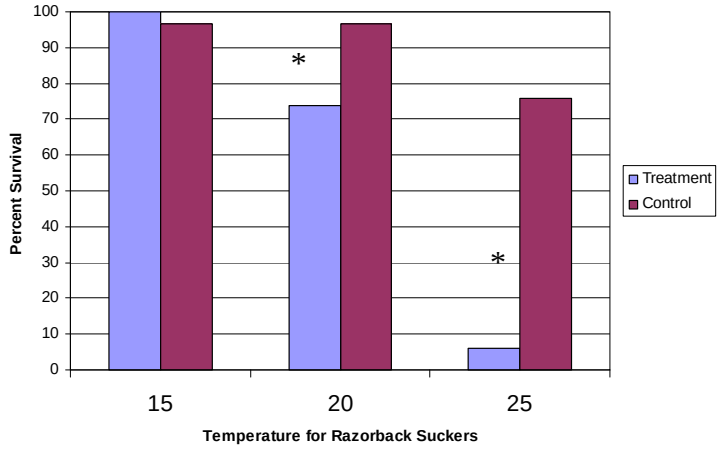


Figure 1.2: Percent survival of razorback suckers at 15 °C, 20 °C, and 25 °C for fish entangled in a trammel net for two hours (treatment) versus fish captured with a seine net (control). Asterisks denote significant differences ($p < 0.05$, Fisher's Exact test).

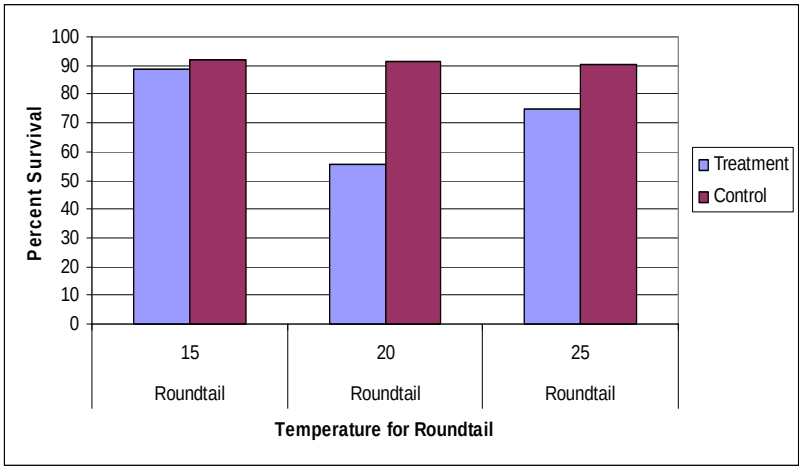


Figure 1.3: Percent survival of roundtail chub at 15 °C, 20 °C, and 25 °C for fish entangled in a trammel net for two hours (treatment) versus fish captured with a seine net (control). There were no significant differences found for these results ($p < 0.05$, Fisher's Exact test).

