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The Combined Effects Of Copper Sulfate And Hypoxia Stress On The Swim Performance Of Bluegill Sunfish (lepomis Macrochirus)

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THE COMBINED EFFECTS OF COPPER SULFATE AND HYPOXIA STRESS ON THE SWIM PERFORMANCE OF BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*)

by

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This thesis, submitted by Dustin Anderson in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Dustin Anderson
October 10, 2014

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To my family: Whose overwhelming encouragement and support made this work possible.

ABSTRACT

Copper (Cu) is a component of several aquatic pesticides often used to target noxious algae, but at high concentrations may elicit toxic effects on non-target organisms such as fish. The severity of Cu toxicity varies widely by fish species, Cu concentration, duration of exposure, and water quality. Hypoxia is a widely occurring aquatic stressor that is also a potential side effect of Cu application due to increased oxygen demand from algae decay. Because Cu and hypoxia stress may occur in concert, it is important to understand and quantify their combined effects. Critical swimming speed (Ucrit) tests are an efficient and non-lethal means of quantifying the acute effects of environmental stressors on a fish's metabolic scope for activity or available energy. The change in swimming speed (ΔUcrit) as opposed to raw Ucrit of bluegill sunfish (*Lepomis macrochirus*) was measured in response to Cu and hypoxia stress in an attempt to remove variation in individual swimming ability. Using a variable speed swim chamber, ΔUcrit was measured in a 2x3x2 factorial design with Cu concentration (0 and 1 mg Cu/L), duration of Cu exposure (24, 48, and 96 hrs) and dissolved oxygen (DO) concentration (>8 (saturated) and 2 mg O_2/L (low)) as fixed factors. Hematocrit and gill sections were also analyzed. A significant, non-additive interaction between [Cu] and [DO] was observed. Duration of Cu exposure did not appear to be a substantial factor. At saturated DO, ΔUcrit was significantly greater in bluegill exposed to 1mg Cu/L. At low DO, ΔUcrit was significantly lower in bluegill exposed to 1mg Cu/L. Gill damage and increased hematocrit with exposure to Cu and hypoxia were also observed. Gill damage likely increased gill permeability that may have

resulted in ion efflux, causing hemoconcentration by plasma water displacement ultimately resulting in increased cardiac stress. Increased ΔUcrit at saturated DO may have been due to an antibiotic or limiting nutrient effect coupled with decreased ion efflux via decreased lamellar perfusion. Substantially more variation was left unexplained (31.7% vs. 79.1%) by measuring ΔUcrit rather than raw Ucrit which resulted in greater statistical resolution.

CHAPTER I

INTRODUCTION

Copper (Cu) is an essential metabolic micronutrient because of its reduction potential and ability to bind to gaseous substrates like oxygen (MacPherson & Murphy 2007). However, Cu levels in the aquatic environment that exceed basal levels are known to be toxic (Sorensen 1991, Heath 1995, Grosell 2012). One such way that Cu enters the aquatic realm is through the application of Cu-containing pesticides meant to control cyanobacteria and aquatic nuisance species. The application of Cu based pesticides may result in unintended toxic effects on non-target species.

Historically, Cu was used liberally to control algal populations in surface waters used for public water supply and recreation. For example, in 1982 the Fairmont chain of lakes in southern Minnesota was one of 330 lakes in a 4 state area (MN, WI, MI, IL) permitted to use copper sulfate ($CuSO_4$) to control algae. Over a 58 year period beginning in 1921, approximately 1.5 million kilograms of the pesticide was added to the chain of lakes causing drastic changes in the lake's biotic structure and chemical characteristics (Hanson & Stefan 1984).

Cu pesticides are used much more sparingly today because of our knowledge of their deleterious effects. However, Cu pesticides such as $CuSO_4$ and Cutrine© are not only still in use, but inexpensive, readily available to the general public, and more variable in their uses. Copper is known to be effective terrestrially in agriculture to treat various plant diseases and aquatically to control various algal species, cyanobacteria, freshwater snails (Eisler 1998, Cyrino de

Oliveira-Filho et al. 2004), zebra mussel veligers (*Dreissena polymorpha*, Kennedy et al. 2006), and submerged macrophytes (Mal et al. 2002).

Despite these potential benefits, Cu has been shown to be toxic to fish (Sorensen 1991, Heath 1995, Grosell 2012). The target of copper's toxic effects is the gill-water interface where it interferes with ion exchange, osmoregulation, and a fish's ability to acquire oxygen from the water. The toxicity of Cu is highly variable and influenced by its concentration, duration of exposure, and the chemical makeup of the target waters. In addition to the potential direct physiological challenges of Cu, fish populations must face potential indirect effects of Cu application.

Decreased oxygen availability (hypoxia) is a common indirect effect of Cu application. Hypoxia is an environmental stressor that temperate fish commonly encounter naturally through events such as thermal stratification and eutrophication. However, hypoxia can be exacerbated by human practices that increase the biological/chemical oxygen demand.

The resulting decaying mass of algae from a dosage of $CuSO_4$ can deplete oxygen from the water column. Moreover algae blooms typically occur in summer months during which higher water temperatures reduce oxygen solubility and minimize mixing due to thermal stratification. This situation can lead to stressful and potentially lethal conditions for fish. Because of the functional relationship between Cu application and hypoxia, one stressor may influence the magnitude of the second. Thus, it is important to study the effect of Cu and hypoxia in combination.

Copper toxicity studies are common (Sorensen 1991, Grosell 2012) but the impact of multiple stressors is less understood. The first aim of my study was to understand the combined, acute effects of copper sulfate and hypoxia on the scope for activity of bluegill sunfish (*Lepomis*

macrochirus). The bluegill is a suitable model species because it is abundant, wide-ranging and likely to coincide with sites of copper sulfate application. The bluegill is also well adapted to laboratory performance experiments.

Swim performance tests are an efficient means of measuring the impact that sub-lethal environmental stress has on the scope for activity of fish, but alone lacks the physiological mechanistic explanation for the change in scope for activity. Thus, along with swim performance I also examined hematology and gill histology in an attempt to explain the mode of Cu toxicity physiologically. These methods were used to test the hypothesis that the combined effects of sub-lethal Cu and hypoxia stress on bluegill scope for activity are additive and independent.

The second aim was to elucidate the effectiveness of a novel method to measuring swimming performance that attempts to decrease individual variation and increase statistical inference. I hypothesized that measuring the change in swimming speed in response to stress from a baseline measure of performance explains more variation than the currently accepted method of a single performance measure in response to stress.

CHAPTER II

BACKGROUND

Critical Swimming Speed

A fish's first line of defense against environmental stress is to relocate to a more favorable environment, which is a behavioral response highly dependent on swimming ability and accessibility to alternate conditions (Nelson et al. 2002). Additionally, a fish's ability to hunt, escape predators, and compete for mates is highly dependent on swimming ability (Plaut 2001). Thus swimming ability, or performance, has major implications related to fitness (Reidy et al. 2000; Nelson et al. 2002).

Beamish (1978) narrowed fish locomotion to three categories: sustained, burst, and prolonged. Sustained swimming relies exclusively on slow oxidative muscle fibers to propel a fish at a constant velocity for an extended period of time without fatigue (Webb, 1998). Sustained swimming is dominated by aerobic metabolism whereas burst swimming recruits fast glycolytic muscle fibers to generate the maximum velocity a fish can maintain for a short period of time. Burst swimming is most likely observed during predation or predator avoidance. The third category described by Beamish, prolonged swimming, requires the use of both aerobic and anaerobic metabolic processes and consists of periods of cruising and burst swimming resulting in exhaustion (Webb 1975).

Maximum critical swimming speed as described by Brett (1964) is a sub-type of the prolonged swimming locomotion category that is often used to estimate the maximum aerobic capacity of an individual. Critical swimming speed (CSS) tests are an efficient and non-lethal

method for determining the sub-lethal effect of a fish's integrated environment on its available energy (Plaut, 2001). Critical swimming speed tests encourage fish to swim against an increasing current of water until exhaustion, where the individual is no longer able to swim at pace. The point of exhaustion, typically resulting from the inability of the individual to meet oxygen demand in the tissues, is termed the maximum critical swimming speed or Ucrit (Plaut 2001). The suite of environmental stressors that impact an individual's aerobic capacity can thus be quantified by a change in Ucrit (Sprague 1971, Plaut 2001).

Biologists have used CSS and its several variations to quantify the aerobic swimming performance of fish under stress. However, there are several common variations in CSS tests that may limit the comparison of results between independent studies. The number of fish performing at a single time may vary, for example Deslauriers & Kieffer (2012) used groups of 5 shortnose sturgeon (*Acipenser brevirostrum*) at a single time. A more common variation in CSS tests is the interval and speed at which water velocity is increased. Burgetz et al. (1998) increased velocity in steps of 30-min by 0.25 to 0.5 body lengths/second whereas Jain et al. (1997) proposed a "ramp" increase in velocity where the velocity is ramped up at a fast pace until a percentage of Ucrit is attained (determined by previous experimentation) and then slowed until the subject fails.

The procedure for which the final metric is determined also varies by experiment. Methods that implement longer step increases in velocity (>10 minutes per increase in velocity) adjust the critical swimming speed by the amount of time elapsed during the last interval the fish failed (Brett 1964) and Jones et al. (2008) attempted to evaluate aerobic capacity exclusively by measuring the speed that bluegill were no longer able to maintain steady swimming.

Critical swimming speed tests not only include the variation of swim performance in response to environmental stressors but also individual variation. Critical swimming speed methodologies explain variation in length by normalizing Ucrit to body lengths per second. However, smaller individuals have been shown to have faster swimming speeds after normalization compared to larger individuals (Brett 1965) and this doesn't take into account the natural variation that exists within similarly sized individuals. In an attempt to account for individual variation in swimming performance to increase statistical inference, this study measured each individual's ability and corrected for it in the final metric, termed ΔUcrit.

Scope for Activity & its Factors

The maximum physical activity that an individual is able to perform is governed by the energy available to the individual. The energy available to an individual at a given time is not only reflective of its overall health and physical fitness, but also its integrated environment. The maximum scope (MS) for activity, or available energy, can be described as the difference between the active metabolic rate (AMR) and the standard metabolic rate (SMR) (Fry, 1947). The SMR, measured for a fasted and rested ectotherm, is the minimum metabolic rate that an organism is able to sustain normal physiologic function, whereas the AMR is the maximum aerobic activity an organism is able to sustain at ambient conditions (Priede, 1985). The optimum condition (in the case of figure 1, the temperature for maximum scope (TMS)) that allows for the greatest scope for activity is said to be the preferred temperature for that individual. Deviation from the preferred temperature will result in a decrease in metabolic scope (Fry, 1947). Because the CSS test requires the fish to put forth all of its available energy into swimming, the Ucrit can be used as a proxy for metabolic scope (e.g. Reidy et al. (2000) showed a significant positive correlation of Ucrit with scope for activity in Atlantic cod (*Gadus morhua*)). However, only the

directionality and amplitude of the environmental stressor can be deduced, leaving the mechanism largely undefined.

Fry (1947) proposed that all stressors acting on an individual could be grouped into six categories of factors: lethal, directive, controlling, limiting, accessory, and masking. These factors will serve to help explain how Cu and hypoxia affect the metabolic scope of bluegill and consequently, their swim performance. Lethal factors are conditions that arise when an individual is required to live outside of its lethal limits. In Figure 1, lethal conditions are those temperatures beyond where the AMR curve intersects the SMR curve (UL and LL). In this situation, an individual does not have the available energy to undergo obligatory maintenance processes and is unable to sustain its life. Directive factors elicit a behavioral or physiological response, for example a sight stimulus may require an individual to utilize energy to relocate to a different area.

Controlling factors govern the metabolic rate. A common controlling factor in fish is temperature. The internal temperature of poikilotherms closely follows that of the external ambient temperature. Therefore, metabolic reaction rates are controlled by the external temperature.

Limiting factors generally don't affect the SMR, only the AMR (Figure 1). A common limiting factor in fish is oxygen. When oxygen is limiting, activity is limited by the demands of systemic tissues. Accessory factors involve a stressor, that when combined with a stressful level of a controlling factor results in mortality. For example, if a fish is exposed to high temperature, proteins denature and metabolic processes are hindered. In this situation, protein denaturation (accessory factor) is causing the mortality rather than temperature (controlling factor).

Masking factors impact the regulation of additional processes and aren't the cause for the change in available energy per se. For example, exposure to Cu may inhibit a fish's ability to ionoregulate resulting in decreased swimming speed. The Cu presence alone is not eliciting the negative effect, but is rather masking the effect of inhibited ionoregulation on swim performance. The effects of metal toxicity on fish's metabolism likely fall into the categories of masking and limiting factors (Hammer 1995).

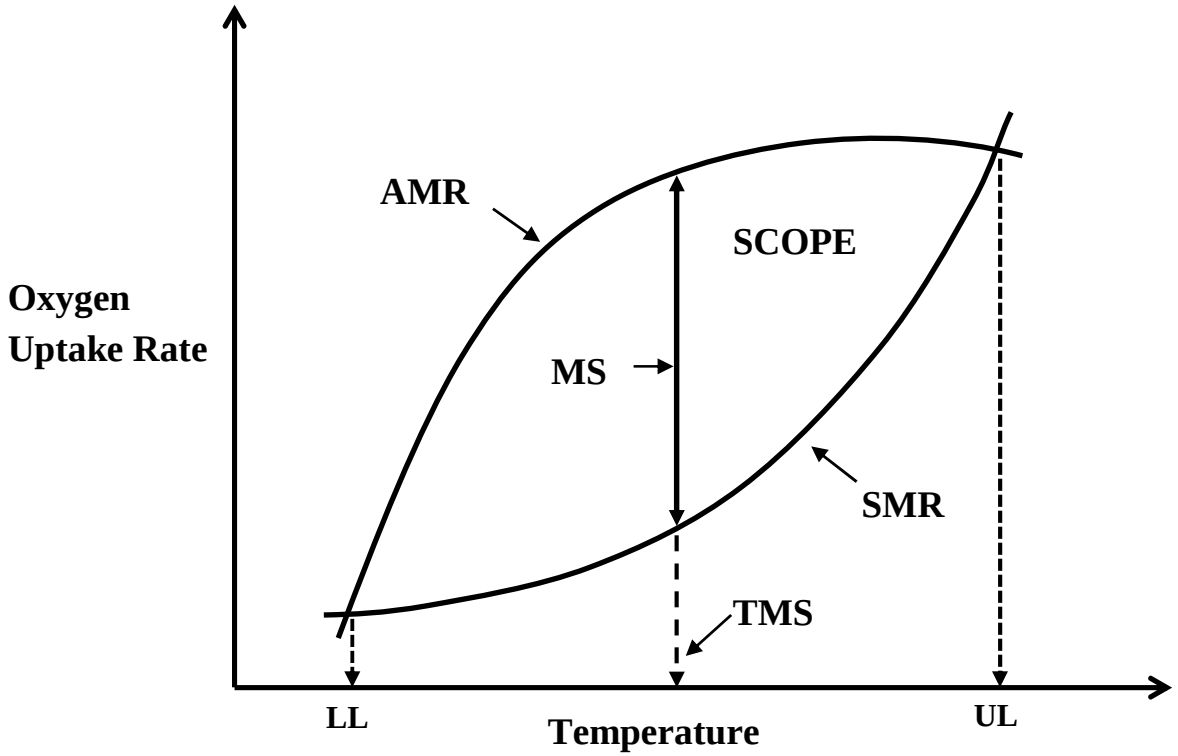

Figure 1. Scope for activity (SCOPE), as defined by Fry (1947), shown as the region bound below by the standard metabolic rate (SMR) and above by the active metabolic rate (AMR), each as a function of temperature. The greatest distance between these two curves is the maximum scope for activity (MS). The lowest and highest temperatures that result in the two curves intersecting (zero scope for activity) are respectively termed the lower (LL) and upper (UL) lethal limits. The temperature that allows for maximum scope (TMS) is predicted to be the preferred temperature (figure from Kelsch & Neill 1990).

