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## Comparing Methods of Euthanasia and Gill Culture for Hypoxia Research on the Gulf Killifish, *Fundulus grandis*

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Comparing Methods of Euthanasia and Gill Culture for Hypoxia Research on the Gulf Killifish,  
*Fundulus grandis*

A Thesis

Submitted to the Graduate Faculty of the  
University of New Orleans  
in partial fulfillment of the  
requirements for the degree of

Master of Science  
in  
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by

Kristina M. Farragut

B.S.E. Tulane University, 2013  
B.S. Tulane University, 2013

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## ABSTRACT

Aquatic hypoxia, or low dissolved oxygen, is a growing environmental concern and has detrimental effects on many fishes. Research on fish responses to hypoxia includes whole animal studies as well as organ culture systems. In this thesis, the gulf killifish, *Fundulus grandis*, was used to determine the interaction between hypoxic exposure and four common euthanasia techniques on selected blood and gill variables and to develop an *in vitro* gill incubation system. Euthanasia techniques had differential effects on blood and gill, with the common fish anesthetic MS-222 having the greatest effects, but none altered the response to hypoxia. During the development of gill culture, the highest gill viability was measured in isosmotic seawater medium and shorter incubation times. This study provides insight into the applicability of different euthanasia techniques in hypoxia research, as well as preliminary observations on an incubation system for isolated gills.

Key words: euthanasia, hypoxia, gill culture, *Fundulus grandis*, and gulf killifish.

# **Chapter 1: Interactions Between Euthanasia Technique and Hypoxia Exposure in the Gulf Killifish, *Fundulus grandis***

## **1.1. Introduction**

### **1.1.1. Hypoxia in the Aquatic Environment**

Biological organisms may be negatively affected by unfavorable environmental conditions such as extreme levels of temperature, oxygen, pH, and pollutants. Extreme habitats can be wide-ranging such as desert regions, but can also exist on a smaller scale such as hydrothermal vents on the ocean floor. Some organisms are able to live in these extreme environmental conditions because of specialized adaptations and phenotypes leading to distinctive ecological communities (Tobler *et al.*, 2007; Tobler *et al.*, 2008; Chapman, 2015). Fishes have inhabited almost all aquatic environments including habitats that test the limits of tolerance. Aquatic hypoxia (or low dissolved oxygen) tests the oxygen tolerance of fishes and provides a system for studying life under extreme conditions. In aquatic environments, dissolved oxygen (DO) can vary over time and space and can range from zero (anoxic) to levels above normal air-saturation (hyperoxic)(D'Avanzo and Kremer, 1994; Diaz, 2001; Diaz and Rosenberg, 2008; Tyler *et al.*, 2009). Hypoxic zones are areas of water with low oxygen concentrations and can occur intermittently or can become permanent (Breitburg, 1990; D'Avanzo and Kremer, 1994; Diaz, 2001; Diaz and Rosenberg, 2008; Smith and Able, 2003; Tyler *et al.*, 2009). They occur naturally but can also be created or enhanced by human activity. Some hypoxic zones can reach such low levels of oxygen that most animal life suffocates and dies. Such extreme hypoxic zones are referred to as "dead zones." Dead zones have increased drastically since the 1960s and are reported to comprise an area of more than 245,000 km<sup>2</sup> globally (Diaz and Rosenberg, 2008). Therefore, these spreading dead zones are creating extreme hypoxic environments on a large spatial scale potentially resulting in massive loss of life (Diaz and Rosenberg, 1995).

### *Properties of Water*

Oxygen solubility in water is 1/30th of its solubility in air, and oxygen diffuses 10,000 times more slowly through water than air (Nikinmaa and Salama, 1998). Oxygen solubility also decreases as temperature or salinity increases (Wetzel, 2001). Water obtains oxygen by diffusion from the atmosphere and by photosynthetic production, which depends upon photoperiod, water clarity, and the abundance of primary producers. On the other hand, oxygen is consumed primarily by respiration of aquatic organisms (Wetzel, 2001). If the rate of oxygen depletion is not balanced with the rate of oxygen replenishment, hypoxia results. Aquatic hypoxia commonly reflects habitats with a DO concentration of 2 mg/l or below; however this definition is deceiving because it does not reflect a level of DO that negatively affects all organisms, some of which can survive lower levels of oxygen before experiencing detrimental effects (Vaquer-Sunyer and Duarte, 2008; Pollock *et al.*, 2007).

### *Natural Hypoxia*

Naturally occurring hypoxic zones can occur in several ways. Hypoxic conditions can be generated and worsened by physical means such as by pool isolation, lake stratification, and inadequate light for photosynthetic production of oxygen (Congleton, 1980; Innes and Wells, 1985; Chapman and Kramer, 1991; Chapman, 2015; Diaz, 2001). Oceanic oxygen minimum zones (OMZs) are some of the largest well-known examples of naturally occurring hypoxic zones on Earth, occupying approximately 102 million km<sup>3</sup>, and are located in oceans at intermediate depths (400–1,000 m) (Childress and Seibel, 1998; Wright *et al.*, 2012). Waters above OMZs are well oxygenated, but once the OMZ is entered, the DO drops due to the imbalance of oxygen consumption and oxygen production. Hypoxic conditions can also be

generated and worsened by diurnal or seasonal means. In eutrophic lakes, photosynthetic activity and biological respiration may cause a diel variation in DO resulting in normoxia at midday and hypoxia or anoxia at night (Congleton, 1980; Chapman, 2015; Breitburg, 1992; Diaz, 2001; Tyler *et al.*, 2009; Wetzel, 2001). Oxygenated streams in the rainy seasons may become small isolated hypoxic pools during drier seasons (Chapman and Kramer, 1991). During winter, lakes covered by ice have a reduction in aeration, which in combination with biological respiration and organic decomposition, can result in severe hypoxia or anoxia for extended periods. During the summer, elevated temperatures cause oxygen to become less soluble and increase rates of organic decomposition and biological respiration thereby decreasing DO levels (Chapman, 2015).

#### *Human-Induced Hypoxia*

Aquatic hypoxia has been exacerbated globally by human activities in the last 25 years and is expected to continue to worsen in the future (Diaz and Rosenberg, 2008; Diaz and Rosenberg, 1995; Rabalais *et al.*, 2010; Diaz, 2001; Ficke *et al.*, 2007). Human-induced environmental degradation through pollution, urbanization and other anthropogenic causes is increasing the occurrence of hypoxia zones leading to mass mortality and thus population declines and changes in community structure (Diaz, 2001; Diaz and Rosenberg, 2008; Dybas, 2005; Rabalais *et al.*, 2002).

#### *Aerobic and Anaerobic Metabolism*

All fishes require oxygen for long-term survival. Fishes use oxygen in the process of mitochondrial respiration to provide energy in the form of ATP, which is needed for processes

such as growth, movement, and reproduction. At low oxygen, ATP supply via aerobic metabolism decreases and the resulting energy deficit can cause physiological dysfunction and, if not corrected, death. Fish encountering hypoxia often try to maintain their cellular ATP supply by either increasing O<sub>2</sub> transport to support aerobic respiration, by increasing the use of anaerobic ATP-producing energy metabolism, or by metabolic suppression (Boutilier, 2001; DeKoning *et al.*, 2004; Pollock *et al.*, 2007; Land *et al.*, 1993; Jibb and Richards, 2008b). The minimum oxygen level required to maintain a constant rate of oxygen consumption is defined as critical tension ( $P_{crit}$ ), below which oxygen consumption decreases linearly with oxygen tension. At any oxygen tension ( $PO_2$ ) above  $P_{crit}$ , tissue O<sub>2</sub> supply is sufficient to support aerobic metabolism (Burton and Heath, 1980; Pörtner and Grieshaber, 1993). However, below  $P_{crit}$ , aerobic metabolism decreases and anaerobic metabolism is utilized to help supplement ATP supply (Dunn and Hochachka, 1986; Pörtner and Grieshaber, 1993; Richards, 2009). However, this is just a temporary fix. Anaerobic metabolism is limited by substrate availability and results in the accumulation of harmful end products. These attributes mean the duration of anaerobic metabolism is limited (Seibel, 2011).

### **1.1.2. *Fundulus grandis* as a Superior Model Organism**

*Fundulus* are a diverse and widespread genus of small nonmigratory teleost fishes, with species inhabiting freshwater streams and coastal marshes of North America and the Gulf of Mexico (Lee *et al.*, 1983; Scott and Crossman, 1998; Skinner *et al.*, 2005). Certain estuarine species such as *F. heteroclitus* (Atlantic Killifish) and *F. grandis* (Gulf Killifish) are able to tolerate a broad range of environmental conditions, including wide fluctuations in temperature, salinity, oxygen, and pH making these fishes a powerful scientific model for studying physiological, biochemical,

ecological, and developmental responses to varying environmental conditions (Burnett *et al.*, 2007; Diaz, 2001; Smith and Able, 2003). In particular, several studies indicate *F. heteroclitus* and *F. grandis* to be among the most tolerant estuarine fishes to low dissolved oxygen levels (D'Avanzo and Kremer, 1994; Smith and Able, 2003; Cochran and Burnett, 1996; Love and Rees, 2002; Stierhoff *et al.*, 2003; Wannamaker and Rice, 2000) with little or no mortality occurring until dissolved oxygen drops below 1 mg/l (Smith and Able, 2003; Voyer and Hennekey, 1972). The  $P_{crit}$  for *F. heteroclitus* and *F. grandis* is approximately 1.6 mg/l (Cochran and Burnett, 1996; Virani and Rees, 2000). Below 1.6 mg/l, *F. heteroclitus* and *F. grandis* undertake behavioral, physiological and biochemical changes that improve oxygen uptake and delivery to tissues, reduce aerobic metabolism, and increase anaerobic metabolism (Smith and Able, 2003; Cochran and Burnett, 1996; Stierhoff *et al.*, 2003; Wannamaker and Rice, 2000; Greaney and Powers, 1977; Greaney and Powers, 1978; Greaney *et al.*, 1980). Therefore, studies on *F. heteroclitus* and *F. grandis* can offer valuable insight into the physiological and molecular mechanisms that underlie this hypoxia tolerance. Other factors that make *F. heteroclitus* and *F. grandis* ideal organisms for hypoxic studies are their abundance, accessibility, small size, and ease of laboratory maintenance. *F. grandis* is a species of *Fundulus* native to the Gulf of Mexico that experiences periodic hypoxia to varying degrees (Engle *et al.*, 1999) and is the organism chosen for the experiments described within this thesis.

### **1.1.3. Physiological Responses to Hypoxia**

Acute hypoxia induces a stress response in many fishes. This stress response involves the activation of the hypothalamo-pituitary-interrenal (HPI) axis resulting in the release of cortisol (van Raaij *et al.*, 1996; Wendelaar Bonga, 2011), as well as downstream changes in the

circulating concentrations of metabolites such as glucose (Barton, 2002). If insufficient oxygen is available to support aerobic ATP production, fish may resort to anaerobic metabolism resulting in the accumulation of lactate (van Raaij *et al.*, 1996; Zhou *et al.*, 2000; Greaney and Powers, 1978; Dunn and Hochachka, 1986; Greaney *et al.*, 1980; Cochran and Burnett, 1996) and a decline of ATP levels (Greaney and Powers, 1977; Smit and Hattingh, 1981). Hypoxic exposure also elevates hematocrit levels to improve blood oxygen carrying capacity. This is done either by splenic contraction releasing more red blood cells or by erythropoietin stimulation of new red blood cell formation (Chapman, 2015; Greaney *et al.*, 1980; Lai *et al.*, 2006). Changes in cortisol levels, metabolite concentrations, or red blood cell count, therefore, provide a means by which the severity and duration of the response of fishes to hypoxia can be quantified. Fishes may avoid these hypoxia-induced physiological changes by undergoing various behavioral responses such as aquatic surface respiration, a process in which fishes acquire oxygen by respiring at the water column's surface.

#### **1.1.4. Euthanasia in Fish Research**

Euthanasia is required in many experiments, especially those designed to collect tissue and blood samples for analysis. Some of the most common euthanasia techniques used in fish research are the drugs tricane methanesulfonate (MS-222) and clove oil, physical blunt trauma to the head, and rapid cooling. MS-222 and clove oil are also commonly used as anesthetics at lower doses with induction time inversely proportional to dosage. These two methods have been shown to affect hematocrit, lactate, cortisol, and glucose levels in various fishes within a broad range of dosages (Houston *et al.*, 1971; Reinitz and Rix, 1977; Lowe-Jinde and Niimi, 1983; Bowzer *et al.*, 2012; Velisek *et al.*, 2011; Weber *et al.*, 2011; Thomas and Robertson, 1991). Adverse

physiological effects are thought to be correlated with longer anesthetic induction times.

However, what is not known is how doses of MS-222 and clove oil needed to achieve euthanasia as well as the methods of rapid cooling and blunt trauma to the head alter variables that are affected by hypoxia.

### *Euthanasia modes of action*

MS-222 is an ester-type synthetic that acts systemically when absorbed through the gills and skin of fish in an anesthetic bath (Carter *et al.*, 2011). Once in the gills, MS-222 enters the bloodstream and is distributed throughout the body where it is metabolized rapidly and excreted through the gills (Wayson *et al.*, 1976; Carter *et al.*, 2011). While in the body, the lipid soluble MS-222 crosses the cell membranes of nerves and blocks voltage-gated sodium channels (Carter *et al.*, 2011; Butterworth and Strichartz, 1990). This results in the inhibition of nerve conduction. Unmetabolized MS-222 is rapidly excreted via the kidneys (Wayson *et al.*, 1976). Clove oil is an essential oil comprised of 85-95% of the active ingredient Eugenol. The anesthetic mechanism of clove oil is poorly understood but it appears to act similarly to MS-222 by inhibiting voltage-sensitive sodium channels within the nervous system (Leary *et al.*, 2013). The nerve inhibition produced by prolonged exposure to MS-222 and clove oil causes respiratory arrest and eventual death due to asphyxiation.

Rapid cooling is a euthanasia technique performed by rapidly chilling the fish in an ice bath (~2-4°C) until loss of orientation (Leary *et al.*, 2013). As the temperature drops, the metabolism of the fish slows down until reaching osmoregulatory shock (Matthews and Varga, 2012). Rapid



cooling has been shown to have a quicker induction time than MS-222 and is considered to be a more humane method of euthanasia for some species (Blessing *et al.*, 2010).

Blunt trauma to the head is an euthanasia technique involving a single sharp blow delivered to head with sufficient force to produce immediate depression of the CNS and destruction of the brain tissue making loss of consciousness rapid (Leary *et al.*, 2013). Although blunt trauma to the head is an easy and fast method of euthanasia, it may be visually unpleasant for the person using the technique.

#### **1.1.5. Goals**

The goal of this study is to determine if there is an interaction between euthanasia technique and hypoxic exposure on blood composition and gill viability in *F. grandis*. Specifically, the study tested if different euthanasia methods resulted in normoxic fish having blood and gill variables consistent with hypoxia. *Fundulus grandis* were exposed to either normoxic or hypoxic conditions ( $DO < P_{crit}$ ) for ~ 24 h and then euthanized by one of the following techniques: overdose of MS-222, overdose of clove oil, blunt trauma to the head, or rapid cooling. Blood composition was determined by measuring hematocrit, cortisol, lactate, and glucose levels. Gill viability was determined by trypan blue (TB) exclusion and relative ATP to ADP ratios.

### **1.2. Materials and Methods**

#### **1.2.1. Husbandry**

*Fundulus grandis* (n=56) were purchased from Joe's Landing Marina in Barataria, LA, and transported to the University of New Orleans, where they were randomly distributed among

several 40-l aquaria at a density less than 1 fish per 2.5 l in filtered, aerated, dechlorinated tap water made up to 1/3 strength seawater (1/3 SW) with Instant Ocean Synthetic Sea Salt. Fish were treated for ectoparasites and bacterial infection with API General Cure and API Furan-2 according to the manufacturer's directions. Water temperature and salinity were measured with an oxygen-temperature-salinity probe (Yellow Springs Instruments Model Pro2030) and ranged from 17°C to 25°C and 10 to 13, respectively, well within the range of tolerance of *Fundulus grandis* (Griffith, 1974). Photoperiod was 14:10 (light:dark) and fish were fed dried flake food (TetraMin Tropical Flakes) equivalent to approximately 1% of their body mass 6 or 7 times per week. To maintain water quality, 30-50% of the aquarium water was replaced every week and filters were changed every 2 weeks. Fish acclimated to these conditions for a minimum of 2 weeks before experiments. Food was withheld for 24 h prior to experiments. All handling and procedures with live animals were performed according to guidelines for research on vertebrate animals (University of New Orleans IACUC Protocol 15-004).

### **1.2.2. Exposures, Euthanasia, and Tissue Sampling**

The day prior to an experiment, fish were placed individually into 2.5-l aquaria containing 1.5 to 1.75 l of aerated, 1/3 SW and allowed to adjust to their new enclosure for 18-20 h. Routinely, two fish were used in parallel, one male and one female. Each aquarium was shielded by a blind to minimize disturbance to the fish. Experiments began between 10:00 and 11:00 by removing a 60 ml water sample to measure dissolved oxygen (DO) using a LaMotte Dissolved Oxygen Test Kit. For normoxic exposures, fish were then euthanized by one of four approaches described below. For hypoxic exposures, aeration was terminated and nitrogen gas was introduced at a flow rate (80 - 120 ml/min) to achieve a nominal DO of 0.8 mg/l (approximately 10% of the air-

saturated value at these temperatures and salinities). For these exposures, 60 ml water samples were removed at 1 h, 3 h, and 24 h after the initiation of nitrogen gassing for DO determination as above. After the 24 h water sample, hypoxic fish were euthanized as described below.