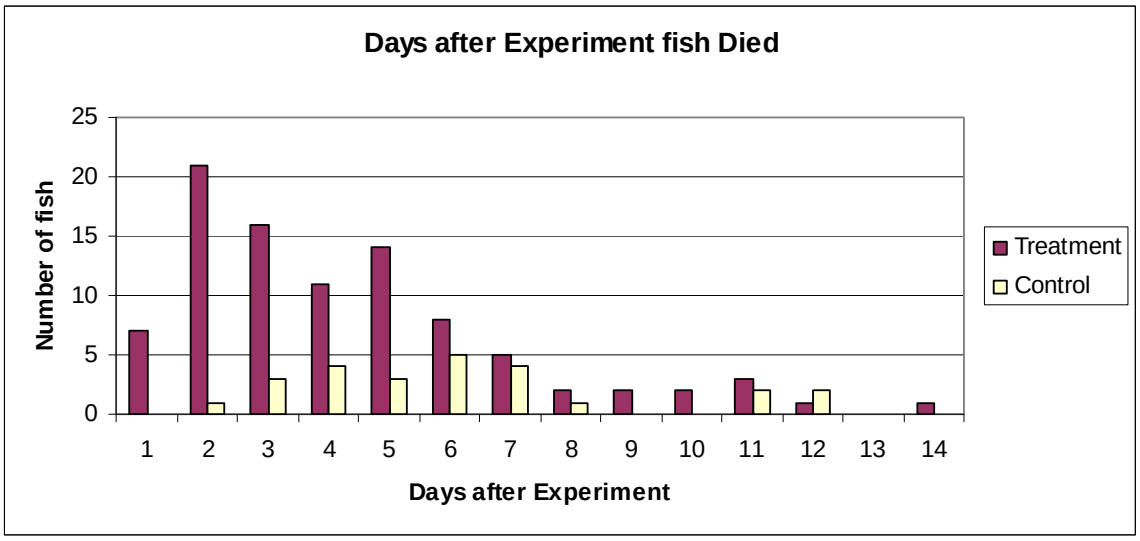


Figure 1.4: Number of bonytail, razorback suckers, and roundtail chub that died within 14 days after capture in either a trammel net or a seine net at all temperatures.