Copper Toxicity

Several water quality parameters, including dissolved organic carbon, redox potential, alkalinity, hardness, and pH, have an influence on copper toxicity. Models have been generated in an attempt to explain the variability of metal toxicity in response to biological and abiotic factors with some success (Pagenkopf 1983; Playle et al. 1993; Allen & Janssen 2006). In addition to water quality parameters, toxicity varies by fish species. The 96-hr LC50 (lethal concentration for 50% of subjects after 96 hours of exposure) for rainbow trout (*Oncorhynchus mykiss*) and bluegill (*Lepomis macrochirus*) in similar water quality is estimated at 200 µg/L (Waiwood & Beamish 1978) and 4,300 µg/L (Dobbs et al. 1994) respectively. Concentrations as low as 4 µg/L have been shown to negatively affect some fish (Eisler 1998).

Water hardness (the concentration of calcium and magnesium, typically expressed as units of calcium carbonate) has a negative effect on Cu toxicity. This is thought to be a result of calcium (and to a lesser extent, magnesium) ions competing with Cu for cation binding sites on the gill surface. In soft water, Cu ions outcompete and displace calcium on the gill surface decreasing cell stability and increasing ion permeability (Laurén & McDonald 1987a, b).

The pH of water affects copper speciation. Generally speaking, metal toxicity is increased by low pH where the metal's free ionic form is favored (Pyle et al. 2000). Thus fish in waters low in pH and water hardness are more vulnerable to the toxic effects of copper. Waiwood & Beamish (1978) used critical swimming speed (CSS) tests to quantify the effects of copper, hardness, and pH on rainbow trout (*Oncorhynchus mykiss*). For trout, copper impacted swim performance most at low hardness and pH. According to the Environmental Protection Agency (EPA) product registration for copper sulfate ($CuSO_4$) crystals, acute non-target aquatic toxicity

may occur in soft (≤120 mg/L) waters with low pH (≤6.5), low dissolved organic carbon (30 mg/L), and low alkalinity (≤50 mg/L, EPA registration number: 56576-1).

Complexing agents, such as dissolved organic carbon, lower copper toxicity by rendering Cu biologically unavailable. The copper-organic complexes that can form in the water column coupled with the adsorption of copper ions to organic matter (humic and fulvic acids, soils) in the substrate can lead to a build-up of the metal in the sediment. Sediment cores in the Fairmont chain of lakes reached concentrations of 5,600 mg Cu/kg sediment in some areas (Hanson & Stefan 1984).

The toxic action of copper on fish begins at the gill surface (Mazon et al. 2002). Fish gills are a multifunctional organ largely responsible for gas exchange, nitrogenous waste excretion, and ion and water balance (Evans et al. 2005). Gills divert blood through a network of vessels that run along the primary filaments to several branching structures called secondary lamellae (Figure 13). Secondary lamellae are comprised of a thin epithelial layer of pavement cells that allow the blood to be oxygenated and diverted to the systemic tissues. The gill surface also contains chloride cells (beta), typically located in the inter-lamellar space of the gill filament. These specialized cells contain a high density of Na/K ATPase, a pump that helps maintain internal ion concentrations (Evans et al. 2005).

Cu uptake through the gills can occur in three ways: (1) Cu-specific ATP powered transporters, (2) sodium channels, (3) and direct diffusion via an electrochemical gradient (Grosell 2012). Once copper has bound to the gill surface, it can exert its toxic effects on site, or pass through the gill membrane and impair the fish internally (Simkiss & Taylor 1989; Playle et al. 1993). Sectioned gills of curimbatá (*Prochilodus scrofa)* exposed to the LC50 for 96 hours showed epithelial lifting, hypertrophy of chloride and pavements cells, hemorrhaging, and

lamellar fusion (Mazon et al. 2002; Cerqueira & Fernandes 2002). It took over a week to see gill tissue regeneration after transference to clean water and full recovery took more than 6 weeks (Cerqueira & Fernandes 2002).

Copper exposure can disrupt ionoregulation. Mozambique tilapia (*Oreochromis mossambicus*) exposed to 203 µg Cu/L for 14 days showed an increase in chloride cell numbers, albeit in favor of fewer mature cells and more necrotic and apoptotic cells with decreased Na/K ATPase activity (Dang et al. 2000). Ion disruption via gill flux has been observed in rainbow trout exposed to 55 µg Cu/L for 24 hours where maximum sodium uptake rate was decreased by 55% and whole body sodium concentration was reduced by 12.5% (Laurén & McDonald 1987b).

Fish have been shown to increase mucous generation in tissues exposed to Cu, including the gills (Sorensen 1991). The mucous increase is thought to protect tissues from the deleterious effects of Cu. However, increased mucous on the gill epithelia increases diffusion distance and reduces the ability of the individual to meet oxygen demands. In addition to mucous build-up, gill damage (e.g. hypertrophy) decreases oxygen exchange ability. Rainbow trout exposed to 105 µg Cu/L showed a significant increase in mean gill epithelial thickness from approximately 3.6 µm in control to 4.7 µm in Cu-exposed fish (Van Heerden et al. 2004).

The aforementioned changes to the gill epithelia may be to blame for changes to internal homeostasis. Mazon et al. (2002) showed that curimbatá exposed to 20-30 µg Cu/L for 4 days had an increased hematocrit, red blood cell count, and hemoglobin concentration. Waiwood (1980) also noted an increase in rainbow trout hematocrit with Cu concentration. Cu may be masking the effects of decreased oxygen sequestration ability leading to the increased hematological response.

Copper sulfate is an effective pesticide when used at the intended levels. However, a fine line may exist between effective concentrations for management and toxicity to fish. For example, Rowland et al. (2009) studied the effective concentration of $CuSO_4$ to control the disease ichthyophthiriasis (caused by the protozoan *Ichthyophthirius multifiliis*), better known simply as ich or white spot disease, in silver perch fingerlings (*Bidyanus bidyanus*). Exposure to 0.2 mg Cu/L as $CuSO_4$ allowed 100% survival of silver perch and eliminated 100% of the disease-causing protozoan responsible for the disease in 14 days. But a slight increase in concentration to 0.25 mg Cu/L showed silver perch survival slip to 82.5% after 14 days and exposure to 0.5 mg Cu/L showed perch survival slip to 10% after 14 days exposure (Rowland et al. 2009).

Although exposure to 0.2 mg Cu/L for two weeks did not kill any perch and completely cleared the fish of ich, the increase in mortality after a small increase in Cu concentration suggests that the effective concentration may be having a sub-lethal toxic effect on the perch. Cu has been shown to inhibit immune function at sub-lethal levels. Baker et al. (1983) showed that Chinook salmon (*Oncorhynchus tshawytscha*) were most susceptible to bacterial infection (*Vibrio anguillarum*) after acute exposure to Cu concentrations well below the 96-Hr LC50.

Hypoxia

The unique temperature-density relationship of water governs the movement and diffusion of gases such as oxygen throughout the water column. Thermal stratification coupled with benthic decomposition can cause chronic hypoxia or anoxia in the deep hypolimnion until a turnover event supplies it with oxygenated water (Diaz & Breitburg 2009). This potentially harsh aquatic environment can have long term and immediate negative impacts on a fish's ability to carry out essential activities such as foraging, reproduction, and predator avoidance.

Fish have several mechanisms to deal with environmental hypoxia. Some fish have been shown to relocate to more oxygen rich waters vertically in the water column or utilize the surface layer of water that maintains constant contact with the higher oxygen concentration of atmospheric air. If unable to relocate to more favorable areas, an alternative strategy is to decrease its oxygen demand by decreasing its overall activity (Kramer 1987).

Some fish may also increase their oxygen uptake efficiency in times of increased oxygen demand. Intimate contact between the blood and external environment is required to facilitate the diffusion of oxygen into the organism, but there is a trade-off of increased ion and osmotic imbalance due to the osmotic gradient that exists between a freshwater fish and its environment (Evans et al. 2005). To deal with this trade-off, fish are known to reduce lamellar perfusion at high oxygen partial pressures (Tuurala et al. 1984, Nilsson & Sundin 1998). When a fish is challenged with low oxygen such as during rigorous exercise or environmental hypoxia, it requires more contact with the source environment to meet its systemic tissue oxygen demand. This can be achieved by increasing gill perfusion via lamellar recruitment. Booth (1978) estimated that on average only 58% of secondary lamellae of rainbow trout were perfused during normoxic conditions at rest. In addition, Tuurala et al. (1984) showed that rainbow trout in well-oxygenated water can shunt significantly more blood via non-respiratory basal channels away from perfused lamellae compared to trout in hypoxia. Fish are also able to increase oxygen carrying capacity in response to decreased oxygen availability via rapid splenic release of erythrocytes (Yamamoto et al. 1980). Rainbow trout have been shown to release more than 70% of their stored splenic hemoglobin reserves within 15 minutes of forced exercise (Kita & Itazawa 1989).

Oxygen is crucial for the aerobic energy producing pathways in fish (Driedzic & Hochachka 1978). When a fish is exposed to hypoxia and oxygen is limiting, the mitochondria are unable to keep up with energy demands and the body is forced to undergo alternative ATP producing pathways that are not nearly as efficient (Richards 2009). A resulting lack of available ATP for crucial ATP consuming metabolic processes is thought to be the mechanism of death by hypoxia (Richards 2009). The decrease in energy output from non-lethal hypoxia exposure results in less energy available to the individual that it could put towards growth, reproduction, or disease resistance.

Bluegill *(Lepomis macrochirus)*

Bluegill sunfish (*Lepomis macrochirus*) are freshwater poikilotherms that have been the focus of several performance tests (Kelsch 1996; Jones et al. 2007; Kendall et al. 2007; Jones et al. 2008; Lim & Ellerby 2009; Ellerby & Gerry 2011). Bluegills are abundant in lakes and rivers and broadly distributed throughout the upper Midwest and much of the United States (Page & Burr 2011). In many aquatic ecosystems the bluegill is a major prey species of apex predators such as largemouth bass (*Micropterus salmoides*) and northern pike (*Esox lucius*), as well as a target of sport anglers, making bluegill a species of management and recreational focus.

Bluegill were chosen as the subjects for this study due to their obtainability, ease of husbandry, and use in previous swim performance tests. Copper toxicity studies specific to bluegill have shown decreased prey consumption (Sandheinrich & Atchison 1989), decreased growth and inhibited spawning (Benoit 1975), and increased plasma glucose (Heath 1991).

Metabolic compensation to Cu exposure has been shown for bluegill. O'Hara (1971) exposed bluegill to sub-lethal Cu concentrations (0.5 – 3.0 mg/L) which resulted in increased standard metabolic rate with Cu concentration. However, after the initial 24-hr exposure period,

rates decreased to and subsequently fell below the basal metabolic rate. Metabolic rates remained depressed below the basal until transference to clean water, where rates began to increase to normal (O'Hara 1971). I have also shown decreased metabolic rate of bluegill exposed to Cu compared to controls, although no statistically significant decrease was observed (Anderson 2013, unpublished).

Bluegill critical swimming speed has also been shown to decline in response to hypoxic stress. A drop in dissolved oxygen (DO) concentration from near saturation (>8 mg/L) to 4 mg/L did not significantly reduce Ucrit, but there was a significant reduction at an oxygen concentration of 2 mg/L (Anderson 2012, unpublished data). These findings are in agreement with the U.S. Fish and Wildlife Service habitat suitability models that suggest bluegill prefer DO concentrations >5 mg/l and avoid concentrations <3 mg/l (Stuber et al. 1982).

Objectives

Understanding and quantifying the metabolic cost of various stressors is important to directing future management efforts and minimizing mortality. If metabolic scope is already depressed, the addition of seemingly sub-lethal stressors could result in mortality if such stressors are additive or interactive. The potential of mortality at the effective concentration for $CuSO_4$ is increased if individuals are challenged with an additional stressor; for example, hypoxia as a result of a decomposing algal mass from Cu exposure. The objectives of this study were to:

(1) Elucidate the interaction of hypoxia and Cu stress on bluegill scope for activity. Critical swimming speed tests were used with a 3x2x2 factorial ANOVA design, using duration of Cu exposure (24, 48, 96 hrs), presence/absence of Cu (0 and 1 mg Cu/L as $CuSO_4$), and hypoxic stress (saturated (>8 mg/L) and 2 mg/L dissolved O_2)

as the respective fixed treatment factors. I hypothesized that the combined effects of Cu and hypoxia were additive, meaning their combined effects are equal to the sum of their individual effects. In an attempt to physiologically explain the toxic effects of Cu on bluegill, hematocrit was measured to detect any disruption of ion balance and gill sections were stained to observe any morphological damage in response to Cu exposure.

(2) Determine the effectiveness of a novel method of measuring critical swimming speed at removing variation in individual swim performance. By measuring each individual fish's baseline swim performance and correcting for it after exposure to the stressors, the statistical power and resolution of the CSS test may be increased. I hypothesized that less variation will be left unexplained by measuring change in swimming speed (ΔUcrit).

CHAPTER III

METHODS

Fish Husbandry

Two one-hundred count batches of young of the year bluegill (*Lepomis macrochirus*) were obtained from 10,000 Lakes Aquaculture, Inc. in Osakis, Minnesota, and transported to Starcher Hall at the University of North Dakota (UND) in Grand Forks, ND. The first batch was obtained in August 2011 and the second batch was obtained in October 2012. The bluegill hatchery populations were certified to be free of viral hemorrhagic septicemia (VHS) and heterosporis, and may have been exposed to previous treatments of Cutrine©, a Cu-containing pesticide. Subjects were housed in 200-L fiberglass aquaria containing aerated UND tap water treated for chloramines and fed Hikari Cichlid Staple fish food daily to satiation on a 12-hr light:dark cycle.

A flow-through aquarium system pumped water from a 1000-L main reservoir to each individual 200-L aquarium. The 200-L aquaria contained bottom-draw center standpipes that gravity drained to a communal tank where water was elevated via a sump pump back to the main reservoir. The communal tank contained several hundred individual plastic artificial substrate cylinders to provide surface area for bacteria to mediate ammonia and nitrate levels. Water from the main reservoir was also pumped through 3 particulate filters, one pH buffering filter, and one ultraviolet filter. The temperature, hardness, pH, and conductivity of the aquarium water are shown in Table 1.

