THE EFFECTS OF MULTIPLE STRESSORS (DILTIAZEM, HYPOXIA, AND TEMPERATURE) ON THE CARDIOVASCULAR FUNCTION OF RAINBOW TROUT

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Dedication

To my mother: thank you for your unconditional support and encouragement. Leaving home and my son Eduardo Keller back in Brazil to go abroad for quality education was emotionally and financially challenging.

Thank you to my wife Gabriela Toscano for all the incredible support and partnership in this new step of my life.

Abstract

The effects of climate change, hypoxia of surface waters, disposal of pharmaceuticals, among other stressors to freshwater ecosystems, are important concerns of modern civilization. Diltiazem (DTZ) is a calcium channel blocker prescribed for heart diseases in humans; however, the knowledge of the effects of DTZ in the aquatic environment is limited. The present study investigated the cardiovascular effects of DTZ in rainbow trout. Fish were exposed in the laboratory to DTZ (0, 1, 10, 100 and 1000µg/L) for 96 hours at different temperatures (4ºC, 10ºC and 18ºC) and different dissolved oxygen concentrations (4mg/L and 8mg/L). DTZ (1 and 10 µg/L) impaired the increase of hemoglobin under hypoxia, at 1 µg/L impaired the increase of the ventilation rate, and at 10 µg/L increased the immature RBC counts, suggesting impairment of the cardiovascular system of rainbow trout. No statistically significant effects of DTZ on heart rate, oxygen consumption or hematocrit were detected.

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“Keep your eyes on the stars, and your feet on the ground.”

– Theodore Roosevelt

Chapter One: **Effects of diltiazem, hypoxia and temperature in rainbow trout (Literature Review)**

1.1 Introduction

Alterations of flow regimes in rivers have the potential to cause great changes in key characteristics of surface waters including water temperature, oxygen availability and concentration of pollutants. Pollutants enter the system either by point sources (e.g. sewage, storm water system and wastewater treatment plants effluents) or diffuse sources (e.g. agricultural runoff). It is important to understand how alterations of a flow regime and the resulting potential changes in water temperature and concentration of dissolved oxygen affect key ecosystem services provided by river systems. Aquatic organisms, including fish, have the capacity to acclimate to a slight to moderate adverse condition (McBryan *et al.*, 2013) and the effects of individual pollutants (e.g., pharmaceuticals), or changes in temperature or oxygen concentrations have been investigated in numerous studies, both in the field and in the laboratory. However, our understanding of how aquatic organisms, such as fish, respond to multiple, combined, flow-related changes in water quality or quantity (e.g., dissolved oxygen and temperature) remains limited. Establishing physiological thresholds for temperature regimes, dissolved oxygen, and tolerance to pollutants for a model species, such as rainbow trout (*Oncorhynchus mykiss*), can contribute important data to identify the threshold flow required to maintain ambient water quality and support biodiversity.

1.1.1 Pharmaceuticals

From the early 19th century, the aquatic environment has been affected by constant anthropogenic threats such as the disposal of pharmaceuticals and their metabolites in surface waters – most commonly in rivers – either via sewage water treatment systems or inadequate disposal (Garcia *et al.*, 2012; Holeton *et al.*, 2011; Quinn *et al.*, 2010). It is important to understand the effects of ecologically relevant stressors such as pharmaceuticals, hypoxia and seasonal temperatures, at values that occur in the natural environment. Predicting the effects of these stressors in the aquatic environment and setting protective guidelines is important to avoid larger losses (e.g., eradication of endemic species) due to toxicity and impairment in invertebrates, fish and riparian vegetation.

Wastewater treatment plant (WWTP) effluents, important point sources of contaminants in aquatic ecosystems, are main sources of human pharmaceuticals (e.g., diltiazem) in surface waters (Bartram *et al.*, 2012; Garcia *et al.*, 2012). These plants are not designed to filter and completely remove pharmaceuticals from the plant's effluent, and a wide variety of pharmaceuticals have been detected in the effluents (Boxall *et al.*, 2012; Garcia *et al.*, 2012; Kim *et al.*, 2007). Most medications currently available in the market are well studied and tested following strict and rigid guidelines that comply with what is considered safe for human consumption or veterinary use. However, the fate of pharmaceuticals after being metabolized and their impact on the aquatic environment remains a concern because despite numerous studies that characterized the effects of pharmaceuticals on non-target species (Ings *et al.*, 2012; Massarsky *et al.*, 2011), the

number of new medications and their projected use as human populations age, is tremendous.