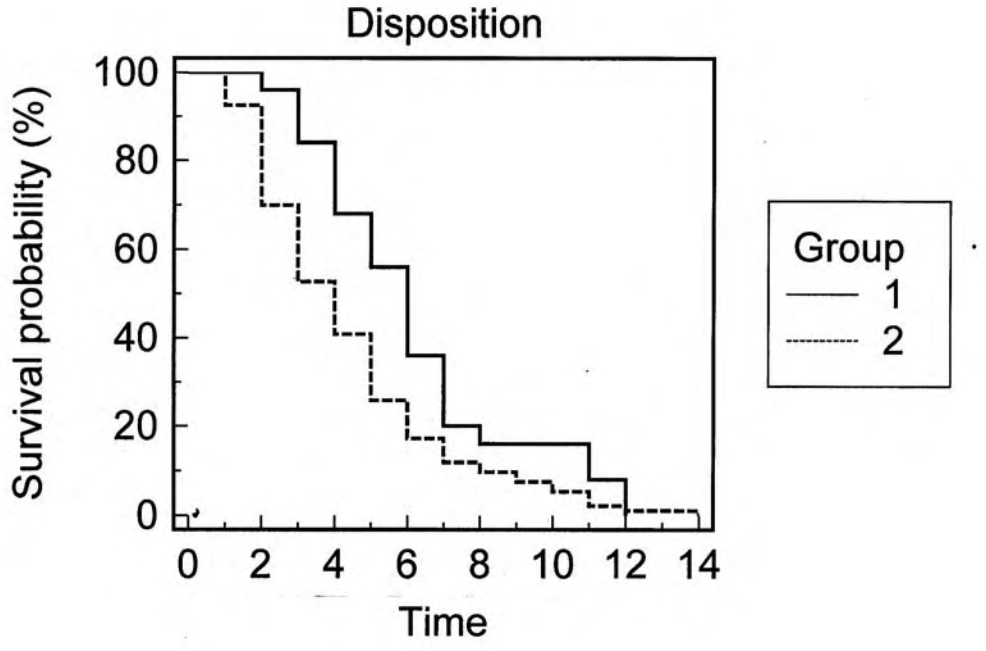
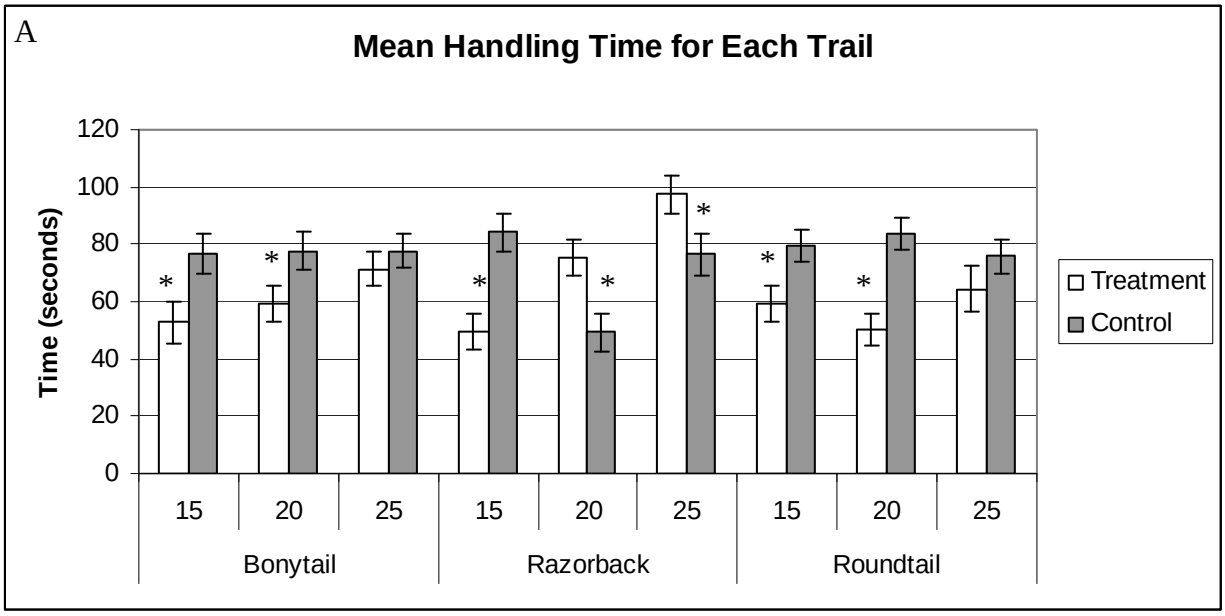
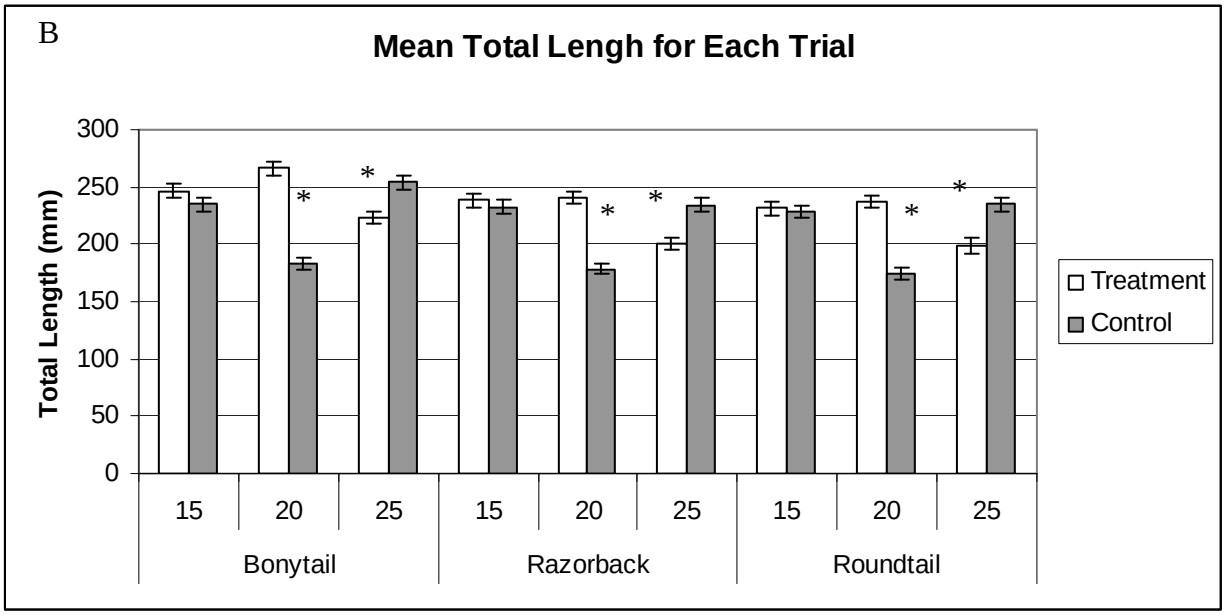


Figure 1.5: Survival probability (%) of fish between 0 and 14 days after capture with either a trammel net (group 1) or a seine net (group 2) (Kaplan-Meier Survival curve).