Tagging Procedure

Subjects were allowed to acclimatize to aquarium conditions for a minimum of 10 days before being tagged to track individual swim performances. Food was withheld 24 hours prior to surgery. Individuals shorter than 80 mm were excluded from the experiment to prevent potential confounding effects from tagging. Implanted tags with a unique code allowed for the identification of individual fish. Prior to implantation, all equipment, tags, and implanters were sterilized in 99% ethanol for 10 minutes and washed with a 1% saline solution. Fish were anaesthetized with a 100 mg/L solution of tricaine methanesulfonate (MS-222) until muscle movement subsided and ventilation had ceased. The injection site was then dried with gauze and sterilized with iodine. A passive integrated transponder (PIT, Northwest Marine Technology, Inc.) tag, 12mm x 2.1 mm, was injected into the body cavity using a 12-gauge implanter syringe. The insertion site was ventral, immediately anterior to the anal vent (Kaemingk et al. 2011, Figure 2). The fish was then revived by flushing water through the mouth and over the gills until muscle movement and ventilation returned. Post-surgery bluegills were observed for 1 hour in a separate holding tank to ensure tag retention and normal recovery prior to being returned to the aquarium. Feeding resumed 24 hours after surgery and tagged fish were allowed a minimum of 10 days to recover before becoming eligible for swim performance tests.

Swim Chamber

An automated, variable-speed swim chamber, developed by Kelsch (1996) was used to measure critical swimming speed and estimate available energy (Figure 3). Attached to the swim chamber was a 500-L main reservoir outfit with nitrogen gas diffusers to control dissolved oxygen (DO) concentration and a Goldline® Model SP-322 heat pump controller with a temperature set-point ranging from -32˚C to +32˚C. Via a propeller, a Dayton® Permanent

Magnet 90-V DC motor forced water from the main reservoir through a 20-cm diameter PVC pipe, through the 20- x 20- x 30-cm designated swimming area and back into the reservoir. Fish were confined to the swim chamber by a mesh screen upstream and a mesh funnel downstream that leads to a servomotor-controlled gate leading to the reservoir. Collimators positioned upstream from the swim chamber promoted laminar flow and decreased turbulence (Figure 3).

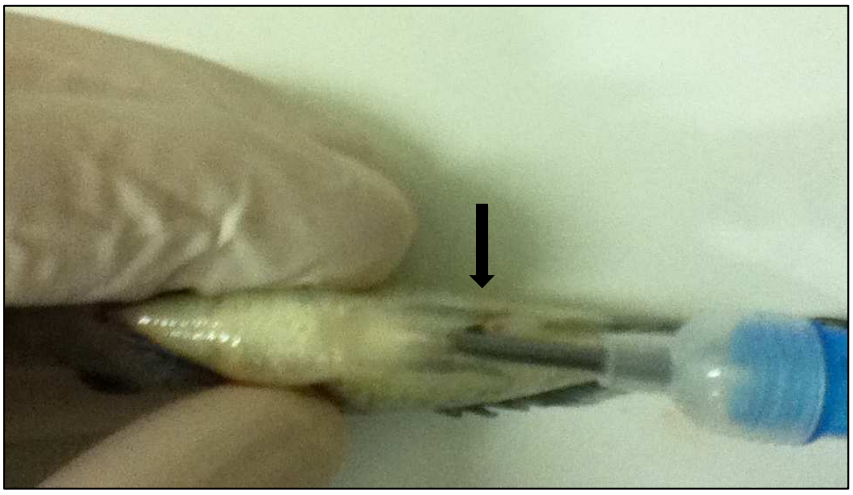

Figure 2. Site of PIT tag insertion. The tag (12mm x 2.1 mm) was injected immediately anterior to the anal vent (black arrow) towards the head of the bluegill.

Subjects were only exposed to hypoxia in the swim chamber. Dissolved oxygen was lowered by forcing pure nitrogen gas through the fine bubbler diffusers located at the bottom of the main reservoir. The flow of nitrogen was controlled with a regulator/flowmeter combination valve from a 300-L compressed nitrogen cylinder and adjusted based on the DO readings from a YSI Incorporated Model 57 DO meter.

The automated swim chamber apparatus was controlled and monitored by a computer program using LabVIEW version 7.0 (National Instruments Corporation, 2003). LabVIEW controlled the rate of increase in water velocity and recorded water velocity, dissolved oxygen

(DO), temperature, the number of times post-chamber barriers were crossed, and time of failure of each individual test subject. LabVIEW controlled and recorded these variables using a National Instruments PCI-6024E data acquisition (DAQ) card. Signals from instruments (e.g. change in voltage) were relayed through a printed circuit board (Advanced Circuits, Inc.) where it was converted into a signal compatible with LabVIEW and translated into a figure that was recorded in a spreadsheet.

During a swim performance trial, water velocity was increased linearly over time. LabVIEW increased water velocity by sending an analog signal via the DAQ board to a Hewitt-Packard 6129C digital voltage source which amplified the signal and sent it to the Dayton® motor powering the propeller. The signal to the motor increased in 0.01 volt increments, which translated to a velocity increase of approximately 0.1 cm/s per pre-determined time interval. Water velocity in the swim chamber was measured using a Swoffer Instruments, Inc. Model 2100 current velocity meter located just downstream from the collimators. A 2-inch propeller fastened to the velocity meter rotated with the current, sending a pulse rate that is proportional to the rotations per minute of the propeller that LabVIEW converts to cm/s.

The swim chamber contained two sets of sensors and a Sony video camera to determine the location of the test subject. When a fish passed by the photo-electric sensors a signal was sent to LabVIEW to be recorded. The first set of sensors is located directly posterior to the designated swimming zone and anterior to the downstream mesh funnel (Figure 3). The second set of sensors was located at the posterior end of the funnel, just anterior to the gate leading to the main reservoir. The video camera is mounted above the chamber and documents the activity of the fish throughout the entire performance test.

The swim chamber has three mechanisms that encourage the fish to continue to swim in the designated swimming zone as the current increases. First, the designated swimming zone is kept darker than the downstream portion. This made use of the fish's natural behavior to move towards a darker rather than brighter environment. The second mechanism was a light located posterior to the gate mechanism (illuminated the mesh funnel) and was set to flash several times when the first set of sensors was crossed. The third mechanism was a pulsed electric shock from a Grass muscle stimulator. When the fish fell back into the mesh funnel, it was encouraged to swim forward by an electrical current that increased in voltage from upstream to downstream.

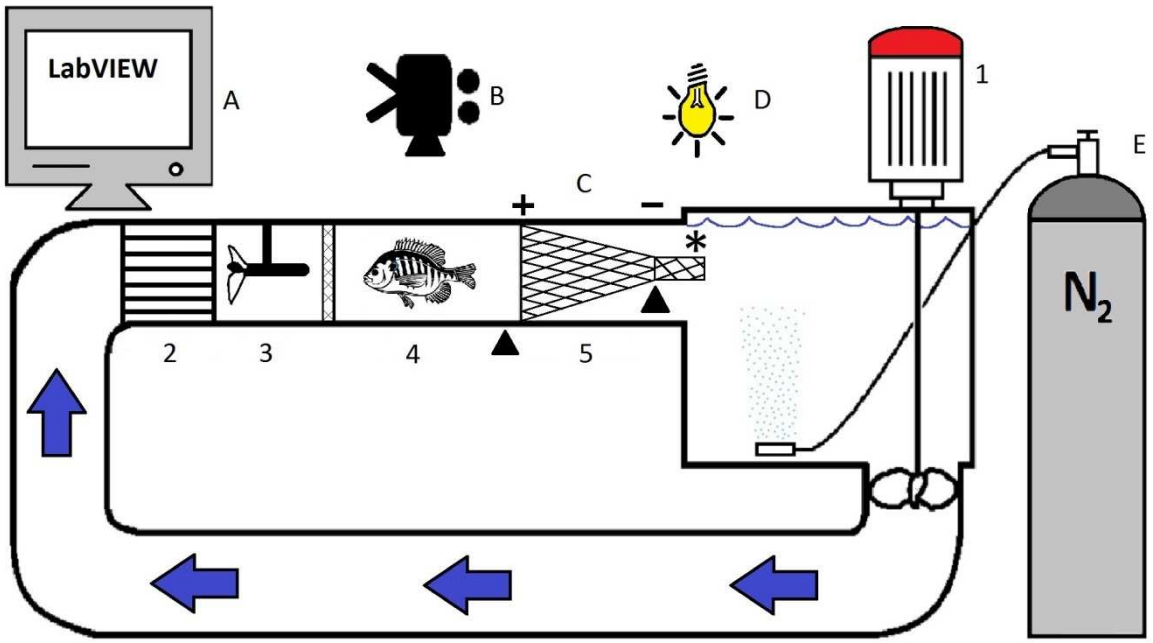

Figure 3. Variable speed swim chamber apparatus schematic. Water velocity is generated by an electric motor (1) that forces water from the main reservoir through pipe via a propeller. The water flows through the upstream collimators (2), passed the velocity sensor (3) and through the designated swimming area that houses the test subject (4). Water then returns to the main reservoir through the downstream mesh funnel (5). (A) Computer running LabVIEW that controls velocity and records performance. (B) Video camera. (C) Pulsed electric shocking mechanism in downstream mesh. (D) Downstream light. (E) Compressed nitrogen gas cylinder and connected fine bubbler diffusor. (▲) Photoelectric barriers. (*) PIT tag reader.

LabVIEW terminated the performance trial when the test subject failed. Failure was determined when an individual failed to keep pace in the designated swimming area, falling back into the funnel triggering the flashing light and electrical stimulation, and then falling back against the posterior gate, blocking the sensors for 10 seconds. At that point, the failure time was recorded and the gate opened. The PIT tag reader sent the information from the unique PIT tag code to LabVIEW where the time of failure for the individual fish was recorded. A failure was also recorded when a subject is impinged on the downstream mesh for 10 seconds, which was determined by personal observation or by referencing the saved video footage from the camera.

LabVIEW controls the rate of velocity increase and duration of the habituation stage (explained below), which were set prior to each trial. Once the parameters were set, LabVIEW asked for PIT tag data. At this point, the test subject was passed by the PIT tag reader and subsequently lowered into the swim chamber. The program was then ready to begin. Starting the program triggered the motor to turn the propeller and the video camera to begin recording.

Experimental Procedure

The implemented velocity program consisted of a 30-min habituation period at a water-velocity of ~5cm/s. Once the initial 30-min pre-stage was completed, LabVIEW increased the voltage to the electric motor that turns the propeller by 0.01-V increments every 25 seconds. The voltage increase translates to a water velocity increase of approximately 0.1 cm/s every 25 seconds. The same velocity parameters were maintained for all baseline and experimental measures.

Each individual performed two swimming trials. The initial trial, termed the baseline Ucrit, quantified the fish's individual performance. A bluegill was randomly removed from the population and placed in a 100-L fiberglass tank 24 hours prior to the baseline Ucrit test.

Individuals were checked for visible defects and feeding was suspended at this time. After the 24 hours had passed, the individual fish was removed from the 100-L holding tank, its unique code input into the LabVIEW program by passing the subject by the PIT tag reader and gently lowered into the swim chamber. If the individual exhibited a stress response (e.g. darting, bumping into walls) after an initial 5-min observation period, the subject was removed and another fish was randomly selected. Upon failure of the baseline Ucrit test, the subject was returned to its holding tank where feeding resumed 24 hours later.

The second performance test, termed the experimental Ucrit, occurred 7 days after the baseline Ucrit test and after the exposure duration to the pre-determined treatment levels of Cu. A factorial experimental design was used for each unique combination of stressors. The variables included duration of exposure (24, 48, and 96 hours), Cu concentration (0 and 1 mg/L) and oxygen concentration (saturated and 2 mg/L), for a total of 12 groups. I chose these treatment levels because it was my intent to observe the acute ($\leq$ 96 hrs) effects of Cu at application rates (1mg/L of elemental Cu is the maximum rate) observed in management regimes. The duration of exposure refers to the amount of time the subjects spent in a 30-L exposure aquarium. It is in these aquaria that the Cu stressor was introduced. Subjects were transferred to the 30-L exposure aquarium for the treatment duration so that the end of the exposure period coincided with the 7-day rest period (e.g. for a 24 hour duration of exposure treatment, the subject was transferred to the exposure aquarium 6 days after the baseline Ucrit test). The 30-L glass exposure aquarium contained the pre-determined test levels of copper.

Copper stress was imparted on test subjects in the form of crystalline copper sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$). Copper was added from a stock solution of 400 mg/L $CuSO_4 \bullet 5H_2O$ and was dissolved in the 30-L aquarium to a concentration of 4 mg/l. Elemental copper

only approximates 25% of copper sulfate pentahydrate, thus the concentration of copper in the aquarium will be 1 mg/l. This concentration of copper is the upper range of copper sulfate application rates recommended for use in freshwater lakes (USEPA Pesticide Registration #56576-1; Montz et al. 2010). To ensure the test water contains the target concentration, total copper was determined colorimetrically (USEPA bicinchoninate method 8506). The Cu concentration was monitored daily and supplemented with the $CuSO_4$ stock solution to maintain the target concentration. In addition to the added copper, water hardness was lowered by diluting aquarium water 1:1 with reverse osmosis water supplemented with sodium chloride for electrical conductivity. The hardness in the exposure aquaria was lowered because preliminary CSS trials indicated that hardness levels observed in the aquarium (Table 1) were providing bluegills protection from Cu. Air was continuously bubbled to maintain oxygen saturation.

After the 7-day rest period, the subject was removed from the 30-L copper exposure aquarium and gently lowered into the swim chamber containing the determined level of dissolved oxygen. When the experimental Ucrit had been determined, the subject was removed from the chamber and euthanized in a 500 mg/L solution of MS-222. The subject was then weighed to the nearest 0.1 grams and the total length was measured to the nearest mm. Subjects were not weighed and measured after the baseline Ucrit to limit handling stress. Relative weight served to ensure there were no differences in fish condition between the 12 groups. Relative weight (*Wr*) was calculated from the measured weight (g) and length (mm, minimum length = 80 mm) of each individual where *a'* and *b* for bluegill are -5.374 and 3.316 respectively (Hillman 1982):

$$Wr = (\text{Weight}/Ws) \times 100$$

$$\log_{10}(Ws) = a' + b \cdot \log_{10}(\text{Length})$$

The effects of the stressors on bluegill scope for activity are reflected in the ΔUcrit metric, which is the baseline Ucrit subtracted from the experimental Ucrit and normalized to body lengths per second. ΔUcrit measures greater than 0 indicate an increase in scope for activity whereas ΔUcrit measures lower than 0 indicate a decrease.

Hematocrit Determination

A capillary tube of blood was then collected via a cut gill arch and the PIT tag retrieved via an incision into the body cavity using a scalpel. The capillary tube was then capped and centrifuged with a Becton Dickinson Clay Adams Autocrit Ultra 3 centrifuge (11,700 maximum rpm) for 6 minutes. Using a caliper, the ratio of packed red blood cell volume to total volume was calculated from the separated fluids in the capillary tube.

Gill Tissue Analysis

Gills were excised from bluegills after hematocrit was measured. Gill samples were collected from bluegill exposed to 0 and 1 mg/L $CuSO_4$ for 48 and 96 hours as well as a batch of bluegill exposed to 0 and 4 mg/L $CuSO_4$ for 48 hours. The group exposed to 4 mg/L $CuSO_4$ did not perform any swim performance trials.