All fish were euthanized between 11:00 and 13:00 by one of the following approaches: blunt trauma to the head (BT), overdose of MS-222 (MS), overdose of clove oil (CO), or rapid cooling (RC). For BT, unanesthetized fish were netted as quickly as possible, removed from the aquarium, and struck forcefully on the head with a hand tool. For MS, 500 ml 1/3 SW containing 0.5 g MS-222 (neutralized by 2.0 g  $\text{NaHCO}_3$ ) were added to the aquarium (~1.5 l after accounting for water removed for DO measurements) to achieve a final concentration of 0.25 g/l MS-222 and 1.0 g/l  $\text{NaHCO}_3$ . For CO, 250 ml 1/3 SW containing 87.5  $\mu\text{l}$  clove oil (NOW essential oils; ~85% eugenol) were added to the aquarium to achieve a final concentration of 50  $\mu\text{l}$  clove oil/l. The dosages for MS-222 and clove oil were chosen as the upper level of doses used for anesthesia in a variety of fishes (Matthews and Varga, 2012; Leary, 2013; Mylniczenko *et al.*, 2014). For RC, frozen, crushed 1/3 SW was added to the aquarium in a ratio of approximately 1 l of frozen 1/3 SW to 1.5 l aquarium water. At this ratio, the temperature of the water dropped to  $\leq 4^\circ\text{C}$  within 2 min. For MS, CO, and RC, fish were netted and removed from the aquarium after they could no longer right themselves (loss of equilibrium, LOE). The time between the initial disturbance of the fish and death (BT) or LOE (MS, CO, and RC) was recorded for each fish. For all fish, euthanasia was ensured by cutting through the spinal cord immediately posterior to the head.

Blood was sampled from MS, CO, and RC treatments immediately after LOE by severing the caudal peduncle. Whenever possible, two blood samples were collected in heparinized microhematocrit tubes by capillary action. One blood sample was used to measure hematocrit by centrifugation for 3 min in a hematocrit centrifuge. The plasma from this sample was collected, transferred to a clean, labeled, pre-weighed micro-centrifuge tube, frozen in liquid nitrogen, and kept at -80°C until assayed for cortisol. The second blood sample was added to 300 µl ice-cold 6% perchloric acid in a clean, labeled, pre-weighed micro-centrifuge tube. After vortex mixing, this sample was centrifuged at 15,000 x g for 10 min at 4°C. The supernatant (300 µl) was removed and combined with 2 µl 0.05% methyl orange and sufficient 2M KOH/0.4 M imidazole to neutralize the sample (between 115 and 135 µl). The neutralized sample was frozen at -20°C. The time between LOE and the collection of blood samples was recorded for each fish. Blood samples could not be taken for the fish killed by BT, presumably because internal bleeding in the cranium reduced blood flow through the dorsal aorta and caudal artery. For some MS, CO, and RC fish, only one blood sample was obtained and additional fish were used as needed to achieve appropriate sample sizes for analysis of blood variables.

Immediately after euthanasia for BT and after blood sampling for MS, CO, and RC, the left gill basket was dissected, freeze-clamped in liquid nitrogen, and stored at -80°C until assayed for ATP and ADP. The time between death (BT) or LOE (MS, CO, and RC) and freeze-clamping the gills was recorded for each fish. The right gill basket was further dissected into four individual gill arches labeled 1-4 from anterior to posterior for trypan blue (TB) staining (see below).

In a subset of experiments, the osmolality of the aquarium water was measured using a vapor pressure osmometer (Wescor) to ensure that it had not been altered by the addition of frozen 1/3 SW or chemical euthanasia agents.

### **1.2.3. Blood Variables**

Note: the measurements described in this section were performed by Dr. Bernard Rees and Ariel Hernandez. The statistical analyses of the resultant data were carried out by K. Farragut and are reported as part of this thesis.

#### **1.2.3.1. Plasma Cortisol**

Micro-centrifuge tubes containing plasma were thawed and weighed. The volume of the plasma was determined as the difference between the tube weight before and after adding the plasma (see above) and assuming a density of 1.0 g/ml. Cortisol was extracted from plasma proteins by organic extraction. Briefly, plasma samples (10 – 30 µl) were acidified by the addition of 100 µl 30 mM HCl and mixed by vortexing. Then, 500 µl dichloromethane was added to each sample and mixed by vortexing. Aqueous and organic phases were allowed to separate, after which the organic phase was removed to a clean, labeled glass tube. The aqueous phase was extracted 3 more times with 500 µl dichloromethane, pooling the organic phases. Dichloromethane was evaporated at 30°C overnight, and the residue was dissolved in 500 µl of ELISA buffer (Cayman Chemical Company Kit #500360). All samples were diluted 10-fold in ELISA buffer, and plasma cortisol was determined in parallel with cortisol standards as specified by the manufacturer. A small number of samples had cortisol below the level of accurate determination;

for these, cortisol was measured in the sample without prior 10-fold dilution. Plasma cortisol in ng/ml was determined after accounting for plasma volume and dilution.

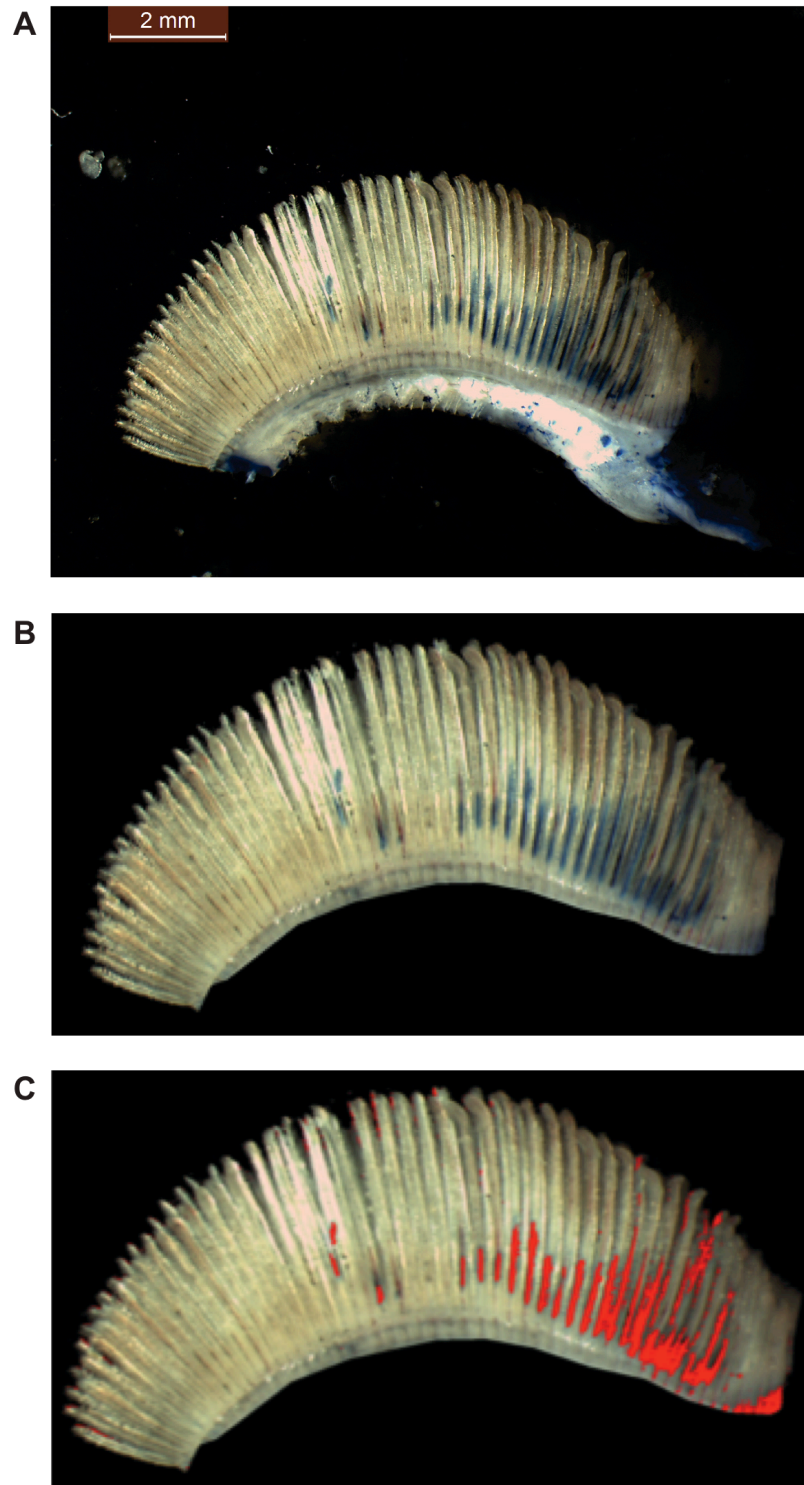
#### 1.2.3.2. Whole Blood Glucose and Lactate

Micro-centrifuge tubes containing neutralized perchloric acid extracts of whole blood samples were thawed. Prior to glucose and lactate assays, samples were centrifuged at 15,000 x g for 10 min at 4°C to remove potassium perchlorate salts. Glucose was determined by a colorimetric assay (Cayman Chemical Company Kit #10009582). Lactate was determined as described in Virani and Rees (2000). The concentrations of glucose and lactate in whole blood in mM were determined based upon the volume of the original blood sample, determined as the difference between the tube weight before and after adding the blood sample assuming a density of 1.1 g/ml (Virani and Rees, 2000) and accounting for dilutions due to the addition of perchloric acid, methyl orange, and KOH/imidazole.

#### 1.2.4. Gill Variables

##### 1.2.4.1. Trypan Blue Exclusion

Immediately after dissection, gill arches isolated for TB exclusion were individually incubated in 1ml 1/3 SW containing 0.04% TB for 7 min (Perry *et al.*, 1997). Gill arches were removed from the TB solution, cleaned of extraneous tissue and mucus, and imaged on both sides using a Leica MZ75 dissection scope with an attached camera in conjunction with the Leica Application Suite software (Figure 1.1A).



**Figure 1.1:** Illustration of how trypan blue staining on the gills of *F. grandis* was selected by ImageJ. (A) The raw gill image. (B) The gill isolated from the background and surrounding cartilage. (C) Areas stained with TB highlighted in red pseudo color to calculate percent stained.

The gill images were analyzed for percent TB stained using ImageJ as follows (Schneider *et al.*, 2012). First, the total area occupied by gill filaments was measured (Figure 1.1B). Then, the area stained with TB was selected using the color threshold option (Figure 1.1C). The settings for the color threshold were defined within a narrow range by the user to best capture the blue regions of the gill. The percent stained of each side of each gill arch was calculated by dividing the area stained by the total area and multiplying this ratio by 100. The percent stained for both sides of an individual gill arch were averaged to give a single value for percent TB staining for each gill arch. Analysis were done on the second gill arch (TB<sub>2</sub>) and an aggregate average of all four gill arches of the right gill basket (TB<sub>avg</sub>).

#### 1.2.4.2. ATP and ADP Measurements

The freeze-clamped left gill baskets were used to measure ATP and ADP as described by Passonneau and Lowry (1993) with modification. Intact, frozen gill baskets (100 – 200 mg fresh weight) were pulverized under liquid nitrogen using a mortar and pestle. The tissue powder was homogenized in 2.0 – 2.5 ml ice-cold 6% perchloric acid using a glass homogenizer with a motor-driven Teflon pestle (Thomas Technological Services 3431-E04 series). Tissue powder was homogenized for 40 s three times, with 60 s cooling on ice in between. To assess recovery of ATP and ADP, a subset of 3 extracts were split in half, with one half of each sample spiked with 3.8 nmol ATP and 3.8 nmol ADP (see Results). Acid extracts were centrifuged for 15 min at 10,000 x g at 4°C. Supernatants were removed to clean microcentrifuge tubes and pellets were dissolved in 1.0 ml 1 M NaOH and stored at -80°C for later protein quantification. Because the gill baskets have bone tissue, and therefore high concentrations of calcium ions, EDTA was added to the perchloric acid supernatant to chelate free calcium and prevent precipitation of Ca-ATP during the subsequent neutralization step (Passonneau and Lowry, 1993). A range of EDTA



concentrations (0 mM-100 mM) was tested in a subset of samples to determine the lowest concentration of EDTA that allowed quantitative recovery of ATP and ADP. This concentration was 50 mM (see Results), and therefore EDTA was added to acid supernatants to achieve a final concentration of 50 mM for the remainder of the analyses. After addition of EDTA, the acid supernatants were neutralized by adding 5 M  $K_2CO_3$  and stored at  $-80^\circ C$ .

Enzyme-coupled fluorometric assays were used to determine the concentrations of ATP and ADP (Passonneau and Lowry, 1993). Prior to assay, neutralized extracts were thawed and centrifuged at  $13,000 \times g$  for 10 min at  $4^\circ C$ . The 2.0 ml assay for ATP contained: 100  $\mu l$  extract; 50 mM Tris-HCl, pH 8.1; 6 mM  $MgCl_2$ ; 0.5 mM dithiothreitol; 50  $\mu M$   $NADP^+$ ; 100  $\mu M$  glucose. Fluorescence was measured for a minimum of 60 s for each sample, after which 0.36 U glucose-6-phosphate dehydrogenase (Roche, cat# 10165875001) was added, and the fluorescence was measured until it reached a stable value ( $\sim 10$ -15 min). Then, 1.2 U hexokinase (Roche, cat# 11426362001) was added, and fluorescence was again measured until it reached a stable value ( $\sim 10$ -15 min). ATP was determined as the change in fluorescence after the addition of hexokinase, and the total ATP in the gill (nmol) was determined after accounting for dilution due to the addition of perchloric acid, EDTA, and  $K_2CO_3$ .

The 2.0 ml assay for ADP contained: 200  $\mu l$  extract; 50 mM imidazole-HCl, pH 7.0; 6 mM  $MgCl_2$ ; 75 mM KCl; 10  $\mu M$  NADH; 20  $\mu M$  phospho-enol pyruvate. Fluorescence was measured for a minimum of 2 min for each sample, after which 2.2 U lactate dehydrogenase (Roche cat# 1012876001) was added, and the fluorescence was measured until it reached a stable value ( $\sim 10$ -15 min). Then, 2.0 U pyruvate kinase (Sigma cat# P1506) was added, and fluorescence was again measured until it reached a stable value ( $\sim 10$ -15 min). ADP was determined as the change

in fluorescence after the addition of pyruvate kinase, and the total ADP in the gill (nmol) was determined after accounting for dilution due to the addition of perchloric acid, EDTA, and  $K_2CO_3$ .

All fluorescence measurements were made at room temperature with a Perkin Elmer LS55 Fluorescence Spectrometer using the default time drive method application in the FL WinLab software. The excitation  $\lambda$  was 340 nm, with a slit width of 15.0 nm. The emission  $\lambda$  was 460 nm, with a slit width of 10.0 nm. The data interval was 0.5 s.

#### 1.2.4.3. Protein Measurement

Pellets from perchloric acid extracts and dissolved in NaOH were used to determine the total protein in the gill samples with the bicinchoninic acid protein assay (Pierce, ThermoFisher Scientific) with bovine serum albumin standards (Smith *et al.*, 1985). Standards and samples were assayed in triplicate and absorbance was measured with a Beckman DU-640 spectrophotometer. For each gill basket, ATP and ADP values were divided by the protein content to get nmol ATP/mg protein or nmol ADP/mg protein.

#### 1.2.5. Statistical Analysis

Experimental variables included DO, room temperature, water osmolality, fish mass, time to LOE, time required to sample blood, time required to freeze-clamp gills, percent hematocrit, plasma cortisol, blood glucose, blood lactate,  $TB_2$ ,  $TB_{avg}$ , gill ATP/mg protein, gill ADP/mg protein, and gill ATP:ADP. All experimental variables were tested for normality using the Anderson Darling test, along with visual inspection of residuals (Ghasemi and Zahediasl, 2012). The change in DO over time during the hypoxia exposures was analyzed using a Friedman rank

sum test. Final DO, RT, water osmolality, fish mass, time required to sample blood, time required to freeze-clamp gills, percent hematocrit, and blood glucose were normally distributed ( $p > 0.05$ ). Other variables were transformed to achieve normal distributions using the Box-Cox procedure in Rstudio (Box and Cox, 1964; Millard, 2013). The following exponential transformations were normally distributed: (time to LOE)<sup>0.6</sup>; (plasma cortisol)<sup>0.4</sup>; (blood lactate)<sup>0.4</sup>; (TB staining of gill 2)<sup>0.1</sup>; (TB<sub>avg</sub>)<sup>0.1</sup>; (ATP/mg protein)<sup>1.5</sup>; (ADP/mg protein)<sup>0.5</sup>; and (ATP:ADP)<sup>-0.2</sup>. Homogeneity of group variances was verified using Levene's tests. Exploratory data analysis used one-way ANOVAs to test the effect of sex and least squares linear regression to test the effects of mass on all experimental variables. Sex did not affect any variable and was left out as a factor during further statistical analyses. Mass was found to be significantly correlated with blood glucose and TB staining (both in gill 2 and TB<sub>avg</sub>). To test the effects of gill arch location on TB staining, a nested ANOVA was performed with oxygen condition, euthanasia approach, and gill arch nested within fish as factors. Two-way ANOVAs were used to assess the effects of oxygen condition and euthanasia approach on all experimental variables (after appropriate transformation) and the mass-corrected residuals of blood glucose, TB staining of gill 2, and TB<sub>avg</sub>. When euthanasia approach was statistically significant, Tukey's post hoc tests were used to test for differences among euthanasia approaches. Unless otherwise stated, interactions between oxygen condition and euthanasia approach were not significant ( $p > 0.05$ ). In some cases, one-way ANOVAs were done for each oxygen condition (normoxia and hypoxia) to assess the effects of euthanasia approach. All analyses were conducted using R 3.2.3 (cran.r-project.org) and SYSTAT with a confidence level of 95% ( $\alpha = 0.05$ ). Means are expressed with one standard deviation and graphs were generated with Graphpad Prism 7.0a.