Lethbridge has a growing, aging population who are likely to receive medicinal treatments for various conditions, including those related to the heart. Diltiazem (DTZ) is a calcium channel blocker commonly prescribed for heart treatment in seniors, and recently DTZ has been detected in the Lethbridge WWTP effluent (Keller et al., in preparation; Chapter 2). The current understanding of the effects of DTZ in fish is limited, especially when one considers its potential combined effects with climate change (e.g., wide temperature ranges and large water level fluctuations in surface waters). The Oldman River is one of the few water bodies in southern Alberta that is a natural habitat to a wide variety of fish species (including rainbow trout), and it is also an important river for the human population surrounding it.

Lethbridge, like several cities and towns close to the Oldman River's drainage, relies on nearby water bodies (e.g. Oldman River) for their water supply, sewage treatment and recreational activities. However, numerous pollutants, including pharmaceuticals such as DTZ, are released from wastewater treatment plant effluent, potentially posing risks to aquatic organisms, such as fish, and the human populations that use the river for source water.

1.1.2 Diltiazem

DTZ (2S,3S)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxy

phenyl)-1,5-benzothiazepin-4(5H)-one is a calcium channel blocker used in the treatment of hypertension, angina pectoris and some types of arrhythmia in humans (Dumont *et al.*, 1991). Calcium channel blockers restrain ions of calcium from crossing cell membranes by L-type calcium channel blockade. L-type calcium channels are mainly found in the heart, aorta and viscera in humans. The effect of the calcium channel blocking by DTZ is the relaxation of the target tissue, including reducing the heart rate and the force of contraction; therefore, it is prescribed as a heart medication. Calcium channel blockers are also reported to cause vasodilation (Grossman & Messerli, 2004). The effects of DTZ in dogs include peripheral and coronary vasodilation, and decreased heart rate (Dumont *et al.*, 1991). Even though DTZ has been used as a heart medication in humans for a long time, little is known about the effect of DTZ in the aquatic environment, including fish.

The bioconcentration factor (BCF) represents the concentration ratio of a given chemical in an organism and in water at a steady state. It has been reported that DTZ can bioconcentrate in rainbow trout's tissues including kidney, liver, muscle and plasma, though it was rated as a chemical with low capacity to bioconcentrate in fish (BCFs observed ranging from 0.5 to 194) (Steinbach, Grabic, *et al.*, 2016). The broad range of BCF reported is due to variations in DTZ concentration (0.03, 3 and 30 µg/L DTZ), length of exposure (21 and 42 days), and target tissue (plasma, muscle, liver and kidney). It has been suggested that DTZ can also bioconcentrate in osprey that are feeding on fish exposed to DTZ (Lazarus *et al.*, 2015). DTZ has shown to have antithrombotic properties in zebrafish (Zhu *et al.*, 2016), however, DTZ can have adverse effects in fish at high concentrations. The LC_{50} (lethal concentrations causing death in 50% of the test population) of DTZ to Japanese medaka (*Oryzias latipes*) ranges from 15 to 25.6 mg/L

for 96-h and 48-h, respectively (Kim *et al.*, 2007); exposure to DTZ at 8.2 mg/L for 96-h and 14 mg/L for 48-h was lethal to 50% of the *Daphnia magna* population (Kim *et al.*, 2007). Even though it is known that DTZ has lethal effects in fish at high concentrations (Kim et al., 2007), there are knowledge gaps on how it affects fish populations at ecologically relevant concentrations. A study carried out by Steinbach, Burkina, *et al.* (2016), in a subacute setting (21 – 42 days), tested hematological parameters, including hematocrit (Ht), hemoglobin (Hb), red blood cell count, white blood cell count and differential blood cell counts in rainbow trout, and demonstrated that hematocrit and mature neutrophil granulocytes increased in fish exposed to 3 and 30 µg/L of DTZ (waterborne).