Excised gill tissue was fixed in a 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, and phosphate buffered saline solution for 24 hours at 4° Celsius. Fixed gills were rinsed in phosphate buffered saline (3 times for 5 minutes each) before a series of dehydrations with ethanol at room temperature. Ethanol was diluted with distilled water to achieve the desired concentrations (% concentration of ethanol followed by duration): 50%-10 min, 70%-10 min, 85%-10 min, 95%-15 min, and 100%-twice for 15 min.

Following the last dehydration step with 100% ethanol, the gills were rinsed twice for 15 min with acetonitrile. Gills were then infiltrated with a 1:1 mixture of acetonitrile and Epon

Araldite resin for 2 hours, covered, and slowly rotated at room temperature for 2 hours. The 1:1 mixture was then replaced with 100% Epon Araldite resin and allowed to sit uncovered, overnight, at room temperature while being slowly rotated to ensure infiltration with resin. Gills were then rinsed in 100% Epon Araldite resin, placed in molds with 100% resin and baked for 2 days at 65° Celsius. Embedded gills were sectioned (1 µm) with a Reichert-Jung Ultracut E microtome and stained for 5 minutes with a solution of 1% Azure II, 1% methylene blue, and 1% borax. Stained sections were cover slipped with Fisher Permount™ mounting media and observed and photographed under 63x and/or 100x magnification with a Leica DM5000B microscope. Gills were observed for histopathological changes such as epithelial lifting or sloughing, cell hypertrophy, and cell necrosis.

Statistical Analysis

A total of nine fish were removed from the dataset. A batch effect was suspected with 5 (fish #132-136, Table 5, Appendix A) fish that were exposed to 1 mg/L Cu for 96 hours and swam at low oxygen. Although the individuals did not visually appear to be in poor condition, they were unable to maintain their position in the swim chamber during the low velocity habituation period. The performances of this batch of fish were also much lower than that of others in the group. Fish #80 (Table 5, Appendix A) was removed due to being dropped on the floor while being transferred from the exposure tank to the swim chamber.

Fish #70, 110, and 140 (Table 5, Appendix A) were also removed as suspected outliers. Each fish belonged to a separate group and had a swim performance well below that of other fish in their groups, indicating poor condition unrelated to the imparted stress from the experiment. The 95% confidence interval was calculated with each of the three fish removed from their

respective groups. Each fish's performance (fish # 70, 110, and 140) was well below the 95% confidence interval of the remaining group.

All analyses were conducted using program R (R Core Team 2012). To determine whether there were any differences in fish condition or length between the groups, two-single-way ANOVAs were produced with relative weight and length as the response variables. The three fixed factors were combined to make a single factor with 12 levels (2 levels of DO x 3 levels of duration of exposure x 2 levels of Cu). Histograms and Q-Q plots of the residuals were used to assess normality and variances were calculated to ensure homogeneity between groups.

Critical Swimming Speed. Swim performance was assessed with a (N=82) 2x3x2 factorial ANOVA (α=0.05, type III sums of squares) with dissolved oxygen, duration of exposure, and [Cu] as fixed factors and ΔUcrit as the response. Histograms and Q-Q plots of the residual error were plotted to assess the assumption of normality and variances within each group were compared to ensure homogeneity. Stepwise backward selection was used to reduce the full model by removing the term with the highest p-value until all remaining terms were significant (α=0.05). Significant terms from the reduced model were followed by pairwise t-tests with the family-wise error rate adjusted by the Holm method due to the unbalanced design.

Efficacy of Δ*Ucrit*. A factorial ANOVA was produced using experimental Ucrit normalized to body lengths per second as the response variable in order to determine the efficacy of ΔUcrit to explain individual variation. The full ANOVA was reduced by stepwise backward selection as explained above. The experimental Ucrit and ΔUcrit reduced models were then compared by the amount of variation left unexplained by the model using sums of squares (SS):

$$\%\ variation\ unexplained = (residual\ SS / total\ SS) * 100$$

Hematocrit. A factorial ANOVA similar to the one described above was used to determine whether the three treatments had an effect on hematocrit. Assumptions were assessed and the model was reduced using the same stepwise backward selection procedures. Test subjects that hematocrit was not collected for were removed from this analysis (N=57).

CHAPTER IV

RESULTS

The water quality remained relatively constant throughout the experimental procedure (Table 1). Variation in water quality of the aquarium can be attributed to variation in tap water composition, time of testing, and a concentration effect due to evaporative loss. In between water changes, evaporative loss was supplemented by the addition of reverse osmosis water. Copper concentration in the exposure aquaria was the most variable parameter. The soluble Cu concentration generally decreased with time. Sources of Cu loss were likely from chemical complexation, adsorption to organic matter, or uptake by the fish. At times, a small amount of a faint, green precipitate was observed at the bottom of the exposure tank which could be the result of chemical complexation. Cu was supplemented daily to keep the concentration as close to 1 mg/L as possible.

Preliminary data indicated that the level of hardness in the University of North Dakota's tap water (Table 1) was providing the bluegill with a protective effect from Cu. To combat this, the hardness level in the exposure tanks was halved for the duration of exposure. Half hardness was achieved by diluting filtered aquarium water with reverse osmosis water. In order to maintain the electrical conductivity the subjects were acclimated to in the aquarium, the exposure tanks were supplemented with NaCl. The potential increase in sodicity of the exposure tanks could also be having a protective effect against Cu by competing with Cu for uptake sites (as Cu is thought to be a sodium mimic, Grosell 2012).

Table 1. Mean (range) of water quality variables of the main aquarium and exposure tanks during the course of the experiment. Hardness is measured in mg/L as calcium carbonate.

	Aquarium	**Exposure Aquaria**	
		Copper	*Control*
Temperature (°C)	20.33 (19.0-22.0)	19.95 (18.5-23.0)	19.65 (18.0-23.0)
pH	7.67 (7.4-7.9)	7.76 (7.4-7.9)	7.72 (7.5-7.9)
Conductivity (µmhos/cm)	530.9 (480-600)	499.2 (480-560)	505.7 (470-540)
Hardness (mg/L)	174.8 (159-201)	93.3 (84-114)	94.1 (83-112)
Total Copper (mg/L)	*<0.04	^1.005 (0.942-1.085)	*<0.04

*Values are below the detection limit of the test
^ Range of means of [Cu] over course of individual experiment.

There were no differences in relative weight (One-way ANOVA, $F_{11,70}$=1.5641, p=0.1290) and length (One-way ANOVA, $F_{11,70}$=1.3476, p=0.2177) of the bluegill among the 12 groups. Relative weight was added to the analysis as a covariate but was subsequently removed because it did not explain a significant amount of variation in ΔUcrit.

Critical Swimming Speed

The following results are presented as mean (± 95% confidence interval) with swim performance measures normalized to body lengths per second (bl/s). There was a significant effect of copper (Cu), duration of exposure, and dissolved oxygen (DO) on ΔUcrit (Table 2). The reduced model contained 5 significant terms, including two single factor terms (oxygen and hour) and all three two-way interactions. The 3-way interaction was not significant in the full or reduced ΔUcrit models, but the 3-way interaction plot was included to visualize the overall trend of the three treatments (Figure 4). The non-additive relationship of the three treatments is important to keep in mind when presenting the results of the 1 and 2-way interactions.

Table 2**.** Results of the factorial ANOVA with ΔUcrit as the response variable (n=82). The model was reduced by backwards, stepwise selection until only significant terms remained. The residual error and factor sums of squares (SS) were included to calculate the % variation unexplained.

	Factors	**DF**	**SS**	**F-value**	**P-value**
Full	Oxygen	1	4.2250	20.7555	<0.0001
Model	Copper	1	0.0908	0.4462	0.5064
	Hour	2	4.2812	10.5157	0.0001
	Oxygen:Copper	1	2.0442	10.0421	0.0022
	Oxygen:Hour	2	2.6276	6.4540	0.0027
	Copper:Hour	2	1.6825	4.1328	0.0201
	Oxygen:Copper:Hour	2	0.0635	0.1561	0.8558
	Residuals	70	14.2492	-	-
Reduced	Oxygen	1	7.4940	37.6986	<0.0001
Model	Hour	2	5.4423	13.6887	<0.0001
	Oxygen:Copper	2	8.4507	21.2554	<0.0001
	Oxygen:Hour	2	5.9412	14.9435	<0.0001
	Hour:Copper	2	3.5508	8.9310	0.0003
	Residuals	72	14.3128	-	-

From the Holm adjusted pairwise t-test post hoc analysis, performance was significantly decreased in response to hypoxia ($p<0.0001$, Figure 5). The mean ΔUcrit for bluegill performing at low DO (2 mg/L) was -1.276 bl/s (± 0.329) while bluegill exposed to saturated DO tended to have an increased ΔUcrit with an average performance of 0.208 bls (± 0.145). The increase in ΔUcrit at saturated DO is likely a training effect from performing the initial baseline Ucrit.

There appears to be a positive relationship of duration of exposure with ΔUcrit, but the only significant difference is between the 24 (-0.686 ± 0.577 bl/s) and 96 (0.021 ± 0.333 bl/s) hour time points ($p=0.0250$, Figure 6). There was no difference between the 48 (-0.272 ± 0.231 bl/s) and the 24 or 96-hr durations.

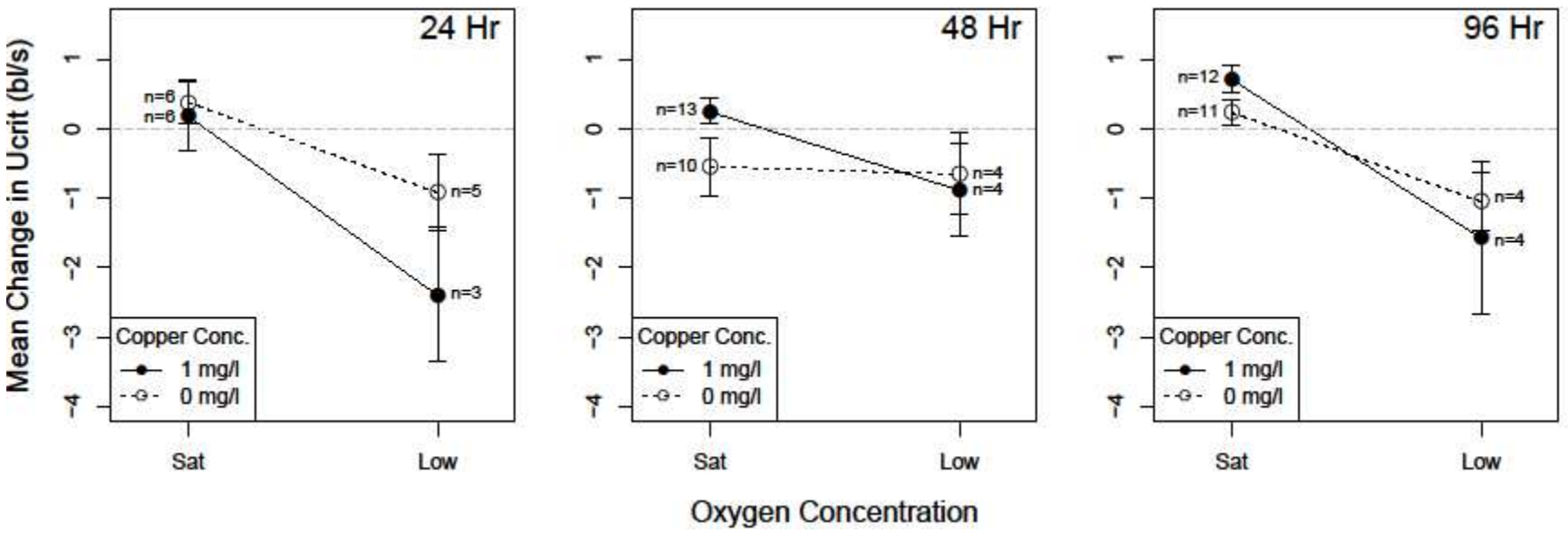

Figure 4. Effects of [Cu], duration of Cu exposure, and hypoxia on the ΔUcrit of bluegills. The 3-way interaction was not a significant term in the full or reduced model and is only presented to show the interactive trend of [Cu] and hypoxia at the three different time points.

The increased performance with duration of exposure may be due to an acclimatory adjustment to being relocated from the aquarium to the exposure tanks.

There was a significant interaction between duration of exposure and DO on ΔUcrit (Table 2). Although this was a significant interaction, the DO factor is most likely driving the significance. The mean ΔUcrit was significantly higher for bluegill exposed to saturated DO compared to low DO regardless of duration of exposure (Figure 7). This follows the expected trend in nature and agrees with the results of the one-way effect of DO on ΔUcrit (Figure 5). However, the positive trend of ΔUcrit with duration of exposure shown in Figure 6 is not observed in the Oxygen:Hour interaction.

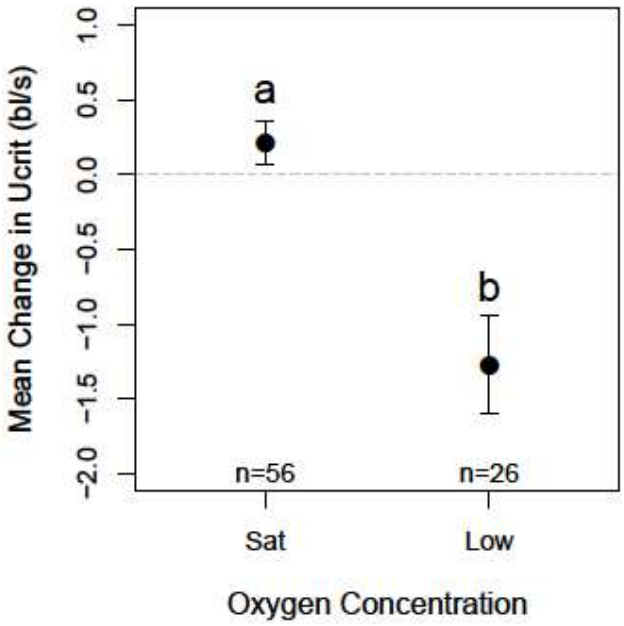

Figure 5. Results of the post hoc Holm adjusted pairwise t-test with ΔUcrit as a function of oxygen concentration ((Sat=Saturation/>8 mg/L, Low=2mg/L). Points indicate mean ΔUcrit bound by the 95% confidence interval. Different letters denote statistically different means.

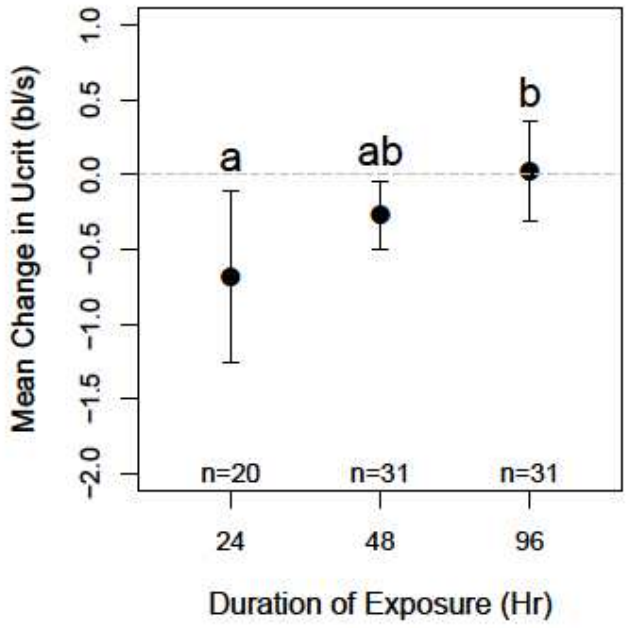

Figure 6. Results of the post hoc Holm adjusted pairwise t-test with ΔUcrit as a function of duration of exposure (time (Hr) spent in the exposure tanks). Points indicate mean ΔUcrit bound by the 95% confidence interval. Different letters denote statistically different means.