## 1.3. Results

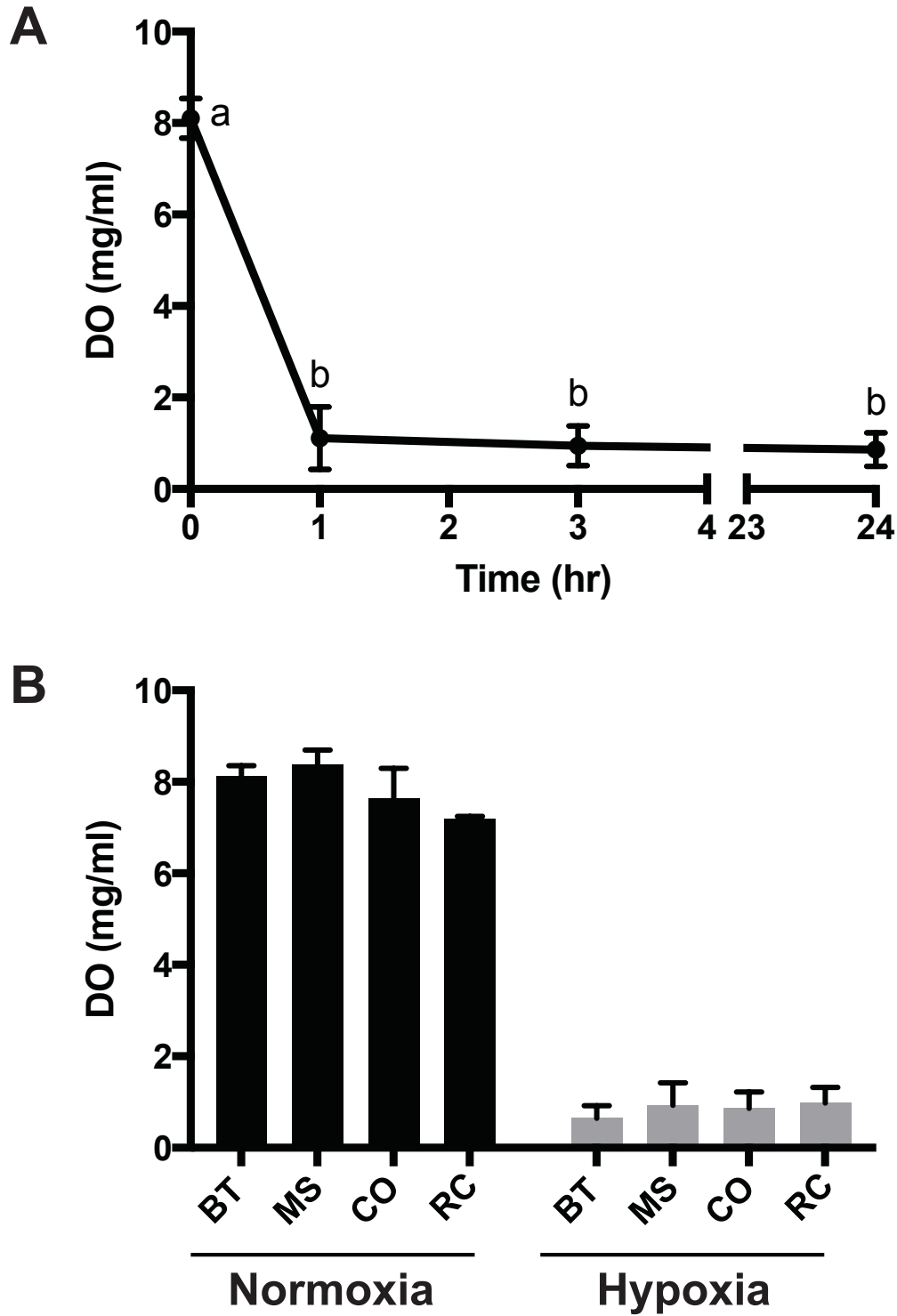
### 1.3.1. General Experimental Conditions

#### 1.3.1.1. Dissolved Oxygen

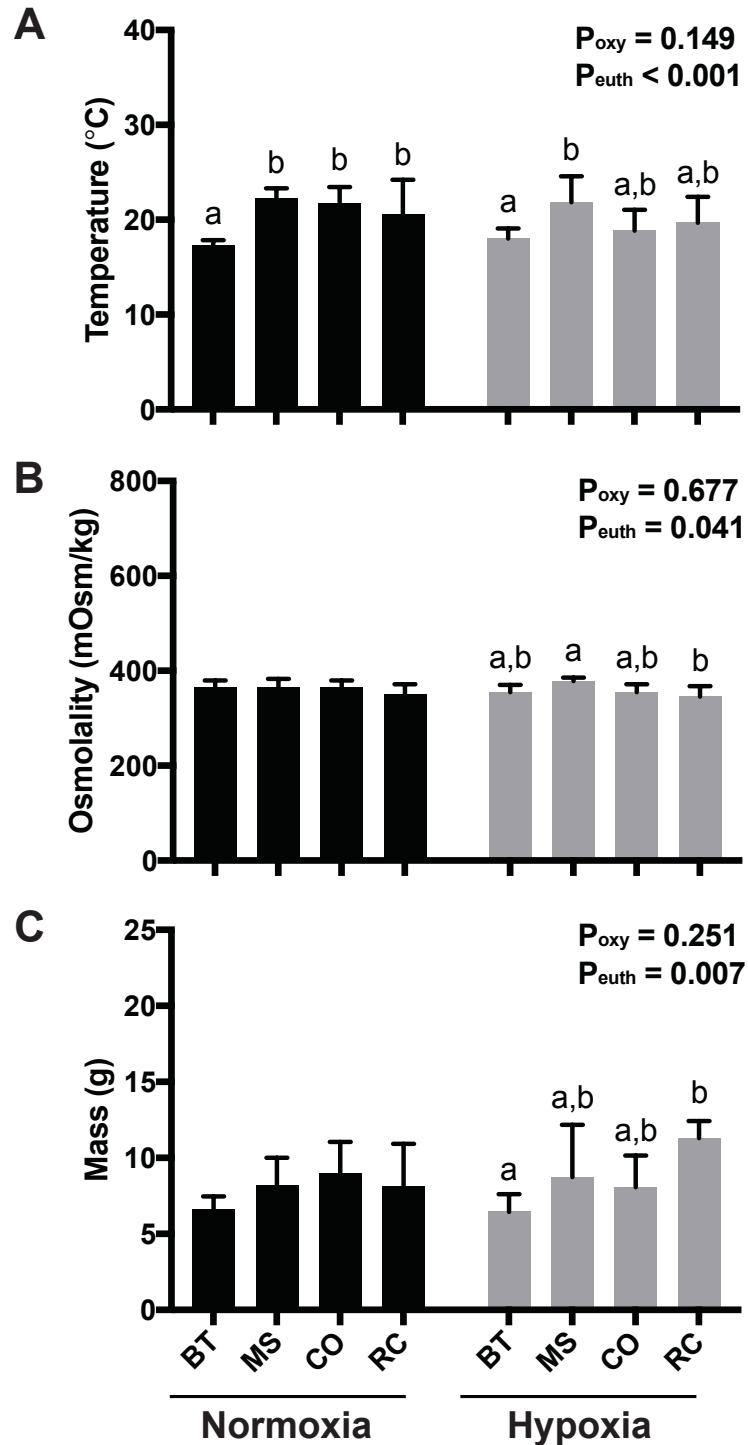
As expected, the DO of the hypoxic aquaria decreased rapidly following the introduction of nitrogen gas and remained stable for the remainder of the 24-h exposure period (Figure 1.2A). Immediately prior to euthanasia, the mean DO of normoxic aquaria was  $7.93 \pm 0.50$  mg/l, or > 95% of the air-saturated value (ca. 8.1 mg/l), whereas the mean DO of hypoxia aquaria was  $0.86 \pm 0.37$  mg/l, near the target of 10% of the air-saturated value (Figure 1.2B). Values among replicate aquaria for a given euthanasia approach fell within a narrow range: these were not statistically tested because of an unequal number of DO measurements in each group. It is important to point out that the DO corresponding to the critical oxygen tension ( $P_{crit}$ ) of *F. grandis* is 1.6 mg/l (Virani and Rees, 2000), showing fish held under hypoxia were exposed to a level of oxygen below that required to sustain aerobic metabolism for  $\geq 23$  h.

#### 1.3.1.2 Temperature, Osmolality, and Fish Mass

Room temperature did not differ between oxygen conditions ( $F_{1,46} = 2.151$ ;  $p = 0.149$ ), but it was significantly different among euthanasia approaches ( $F_{3,46} = 9.172$ ;  $p < 0.001$ ) (Figure 1.3A). Due to the lack of control of room temperature, fish killed by BT were sampled, on average, at temperatures 3°C cooler than fish euthanized by the other approaches. In addition, water osmolality did not differ between oxygen conditions ( $F_{1,31} = 0.177$ ;  $p = 0.677$ ), but it did differ among euthanasia approaches ( $F_{3,31} = 3.095$ ;  $p = 0.041$ ) (Figure 1.3B). Despite being statistically significant, the difference among euthanasia approaches was small in magnitude and all water



**Figure 1.2:** Dissolved oxygen (mg/ml) in the aquaria used for normoxic and hypoxic exposures. (A) Time course of DO decrease in the aquaria used for hypoxia exposures just prior to nitrogen gas induction (0 h) and 1 h, 3 h, and 24 h post nitrogen gas induction. Different letters indicate a statistical difference as determined by a Friedman rank-sum test. (B) The final DO measurements of the normoxic aquaria (black bars,  $n = 2-7$ ) and hypoxic aquaria (grey bars,  $n = 6-7$ ) for the four euthanasia methods: BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Bars indicate the mean  $\pm$  1 SD.

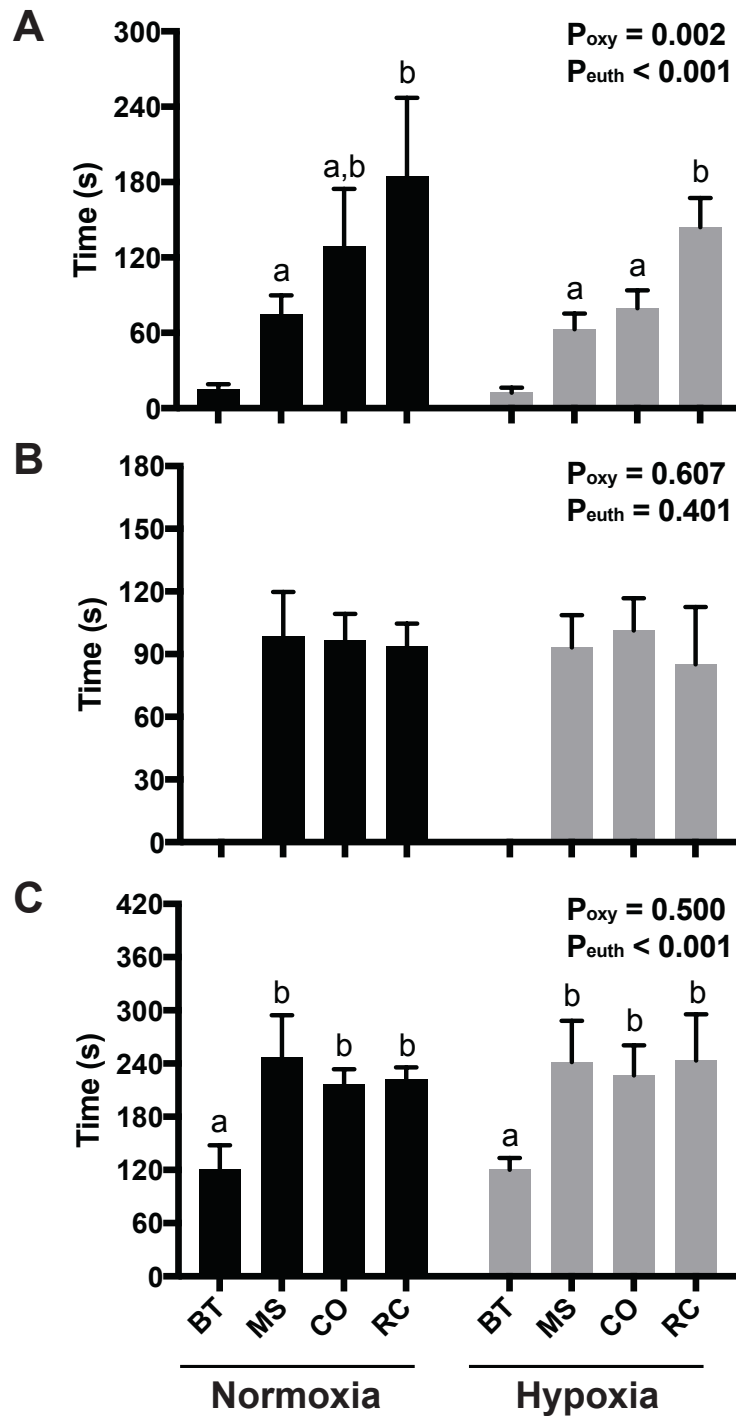


**Figure 1.3:** Room temperature, water osmolality, and fish mass measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A) Room temperature the day of fish euthanasia. (B) Osmolality of aquaria water post euthanasia. (C) Masses of fish in each oxygen condition for all euthanasia methods. BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes range from 4-8 fish for each subgroup and the values reported are the means  $\pm$  1 SD. Black and grey bars indicate normoxic and hypoxic fish or aquaria respectively. Different letters indicate a significant difference determined by a one-way ANOVA followed by a Tukey's pairwise comparison between treatments within a given oxygen condition. P-values are given for oxygen condition ( $P_{oxy}$ ) and euthanasia method ( $P_{euth}$ ).

osmolalities were close to being isosmotic with *F. grandis* plasma (300-400mOsm/kg) (Kolok and Sharkey, 1997; Boily *et al.*, 2007) suggesting that fish sampled by all euthanasia approaches experienced minimal osmoregulatory stress. Finally, fish body mass did not differ between oxygen conditions ( $F_{1,46} = 1.352$ ;  $p = 0.251$ ), but it did differ among euthanasia approaches ( $F_{3,46} = 4.600$ ;  $p = 0.007$ ). Despite efforts to sample fish randomly, fish killed by BT were the smallest on average (Figure 1.3C).

#### 1.3.1.3. Time Variables

The time between when fish were netted and killed by BT averaged  $13 \pm 4$  s and did not differ between normoxic and hypoxic exposures. The other euthanasia approaches required fish to first become unresponsive after adding MS-222, clove oil, or ice to their aquaria. The time between this addition and LOE depended upon both oxygen condition ( $F_{1,36} = 11.691$ ;  $p = 0.002$ ) and euthanasia approach ( $F_{2,36} = 32.966$ ;  $p < 0.001$ ) (Figure 1.4A). On average, LOE took about 30 s longer for fish sampled after normoxic exposure than for fish sampled after hypoxic exposure. Among the euthanasia approaches, MS reached LOE quickest, followed by CO and RC. The time that elapsed between LOE and the completion of blood sampling was  $95 \pm 17$  s and did not differ between the oxygen conditions ( $F_{1,36} = 0.269$ ;  $p = 0.607$ ) or across euthanasia treatments ( $F_{2,36} = 0.936$ ;  $p = 0.401$ ) (Figure 1.4B). The time from death (BT) or LOE (MS, CO, and RC) until gills were dissected and freeze-clamped did not differ between oxygen conditions ( $F_{1,46} = 0.463$ ;  $p = 0.500$ ), but it did differ among the euthanasia approaches ( $F_{3,46} = 32.881$ ;  $p < 0.001$ ) (Figure 1.4C). For BT, gills were dissected and freeze-clamped on average  $120 \pm 21$  s after death, compared to  $233 \pm 38$  s after LOE for the other three approaches, a direct reflection of the time required to sample blood from fish euthanized by MS, CO, and RC.



**Figure 1.4:** Time variables measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A) The time to death for BT fish and the time to LOE in MS, CO, and RC. (B) The time between LOE and blood sampling. (C) The time between death (BT) or LOE (MS, CO, and RC) and freeze-clamping gill tissues. BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes range from 6-8 fish for each subgroup and the values reported are the means  $\pm$  1 SD. Different letters indicate a significant difference determined by a one-way ANOVA followed by a Tukey's pairwise comparison between treatments within a given oxygen condition. P-values are given for oxygen condition ( $P_{oxy}$ ) and euthanasia method ( $P_{euth}$ ).