A


Mean Handling Time for Each Trail
Time (seconds)
120
100
80
60
40
20
0
15
20
25
Bonytail
Razorback
Roundtail
Treatment
Control

B


Mean Total Lengh for Each Trial
Total Length (mm)
300
250
200
150
100
50
0
15
20
25
Bonytail
Razorback
Roundtail
Treatment
Control

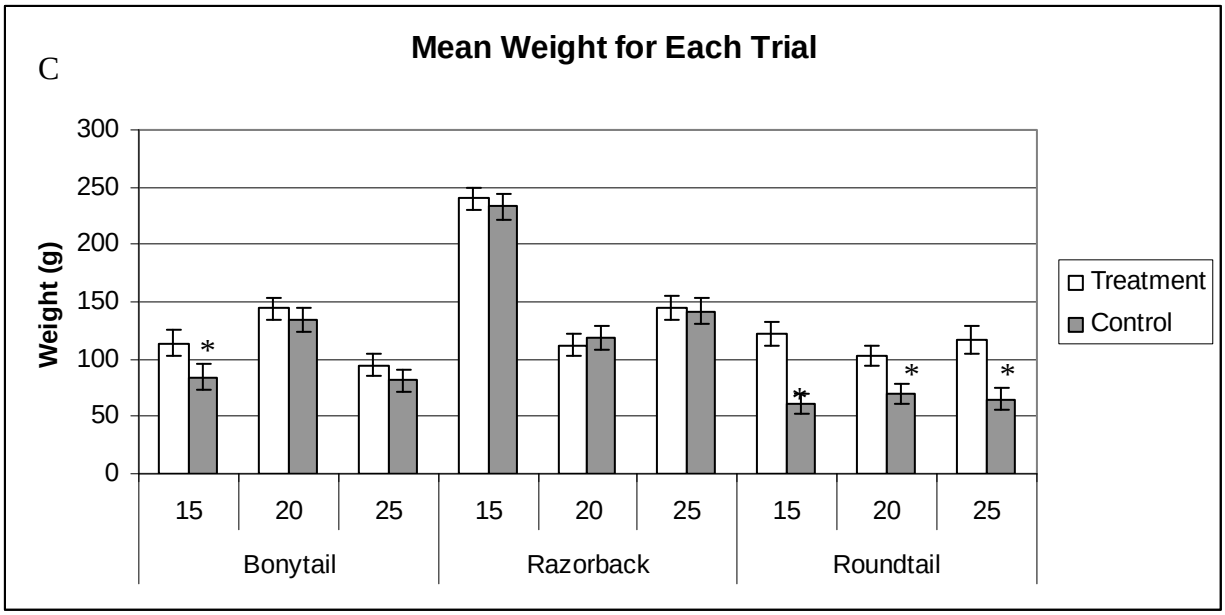


Figure 1.6: Mean handling time (A), total length (B), and weight (C) for bonytail, razorback suckers, and roundtail chub captured in either a trammel net or a seine net. Error bars represent standard error and asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

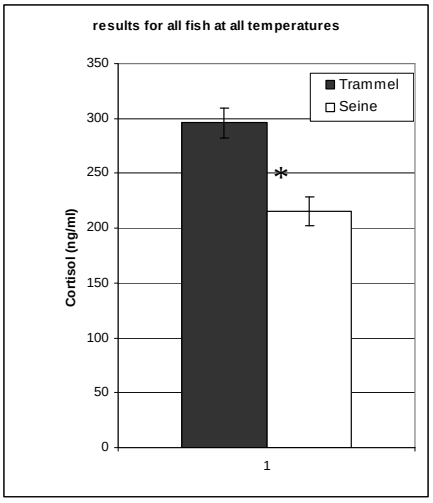


Figure 2.1: Mean cortisol amounts at all temperatures (15 °C, 20 °C, and 25 °C) for all the species pooled. Fish captured in a trammel net for two hours versus fish captured with a seine net. Asterisk denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