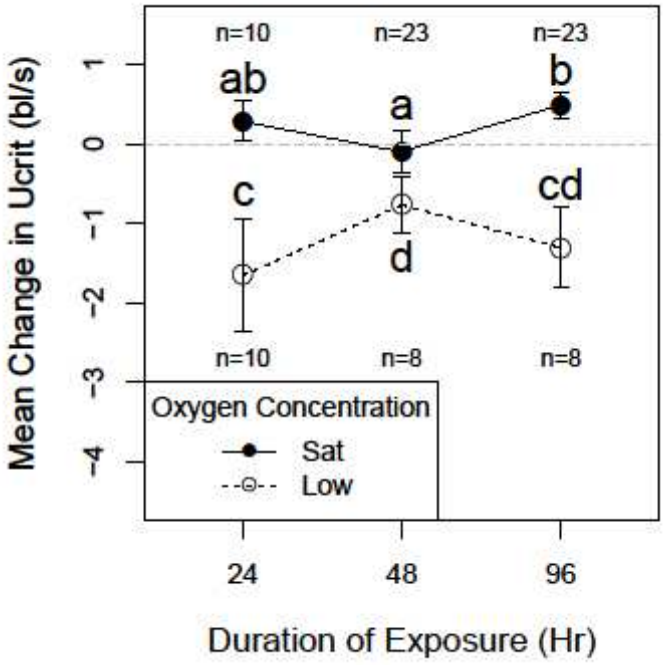

Figure 7. Results of the post hoc Holm adjusted pairwise t-test with ΔUcrit as a function of duration of exposure (Hr) and oxygen concentration (Sat=Saturation/>8 mg/L, Low=2mg/L). Points indicate mean ΔUcrit bound by the 95% confidence interval. Different letters denote statistically different means.

There was a significant interaction between Copper and Hour on ΔUcrit (Table 2). From the post hoc analysis, there was a significant difference amongst Cu exposed bluegill, with fish swimming significantly slower at 24 hr (-1.101 ± 1.056 bl/s) compared to 48 (-0.020 ± 0.306 bl/s, p=0.0420) and 96 hr (0.143 ± 0.588 bl/s, p=0.0120, Figure 8). There was no difference in ΔUcrit between control and Cu-exposed bluegill at any time point. However, the same increasing trend of ΔUcrit with duration of exposure is apparent in Cu treated fish.

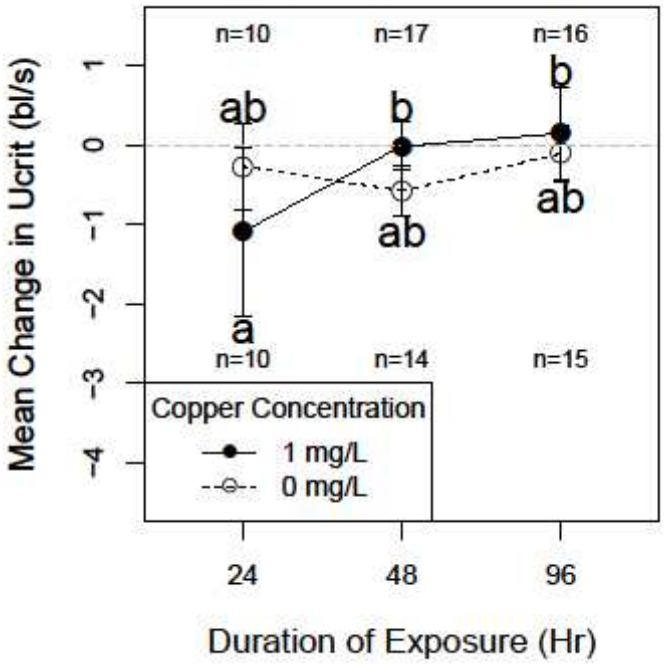

Figure 8**.** Results of the post hoc Holm adjusted pairwise t-test with ΔUcrit as a function of duration of exposure (Hr) and copper concentration (as $CuSO_4$). Points indicate mean ΔUcrit bound by the 95% confidence interval. Different letters denote statistically different means.

I tested the hypothesis that the combined effects of Cu and hypoxia were additive. Although the 3-way interaction between the three factors was not significant (Figure 4), the interaction between Cu and DO was (Table 2). The relationship between Cu and DO was not additive with Cu having the opposite effect on swimming speed at saturated versus low DO (Figure 9). When exposed to saturated DO in the swim chamber, Cu-exposed bluegill swam significantly faster (0.423 ± 0.148 bls/s) than control fish (-0.040 ± 0.235 bl/s, p=0.0035). But

when swum at low DO (2 mg/L), Cu-exposed fish (-1.674 ± 0.561 bl/s) swam significantly slower than control fish (-0.879 ± 0.250 bl/s, p=0.0014). This interactive effect indicates that the two stressors are not independent of each other. This interactive trend is fairly consistent among all three time points of duration of exposure (Figure 4), leading to the view that at acute levels, duration of exposure is not an overly important factor.

Hematocrit

A total of 52 hematocrit samples were collected with a minimum of 3 samples in each group. There were no significant terms in the 3-way ANOVA full model with hematocrit (%) as a function of Cu, duration of exposure, and DO. However, the reduced model contained one significant term (Table 3). Oxygen concentration had a significant effect on hematocrit (Figure 10). Bluegill swam at low DO had a significantly higher mean hematocrit (45.68 ± 1.95) than fish swam at saturated DO (41.90 ± 1.85, Holm adjusted pairwise t-test, p=0.0062).

Table 3. Results of the factorial ANOVA with hematocrit (%) as the response variable. The model was reduced by backwards, stepwise selection until only significant terms remained.

	Factors	**DF**	**F value**	**P-value**
Full	Oxygen	1	1.3747	0.2472
Model	Copper	1	0.0055	0.9410
	Hour	2	0.2183	0.8047
	Oxygen:Copper	1	0.1490	0.7013
	Oxygen:Hour	2	0.3949	0.6761
	Copper:Hour	2	0.3120	0.7335
	Oxygen:Copper:Hour	2	1.4009	0.2569
Reduced Model	Oxygen	1	8.1173	0.0062

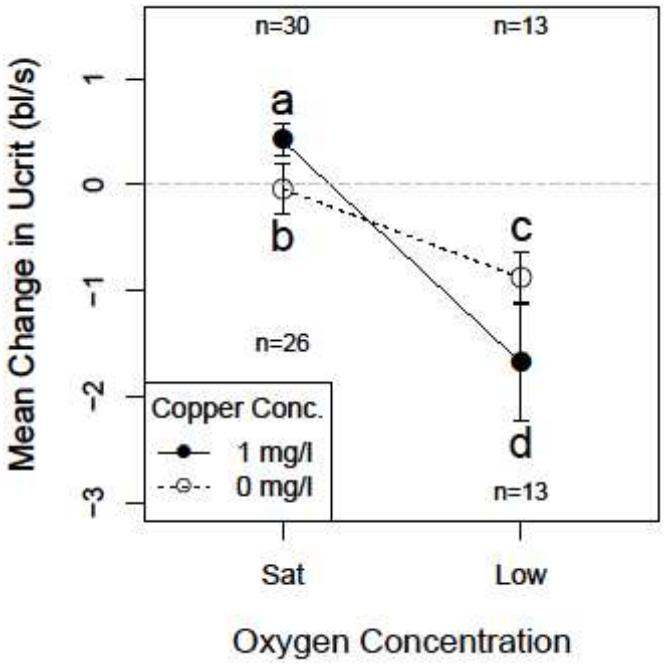

Figure 9. Results of the post hoc Holm adjusted pairwise t-test with ΔUcrit as a function of copper (as $CuSO_4$) and oxygen concentration (Sat: >8 mg/L, Low: 2mg/L). Points indicate mean ΔUcrit bound by the 95% confidence interval. Different letters denote statistically different means.

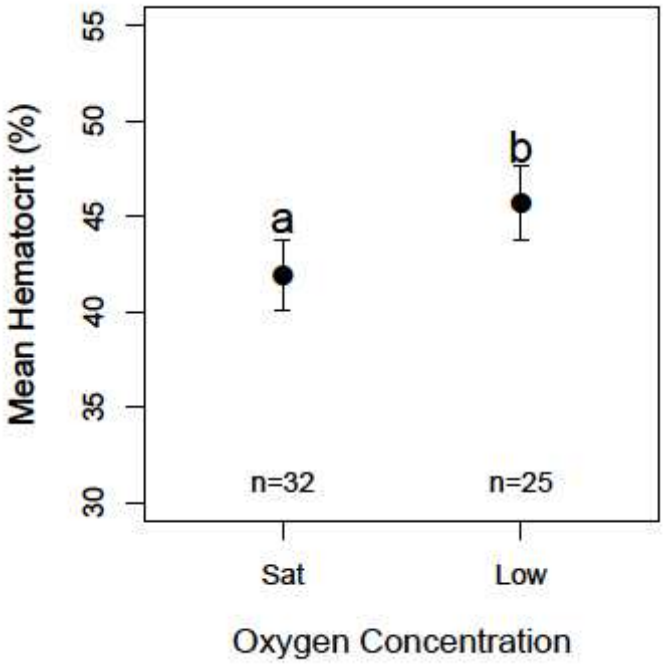

Figure 10. Results of the post hoc Holm adjusted pairwise t-test with hematocrit as a function of oxygen concentration (Sat=Saturation/>8 mg/L, Low=2mg/L). Points indicate mean hematocrit (%) bound by the 95% confidence interval. Different letters denote statistically different means.

Although not a significant term in the full or reduced model, the interaction of Cu and DO on hematocrit was included due to an obvious trend in the data. Copper and DO appear to have and additive effect on bluegill hematocrit, with both Cu and hypoxia independently resulting in an increase in hematocrit (Figure 11). The high degree of variability in hematocrit has limited our statistical inference and may be a function of collection methodology. However, the trend is worth noting for its potential biological significance.

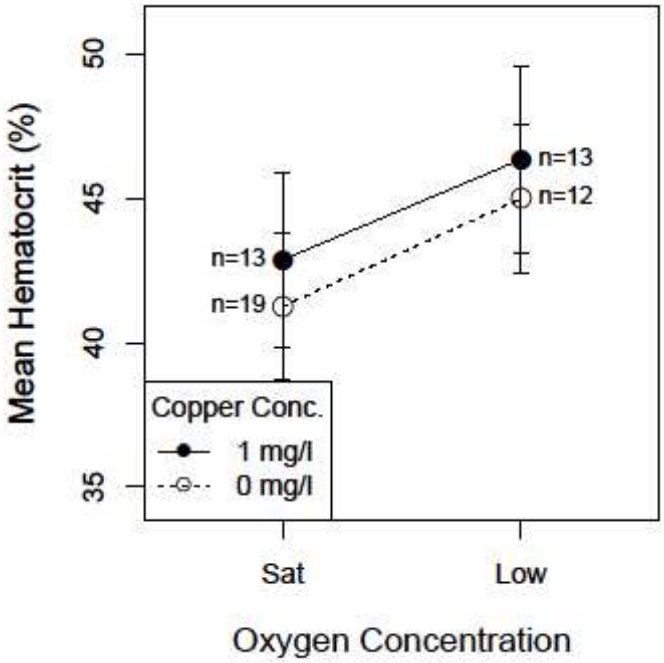

Figure 11. Effect of [Cu] and [DO] on hematocrit. Points indicate mean hematocrit bound by the 95% confidence interval. There is no significant difference between the groups.

Gill Morphology

Representative gill samples of bluegill exposed to 0, 1, and 4 mg/L Cu as $CuSO_4$ for 48 hours in half hardness were collected and observed for damage. Swim performance measures were not produced for highly dosed bluegill. The target concentration for the highly dosed group was 4 mg/L Cu, however the actual concentration was much lower (~2.5 mg/L Cu). A blue-green

precipitate was observed at the bottom of the high dosed tanks and likely explains the disparity between the target and measured Cu concentrations.

Gill damage was recorded at 1 mg/L $CuSO_4$ (Figures 12, 13). There appeared to be a prevalence of “spurs” protruding from the secondary lamellae that give the structures a rough consistency compared to control lamellae. The spurs may indicate epithelial detachment due to epithelial instability as a result of Ca displacement by Cu at intercellular junctions. A similar effect was observed in white seabass (*Lates calcarifer*) in response to sub-chronic cadmium exposure (Thophon et al. 2003). Also present (even more so in the 4 mg/L dosed subjects) appear to be sloughed cells that were completely detached from the lamellae (Figure 13).

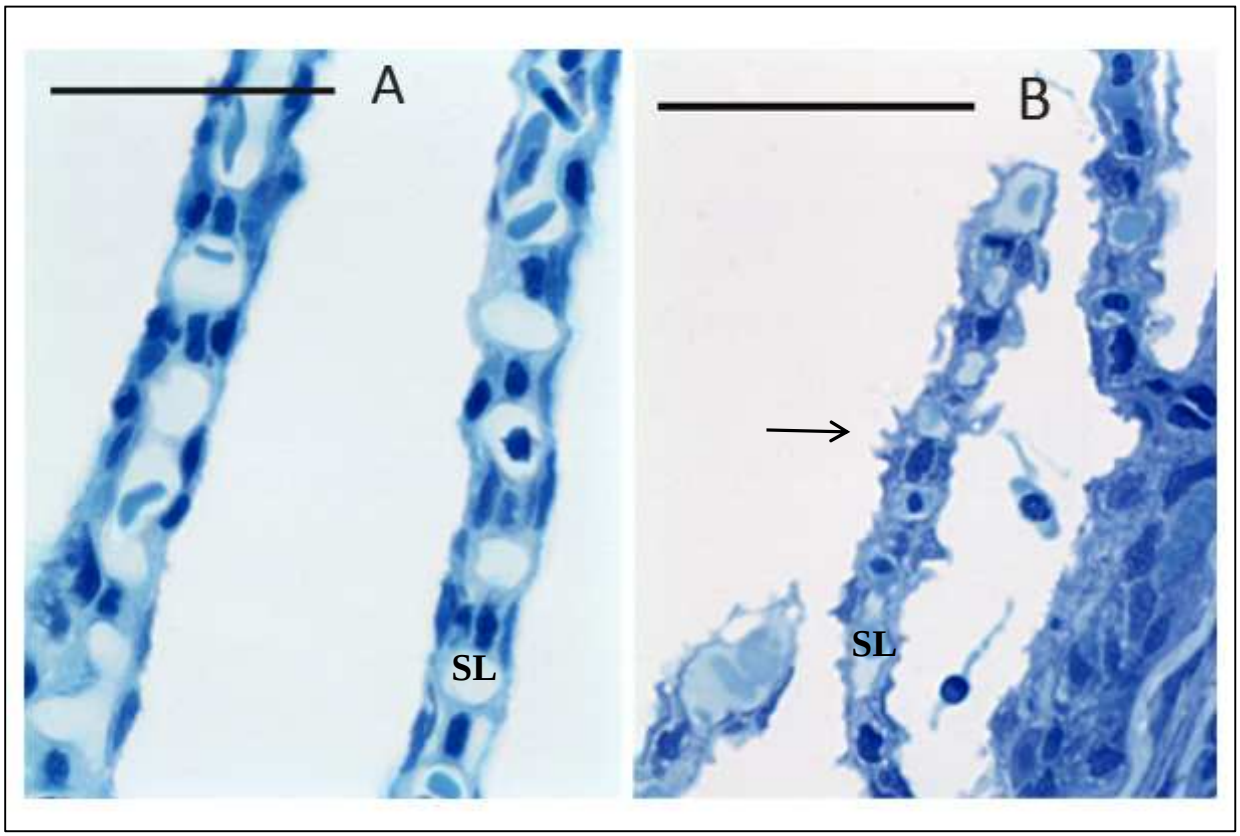

Figure 12. Photographs of sectioned secondary lamellae (SL) of bluegill sunfish under 100x magnification. Panel A shows the gills of a control fish whereas panel B shows the gills of a bluegill exposed to 1 mg/L Cu for 48 hours. Cu-exposed gills were marked by a prevalence of spurs (→) giving them a rough appearance.