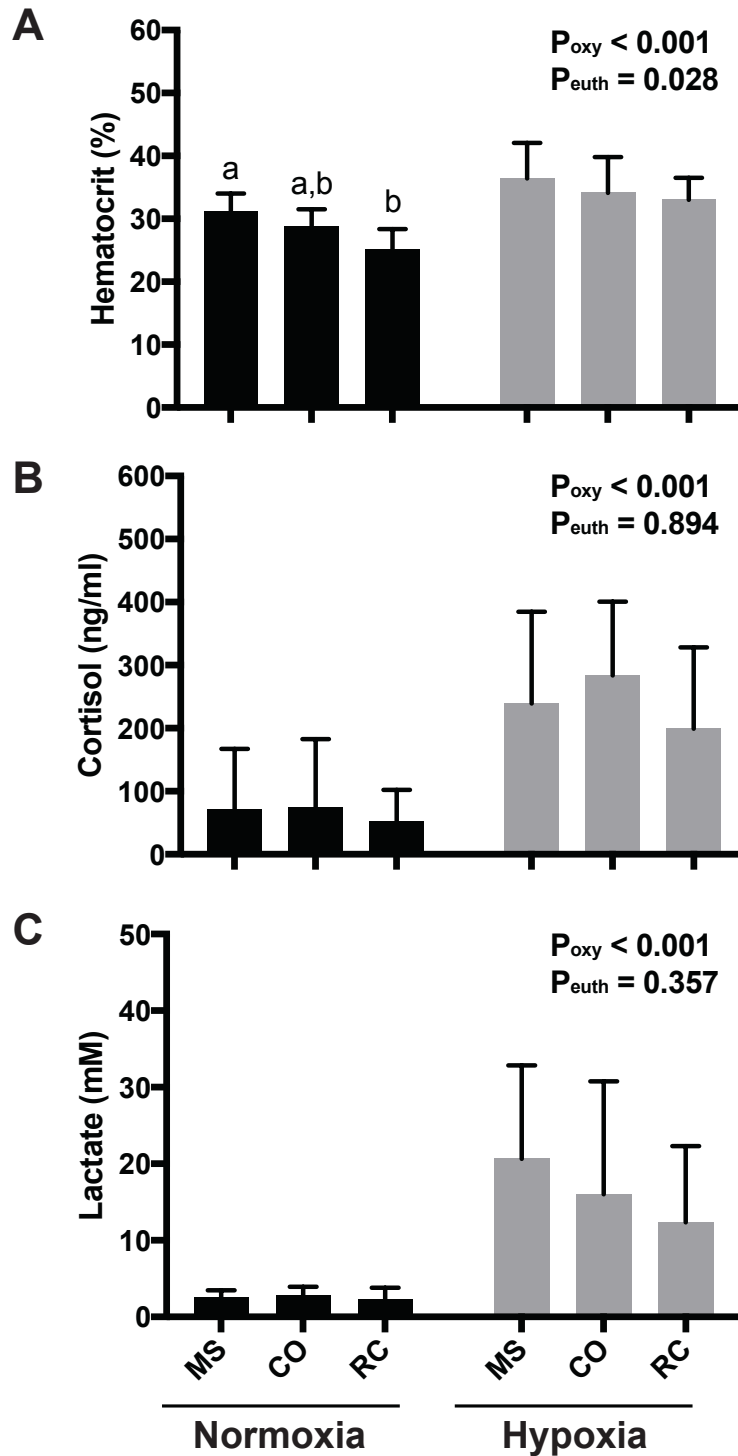
### **1.3.2 Blood Variables**

#### **1.3.2.1. Hematocrit, Cortisol, and Lactate**

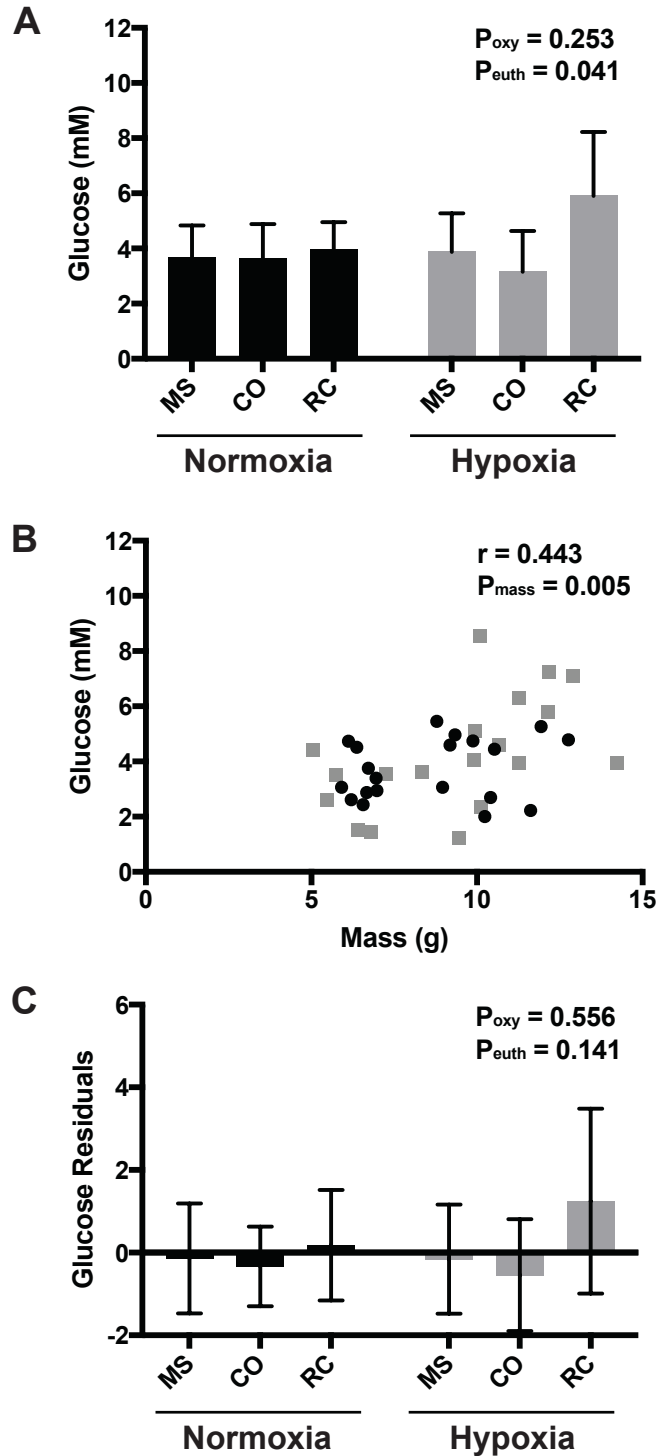
Hematocrit was significantly different between oxygen conditions ( $F_{1,34} = 21.446$ ;  $p < 0.001$ ), with hypoxic exposure causing an increase in hematocrit as expected. Euthanasia approach also affected hematocrit ( $F_{2,34} = 3.998$ ;  $p = 0.028$ ) (Figure 1.5A). This effect was that fish euthanized by RC had lower hematocrit than fish euthanized by MS. When the data were divided by oxygen treatment, this effect was only significant in normoxia (Tukey's,  $p=0.021$ ). Plasma cortisol was significantly different between oxygen conditions ( $F_{1,31} = 17.064$ ;  $p < 0.001$ ), with hypoxic exposure causing an increase of nearly 4-fold. Plasma cortisol did not differ among euthanasia approaches ( $F_{2,31} = 0.113$ ;  $p = 0.894$ ) (Figure 1.5B). Blood lactate was also significantly different between oxygen conditions ( $F_{1,32} = 38.813$ ;  $p < 0.001$ ), with hypoxia causing more than a 6-fold increase. Blood lactate did not differ among euthanasia approaches ( $F_{2,32} = 1.064$ ;  $p = 0.357$ ) (Figure 1.5C). Of note, blood lactate concentrations were highly variable among fish exposed to hypoxia, although this variation could not be explained by euthanasia approach, time to LOE, time to blood sampling, or body mass.

#### **1.3.2.2. Blood Glucose**

Blood glucose was not significantly different between oxygen conditions ( $F_{1,33} = 1.353$ ;  $p = 0.253$ ), but it was apparently influenced by euthanasia approach ( $F_{2,33} = 3.525$ ;  $p = 0.041$ ) (Figure 1.6A). However, blood glucose was positively correlated with body mass ( $r = 0.443$ ,  $p = 0.005$ ) (Figure 1.6B), and body mass differed among euthanasia approaches (Figure 1.3C). When blood glucose was corrected for body size variation, the mass-corrected residuals were not significantly different between oxygen conditions ( $F_{1,33} = 0.353$ ;  $p = 0.556$ ) or among euthanasia



**Figure 1.5:** Blood variables measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A) Hematocrit. (B) Plasma cortisol. (C) Blood lactate. MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes range from 5-8 fish for each subgroup. Black and grey bars indicate normoxic and hypoxic samples. Different letters indicate a significant difference determined by a one-way ANOVA followed by a Tukey's pairwise comparison between treatments within a given oxygen condition. P-values are given for oxygen condition ( $P_{oxy}$ ) and euthanasia method ( $P_{euth}$ ).



**Figure 1.6:** Blood glucose measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A) Blood glucose. (B) Mass effects on blood glucose levels. (C) Mass-corrected residuals of glucose. MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes range from 5-8 fish for each subgroup. Black circles and bars indicate normoxic samples and grey squares and bars indicate hypoxic samples. Bars indicate the mean  $\pm$  1 SD and the symbols indicate the mean. P-values are given for oxygen condition ( $P_{oxy}$ ), euthanasia method ( $P_{euth}$ ), and mass ( $P_{mass}$ ).

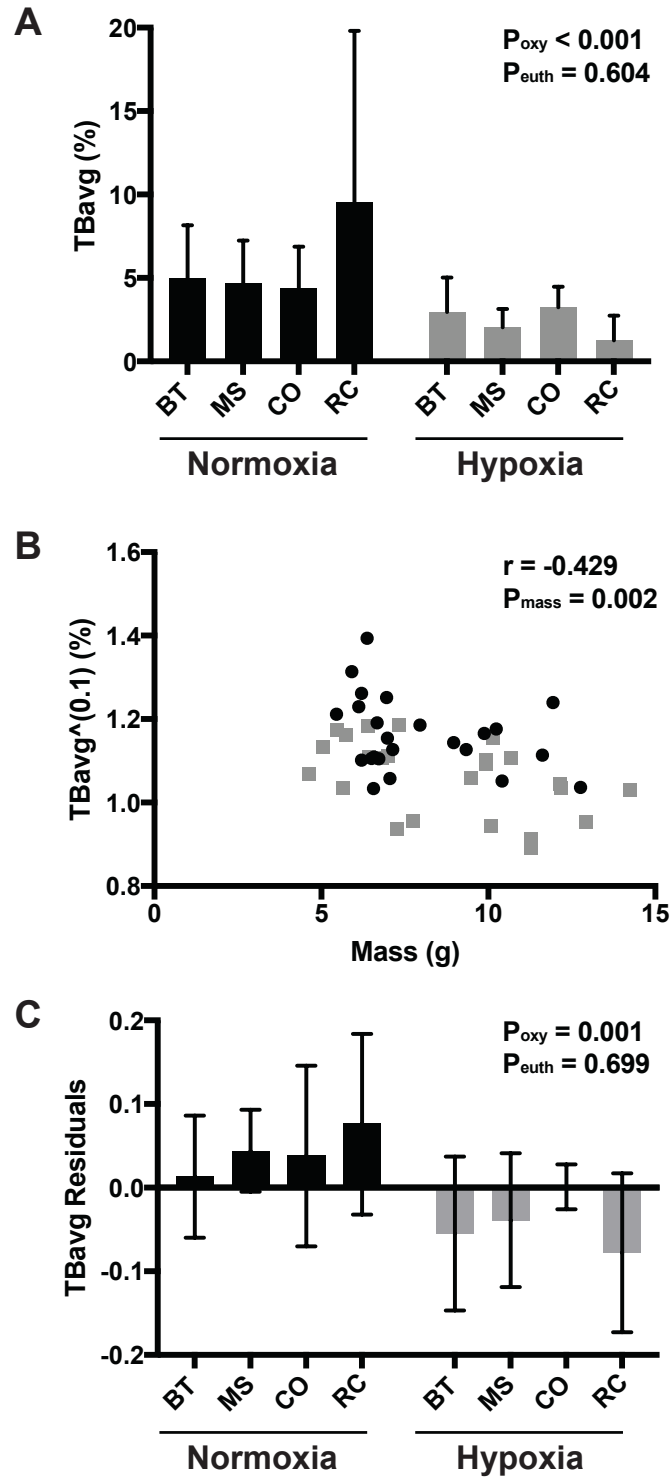
approaches ( $F_{2,33} = 2.08$ ;  $p = 0.141$ ) (Figure 1.6C). Therefore, the effects of euthanasia approach can be explained by hypoxic fish euthanized by RC being the largest (see Figure 1.3C), and having the highest blood glucose, rather than an effect of RC on blood glucose *per se*.

### **1.3.3. Gill Variables**

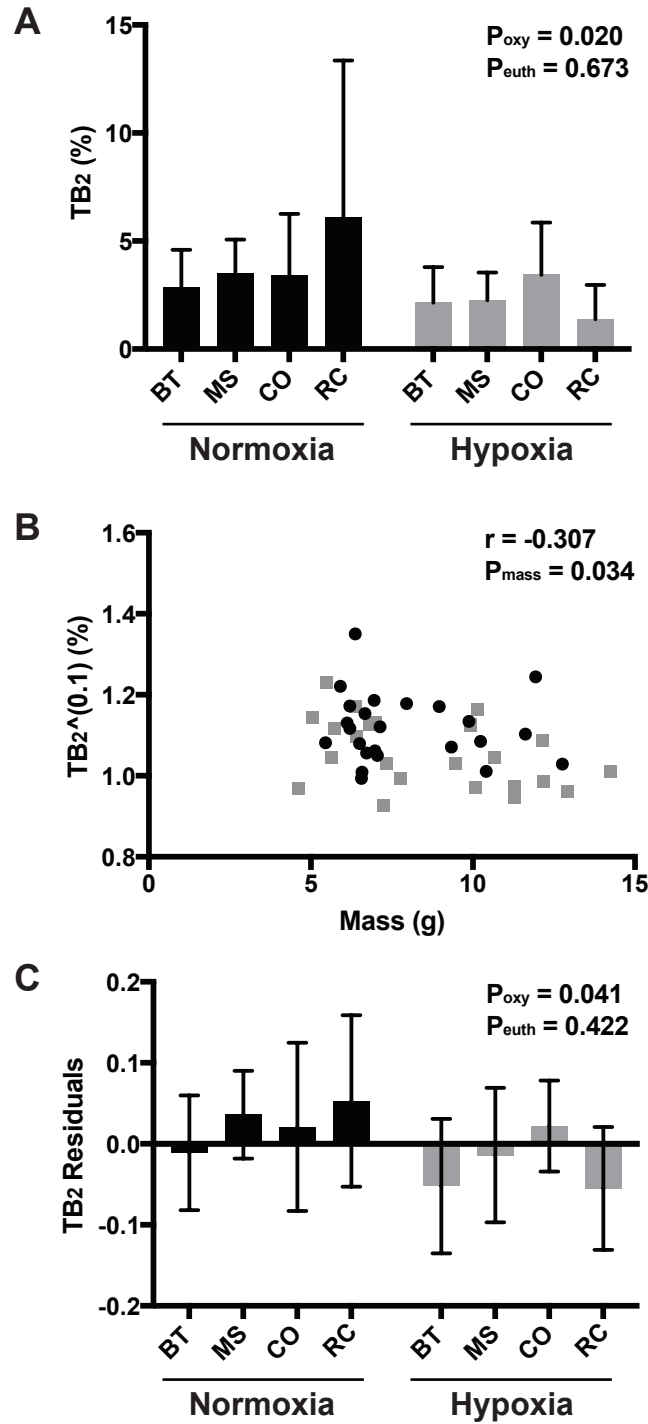
#### **1.3.3.1. Trypan Blue Staining**

The exclusion of TB was used as an index of tissue integrity and the percent of the gill area stained by TB was determined individually for all four gills arches on one side of every fish. A preliminary analysis showed that the percent of the gill filament area that was stained was not significantly different among gill arches ( $F_{144,40} = 0.160$ ;  $p = 1.000$ ). Therefore, the analyses below were done on the average TB staining of all four gill arches ( $TB_{avg}$ ), as well as the TB staining of a single gill arch, the second from the front of the fish ( $TB_2$ ). Similar results were obtained with both analyses.

$TB_{avg}$  was significantly different between oxygen conditions ( $F_{1,40} = 16.028$ ;  $p < 0.001$ ) but not among euthanasia approaches ( $F_{3,40} = 0.623$ ;  $p = 0.604$ ) (Figure 1.7A). Similarly  $TB_2$  was significantly different between oxygen conditions ( $F_{1,40} = 5.837$ ;  $p = 0.020$ ) but not across euthanasia treatments ( $F_{3,40} = 0.517$ ;  $p = 0.673$ ) (Figure 1.8A). However, both  $TB_{avg}$  and  $TB_2$  were significantly correlated with body mass ( $r = -0.429$ ,  $p = 0.002$ ;  $r = -0.307$ ,  $p = 0.034$ ) (Figures 1.7B and 1.8B), and mass corrected-residuals were calculated for each. The mass-corrected residuals for  $TB_{avg}$  and  $TB_2$  were significantly affected by oxygen condition ( $TB_{avg}$  :  $F_{1,40} = 12.679$ ,  $p = 0.001$ ;  $TB_2$ :  $F_{1,40} = 4.441$ ,  $p = 0.041$ ) but not by euthanasia approach ( $TB_{avg}$  :  $F_{3,40} = 0.479$ ,  $p = 0.699$ ;  $TB_2$ :  $F_{3,40} = 0.957$ ,  $p = 0.422$ ) (Figure 1.7C and 1.8C). The effect of



**Figure 1.7:** Average trypan blue staining measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A)  $TB_{avg}$ . (B) Mass effects on normalized  $TB_{avg}$ . (C) Mass-corrected residuals of  $TB_{avg}$ . BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes were 6 samples for each subgroup. Black circles and bars indicate normoxic samples and grey squares and bars indicate hypoxic samples. Bars indicate the mean  $\pm$  1 SD and the symbols indicate the mean. P-values are given for oxygen condition ( $P_{oxy}$ ), euthanasia method ( $P_{euth}$ ), and mass ( $P_{mass}$ ).



**Figure 1.8:** Trypan blue staining for gill arch 2 measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure of *F. grandis*. (A) TB<sub>2</sub>. (B) Mass effects on normalized TB<sub>2</sub>. Grey squares indicate hypoxic samples and black circles indicate normoxic samples. (C) Mass-corrected residuals of normalized TB<sub>2</sub>. BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes were 6 samples for each subgroup. Black circles and bars indicate normoxic samples and grey squares and bars indicate hypoxic samples. Bars indicate the mean  $\pm$  1 SD and the symbols indicate the mean. P-values are given for oxygen condition ( $P_{oxy}$ ), euthanasia method ( $P_{euth}$ ), and mass ( $P_{mass}$ ).