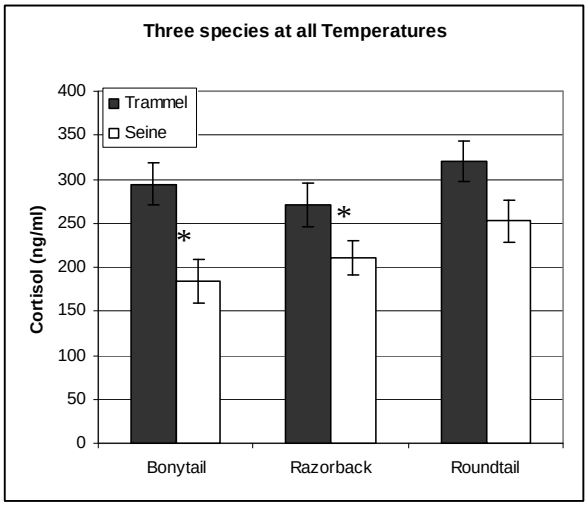


Figure 2.2: Mean amounts of cortisol for each of the three species entangled in a trammel net for two hours versus fish captured with a seine net, with all temperatures (15 °C, 20 °C, and 25 °C) pooled together. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

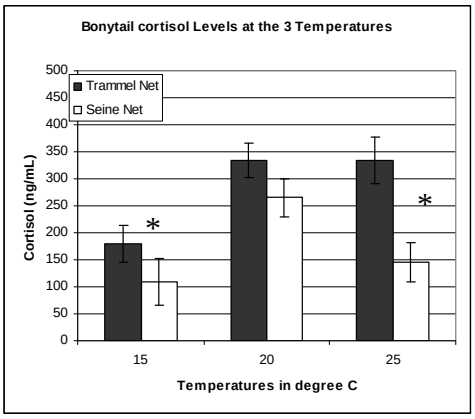


Figure 2.3: Mean cortisol levels for bonytail at the three different temperatures (15 °C, 20 °C, and 25 °C) for fish entangled in a trammel net for two hours versus fish captured in a seine net. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

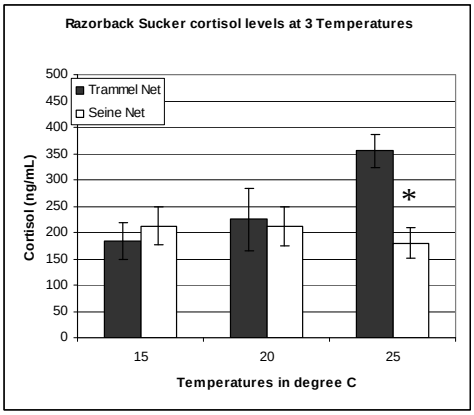


Figure 2.4: Mean cortisol levels for razorback sucker at the three different temperatures (15 °C, 20 °C, and 25 °C) for fish entangled in a trammel net for two hours versus fish captured in a seine net. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

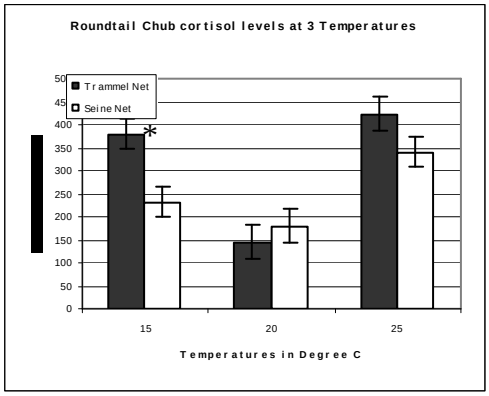


Figure 2.5: Mean cortisol levels for roundtail chub at the three different temperatures (15 °C, 20 °C, and 25 °C) for fish entangled in a trammel net for two hours versus fish captured in a seine net. Asterisks denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

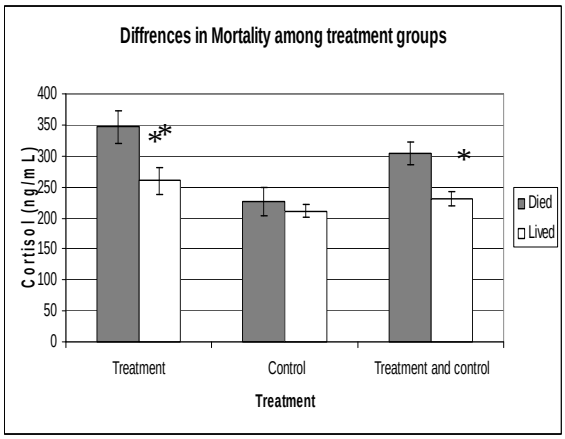


Figure 2.6: Mean cortisol levels comparing the difference in mortality for treatment group (fish entangled in a trammel net for two hours), control group (fish captured in a seine net), and combined treatment and control. Asterisk denote significant differences ($p < 0.05$, ANOVA), and error bars represent standard error.