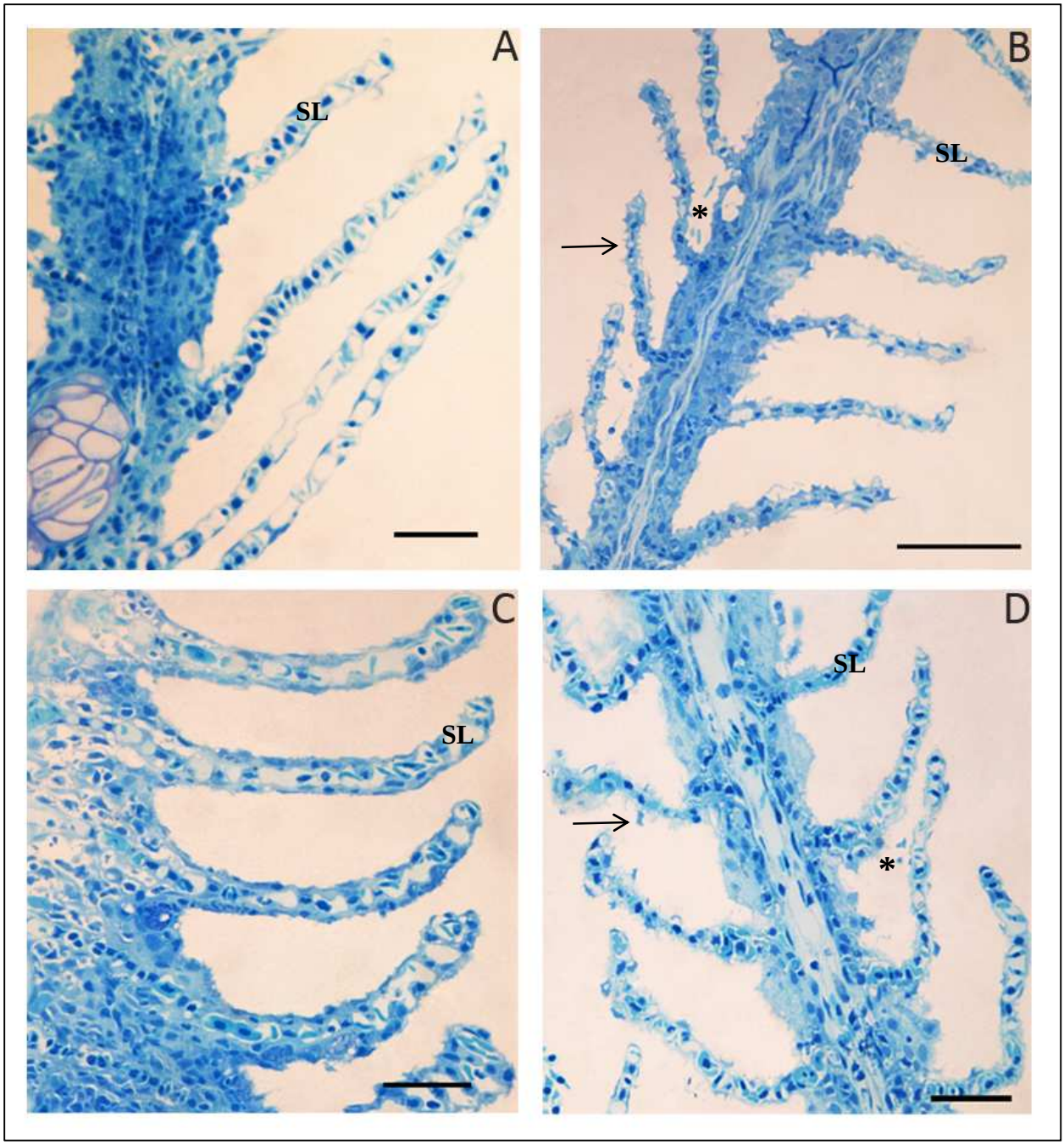

Figure 13. Photographs of sectioned gill filaments and secondary lamellae (SL) of bluegill sunfish under 63x magnification. Panels A and C show the gills of control fish whereas panel B shows gills exposed to 1 mg/L for 48 hours and Panel D shows gills exposed to 4 mg/L Cu for 48 hours. Cu-exposed gills were marked by a prevalence of spurs (→) giving them a rough appearance. Sloughed epithelial (*) cells were also apparent, especially in gills collected from bluegill exposed to 4 mg/L Cu.

Efficacy of ΔUcrit

The factorial ANOVA full model with experimental Ucrit (bl/s) as the response variable yielded two significant terms (Table 4). The reduced model resulted in the same two significant terms from the full model: Oxygen and Oxygen:Copper. The 3-way interaction was not significant, but the 3-way interactive plot (Figure 15) is included to illustrate the trend. I hypothesized that less variation would be left unexplained by measuring the change in swimming speed (ΔUcrit) as opposed to a single experimental performance measure (experimental Ucrit). From the ΔUcrit (Table 2) and experimental Ucrit (Table 4) ANOVA tables, the ΔUcrit reduced model had a substantially less amount of variation left unexplained (31.7% compared to 79.1%).

Table 4. Results of the factorial ANOVA with experimental Ucrit as the response variable. The model was reduced by backwards, stepwise selection until only significant terms remained. The residual error and factor sums of squares (SS) were included to calculate the % variation unexplained.

	Factors	**DF**	**SS**	**F value**	**P-value**
Full	Oxygen	1	1.5190	4.9724	0.0290
Model	Copper	1	0.4090	1.3387	0.2512
	Hour	2	0.5180	0.8472	0.4330
	Oxygen:Copper	1	1.2190	3.9903	0.0497
	Oxygen:Hour	2	0.4180	0.6841	0.5079
	Copper:Hour	2	0.5690	0.9317	0.3987
	Oxygen:Copper:Hour	2	0.4030	0.6605	0.5198
	Residuals	70	21.3790		
Reduced	Oxygen	1	1.6000	4.9976	0.0282
Model	Oxygen:Copper	2	5.0200	7.8194	0.0008
	Residuals	78	25.0300		

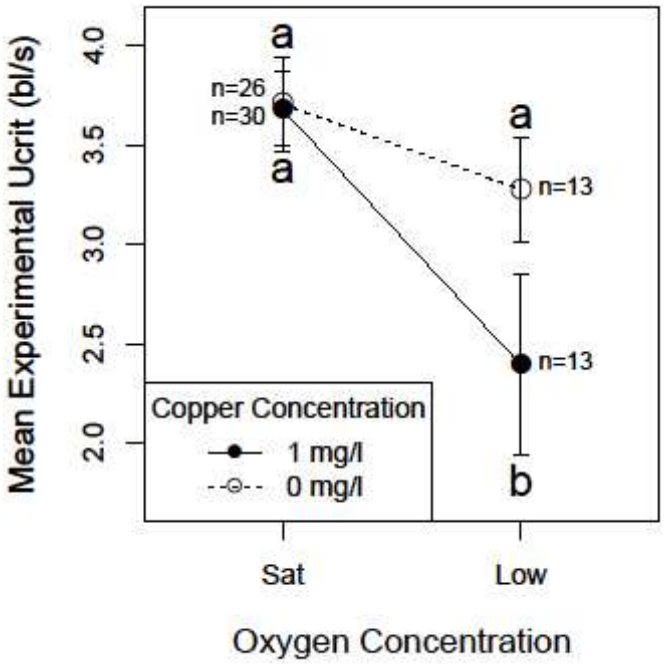

Figure 14. Results of the post hoc Holm adjusted pairwise t-test with experimental Ucrit as a function of copper (as $CuSO_4$) and oxygen concentration (Sat: >8 mg/L, Low: 2mg/L). Points indicate mean Experimental Ucrit bound by the 95% confidence interval. Different letters denote statistically different means.

Both models showed a significant decrease in swimming speed in response to hypoxia (Tables 2, 4). However, the trends observed in the Oxygen:Copper interaction between the two models are not in agreement (Figure 14). There was no statistically significant increase in experimental Ucrit in response to Cu at saturated DO. In fact, the mean experimental Ucrit of Cu exposed fish at saturated DO (3.682 ± 0.186 bl/s) is slightly slower compared to controls (3.705 ± 0.236 bl/s). The only statistically significant difference involved Cu exposed fish in hypoxia. The mean experimental Ucrit of this group was significantly lower than any other group (Figure 14). No difference was detected between the Cu control bluegill at saturated DO and the Cu control bluegill at low DO (Holm adjusted pairwise t-test, p=0.0847).

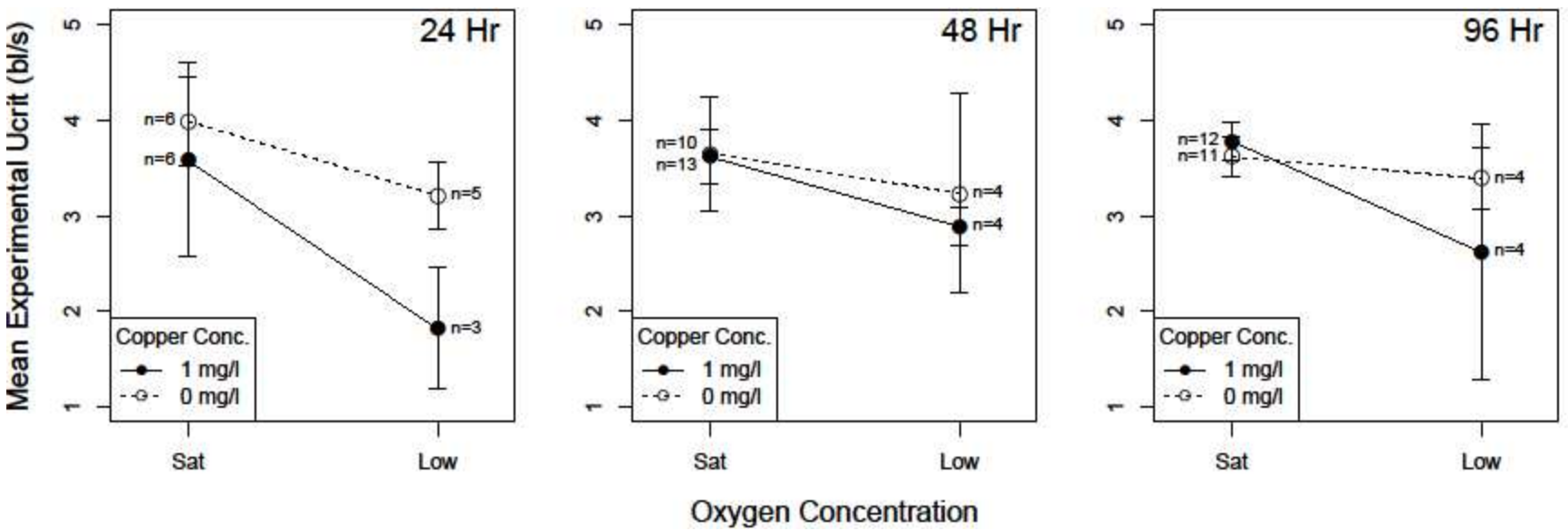

Figure 15. Effects of [Cu], duration of Cu exposure, and hypoxia on the experimental Ucrit of bluegills. The 3-way interaction was not a significant term in the full or reduced model and is only presented to show the interactive trend of [Cu] and hypoxia at the three different time points.

CHAPTER V

DISCUSSION

Effects of Copper & Hypoxia on ΔUcrit

The development of this experiment is centered around the combined effects of hypoxia and Cu stress on scope for activity. It was thought that the duration of Cu exposure at the acute level may be impactful, which prompted the addition of the duration of exposure factor to the design. Although duration of exposure is significant in the reduced model, it does not appear to be a major factor in the combined effects of hypoxia and Cu story.

I hypothesized that the combined effects of hypoxia and Cu stress would negatively affect bluegill swim performance in an additive manner. More simply stated the effects of Cu and hypoxia would be negative and independent of each other. The seemingly conflicting interactive effect of these two stressors leads me to reject the hypothesis of a simple additive relationship.

Cu affects bluegill swim performance differently depending on DO (Figure 9). There are two observations from the relationship: 1) At saturated DO, exposure to Cu actually increases ΔUcrit compared to controls not exposed to Cu, and 2) during hypoxia, exposure to Cu decreases ΔUcrit compared to controls not exposed to Cu. From an energetics standpoint, an increase in swim performance translates to an increase in the scope for activity and vice versa. The scope for activity, or available energy, can be increased or decreased by a shift in the standard metabolic rate (SMR) or active metabolic rate (AMR) (Figure 1).

The study of the combined effects of multiple stressors can be difficult to quantify and understand due to the potential for non-additive and complex inter-relationships at many levels of biological organization. Based on the evidence available, I have proposed several hypotheses that may explain the interactive effect observed. However, more extensive and definitive evidence is needed to test these hypotheses.

Based on the presented hematocrit and gill histology evidence, the most likely mechanism to explain the interactive effect of copper and hypoxia on swim performance is hemoconcentration. Hemoconcentration is the result of an increase in hematocrit and several mechanisms may result in the effect. Grosell (2012) discussed the potential of hemoconcentration due to Cu exposure to result in cardiac failure. The following hemoconcentration hypothesis has been modified from Grosell (2012) to possibly explain the interactive effect of Cu and hypoxia. It is important to note that the hemoconcentration hypothesis hinges on the premise that for a given fish species under given conditions therein lays an optimal hematocrit balanced by a need to bind and transport oxygen without increasing blood viscosity (Wells & Weber 1991). In this setting, hemoconcentration is a collective result of the physiological responses to hypoxia, and gill damage.

*Hemoconcentration Hypothesis***.** Hypoxia increased bluegill mean hematocrit (%) by 3.8 (Figure 10), even though the bluegill were only exposed to low DO for the duration of the experimental swim trial totaling no more than 3.5 hours (30-min habituation + time to exhaustion). This finding suggests that bluegills are able to rapidly respond to drops in environmental oxygen partial pressures and do so through a mechanism that results in hemoconcentration such as splenic release. Exercised-induced urine flow has also been shown to

contribute to hemoconcentration (Yamamoto et al. 1980, Wood 1988). Nonetheless, hematocrit has been shown to be positively correlated with Ucrit (Gallaugher et al. 1995).