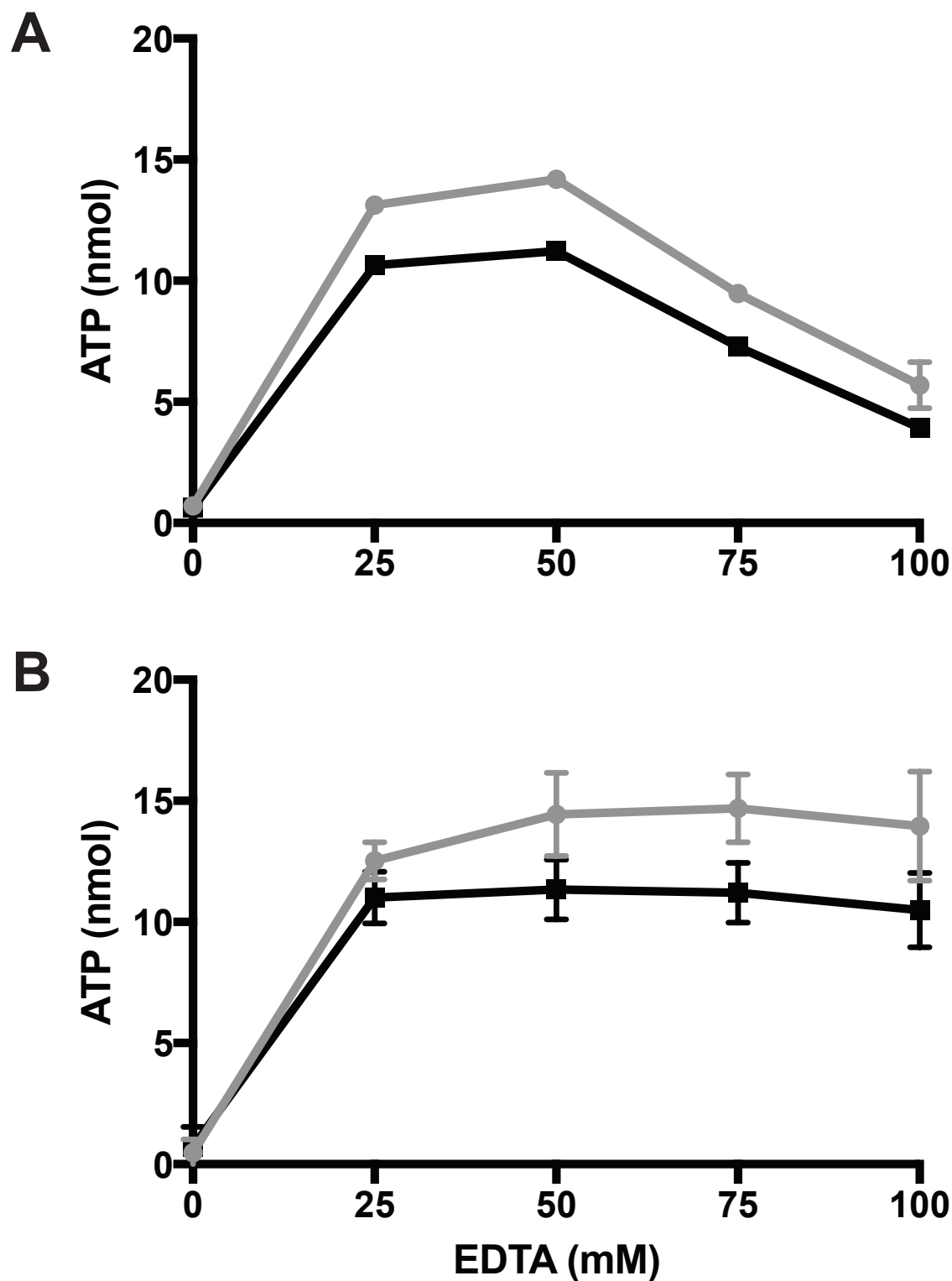
oxygen condition was that  $TB_{avg}$  and  $TB_2$  were both higher in normoxia than after hypoxic exposure.

#### 1.3.3.2. ATP and ADP Measurements in Gill Tissues

##### *Assay Optimization*

When using a published protocol for ATP analysis (Passonneau and Lowry, 1993), ATP concentrations of gill extracts were low and highly variable (data not shown). Because extracts were prepared from intact gill arches, one potential problem was the presence of calcium in the bones supporting the gills, which could lead to the formation and loss of insoluble Ca-ATP during neutralization of acid extracts (Passonneau and Lowry, 1993). Thus, a subset of samples, spiked with a known amount of ATP, was used to assess the recovery of ATP at increasing concentrations of EDTA added to perchloric acid extracts to chelate calcium. However, the enzymatic analysis of ATP requires magnesium ions, and EDTA carried over from the neutralized tissue extracts could potentially interfere with the fluorometric assay if high enough to chelate this ion. Thus, these trials were performed at multiple levels of  $MgCl_2$  in the final assay.

At a concentration of 3 mM  $MgCl_2$  in the ATP assay, increasing the EDTA concentration added to extracts from 0 mM to 50 mM increased the ATP recovered (the difference between gray and black lines, Figure 1.9A). However, at higher EDTA concentrations, the amount of ATP measured decreased, likely due to EDTA carry over from the neutralized perchloric acid extract (contributing up to 5 mM EDTA). When these assays were repeated at a concentration of 6 mM  $MgCl_2$  in the ATP assay, the amount of ATP recovered rose and reached a plateau between 50 mM and 100 mM EDTA (Figure 1.9B). Thus, EDTA was added to perchloric acid extracts to



**Figure 1.9:** ATP assay optimization. (A) The recovery of ATP using 3 mM  $\text{MgCl}_2$  in the assay at various concentrations of EDTA. (B) The recovery of ATP using 6 mM  $\text{MgCl}_2$  in the assay at various concentrations of EDTA. Black squares and lines indicate recovery of endogenous ATP. Grey circles and lines indicate recovery of endogenous ATP plus a known addition of ATP (3.8 nmol per sample).



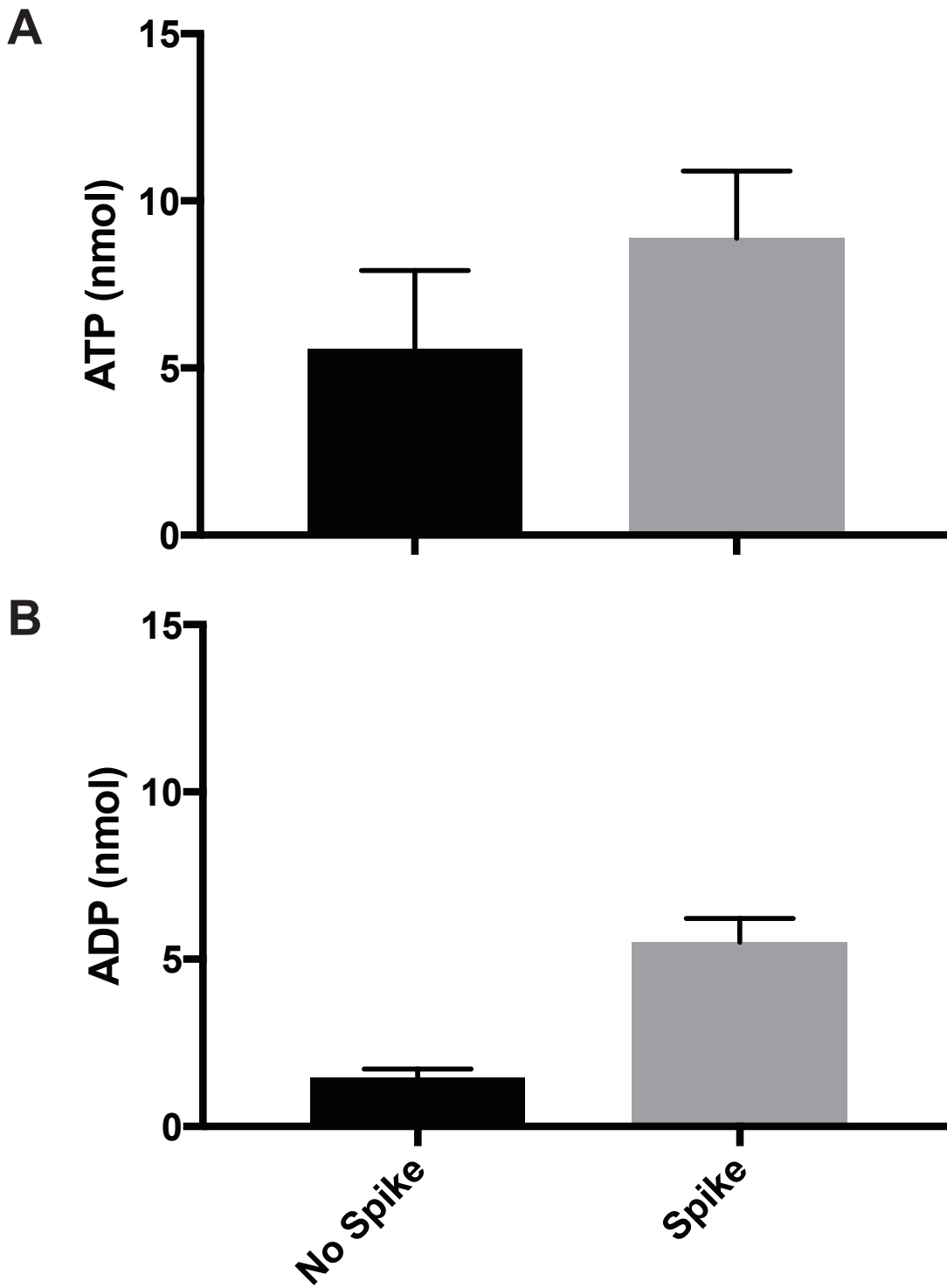
achieve a final concentration of 50 mM, and  $\text{MgCl}_2$  was added to assay buffer to achieve a final concentration of 6 mM. Under these conditions, an average of  $87 \pm 1$  % of the ATP added in spiked extracts was recovered (Figure 1.10A). The same samples were spiked with ADP and the recovery of ADP was  $107 \pm 21$  % (Figure 1.10B). The higher ADP recovery compared to the spiked amount could be due, in part, to limited hydrolysis of ATP to ADP during extract preparation.

### *Experimental Samples*

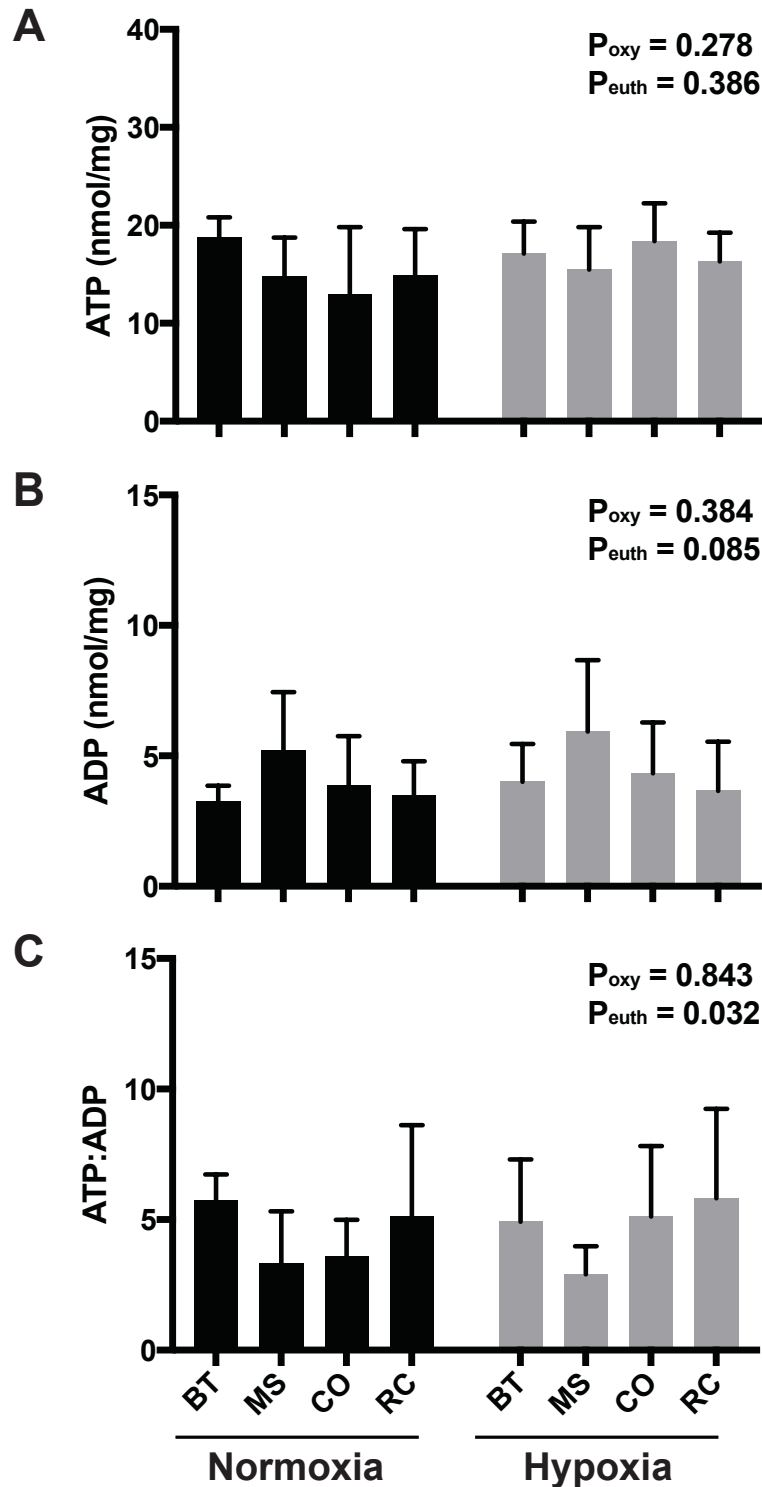
Gill ATP and ADP levels were corrected for variable amounts of tissue used in the extraction process by measuring the amount of protein in each sample. When expressed as ATP/mg protein, there was no differences between oxygen conditions ( $F_{1,40} = 1.210$ ;  $p = 0.278$ ) or among euthanasia approaches ( $F_{3,40} = 1.039$ ;  $p = 0.386$ ) (Figure 1.11A). Similarly, there was no significant difference in the ADP/mg protein between oxygen conditions ( $F_{1,39} = 0.776$ ;  $p = 0.384$ ) or among euthanasia approaches ( $F_{3,39} = 2.370$ ;  $p = 0.085$ ) (Figure 1.11B). Commonly, the ratio of ATP:ADP is used as an index of energetic status of a tissue. This ratio is sensitive to small differences in ATP and ADP that individually might not be statistically significant. When expressed this way, there was a significant difference in the gill ATP:ADP ratio among euthanasia approaches ( $F_{3,39} = 3.261$ ;  $p = 0.032$ ) but not between oxygen conditions ( $F_{1,39} = 0.040$ ;  $p = 0.843$ ) (Figure 1.11C). Fish killed by BT had a higher mean ATP:ADP ratio compared to fish euthanized by MS (Tukey's,  $p = 0.029$ ).

## **1.4. Discussion**

Major findings in this study show variations between the euthanasia techniques in their effects on blood composition and gill viability. In addition, hypoxic exposure was also found to have a



**Figure 1.10:** Recovery of exogenous ATP and ADP in *F. grandis* gill homogenates. (A) Recovery of  $87 \pm 1\%$  of a 3.8 nmol ATP spike. (B) Recovery of  $107 \pm 21\%$  of a 3.8 nmol ADP spike.



**Figure 1.11:** ATP and ADP content in the gills of *F. grandis* measured in experiments to evaluate euthanasia techniques after normoxic or hypoxic exposure. (A) ATP in nmol per mg protein. (B) ADP in nmol per mg protein. (C) ATP:ADP ratio. BT, blunt trauma to the head; MS, overdose of MS-222; CO, overdose of clove oil; RC, rapid cooling. Sample sizes range from 5-6 samples for each subgroup. Black bars indicate normoxic samples and grey bars indicate hypoxic samples. Bars indicate the mean  $\pm$  1 SD. P-values are given for oxygen condition ( $P_{oxy}$ ) and euthanasia method ( $P_{euth}$ ).

profound influence on hematocrit, lactate, and cortisol levels but not on any of the gill variables. Mass was correlated with percent TB staining and circulating glucose levels.

#### **1.4.1. Effects of Mass**

Fish of larger mass had higher levels of blood glucose. In many hypoxia tolerant fish species such as the crucian carp, larger mass is associated with higher glycogen stores (Vornanen *et al.*, 2011). During times of low substrate availability or high anaerobic metabolism, glycogen gets broken down into glucose for cellular respiration. Since there was an overnight fasting period for these fish, the smaller fish may have been relatively more starved compared to larger fish due to the lower levels of glycogen. Therefore, it is not surprising fish of larger mass had higher circulating blood glucose levels compared to smaller fish. Fish of larger mass also had a lower percent TB staining. The reason for this is evident during the dissection process. TB is a viability stain, which is only taken up by cells with compromised plasma membranes and excluded from cells with intact cellular membranes. As gills are individually dissected, some tissue was damaged on the lateral edges and, as a result, became stained when incubated in TB. Gills from fish of differing masses have similar sized lateral edges but significantly different gill surface areas, with heavier fish having larger gill surface areas. It is because of the differences in lateral edge area to gill surface area that results in heavier fish have a lower percent TB staining compared to smaller fish.

#### **1.4.2 Effects of Oxygen Condition**

Hypoxic fish reached LOE sooner than normoxic fish. LOE is an integrated loss of muscle and neurological function and occurs when the fish reaches a stress threshold. The hypoxic fish

experienced a cumulative stress response, as is evident by the elevated levels of lactate and cortisol, to the hypoxic exposure and the euthanasia treatment thereby allowing them to reach the stress threshold quicker. An impaired swim bladder could also be the cause of a faster onset of LOE in the hypoxic fish. Under hypoxic conditions, gasses diffuse out from the swim bladder resulting in a decline of swim bladder lift (Gee and Ratynski, 1988). Therefore, when fish were exposed to hypoxic conditions, they were unable to use their swim bladder to temporarily compensate for the disorienting effects of the euthanasia techniques.