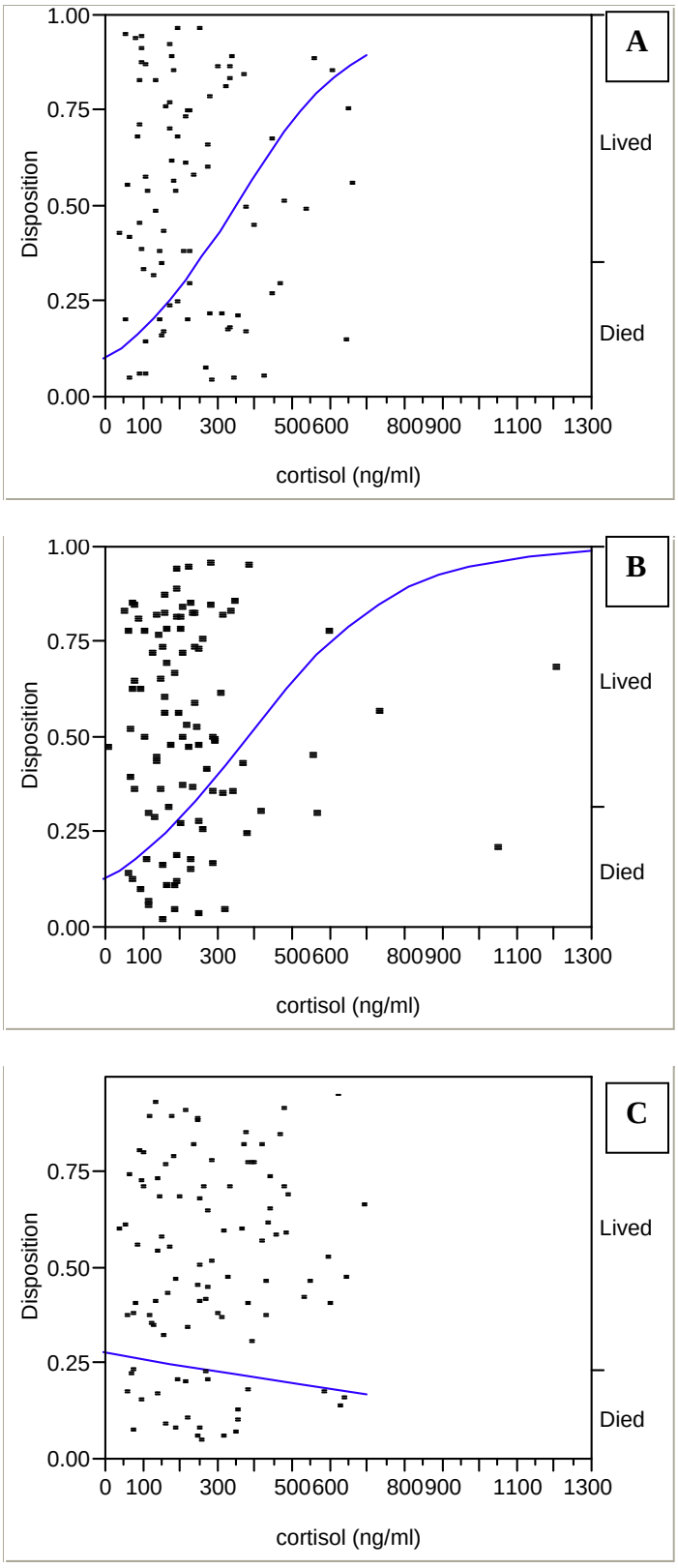


Figure 2.7: Logistic Regressions to compare cortisol levels from fish that lived to fish that died for each of the three species (bonytail (A), razorback suckers (B), and roundtail chub (C)).

Cortisol appears to be a good marker for risk of mortality for bonytail (A) and razorback suckers (B), but not for roundtail chub (C).

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