Although not statistically significant, I have presented an apparent trend of hemoconcentration in response to Cu that is independent of the increase associated with hypoxia. Studies have shown a boost in red blood cell count accompanied by an increase in hemoglobin concentration in response to Cu (Mazon et al. 2002, Cerqueira & Fernandes 2000). The increase in hemoglobin (the protein significantly responsible for oxygen transport) directly translates to increased oxygen transport capacity. Cu in this case appears to have a masking effect on hemoconcentration. The increase in hematocrit in response to Cu is likely to be in response to decreased oxygen uptake through Cu induced damage to gill tissue and/or increased diffusion distance due to mucous excretion on the gills. This study (Figures 12, 13) and several others (Dang et al. 2000, Cerqueira & Fernandes 2002, Mazon et al. 2002, Van Heerden et al. 2004) have shown gill damage as a direct result of Cu exposure.

Hemoconcentration as a result of Cu exposure is not always correlated with increased oxygen carrying capacity. Several studies have shown increased hematocrit in response to Cu exposure and attribute the change to a shift of water from extracellular plasma to intracellular compartments (Waiwood 1980, Heath 1984, Schjolden et al. 2007, Grosell 2012). Cu is thought to inhibit ion regulation by competing for ion uptake pumps and displacing intercellular calcium causing leaky junctions (Laurén & McDonald 1985, Laurén & McDonald 1986, Grosell 2012). Because of the hyperosmotic balance of the blood compared to the freshwater environment, leaky junctions will permit the outward movement of ions from the blood to the freshwater environment. It is then this decrease in osmolality of the blood that is the impetus for water to move from the plasma to the tissues and/or red blood cells resulting in hemoconcentration. So, a

boost in hematocrit may not correlate to an overall increase in oxygen carrying capacity, but simply an increase in packed cell volume to plasma ratio due to displacement of plasma water.

The combined effects of splenic release from hypoxia and plasma water displacement from Cu exposure could explain the additive relationship of the two stressors on hematocrit (Figure 11). As discussed earlier, hematocrit is potentially under stabilizing selection due to a trade-off of blood viscosity and oxygen transport ability. The doubly increased hematocrit in response to Cu and hypoxia may have resulted in a viscous blood that is difficult to circulate, straining the cardiac tissue. Cardiac failure has been documented in Nile tilapia (*Oreochromis niloticus*) in response to acute Cu exposure (Andrade Waldemarin et al. 2012).

Unfortunately, I am unable to deduce whether hemoconcentration observed in response to Cu alone is a result of plasma water displacement or increased oxygen transport capacity, but the increased ΔUcrit shows support for the latter. The question still remains of how normoxia is providing bluegill protection from Cu toxicity even though the degree of gill damage appeared to be independent of exposure to hypoxia. The answer could lay in the iono-respiratory challenge of freshwater fish.

Intimate contact between the blood and external environment is required to facilitate the diffusion of oxygen into the organism, but there is a trade-off of increased ion and osmotic imbalance due to the osmotic gradient that exists between a freshwater fish and its environment (Evans et al. 2005). Although some ion loss may result from lamellar recruitment, freshwater teleosts exhibit very low branchial ionic permeability (Karnaky 1997). Additionally, fish are well equipped with specialized branchial cells for the active transport of ions, but exposure to Cu has been shown to inhibit both ionic permeability and ion transport. Laurén & McDonald (1985) showed a dose-dependent net ion loss in rainbow trout exposed to acute Cu concentrations. The

net loss was a result of inhibited ion influx and the stimulation of ion efflux. The net ion loss from Cu exposure may be exacerbated by the increased lamellar recruitment and perfusion response during hypoxia.

Because of the increased gill permeability that exists from gill damage, increased lamellar perfusion and recruitment is likely to facilitate net ion efflux. I propose that Cu-exposed bluegill in normoxic conditions were able to keep lamellar perfusion depressed for a longer period of time during the excessive exercise of CSS tests. By limiting blood-water contact, bluegill were able to limit ion loss which is reflected in the lower hematocrit compared to hypoxic, Cu-exposed bluegill.

Based on the lamellar perfusion idea, bluegill exposed to Cu are more susceptible to hypoxia. Once Cu-exposed bluegills were placed in the hypoxic swim chamber, the proposed hemoconcentration possibly from from splenic release wasn't enough to meet systemic tissue oxygen demand. Thus, the subjects were prompted to increase oxygen uptake efficiency by increasing lamellar perfusion and recruitment which lead to ion efflux due to the more permeable Cu-damaged gills. The subsequent cardiac stress from increased blood viscosity coupled with the effects of ion loss on skeletal-muscular function likely lead to the decreased scope for activity of Cu-exposed bluegill in hypoxia. In summation, Cu causes gill damage which results in a compensatory response in hemoconcentration which works in normoxic conditions. But when exposed to hypoxia, hematocrit increases to a point where the blood is too viscous leading to inhibited cardiac performance.

There are several studies that highlight the deleterious effect of Cu on swim performance (Waiwood & Beamish 1978, Beaumont et al. 1995, McGeer et al. 2000). The interactive relationship between Cu and hypoxia on swim performance in the present study is difficult to

define due to the lack of findings that show increased performance in response to acute Cu. Taylor et al. (2000) showed a significant increase in the sprint performance of rainbow trout after 30 days exposure to Cu, but proposed no mechanism to explain the result.

Inhibited Stress Response Hypothesis. An additional hypothesis, lacking evidence and explanation in the literature and in this study, is that exposure to Cu inhibits the organism's ability to respond to the additional hypoxic stress. This hypothesis does not explain how Cu may increase Ucrit and the scope for activity, so it is important to discuss other potential explanations (in addition to boosted hematocrit) of how Cu may increase the scope for activity of bluegill at normoxia.

Micronutrient Hypothesis. Copper is an essential micronutrient in the metabolism of bluegill and numerous other organisms, with homeostatic control and various uptake routes (Grosell 2012). It is a component of various enzymes including a cofactor for a cytochrome oxidase in the electron transport chain (Solomon & Lowery 1993). If Cu was a limiting nutrient in the aquarium system, exposure to Cu would increase inward Cu movement through known generic and specific ion pumps. The increased Cu intake would alleviate the limiting nutrient issue and allow for an upward shift of the AMR curve, thus increasing the scope for activity and swim performance. However, it is important to note that the commercial fish pellets that comprised the subject's diet were supplemented with Cu but it is unknown if this concentration is sufficient to meet the nutrient requirements for bluegill.

Antibiotic Hypothesis. Cu containing chemicals have been shown to have anti-biotic effects. Cu sulfate has been shown to cure ich and other fungal diseases. An antibiotic effect (controlling factor) may cause a decreased SMR because disease and parasites typically carry a metabolic load. If the disease is an obligate metabolic load and Cu relieves it, this would shift the

SMR curve (Figure 1) down increasing the scope. However, there was little evidence of disease outbreaks in my aquarium and this would not explain the decreased swimming speed at low DO.

The potential for Cu as a limiting nutrient and/or an anti-biotic to increase the scope for activity of bluegill at saturated DO coupled with Cu inhibiting their ability to physiologically respond to hypoxia may explain the interactive effect observed. Like most fish, bluegills are able to maintain their SMR to a certain environmental threshold. This threshold or the point at which oxygen uptake rate begins to decrease in response to decreasing oxygen partial pressures is termed the critical oxygen concentration (C_c). De Boeck et al. (1995) measured the C_c of juvenile common carp (*Cyprinus carpio*) exposed to increasing [Cu]. They showed a shift in the C_c towards higher oxygen concentrations with increasing Cu. This upward shift in the C_c may indicate the organism's inability to sufficiently respond to changing conditions and may translate to an increased susceptibility to environmental hypoxia with exposure to Cu. The ability of fathead minnows (*Pimephales promelus*) to respond to heat stress has also been shown to be inhibited by Cu exposure (LaPointe et al. 2011).

The physiological mechanism for the inhibited response to hypoxia is unknown, but the effect could be more pronounced in bluegills which are likely more sensitive to hypoxia than common carp. O'Hara (1971) measured the oxygen consumption rate of bluegill at various concentrations of Cu. He showed a spike in the oxygen uptake rate soon after exposure to 1 mg/L Cu, which then decreased below basal rates within 24 hours, leveling off at 60-70% the normal rate. A decrease in the oxygen uptake rate of bluegill in response to Cu may lead one to assume a downward shift in the SMR resulting in increased scope. However, the same trend occurred for bluegill exposed to 3 mg/L Cu which experienced increased mortality, thus discrediting the

decreased SMR hypothesis in favor of the inhibited oxygen regulation hypothesis. Also noted by O'Hara was a rapid recovery of bluegill upon transference to Cu-free water.

Fish have been shown to acclimate to the toxic effects of Cu. Waiwood & Beamish (1978) showed a rebound in Ucrit of rainbow trout after 10 days of depressed Ucrit. Laurén & McDonald (1987a, b) elucidated the Cu-acclimation mechanism of rainbow trout. While continuously exposed to Cu, the rainbow trout were able to return sodium homeostasis by decreasing sodium efflux and increasing sodium uptake by increasing the number of Na/K ATPase transporters. I showed a trend of increasing ΔUcrit with time in bluegill exposed to Cu (Figure 6), but an organism physiologically responding to an environmental toxicant in fewer than 96 hours is unlikely. However, upon transference to Cu-free water, O'Hara (1971) noted evidence of rapid recovery of bluegill that had previously showed signs of erratic respiration in response to Cu.

Efficacy of ΔUcrit

I was able to remove a substantial amount of individual variation by measuring the change in swimming speed rather than raw swimming speed. This allowed for greater statistical inference reflected in the comparison of the reduced models of ΔUcrit (Table 2) and experimental Ucrit (Table 4). The advantage of measuring scope for activity in a laboratory setting is being able to control as many environmental immeasurables as possible in order to isolate variation of the stressors of interest. This research provides evidence that an assay that corrects for individual variation is useful in determining the sub-lethal effects of environmental stressors.

The efficacy of a sound method may be revealed by detecting anticipated trends. In this case, the trend that is very likely to be observed is a decrease in swimming speed with hypoxia.

Both Ucrit models showed a significant decrease in swimming speed in response to hypoxia alone. However, high variability in the experimental Ucrit model prevented likely trends from being detected in the significant interaction of Copper:Oxygen.

No statistically significant difference was detected between control fish at saturated DO and control fish at low DO with the experimental Ucrit method (Figure 14). As previously stated, we have previously shown a significant decrease in Ucrit of bluegill in response to hypoxia in the same laboratory (Anderson 2012, unpublished data). A significant decrease in swimming speed in response to hypoxia is a trend that should be consistently detected with an effectual method. The merely marginal significance of the aforementioned decrease in Ucrit speaks to the potential of individual variation to limit statistical inference.

Despite the exact same bluegill and their performances compiling the data for the comparison of the two methods, the trends of the models presented different results. The inference of this study would be dramatically different had I utilized the experimental Ucrit method that is typically used in critical swimming speed tests. An insignificant decrease in swimming speed rather than a significant increase in swimming speed would have been detected had I used raw swimming speed.

The disparities between the two methods beg the question of which result is more likely to be true? The result of the experimental Ucrit model more closely matches my anticipated outcome which is based on information gained by consulting previous published works. The experimental Ucrit method has been the preferred method for several years and continues to be the standard. However, it is unfair to hastily rebuke a new method that differs from an existing when the results being used are of a novel system where interactive effects are unknown.

To my knowledge, this is the first study of its kind to use change in swimming speed rather than raw swimming speed to measure a fish's response to environmental stress. Several studies have forced subjects to perform multiple performance tests, such as in repeated measures designs (e.g. Ficke et al. 2012) and repeat swim performance tests (e.g. Reidy et al. 2000). Recovery ability is often the target measure in repeat swim performance tests. The measure of recovery ability is termed the recovery ratio, which is the ratio of an initial Ucrit followed by a second Ucrit after the subject is allowed to recover for a duration of time (e.g. $Ucrit_2$:$Ucrit_1$, Jain et al. 1998).

The recovery ratio statistic is very similar to ΔUcrit but there are 2 foundational differences between the two methods: 1) the questions/issues each method is intended to confront are different. Recovery ratio addresses the question of how long it takes for a subject (in response to exercise or an environmental stressor(s)) to recover to baseline levels, whereas the main purpose of ΔUcrit is to remove individual variation to increase statistical inference; 2) ΔUcrit carries more information than recovery ratio. Because ΔUcrit is an actual swimming velocity rather than a ratio of swimming speeds, it is more ecologically relevant as well as more comparable to existing and future critical swimming speed studies.

For the ΔUcrit methodology to be feasible, I propose 3 assumptions/guidelines that should be followed: 1) the baseline Ucrit, or measure of individual ability, must always be followed by the experimental measure. If the experimental Ucrit were performed as the initial test, it is likely that the stress imparted on the individual will have an effect on the following baseline measure. 2) It is assumed that effects of training are homogenous amongst all test subjects. Critical swimming speed tests have been shown to be repeatable, but training effects, both in physical fitness and from test experience have also been documented (see Hammer

1995). This assumption should always be met as long as the correct order of swimming tests is performed. 3) The time between the baseline and experimental measures should be consistent between all test subjects. Deviation from this guideline invalidates the assumption of homogenous training effects.

I feel that ΔUcrit is a valid method as long as the guidelines are followed; however, the increased statistical inference did not come without a cost. Some caveats that come with performing ΔUcrit as opposed to Ucrit alone are increased time, space, and material commitments. There is a need to track individuals in a time sensitive manner. Passive integrated transponder (PIT) tags were used in this study to track individual performances. This step incurs great material costs as well as time required for recovery from the surgery. The subjects were also placed in separate aquaria after the baseline Ucrit to ensure they would perform the experimental Ucrit exactly 7 days later. The separate aquaria greatly added to the space requirements of the method because the subjects need to be separated for easy access. Perhaps the most inconvenient aspect of this method is the time commitment. The amount of work required for a single data point is essentially doubled because each individual must perform two trials.

Several recent variations of Ucrit have emerged in an attempt to shorten Ucrit procedures while still obtaining ecologically and physiologically relevant data. I feel this method is worth the extra time, space, and material because it ultimately leads to greater effect resolution with fewer replications. This study showed that individual variation can inhibit and/or alter statistical inference. Also, this study used hatchery reared fish of similar age, size, and more than likely genetics, potentially resulting in less inter-individual variation than may be encountered using subjects obtained from the wild.

Although ΔUcrit decreased the amount of individual variation in the reduced model, approximately 31.7% was still left unexplained. There are several potential factors that possibly contributed to variation in this study. Although all subjects used in this study were purchased from the same fish hatchery, they were obtained at two different times and likely comprise two different year classes. A batch effect between or within these two different groups likely added to variation in the model. Swimming tests were also variable in time, ranging from June 2013 to March 2014.

The swim chamber apparatus used for this study was extremely useful due its automated functions. However it was limited in its precision of velocity measurements. The velocity meter used only recorded speeds in increments of 0.8 cm/s, which greatly limited the resolution of swimming speeds. Another downfall with the swim chamber apparatus was the potential for subjects to locate refuges from laminar flow.