Hematocrit levels were elevated in hypoxic fish compared to normoxic fish. A similar increase in hematocrit was also observed in studies on *F. heteroclitus* (Greaney and Powers, 1977; Greaney and Powers, 1978; Greaney *et al.*, 1980; Borowiec *et al.*, 2015) in which fish were exposed to a constant level of hypoxia. However, in the present study the normoxic fish showed a slightly elevated hematocrit (~28%) compared to the basal level of ~20-25% reported by Greaney and Powers (1977) and Borowiec *et al.* (2015). This is most likely due to the hypoxic effects of respiratory cessation by euthanasia (see below). This suggests hypoxia has an important role in controlling hematocrit levels by promoting the synthesis and maturation of red blood cells or changing plasma volume, thereby changing the proportion of plasma to red blood cells (Lai *et al.*, 2006).

Plasma cortisol levels were also elevated in hypoxic fish suggesting the fish were undergoing a stress response. Leach and Taylor (1977) reported seasonal variations in cortisol levels in *F. heteroclitus* with a mean basal cortisol level of 72.8 ng/ml during the months of October and November (which is when the vast majority of the present study's samples were taken). This is

similar to the mean cortisol value of 66.8 ng/ml found in the normoxic fish indicating these fish were not undergoing a stress response under normal oxygen conditions. However in other studies done on *F. heteroclitus* by DeKoning *et al.* (2004) and Spotte *et al.* (1991), basal cortisol levels were reported as ~20-35 ng/ml. The difference between these reported cortisol levels and the cortisol levels found in the present study and in the study done by Leach and Taylor (1977) is most likely due to seasonal variation with lower cortisol levels found in fish sampled during the summer and higher cortisol levels found in fish sampled in the winter. In the study done by Spotte *et al.* (1991), acute handling stress (minutes) increased cortisol levels from ~20 ng/ml to ~50-75 ng/ml (a 2- to 4-fold increase). In the study done by DeKoning *et al.* (2004), a single handling stressor increased cortisol levels from ~30-35 ng/ml to ~300-400 ng/ml (~10-fold increase) in a southern subspecies of *F. heteroclitus*. These stress-induced cortisol increases are similar to the cortisol increases found in the present study's hypoxic fish indicating hypoxic exposure is inducing a stress response in *F. grandis*.

Hypoxic exposure also elevated lactate levels. Borowiec *et al.*, (2015) demonstrated a 6.6 fold increase in blood lactate concentration for *F. heteroclitus* exposed to severe constant hypoxia (~0.9 mg/l). Although Boroweic's study was a 7-day hypoxia acclimation study, a similar increase in blood lactate concentration was found in the present study (~6.5 fold increase). Another study done on *F. grandis* by Virani and Rees (2000) demonstrated similar increases in lactate levels under hypoxia and also observed similar basal levels of lactate under normoxia. Based on these studies and the results of the present experiments, the hypoxic fish were accumulating lactate as a result of anaerobic respiration.

Surprisingly, glucose levels were not found to be elevated in hypoxic fish compared to the normoxic fish. Past studies have shown hypoxia causes hyperglycemia in fish due to the breakdown of liver glycogen for its use in anaerobic metabolism (Neiffer and Stamper, 2009). However, the results of the present study show a mean blood glucose concentration of 3.98 mM for hypoxic and normoxic fish, which is similar to the basal circulating glucose levels reported for *F. heteroclitus* (~3.38 mM)(Leach and Taylor, 1977). Therefore these results suggest *F. grandis* maintained a homeostatic equilibrium between glycogen breakdown and glycolysis resulting in no change in basal blood glucose levels.

The gills of the normoxic fish had a statistically higher percent TB staining than the gills of hypoxic fish. This finding is counter to what was expected. Past studies have shown that gills of various fish species undergo morphological changes during hypoxic conditions by increasing lamellar surface area through apoptosis of the interlamellar cell mass (ILCM) (Tzaneva *et al.*, 2014; Dhillon *et al.*, 2013; Sollid *et al.*, 2003; Mitrovic *et al.*, 2009). Although the gill images taken in the present study are not of a high enough magnification to distinguish the ILCM, the effect of apoptosis should have increased the TB staining in the hypoxic gills compared to the normoxic gills. On the other hand, hypoxia is known to suppress metabolic rate (Chapman, 2015). If gills from hypoxic fish also have a lower metabolism then perhaps they would have less tissue death during the time between euthanasia and staining. It is important to note the TB staining was overall low (< 5%) across all gills and the statistical difference between oxygen conditions might not be biologically meaningful.

ATP and ADP levels were similar (and therefore ATP:ADP ratios) between the normoxic and hypoxic groups. This was also not expected especially for ATP, which has been reported in other fish species to decrease with increase use of anaerobic metabolism (Greaney and Powers, 1977; Smit and Hattingh, 1981). However, despite this, other studies have shown stable ATP levels under low oxygen conditions in hypoxia-tolerant fish such as the goldfish, tilapia, and European eel (van Ginneken *et al.*, 1997; van Ginneken *et al.*, 2001; Jibb and Richards, 2008). These fishes are able to maintain high ATP levels through two ways. First, these types of fish have large amounts of glycogen stores for anaerobic metabolism (Vornanen *et al.*, 2009). Secondly, they are able to suppress metabolic demands to conserve energy (Richards, 2011). Therefore it would not be unreasonable for the hypoxia-tolerant *F. grandis* to retain stable adenylate levels during hypoxia. The mean ATP levels for both hypoxic and normoxic fish was measured to be 16.1 nmol/mg protein ( $\sim 2.6 \mu\text{mol/g}$  wet mass). This is similar to the basal ATP levels previously measured in the gills of tilapia ( $\sim 1.1$  to  $3.1 \mu\text{mol/g}$  wet mass) reinforcing the idea that there is no decline in ATP levels during hypoxia (Perry and Walsh, 1989).

#### **1.4.3. Effects of Euthanasia Method**

Time to reach LOE was statistically different between euthanasia methods. MS fish reached LOE sooner than RC fish, which is the opposite of what has been shown in other studies comparing the two euthanasia methods (Wilson *et al.*, 2009). This is probably due to our method of administering the rapid cooling euthanasia technique. Wilson *et al.* (2009) conducted the rapid cooling technique by placing zebrafish within an already pre-chilled water tank whereas in our experiment we put 1/3 SW ice in the tank already containing the experimental fish. We conducted this experiment in this way to prevent stress associated with netting and movement of



fish to another container. Therefore, the time to LOE for RC fish includes the time for water temperature to decrease. In a study similar to the one done by Wilson *et al.* (2009), Blessing *et al.* (2010) found bony bream, *Nematolosa erebi*, reached LOE quicker in an ice slurry than in an anesthetic bath of benzocaine (100 mg/l), a drug with a mechanism of action similar to MS-222. However, in a study by Collymore *et al.* (2014) looking at a lower dose of MS-222 (150 mg/l) and gradual cooling in zebrafish, RC took longer than MS to induce LOE, similar to what was found in the present experiment. Time to LOE for CO fish was intermediate. In a study done on zebrafish by Davis *et al.* (2015), CO fish had a similar time to LOE compared to MS fish. This discrepancy is most likely due to a dosage difference in clove oil [100 mg/l in the study done by Davis *et al.* (2015) vs ~50 mg/l in our study].

MS fish had the highest hematocrit followed by CO fish and then RC fish. This MS-222 induced hematocrit increase is consistent with other studies done on rainbow trout (Houston *et al.*, 1971; Reinitz and Rix, 1977; Lowe-Jinde and Niimi, 1983) and is thought to be due to the hypoxic effect of reduced gill irrigation (Iwama *et al.*, 1988). Although the present study shows an intermediate effect of clove oil on hematocrit, a study done by Bowzer *et al.* (2012) demonstrated similar hematocrit levels between MS and CO fish. However that study was done in grass carp with a lower dose of MS-222 (150 mg/l) and a higher dose of clove oil (60 mg/l). Therefore it appears dosage greatly influences the drug's effects on hematocrit levels. Under normoxia, RC fish exhibited a hematocrit level previously recorded as the basal hematocrit level for *F. heteroclitus* (Borowiec *et al.*, 2015). Therefore, it appears the methods of MS-222 and clove oil overdose cause an increase in hematocrit whereas rapid cooling does not. This may be due to the different mechanism of killing (osmoregulatory shock vs inhibition of the nervous

system) and indicates rapid cooling as the method least intrusive to blood composition despite a longer induction time.

Euthanasia treatment does not affect cortisol, lactate, or glucose levels. The literature contains mixed views on these results showing increases or decreases in any number of these blood factors. For example, a study done on the *Senegalese sole* by Weber *et al.* (2011) showed MS-222 (~200 mg/l) and clove oil (80 mg/l) significantly increased cortisol, lactate, and glucose levels after a short time (< 5 min). Another study done by Thomas and Robertson (1991) on the red drum demonstrated a low dose of MS-222 (~10 mg/l) did not induce a stress response by increasing cortisol and glucose levels whereas a high dose of MS-222 (> 80 mg/l) did. Velisek *et al.* (2011) showed MS-222 (~100 mg/l) and clove oil (30 mg/l) increased glucose and lactate levels in the rainbow trout. Within these studies, lactate is the only blood variable to have increased for all euthanasia methods throughout the various dosages used. All of these discrepancies are a matter of differences in drug dosages and possibly species differences.

RC and BT fish had the highest ATP:ADP ratio while MS had the lowest. This suggests the MS fish were the least able to maintain basal levels of ATP relative to ADP (determined by the BT fish ATP:ADP ratio) due to the utilization of anaerobic metabolism and is consistent with local tissue hypoxia. No euthanasia effects were seen in percent TB staining.

#### **1.4.4. Conclusions and Recommendations**

Altogether, the results of these experiments strongly suggest MS-222 affects blood composition and gill integrity, specifically hematocrit levels and ATP to ADP ratios, through the hypoxic

effects of respiratory cessation. This is surprising since MS-222 had the shortest induction time of the euthanasia methods used with the exception of blunt trauma to the head. Therefore, induction time is not as important as the actual mechanism of the euthanasia method in preserving the *in vivo* status of physiological parameters. These findings display the importance of carefully choosing the euthanasia technique appropriate for the experimental timeframe and the factors being measured. Researchers should look for the euthanasia method least intrusive to their samples and analysis. Blunt trauma to the head preserves the adenylate levels in the gill but does not allow for blood analysis. It is also only appropriate for small fish and can be hard to execute without the right training. MS-222 is fast and well-studied; however, it affects hematocrit and adenylate levels. Clove oil appears to only have intermediate effects on hematocrit and adenylate levels but is hard to work with. Its hydrophobicity makes it hard to dissolve in water causing the fish to experience an unpredicted amount of the drug. This problem can be alleviated by first dissolving the clove oil in ethanol; however, ethanol can have its own side effects. Another problem with clove oil is knowing the exact amount of the active ingredient Eugenol in each bottle. Rapid cooling appears to have the least intrusive effects on blood composition and adenylate measurements despite having the longest induction time; however, it is only appropriate for small tropical freshwater fish. It is also important to note these results may only be accurate for *Fundulus* with the specified drugs dosages. If using another model organism or a chemical dose, a preliminary study should be conducted to determine any interactive and individual effects before conducting any further experiments. Some results should also be viewed with caution due to a few systematic errors in methods. Namely, BT fish were sampled at lower room temperatures and had a lower mean mass compared to fish of the other euthanasia treatments. The results of this study provide insight on how euthanasia interacts (or

does not interact) with environmental stressors. It also provides valuable information to the *Fundulus* community on the effects of the most common euthanasia techniques on blood composition and gill viability.

## **Chapter 2: Optimization of *in vitro* Incubation for *Fundulus grandis* Gills**

### **2.1. Introduction**

#### **2.1.1. Complications of Whole Organism Studies**

The detrimental effects of aquatic hypoxia are well known and hypoxic zones are increasing in number and size globally due to natural and anthropogenic means (see chapter 1). Therefore learning the molecular mechanisms that make some fish hypoxia tolerant is imperative to deciphering and preparing for the future consequences of these growing hypoxic zones. Studying the physiological and behavioral actions of the intact organism provides a holistic picture of hypoxia tolerance. However, despite the benefits of using intact fish as model organisms for hypoxic experiments, the use of whole animals can have some drawbacks compared to simpler models such as cell or tissue culture systems. There are many processes that act simultaneously to regulate complex responses seen at the whole organismal level. Resolving these responses into individual molecular mechanisms from the intact animal is difficult. Therefore, organ or cell culture models are needed to allow researchers to determine specific molecular mechanisms underlying more complex whole-animal responses (Murphy, 1991).

#### **2.1.2. Organ Culture**

There are many advantages of organ or tissue culture systems. A distinct advantage is the ability to test one variable and directly attribute a response to that variable. This is not easily done in whole animals because of possible interactions between different organs and cell types. Another benefit of culture systems is the ability to run multiple experiments using different tissues from a single organism thereby reducing the number of animals needed. For example, a hypoxia focused lab interested in the molecular responses of the gill to low oxygen would be able to use multiple

gill arches from a single fish to perform multiple exposures. Time and maintenance costs are also drastically reduced in cell and organ culture studies (Murphy, 1991).

Many gill culture systems have been developed over the decades. The first phase of gill organ culture began in the 1930s with heart-gill preparations in which the gill was dissected with the heart in order to isolate the gill from the rest of the body but still retain function (Keys, 1931). Despite the successes of heart-gill preparations, the gill's connection to the heart was not viewed as ideal due to complications that arise with intertissue interactions. Therefore, researchers tried to make this system simpler by isolating individual gill arches and incubating them in media supporting viability (Bellamy, 1961; Bellamy and Jones, 1961; Ritchie *et al.*, 1971). *In vitro* perfusion was eventually added to the isolated gill arch approach and, after many trials and improvements, proved to be successful (Richards and Fromm, 1970; Shuttleworth, 1972). In these perfusion experiments, the gills were dissected and the arteries were cannulated and perfused with physiological saline. However despite its success, gill arch perfusion is extremely hard to implement even for the most experienced and can only be done on gills from fishes of a sufficient size. Therefore, many fish researchers began turning to the use of primary gill cell cultures (Part *et al.*, 1993; Wood *et al.*, 1998; Fletcher *et al.*, 2000; Wood *et al.*, 2002a; Wood *et al.*, 2002b). Although useful in determining single cell responses, primary gill cell cultures are unable to recreate the functionality of the gill organ as a whole (Marshall and Bellamy, 2010).

### **2.1.3. Goal**

This study's overall goal is to design an easily implemented and low-cost gill incubation system in order to later study the physiological responses of the gill to hypoxic environments. The main objective is to maximize gill viability *in vitro* using an isolated gill arch approach without the

complications of perfusion. The gills of the hypoxia tolerant Gulf Killifish (*Fundulus grandis*) were used since it is a good model organism for hypoxic studies (Burnett *et al.*, 2007). Two gill incubation apparatus differing by aeration method and incubation medium volume were tested. Gills were incubated in a diluted seawater or physiological saline to resemble their external and internal environment respectively. Gill viability was accessed by trypan blue exclusion as well as by the level of lactate dehydrogenase leakage across the gill membrane into the media. Trypan blue is a vital dye taken up by cells with compromised plasma membranes and excluded from cells with intact membranes. Lactate dehydrogenase is a cytosolic protein able to leak out of cells with permeable plasma membranes. Disrupted plasma membranes are a hallmark of dying cells.

## **2.2. Materials and Methods**

### **2.2.1. Husbandry**

*Fundulus grandis* were acquired from Joe's Landing Marina in Barataria, LA or Rigolet's Bait and Marina in Slidell, LA and maintained according to the specifications listed in section 1.2.1. All research conformed to national and institutional guidelines for research on vertebrate animals (University of New Orleans IACUC Protocol 13-005).

### **2.2.2. Experimental Design**

#### *Incubation media*

Two culture media were assessed. The first medium consisted of dechlorinated tap water made up with Instant Ocean Synthetic Sea Salt to 1/3 strength seawater (1/3 SW), 5.5 mM glucose, 100 I.U./ml Penicillin, 100 I.U./ml Streptomycin, and 0.25 µg/ml Amphotericin B. A stock solution of the 1/3 SW was filter-sterilized (0.2 µm) and stored at 4°C until use. The glucose,

antibiotics, and the antimycotic were added using sterile technique just prior to use. The 1/3 SW medium mimics the external environment of the fish.

The second medium consisted of a physiological saline solution (PS) and mimics the internal environment of the fish. This medium was a variation of the PS created by Genz and Grosell (2011) and the final composition used in this study is shown in Table 2.1. All of the ingredients with the exception of glucose, the antibiotics, and the antimycotic were made in stock solution, filtered-sterilized (0.2  $\mu$ m) and stored at 4°C until use. The glucose, antibiotics, and the antimycotic were added using sterile technique just prior to experimentation.

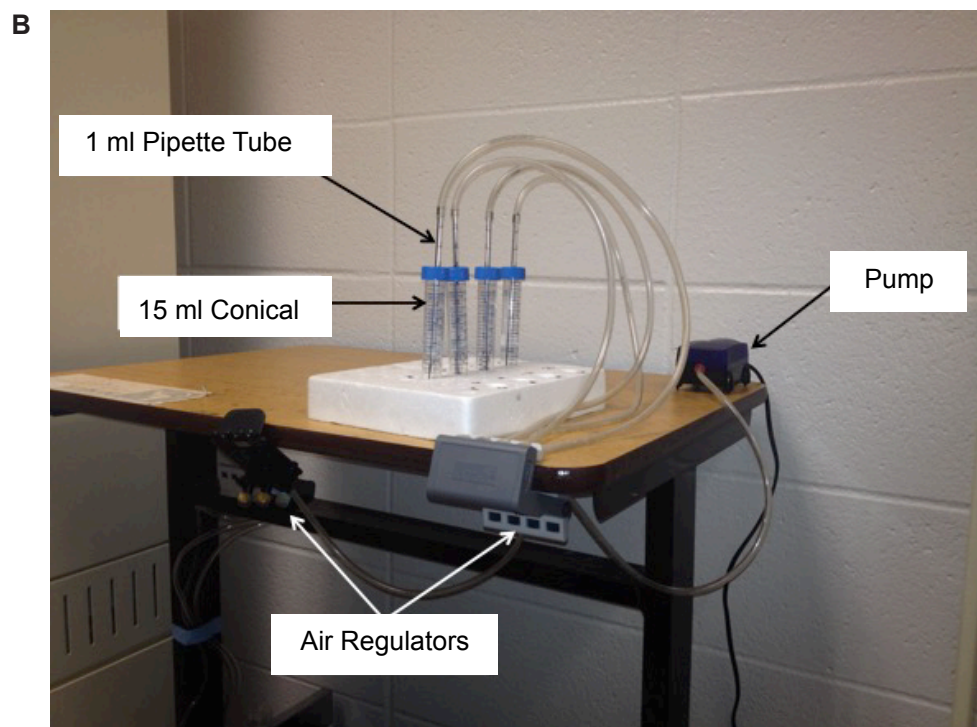
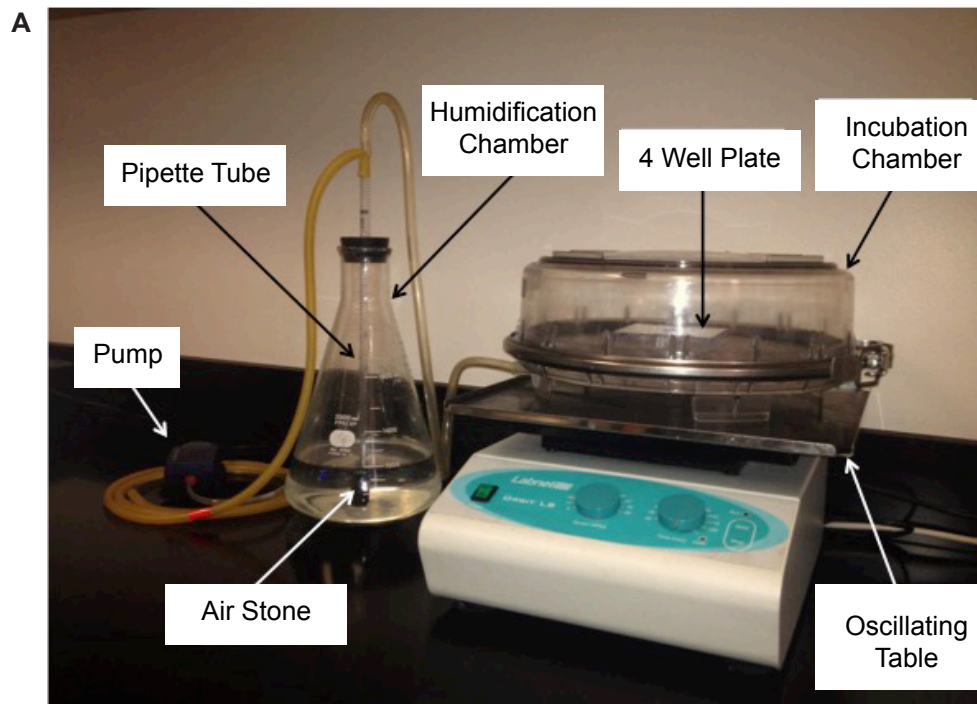
### *Apparatus Design*

There were two incubation apparatus designs. The first apparatus consisted of a four-well cell culture plate placed in a Billups-Rothenburg Modular Incubation Chamber continuously supplied humidified air via a standard aquarium air pump. Each well held 2 ml of 1/3 SW or PS medium plus an individual gill arch. The incubation chamber was placed on an orbital shaker set to 60 rpm (Figure 2.1A). The second apparatus consisted of four capped 15 ml conical tubes, which were fed ambient air via an aquarium air pump connected to a plastic pipette through aquarium tubing. The plastic pipette was inserted in a 5 mm hole within the cap and glued in place with aquarium glue such that the open end of the pipette was resting on the bottom of the conical tube. The pipette had a 1.5 mm hole drilled approximately 3 mm from the tip. A 3 mm hole was also drilled into the cap to prevent pressure buildup. The target bubbling rate of the conical tubes was approximately 10 bubbles per second, or approximately 60 ml per minute per tube. Each conical tube held an individual gill arch with 4 ml of 1/3 SW or PS medium (Figure 2.1B).



Compound	Concentration
NaCl	144.1 mM
KCl	5.1 mM
CaCl <sub>2</sub> x 2 H <sub>2</sub> O	1.6 mM
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	0.9 mM
NaHCO <sub>3</sub>	11.9 mM
NaH <sub>2</sub> PO <sub>4</sub>	2.9 mM
*Glucose	5.5 mM
*Penicillin	100 I.U./ml
*Streptomycin	100 I.U./ml
*Amphotericin	0.25 µg/ml

**Table 2.1:** The ingredients of the physiological saline medium. A sterilized stock solution was made up and stored at 4°C. The asterisks indicate compounds not included in the stock solution and were added just prior to experiments.



**Figure 2.1:** The designs of the two incubation apparatus. (A) The dish apparatus with aeration by continuous shaking. (B) The conical apparatus with aeration through bubbling action.

### *Euthanasia, Dissection and Incubation*

Fish were euthanized with a solution containing 0.25 g MS-222 and 1.0 g NaHCO<sub>3</sub> in 1/3 SW until operculum movement ceased. After euthanasia, fish were blotted dry with a paper towel and weighed followed by the transection of the spinal cord behind the head. Both gill baskets were dissected and placed in a petri dish containing 1/3 SW. The individual gill arches (four per gill basket) were isolated and put in their respective incubation medium and apparatus. Gills were sampled at 0 h, 2 h, or 24 h of incubation for gill viability measurements.

#### **2.2.3. Gill Viability Measurements**

Gill viability was determined using TB exclusion or lactate dehydrogenase (LDH) leakage.

##### **2.2.3.1 Trypan Blue Exclusion**

TB staining and imaging were performed as stated in section 1.2.4.1.

##### **2.2.3.2 Lactate Dehydrogenase Leakage**

Gill tissues designated for LDH measurements were weighed and snap frozen using either a 95% ethanol and dry ice bath or liquid nitrogen. The media from the 2 h and 24 h samples were also snap frozen to assess LDH leakage. Both medium and tissue were then stored at - 80° C for later analysis of LDH enzyme activity.

Lactate dehydrogenase analyses were done in both gill and medium as outlined in Martínez *et al.*, (2006). Briefly, the tissues were homogenized in 100 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM DTT, and 0.2% Triton X-100. Next, the homogenate was centrifuged for 20 min at 10,000 x g. The supernatant was used for LDH and protein assays. LDH measurements were conducted

using a Beckman DU 640 spectrophotometer with the excitation wavelength set at 340 nm. Background absorbance was measured using 950 µl solution containing 100 mM HEPES (pH 7.4), 10 mM KCl, 0.17 mM NADH, and gill homogenate supernatant. Next 50 µl of 20 mM pyruvate was added to the assay for a final concentration of 1 mM bringing the final volume to 1 ml. The change of absorbance was measured and LDH activity was determined using the equation:

$$LDH\ Units = \frac{\text{change in absorbance} \times \text{Volume of cuvette}}{\text{extinction coefficient of NADH} \times \text{path length of cuvette}}$$

The extinction coefficient of NADH is 6.22 mM<sup>-1</sup>cm<sup>-1</sup>, the volume of the cuvette was 1 ml, and the path length was 1 cm. The same process was used to measure the LDH Units in the medium for the 2 h and 24 h samples. Percent LDH leakage was calculated using the following equation:

$$\text{Percent LDH leakage} = \frac{LDH\ Units\ in\ medium}{LDH\ Units\ in\ medium + LDH\ Units\ in\ gill} \times 100$$

#### *Total Protein*

Protein was determined in the supernatant used for the LDH assay by the bicinchoninic acid protein assay (Pierce, ThermoFisher Scientific) with bovine serum albumin standards (Smith *et al.*, 1985). Prior to assay, interfering substances were removed by precipitation with trichloroacetic acid and deoxycholate (Brown *et al.*, 1989). Standards and samples were assayed in triplicate and absorbance was measured with a Beckman DU-640 spectrophotometer. Specific activity was calculated as units LDH/mg protein.

It should be noted Joseph Diaz performed preliminary trials of the experiments listed above.

#### **2.2.4. Statistical Analysis**

All experimental variables were tested for normality using the Anderson-Darling test (Ghasemi and Zahediasl, 2012). Experimental variables include percent TB staining, LDH activity (U/g), LDH specific activity (U/mg protein), LDH U in gill, LDH U in medium, and percent LDH leakage. LDH U/g, LDH U/mg, and LDH U in the gill were normally distributed ( $p > 0.05$ ). Appropriate transformations were found for the other variables using the Box-Cox transformation tool in Rstudio. The following exponential transformations were normally distributed: (TB staining)<sup>-0.02</sup>; (LDH U in the media)<sup>0.2</sup>; and the (percent LDH leakage)<sup>0.06</sup>. For just the 0 h gill arch analysis, the LDH U/mg and mg/g could not be transformed into a normal distribution. All normally distributed variables were checked for homogeneity of variances using a Levene's test. All analyses were conducted using R 3.2.3 (cran.r-project.org) and SYSTAT with a confidence level of 95% ( $\alpha = 0.05$ ). Means are expressed with one standard deviation and graphs were generated with Graphpad Prism 7.0a.

Statistical analysis was performed on 0 h sampled to evaluate selected variables in gills immediately after dissection without any *in vitro* incubation. To test the effects of gill arch location on LDH activity, a nested ANOVA was performed on LDH (U/g and U/mg) of all four gills using gill arch nested within fish as a factor. To test the effect of sex, a one-way ANOVA was performed on the same LDH variables. Since TB staining was not influenced by gill arch location (see section 1.3.3.1), a one-way ANOVA was performed on the TB staining for gill arch 2 to determine the effects of sex.

To test the effects of *in vitro* incubation conditions, three-way ANOVAs were performed on all experimental variables using media, time, and apparatus as factors. When statistical significance was found, a Tukey's post hoc test was used to test the differences between means. Unless otherwise stated, interaction effects were not significant ( $p > 0.05$ ).

## **2.3. Results**

### **2.3.1. Effects of Sex and Gill Arch Position**

No effect of gill arch location (1-4) was found for either LDH activity (U/g) ( $F_{24,7} = 0.105$ ,  $p = 1.0$ ) or LDH specific activity (U/mg) ( $F_{24,7} = 0.045$ ,  $p = 1.0$ ) for the 0 h samples (not shown). However, the statistical results for U/mg protein should be taken with caution due to the inability to transform the 0 h data into a normal distribution. Since LDH content and TB staining (see section 1.3.3.1. in chapter 1) for gill tissue is not affected by gill arch location, individual gill arches can be used for *in vitro* incubation under any experimental condition without regard for their anatomical location prior to dissection.

No sex effects were found for LDH activity (U/g) ( $F_{1,6} = 0.130$ ,  $p = 0.731$ ), LDH specific activity (U/mg) ( $F_{1,6} = 0.035$ ,  $p = 0.858$ ), or TB<sub>2</sub> ( $F_{1,7} = 0.619$ ;  $p = 0.457$ ) for the 0 h samples. These results allow the experimental analysis of TB staining and LDH content without regard for sex of the fish from which the gill was dissected. The mean for the values TB staining, U/g, and U/mg averaged across all gill arches and for just gill arch 2 are shown in Table 2.2.

	$\bar{x}_{1-4}$	$\bar{x}_2$
<b>TB staining (%)</b>	5.8 ± 6.6	2.1 ± 2.1
<b>LDH (U/g)</b>	67.5 ± 24.1	65.6 ± 29.0
<b>Protein (mg/g)</b>	28.8 ± 13.5	28.3 ± 12.0
<b>LDH (U/mg)</b>	2.91 ± 1.64	2.69 ± 1.42

**Table 2.2:** Trypan blue and lactate dehydrogenase variables for gills of *F. grandis* without *in vitro* incubation. The mean ± SD of TB staining, LDH units per gram of tissue (U/g), protein per gram of tissue (mg/g) and LDH units per mg of protein (U/mg) for the average values across all 4 gill arches ( $\bar{x}_{1-4}$ ) and the second most anterior gill arch ( $\bar{x}_2$ ) (n = 9 for TB staining and n= 8 for all LDH and protein measurements).

### **2.3.2 Effects of Time, Apparatus, and Medium**

#### **2.3.2.1. Trypan Blue Staining**

Overall, TB staining of gills after 2 or 24 h *in vitro* incubation was very low (< 2%) indicating high gill viability under all experimental conditions. A 3-way ANOVA of TB staining showed significant effects of incubation apparatus ( $F_{1,39} = 5.936$ ;  $p = 0.020$ ) and incubation medium ( $F_{1,39} = 7.316$ ;  $p = 0.010$ ) (Figure 2.2). Gills incubated in the culture dishes have a lower TB percent staining compared to gills incubated in the conical tubes (Tukey's,  $p = 0.020$ ). For gills incubated in culture dishes or conicals tubes, the mean percent TB staining was  $0.67 \pm 0.64\%$  and  $0.95 \pm 0.65\%$  respectively. In addition, gills incubated with 1/3 SW have a lower TB percent staining than the gills incubated with PS (Tukey's,  $p = 0.010$ ). The mean percent TB staining for gills incubated in 1/3 SW was  $0.58 \pm 0.54\%$  and for gills incubated in PS was  $1.10 \pm 0.68\%$ . Incubation time had no effect on TB staining ( $F_{1,39}=0.204$ ;  $p=0.654$ ). Based on these results, the best incubation conditions for gills were 1/3 SW in the culture dishes.

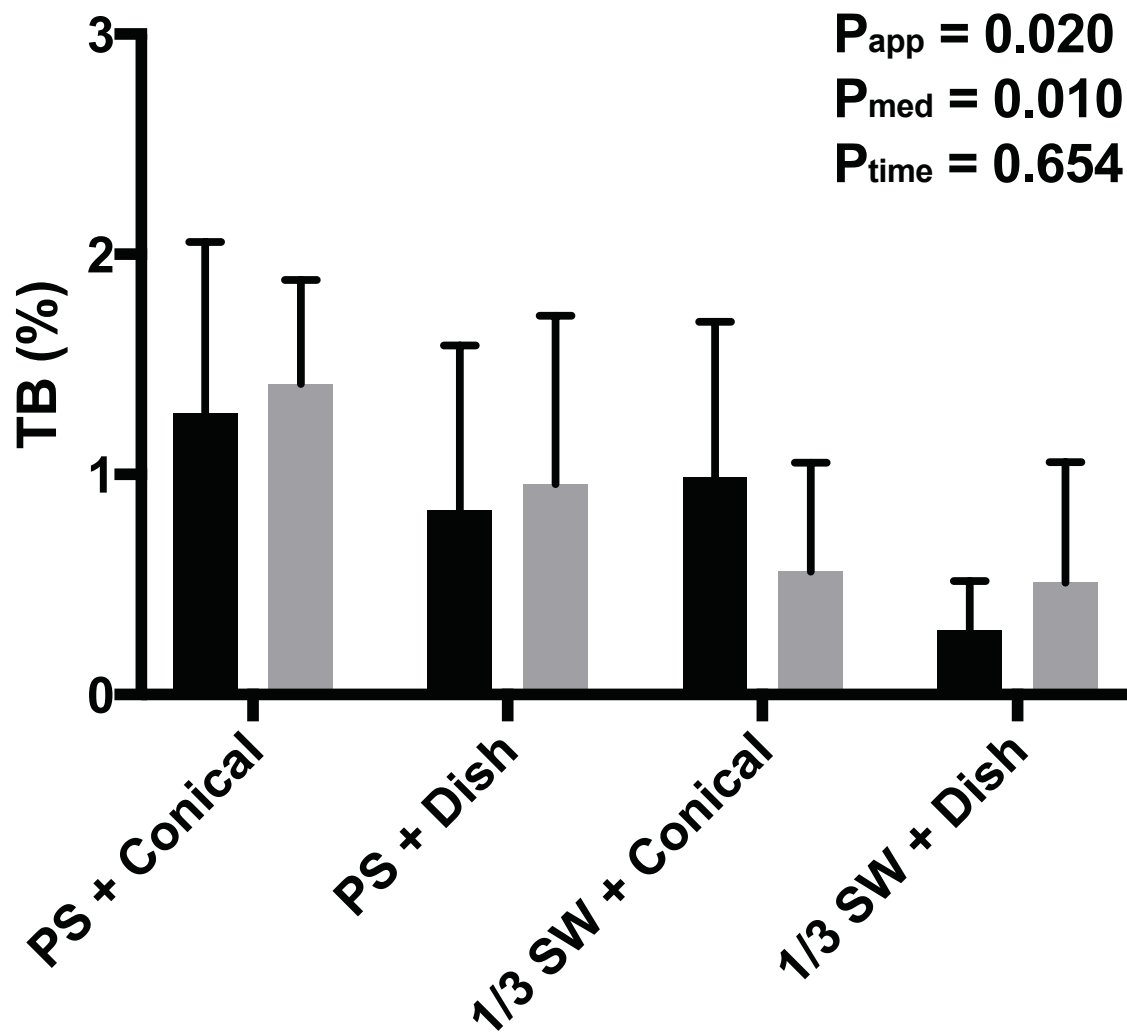
#### **2.3.2.2. Lactate Dehydrogenase Variables**