The electrically charged downstream mesh prevented subjects from resting on the mesh, but there were instances where subjects were observed resting in areas upstream from the mesh. For example, there is a wire porch at the bottom of the chamber with stabilizing cross bars. The bluegill were observed to support themselves in the current by pinning their caudal or anal fins against the cross bars. On occasion, substances (e.g. duct tape, sloughed epoxy resin from the reservoir) would become dislodged and plug the upstream mesh divider, creating turbulence and refuge from a laminar flow. Bluegills were observed to elicit this behavior also. The subjects additionally appeared to have a higher affinity for one side of the swim chamber, indicating a potential heterogeneous flow through the chamber with increased refuge on the side most frequented by subjects.

Management Implications

This study reiterates the need for caution when using copper sulfate in an aquatic treatment regime. Copper is known to be toxic to fish and several other aquatic organisms and the awareness of increased oxygen demand as a result of its application are well-known. However, we have shown that the effects of hypoxia as a result of Cu application go well beyond simple asphyxiation. The combined effect of these two stressors has the potential to greatly reduce the available energy of individual fish. Therefore, there is a need for extra caution and awareness when using Cu-containing products on waters in situations that may facilitate hypoxia (i.e. warm, cloudy, calm conditions). I have demonstrated that the effects of Cu and hypoxia combine to have a greater effect on bluegill scope for activity than either stressor alone. A decrease in scope for activity can translate to reduced growth, fecundity, and/or immune function. Additionally, fish are likely to be impacted by more than one or two environmental stressors at any given time. The seemingly sub-lethal effects of Cu and hypoxia may compound with additional environmental stressors to further decrease scope for activity and increase the probability of mortality. Furthermore, the unknown relationship of multiple stressors combined on scope for activity is likely to be much more complex than a simple additive relationship.

APPENDIX

Appendix A
Critical Swimming Speed Data

Table 5. Raw swimming speed data, including bluegills that were removed from the analysis (highlighted). Each bluegill was assigned a generic number (Fish_#) and belongs to 1 of 12 groups comprised of the combinations of the three treatment factors (O2-hypoxia, Cu-Copper, and Hr-Duration of Exposure). Relative weight (Wr) was calculated from the length (L) and weight (Wt) measured from each individual. Base_T/Exp_T= Duration (minutes) of baseline and experimental swim test. Hct=Hematocrit (%). Base_Vel/Exp_Vel= maximum critical swim speed (cm/s) of baseline and experimental swim test. Base_BL/Exp_BL= Base_Vel/Exp_Vel normalized to body lengths per second. Δ_Ucrit= Exp_BL – Base_BL.

Fish_#	L (cm)	Wt (g)	Wr	Hct	O2	Cu	Hr	Base_T	Base_Vel	Base_BL	Exp_T	Exp_Vel	Exp_BL	Δ_Ucrit
88	10.2	17.2	88.9	34.0	SAT	0	24	128.33	40.58	3.978	141.42	45.35	4.446	0.468
91	10.8	20.1	86.0	52.8	SAT	0	24	107.50	34.22	3.169	127.92	41.38	3.832	0.663
92	10.7	20.5	90.4	46.5	SAT	0	24	136.42	43.76	4.090	137.17	46.15	4.313	0.223
95	10.3	17.8	89.1	34.4	SAT	0	24	111.58	35.01	3.399	121.00	40.58	3.940	0.541
98	10.2	17.5	90.5	35.7	SAT	0	24	109.92	35.01	3.432	106.75	35.01	3.432	0.000
118	10.3	17.1	85.6	34.1	SAT	0	48	136.83	46.95	4.558	151.75	49.33	4.789	0.231
119	9.2	12.8	93.2	36.8	SAT	0	48	132.50	45.35	4.929	145.75	45.35	4.929	0.000
122	10.0	15.0	82.8	39.5	SAT	0	48	116.25	35.81	3.581	91.50	27.05	2.705	-0.876
123	10.6	18.6	84.6	-	SAT	0	48	145.67	50.13	4.729	137.08	41.38	3.904	-0.826
120	9.5	14.6	95.5	43.9	SAT	0	48	119.00	40.58	4.272	117.58	24.22	2.550	-1.722
124	9.8	16.5	97.4	42.9	SAT	0	48	125.50	42.97	4.385	131.17	39.78	4.059	-0.326
125	10.0	17.0	93.9	44.1	SAT	0	48	143.00	48.54	4.854	136.83	41.38	4.138	-0.716
137	9.5	13.4	87.7	40.1	SAT	0	48	95.50	31.03	3.266	102.00	31.83	3.351	0.084
138	9.7	15.2	92.8	46.3	SAT	0	48	112.25	35.81	3.692	102.92	32.62	3.363	-0.329
139	9.5	12.5	81.8	40.0	SAT	0	48	109.67	35.81	3.769	87.58	26.26	2.764	-1.005
69	9.6	14.5	91.7	-	SAT	0	96	108.42	33.42	3.481	117.08	38.19	3.978	0.497
70	12.7	33.2	83.0	-	SAT	0	96	122.75	39.78	3.132	65.25	19.10	1.504	-1.628
72	9.4	14.2	96.2	-	SAT	0	96	101.00	31.83	3.386	100.08	31.03	3.301	-0.085
73	9.9	15.0	85.6	-	SAT	0	96	117.17	37.40	3.778	106.67	34.22	3.457	-0.321

Table 5 cont.

Fish_#	L (cm)	Wt (g)	Wr	Hct	O2	Cu	Hr	Base_T	Base_Vel	Base_BL	Exp_T	Exp_Vel	Exp_BL	Δ_Ucrit
75	10.1	17.7	94.5	-	SAT	0	96	110.67	35.01	3.466	115.17	37.40	3.703	0.237
77	12.6	34.8	89.3	-	SAT	0	96	120.75	38.19	3.031	128.50	40.58	3.221	0.190
78	8.6	10.0	91.0	-	SAT	0	96	101.67	31.83	3.701	113.92	36.60	4.256	0.555
79	10.5	17.3	81.2	42.4	SAT	0	96	112.67	35.01	3.334	123.42	39.78	3.789	0.454
81	9.6	13.9	87.9	41.4	SAT	0	96	96.42	30.24	3.150	107.50	34.22	3.565	0.415
82	10.1	17.5	93.5	49.6	SAT	0	96	102.33	32.62	3.230	114.17	36.60	3.624	0.394
86	10.7	17.8	78.5	41.5	SAT	0	96	104.58	33.42	3.123	113.42	35.81	3.347	0.223
87	10.0	15.8	87.2	37.8	SAT	0	96	112.33	35.81	3.581	111.67	35.81	3.581	0.000
89	11.9	29.7	92.1	42.8	SAT	1	24	118.67	37.40	3.143	138.50	43.76	3.677	0.535
90	10.5	20.2	94.9	43.2	SAT	1	24	123.17	38.99	3.713	117.50	35.81	3.411	-0.303
94	9.5	15.0	98.2	43.6	SAT	1	24	124.42	39.78	4.187	131.17	44.56	4.691	0.503
96	11.3	18.2	67.0	34.4	SAT	1	24	93.33	28.65	2.535	77.08	25.46	2.253	-0.282
97	11.4	26.2	93.7	40.6	SAT	1	24	123.17	38.99	3.420	130.83	44.56	3.909	0.489
41	13.3	39.8	85.3	-	SAT	1	48	95.50	29.44	2.214	109.42	33.42	2.513	0.299
42	10.5	19.9	93.5	-	SAT	1	48	118.58	37.40	3.562	134.50	42.97	4.092	0.531
43	10.7	21.6	95.3	-	SAT	1	48	109.83	34.22	3.198	117.67	37.40	3.495	0.297
44	9.8	17.1	100.9	-	SAT	1	48	115.33	36.60	3.735	124.00	39.78	4.059	0.325
49	10.3	17.0	85.1	-	SAT	1	48	106.08	32.62	3.167	123.83	38.99	3.785	0.618
50	12.6	35.5	91.1	-	SAT	1	48	133.33	42.97	3.410	142.33	46.15	3.663	0.252
51	10.3	18.7	93.6	-	SAT	1	48	111.33	35.01	3.399	106.00	33.42	3.245	-0.154
52	9.6	13.2	83.4	-	SAT	1	48	111.50	35.01	3.647	132.75	42.97	4.476	0.829
57	10.9	22.0	91.3	-	SAT	1	48	126.08	40.58	3.723	116.75	37.40	3.431	-0.292
58	9.3	13.0	91.3	-	SAT	1	48	98.00	30.24	3.252	101.67	31.03	3.337	0.085
140	10.1	16.7	89.2	44.3	SAT	1	48	125.67	42.97	4.254	79.08	23.87	2.363	-1.891
141	9.7	14.8	90.4	37.5	SAT	1	48	97.42	31.03	3.199	106.25	33.42	3.445	0.246
142	9.8	14.8	87.4	43.4	SAT	1	48	114.25	37.40	3.816	113.83	37.40	3.816	0.000

Table 5 cont.

Fish_#	L (cm)	Wt (g)	Wr	Hct	O2	Cu	Hr	Base_T	Base_Vel	Base_BL	Exp_T	Exp_Vel	Exp_BL	Δ_Ucrit
143	9.8	15.3	90.3	42.1	SAT	1	48	111.67	35.01	3.572	112.25	36.60	3.735	0.162
61	9.6	13.9	87.9	-	SAT	1	96	98.83	31.03	3.232	119.00	39.78	4.144	0.912
62	9.7	13.7	83.7	-	SAT	1	96	96.00	29.44	3.035	123.83	40.58	4.184	1.149
63	10.0	15.4	85.0	-	SAT	1	96	102.67	32.62	3.262	123.67	41.38	4.138	0.876
64	10.8	20.7	88.5	-	SAT	1	96	109.75	35.01	3.242	127.17	41.38	3.832	0.590
65	13.4	39.7	83.0	-	SAT	1	96	113.67	36.60	2.731	141.25	46.15	3.444	0.713
67	9.3	12.5	87.8	-	SAT	1	96	97.00	30.24	3.252	117.33	38.19	4.107	0.855
68	11.0	19.4	78.1	-	SAT	1	96	105.67	33.42	3.038	125.17	41.38	3.762	0.724
74	9.8	14.2	83.8	42.5	SAT	1	96	73.42	22.28	2.274	104.33	33.42	3.410	1.137
76	9.7	15.7	95.9	56.1	SAT	1	96	107.58	33.42	3.445	113.17	36.60	3.773	0.328
80	9.6	15.6	98.6	45.3	SAT	1	96	114.75	36.60	3.813	99.92	31.83	3.316	-0.497
83	10.3	18.1	90.6	47.0	SAT	1	96	87.92	27.85	2.704	109.17	35.01	3.399	0.695
84	11.9	27.8	86.2	40.7	SAT	1	96	116.33	36.60	3.076	123.50	39.78	3.343	0.267
85	10.2	16.4	84.8	43.1	SAT	1	96	114.92	36.60	3.588	123.25	39.78	3.900	0.312
111	10.0	15.9	87.8	47.5	LOW	0	24	117.25	40.58	4.058	99.50	33.42	3.342	-0.716
112	9.9	16.2	92.5	47.9	LOW	0	24	118.92	40.58	4.099	105.58	35.81	3.617	-0.482
113	11.9	30.1	93.3	43.3	LOW	0	24	127.83	44.56	3.745	104.92	35.81	3.009	-0.735
114	11.0	24.4	98.2	37.1	LOW	0	24	143.83	50.13	4.557	93.58	31.03	2.821	-1.736
117	10.2	19.0	98.2	46.5	LOW	0	24	124.92	42.97	4.213	99.83	33.42	3.277	-0.936
130	9.3	12.7	89.2	46.5	LOW	0	48	142.33	41.38	4.450	119.25	39.78	4.277	-0.172
131	9.6	14.6	92.3	44.7	LOW	0	48	125.92	36.60	3.813	99.83	28.65	2.984	-0.828
129	9.0	13.0	101.8	40.4	LOW	0	48	131.83	38.99	4.332	99.00	28.65	3.183	-1.149
128	10.2	16.2	83.8	39.6	LOW	0	48	101.83	30.24	2.965	88.50	25.46	2.496	-0.469
103	9.4	15.5	105.1	50.3	LOW	0	96	131.83	46.15	4.910	101.17	34.22	3.640	-1.269
104	9.9	17.0	97.0	49.4	LOW	0	96	122.00	42.17	4.260	102.42	35.01	3.536	-0.723
105	10.6	19.0	86.5	46.6	LOW	0	96	137.75	48.54	4.579	102.08	34.22	3.228	-1.351

Table 5 cont.

Fish_#	L (cm)	Wt (g)	Wr	Hct	O2	Cu	Hr	Base_T	Base_Vel	Base_BL	Exp_T	Exp_Vel	Exp_BL	Δ_Ucrit
106	9.3	12.2	85.7	-	LOW	0	96	105.83	37.40	4.022	86.42	29.44	3.166	-0.856
107	11.6	28.0	94.5	48.7	LOW	1	24	136.58	46.95	4.047	91.25	30.24	2.607	-1.441
108	10.7	23.1	101.9	34.2	LOW	1	24	130.75	44.65	4.173	74.83	23.87	2.231	-1.942
109	10.4	18.0	87.3	50.7	LOW	1	24	121.58	41.38	3.979	66.17	21.48	2.065	-1.914
110	8.3	9.2	94.2	52.5	LOW	1	24	105.67	38.99	4.698	19.40	7.16	0.863	-3.835
115	10.8	22.4	95.8	50.9	LOW	1	24	115.50	39.78	3.683	64.25	19.89	1.842	-1.842
116	10.2	18.6	96.2	44.0	LOW	1	24	131.67	46.15	4.525	66.83	21.48	2.106	-2.419
132	10.4	19.7	95.5	68.2	LOW	1	48	127.25	39.78	3.825	0.00	0.00	0.000	-3.825
133	9.5	14.0	91.6	64.7	LOW	1	48	110.17	32.62	3.434	8.00	8.75	0.921	-2.513
135	10.2	18.0	93.1	67.1	LOW	1	48	137.42	41.38	4.057	0.00	0.00	0.000	-4.057
134	9.9	15.4	87.9	68.7	LOW	1	48	100.75	29.44	2.974	0.00	0.00	0.000	-2.974
136	10.6	21.4	97.4	58.6	LOW	1	48	102.08	29.44	2.777	0.00	0.00	0.000	-2.777
144	9.8	14.0	82.6	47.9	LOW	1	48	107.83	31.83	3.248	90.08	28.65	2.923	-0.324
145	9.4	13.6	92.2	43.6	LOW	1	48	120.92	38.19	4.063	90.92	27.85	2.963	-1.100
146	9.3	11.9	83.6	45.6	LOW	1	48	107.42	34.22	3.680	89.42	27.85	2.995	-0.685
147	9.5	13.4	87.7	52.1	LOW	1	48	119.42	38.99	4.104	81.83	25.46	2.680	-1.424
99	10.2	18.2	94.1	50.8	LOW	1	96	126.17	44.65	4.378	96.67	32.62	3.198	-1.179
100	10.0	17.4	96.1	46.7	LOW	1	96	105.67	36.60	3.660	42.17	11.94	1.194	-2.466
101	10.5	21.2	99.6	43.2	LOW	1	96	127.83	40.58	3.865	99.08	33.42	3.183	-0.682
102	9.0	11.3	88.5	40.0	LOW	1	96	125.00	43.76	4.862	78.08	26.26	2.918	-1.944

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