##### *U/g and U/mg*

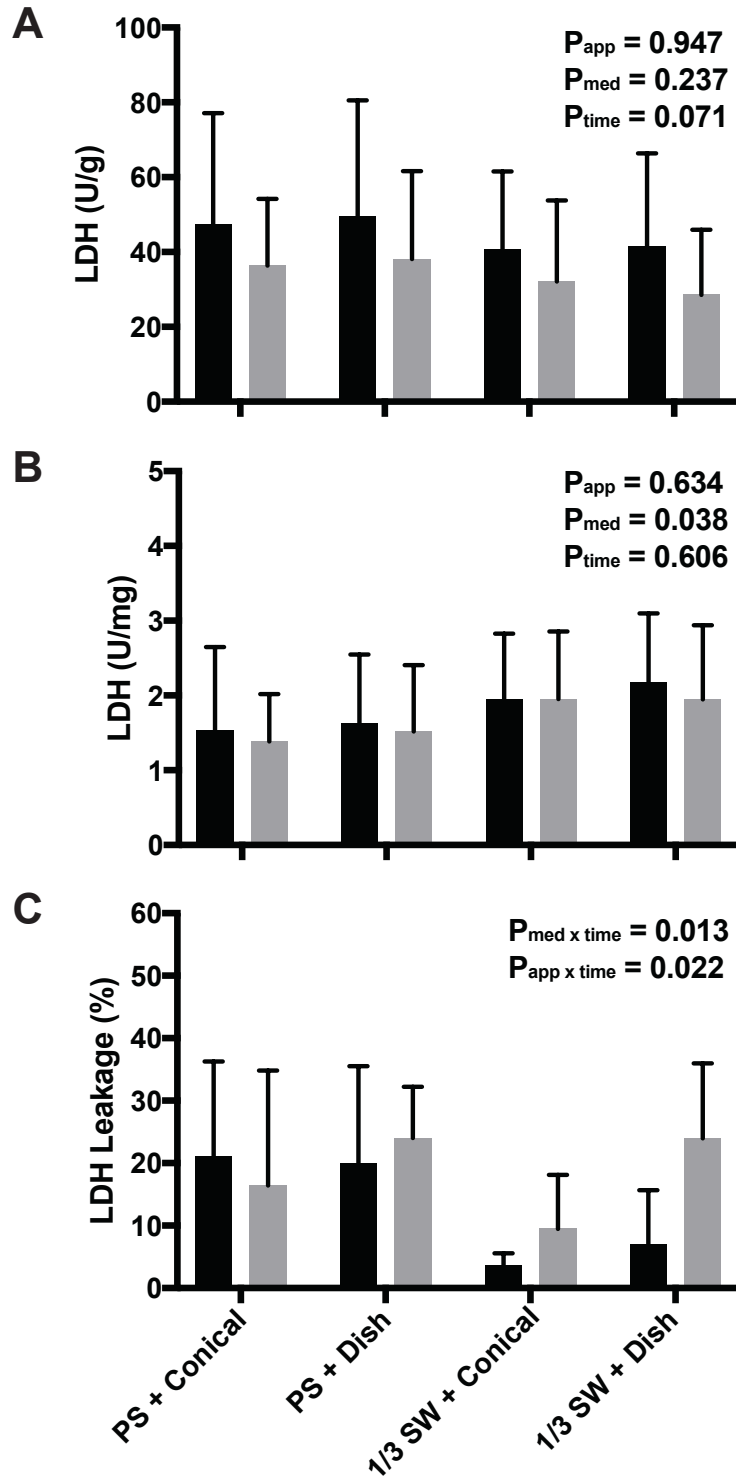
For LDH U/g, there were no significant effects of incubation time ( $F_{1,56}=3.387$ ; 0.071), apparatus ( $F_{1,56}=0.004$ ; 0.947) or medium ( $F_{1,56}=1.429$ ;  $p=0.237$ ) (Figure 2.3A). The overall mean LDH U/g was  $39.27 \pm 23.45$  U/g ( $n=64$ ). This result indicates a similar amount of LDH Units per gram of gill tissue across all incubation conditions.

For LDH specific activity (U/mg), there was a significant effect of medium used ( $F_{1,55} = 4.520$ ;  $p = 0.038$ ) but not apparatus ( $F_{1,55} = 0.229$ ;  $p = 0.634$ ) or incubation time ( $F_{1,55} = 0.270$ ;  $p = 0.606$ )





**Figure 2.2:** Percent trypan blue staining of *F. grandis* gills incubated after *in vitro* incubation. PS, physiological saline medium; 1/3 SW, one-third strength seawater. Black bars indicate values for gills incubated for 2 h and grey bars indicate values for gills incubated for 24 h. Bars indicate the mean  $\pm$  1 SD. Sample sizes range from 3-7 samples for each group. P-values are given for the effect of apparatus ( $P_{app}$ ), media ( $P_{med}$ ), and time ( $P_{time}$ ).



**Figure 2.3:** Lactate dehydrogenase content in *F. grandis* gills after *in vitro* incubation. (A) LDH units per gram of tissue (U/g). (B) LDH units per mg of protein (U/mg). (C) Percent LDH leakage. PS, physiological saline medium; 1/3 SW, one-third strength seawater. Black bars indicate values for gills incubated for 2 h and grey bars indicate values for gills incubated for 24 h. Bars indicate the mean  $\pm$  1 SD. Sample sizes are 8 for each group. P-values are given for the effect of apparatus ( $P_{app}$ ), media ( $P_{med}$ ), and time ( $P_{time}$ ) or the p-values for interactions between media and time ( $P_{med \times time}$ ) and the apparatus and time ( $P_{app \times time}$ ) when significant.

(Figure 2.3B). The gills incubated with 1/3 SW had a higher U/mg compared to gills incubated with PS (Tukey's,  $p=0.038$ ). This result is due to gills incubated in 1/3 SW having a lower mg of protein per g of tissue compared to gills incubated in PS (data not shown). The mean LDH U/mg in the gills incubated in 1/3 SW was  $2.00 \pm 0.89$  U/mg ( $n=32$ ) whereas the mean LDH U/mg in the gills incubated in PS was  $1.51 \pm 0.86$  U/mg ( $n=31$ ). These results indicate the 1/3 SW medium as the better incubation medium.

#### *Percent LDH leakage*

The percent LDH leakage results were variable because of how percent leakage was calculated (see section 2.2.3.2.). This calculation relies on total media LDH U, which were low, highly variable, and difficult to measure accurately. For percent leakage of LDH U from the gills into the medium, a two-way interaction between media and incubation time ( $F_{1,56} = 6.648$ ;  $p = 0.013$ ) was found indicating the effect of medium depends upon time (Figure 2.3C). At 2 h, the percent LDH leakage was lower for the gills incubated in 1/3 SW compared to PS (Tukey's,  $p = 0.001$ ). There was also a two-way interaction between apparatus and time ( $F_{1,56} = 5.526$ ;  $p = 0.022$ ) indicating the effect of apparatus depends upon time. At 24 h, the LDH leakage was lower for gills incubated in conical tubes compared to gills incubated in cell culture dishes (Tukey's,  $p=0.018$ ). These results indicate the percent LDH leakage was lowest for gills incubated in 1/3 SW in conical tubes particularly after 2 h of incubation.

## **2.4. Discussion**

The major finding in this study from the two gill viability measurements is 1/3 SW is the better incubation medium for *F. grandis* gills. The conclusions of the two gill viability measurements can be separated based on the effects of media, apparatus and time.

### **2.4.1. Effects of Medium**

For medium, TB staining and LDH analysis agree: 1/3 SW is better than PS for incubating gills of *F. grandis*. This is not surprising since gills *in vivo* are naturally exposed to the external environment rather than blood or serum (which is the basis for PS) (Marshall & Bellamy, 2010).

### **2.4.2. Effects of Apparatus**

For apparatus, TB staining demonstrated the culture dishes are better than conical tubes in maintaining gill viability whereas LDH analysis indicates the conical tubes are better than the culture dishes. Since TB is low between all incubation conditions, the variation in staining may not be biologically significant. Therefore the better apparatus would be the conical tubes as determined by LDH leakage.

### **2.4.3. Effects of Time**

For incubation time, TB staining surprisingly demonstrated the same level of gill viability between 2 h and 24 h of incubation whereas LDH analysis indicated a decrease in gill viability from 2h to 24 h. This discrepancy is most likely due to the differences of the two measurements. TB staining only occurs over a 7 min timeframe following the incubation period. Therefore if cells have already undergone cell death and sloughed off into the media prior to this staining

process, the percent TB staining would show a lower than genuine value for gill viability.

However, LDH leakage occurs over the entire incubation period thereby providing a cumulative measurement of gill damage. Therefore LDH leakage should be a more accurate measurement of gill viability over time.

It is possible to compare gills without incubation (0 h) to those incubated for 2 or 24 h. The TB staining was lower in the 2 and 24 h samples (see Figure 2.2) compared to the 0 h samples (Table 2.2). The LDH activity and specific activity was highest in the 0 h samples (see Table 2.2) and decreased over time in the 2 h and 24 h samples (see Figure 2.3A and B). This decrease in LDH over time, especially between the 0 h and 2 h time points, suggests there is significant tissue death occurring even over this earliest interval. These results would again indicate the measured variability in TB staining between the various time points is not biologically significant and would therefore indicate LDH measurements as a more accurate measure of gill viability over time.

#### **2.4.4. Conclusions and Recommendations**

The overall conclusion is the 1/3 SW solution is better than PS solution as an incubation medium for the gills of *F. grandis*. However, there is no obvious answer for which incubation apparatus and time is better. The disagreement between LDH analysis and TB staining might be due to two possible reasons. The first reason is LDH is the more accurate measurement of gill viability allowing for the detection of small fluctuations in tissue death over time. If this is the case, results from LDH analysis would indicate the gills should be incubated in conical tubes for shorter time periods. The second reason is the presence of a mass-effect on TB staining similar to

what was seen in the first chapter. Since the masses of all the fish were not recorded, a mass effect on TB staining could not be assessed in this experiment. Therefore, if a particular set of conditions were applied to gills from a fish of significantly different mean mass, this could have affected the TB analysis. Due to the discrepancy between LDH measurements and TB staining, a third measurement of gill viability such as measuring of relative adenylate levels is recommended.

These findings are only accurate for gill incubation and should be taken with caution when using other tissue types. For example, a tissue that is naturally accustomed to an internal environment such as the liver may prefer the PS medium. Although the results of this study may not be applicable across a broad range of tissues, they are useful in gill tissue incubations and can therefore be used to assess the molecular mechanisms of various stressors, in particular hypoxia, on gill tissues.

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### Appendix 1: Summary data for Chapter 1

	NORMOXIA				HYPOXIA					
	BT	MS	CO	RC	BT	MS	CO	RC	P <sub>oxy</sub>	P <sub>euth</sub>
<b>DO (mg/l)</b>	8.13 ± 0.22 (n = 6)	8.38 ± 0.32 (n = 2)	7.63 ± 0.67 (n = 2)	7.19 ± 0.06 (n = 2)	0.65 ± 0.27 (n = 6)	0.93 ± 0.49 (n = 6)	0.86 ± 0.36 (n = 7)	0.98 ± 0.35 (n = 7)	ND	ND
<b>Temperature (°C)</b>	17 ± 1 (n = 6)	22 ± 1 (n = 8)	22 ± 2 (n = 8)	21 ± 4 (n = 6)	18.0 ± 1 (n = 6)	22 ± 3 (n = 7)	19 ± 2 (n = 7)	20 ± 3 (n = 6)	0.149	< 0.001
<b>Osmolality (mOsm/kg)</b>	364 ± 15 (n = 6)	365 ± 18 (n = 4)	365 ± 15 (n = 4)	350 ± 22 (n = 6)	355 ± 15 (n = 6)	378 ± 7 (n = 5)	355 ± 17 (n = 4)	346 ± 22 (n = 4)	0.677	0.041
<b>Mass (g)</b>	6.62 ± 0.84 (n = 6)	8.19 ± 1.81 (n = 8)	8.94 ± 2.11 (n = 8)	8.12 ± 2.82 (n = 6)	6.46 ± 1.16 (n = 6)	8.75 ± 3.44 (n = 7)	8.06 ± 2.09 (n = 7)	11.31 ± 1.11 (n = 6)	0.251	0.007
<b>Time to LOE (s)</b>	ND	74 ± 16 (n = 8)	129 ± 45 (n = 8)	185 ± 62 (n = 6)	ND	63 ± 13 (n = 7)	80 ± 14 (n = 7)	144 ± 23 (n = 6)	0.002	< 0.001
<b>Time to blood sampling (s)</b>	ND	98 ± 21 (n = 8)	96 ± 13 (n = 8)	94 ± 11 (n = 6)	ND	93 ± 15 (n = 7)	101 ± 16 (n = 7)	85 ± 27 (n = 6)	0.607	0.401
<b>Time to freezing gill (s)</b>	120 ± 28 (n = 6)	247 ± 47 (n = 8)	216 ± 17 (n = 8)	222 ± 14 (n = 6)	120 ± 13 (n = 6)	242 ± 47 (n = 7)	227 ± 34 (n = 7)	244 ± 52 (n = 6)	0.500	<0.001
<b>Hematocrit (%)</b>	ND	31 ± 3 (n = 8)	29 ± 3 (n = 7)	25 ± 3 (n = 5)	ND	36 ± 6 (n = 7)	34 ± 6 (n = 7)	33 ± 4 (n = 6)	< 0.001	0.028
<b>Cortisol (ng/ml)</b>	ND	70.9 ± 96.4 (n = 7)	73.7 ± 109.1 (n = 7)	51.5 ± 50.6 (n = 5)	ND	239.3 ± 145.5 (n = 6)	283.7 ± 117.1 (n = 5)	199.3 ± 128.9 (n = 6)	< 0.001	0.894
<b>Lactate (mM)</b>	ND	2.48 ± 0.98 (n = 7)	2.75 ± 1.19 (n = 7)	2.25 ± 1.58 (n = 5)	ND	20.65 ± 12.21 (n = 6)	16.03 ± 14.75 (n = 7)	12.37 ± 9.94 (n = 6)	< 0.001	0.357
<b>Glucose (mM)</b>	ND	3.67 ± 1.16 (n = 7)	3.63 ± 1.26 (n = 8)	3.96 ± 1.00 (n = 5)	ND	3.87 ± 1.40 (n = 6)	3.16 ± 1.48 (n = 7)	5.91 ± 2.32 (n = 6)	0.556	0.141
<b>TB<sub>avg</sub> (%)</b>	5.0 ± 3.2 (n = 6)	4.7 ± 2.6 (n = 6)	4.4 ± 2.5 (n = 6)	9.5 ± 10.3 (n = 6)	3.0 ± 2.1 (n = 6)	2.0 ± 1.1 (n = 6)	3.2 ± 1.2 (n = 6)	1.3 ± 1.5 (n = 6)	0.001	0.699
<b>TB<sub>2</sub> (%)</b>	2.9 ± 1.7 (n = 6)	3.5 ± 1.6 (n = 6)	3.4 ± 2.8 (n = 6)	6.1 ± 7.3 (n = 6)	2.1 ± 1.6 (n = 6)	2.3 ± 1.3 (n = 6)	3.4 ± 2.4 (n = 6)	1.4 ± 1.6 (n = 6)	0.041	0.422
<b>ATP (nmol/mg)</b>	18.7 ± 2.1 (n = 6)	14.8 ± 4.0 (n = 5)	12.9 ± 6.9 (n = 6)	14.9 ± 4.7 (n = 5)	17.1 ± 3.3 (n = 6)	15.5 ± 4.3 (n = 7)	18.4 ± 3.9 (n = 7)	16.3 ± 2.9 (n = 6)	0.278	0.386
<b>ADP (nmol/mg)</b>	3.3 ± 0.6 (n = 5)	5.2 ± 2.2 (n = 5)	3.9 ± 1.9 (n = 6)	3.5 ± 1.3 (n = 5)	4.0 ± 1.4 (n = 6)	5.9 ± 2.7 (n = 7)	4.3 ± 1.9 (n = 7)	3.7 ± 1.9 (n = 6)	0.384	0.085
<b>ATP:ADP</b>	5.7 ± 1.0 (n = 5)	3.3 ± 2.0 (n = 5)	3.6 ± 1.4 (n = 6)	5.1 ± 3.5 (n = 5)	4.9 ± 2.4 (n = 6)	2.9 ± 1.1 (n = 7)	5.1 ± 2.7 (n = 7)	5.8 ± 3.4 (n = 6)	0.843	0.032



## Appendix 2: IACUC approval for Chapter 1

# Institutional Animal Care and Use Committee

U N I V E R S I T Y   O F   N E W   O R L E A N S

**DATE:** May 22, 2015  
**TO:** Bernard B. Rees  
**FROM:** Elizabeth Sigler  
**RE:** IACUC Protocol # 15-004  
*Entitled:* Comparing euthanasia techniques for fish hypoxia research

Your application for the use of animals in research (referenced above) has been approved beginning May 22, 2015 and expiring May 21, 2016. The initial approval period is one year. Near the end of this period, you will be asked to complete and submit an annual review in order to continue animal activities.

The University of New Orleans has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is A3299-01.

### Appendix 3: IACUC approval for Chapter 2

## Institutional Animal Care and Use Committee

U N I V E R S I T Y   O F   N E W   O R L E A N S

**DATE:** 7-17-13

**TO:** Bernard Rees

**FROM:** Elizabeth  
Sigler

**RE:** *IACUC Protocol # 13-005*

*Entitled:* Isolation of fish gills for hypoxia research

Your application for the use of animals in research (referenced above) has been approved beginning 7-13-13\_\_and expiring 7-12-14\_\_\_\_\_. Please note that annual and final reports must be provided to the UNO IACUC.

The University of New Orleans has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is A3299-01.

## **VITA**

Kristina Farragut was born in New Orleans, LA. She obtained her Bachelor's degree in Biomedical Engineering and Bachelor's degree in Neuroscience from Tulane University in 2013. In 2014, she began the Master's of Biological Sciences program at the University of New Orleans where she became a member in the lab of Dr. Bernard Rees.