DTZ has been detected in WWTP's effluents in several river ecosystems in North America (Kolpin *et al.*, 2002), including in the Oldman River in Alberta, Canada. Even though DTZ has a short photo-degradation half-life (less than 48-h) (Li, 2013), in collaboration with Dr. Bryan Brooks (Baylor University), relatively high concentrations of DTZ (ranging from 210 to 250 ng/L) were detected in WWTP effluent in Lethbridge, AB, Canada (unpublished data). The concentration detected in the Oldman River effluent is above the average detected in streams in the USA (20 ng/L) (Kolpin *et al.*, 2002) but below the predicted concentration in English rivers (670 ng/L) (Jones *et al.*, 2002); however, the average concentration in the Oldman River has not been determined yet. Scott *et al.* (2016) used a predictive model (Threshold Hazard Value), and suggested that fish are perhaps being treated similarly to humans with DTZ. Human plasma therapeutic dose of DTZ is 30 ng/mL (Scott *et al.*, 2016); Scott *et al.* (2016) measured DTZ concentration in fish plasma, and a few fish had equal or higher DTZ concentrations

equivalent to a human therapeutic dose. This unexpected finding of DTZ concentrations detected in the Lethbridge WWTP effluent, and the possibility of fish being treated with heart medication, led to the selection of DTZ as the model pollutant for the current proposed study, combined with hypoxia and different water temperatures mimicking exposures under a climate change scenario.

1.1.3 *Hypoxi*a

Fish frequently experience hypoxia in nature and in aquaculture (Eliason and Farrell, 2014; Table 1.1). The usual response of fish to a hypoxic challenge is to increase ventilation rate while maintaining the heart rate and stroke volume proportional to the metabolic demand (Randall, 1982). Some of the main behavioural responses of fish to cope with hypoxia are hyperventilation and surface breathing. Due to increased ventilation frequency, toxicants may become more available due to the increased volume of water (and toxicants) flowing through gills. Other organisms, such as *Daphnia magna*, may cope differently by progressively increasing heart rate proportionally to the severity of hypoxia (Pirow & Buchen, 2004). In contrast to daphniids, rainbow trout can also induce bradycardia when challenged by severe hypoxia (7.3 ± 0.2 kPa) (Sandblom & Axelsson, 2005). Other mechanisms that fish may rely upon during acclimation to hypoxic conditions is through increased production of erythropoietin, the hormone that controls erythropoiesis (red blood cells production) (Zhu *et al.*, 2013). An increase in hematocrit may also be seen in some circumstances under hypoxia (Holeton & Randall, 1967b); however, the increase in hematocrit has also been associated with the swelling of

the red blood cells due to changes in the pH of the blood (Holeton & Randall, 1967b). It is known that hypoxia triggers vasoconstriction in the gills of fish thus increasing blood pressure. This increase in blood pressure may concomitantly increase the gas exchange surface area through lamellar recruitment (Pettersson & Johansen, 1982). Under optimal conditions, blood circulation through gills flows from the afferent filamental artery (CO_2 rich blood), to the afferent lamellar artery, which then flows to secondary lamellae (where the majority of gas exchange occurs).

Table 1.1 - Laboratory studies investigating the cardiovascular responses of rainbow trout to fluctuations in temperature and to hypoxia.

Endpoint	Variable	Species	Response	References
Hematocrit	**Hypoxia**	Rainbow trout	No change	Wells and Weber (1991); Holeton and Randall (1967b)
			Increase	Swift and Lloyd (1974)
		Rainbow trout (Embryo and larva)		Bianchini and Wright (2013)
		General fish		Randall (1982)
	Warmer temperature (>16 ºC)	Steelhead trout (*Oncorhynchus mykiss*)	No change	Keen and Gamperl (2012)
		Rainbow trout		Lewis *et al.* (2012); Valenzuela *et al.* (2008); Wood *et al.* (1979)
			Increase	Sandblom and Axelsson (2007)
	Cooler temperature (<6 ºC)	Rainbow trout	Increase	Houston *et al.* (1996); Wood *et al.* (1979)
		Brown trout (*Salmo trutta)*	No change	Beaumont *et al.* (1995); Butler *et al.* (1992)
Hemoglobin	**Hypoxia**	Rainbow trout (Embryo and Larva)	Decrease	Bianchini and Wright (2013)
		Rainbow trout	No change	Pollock *et al.* (2007); Wells and Weber (1991)
			Increase	Swift and Lloyd (1974)
	Warmer temperature (>16 ºC)	Rainbow trout	Decrease	Houston *et al.* (1996); Lewis *et al.* (2012)
			No change	Valenzuela *et al.* (2008)
	Cooler temperature (<6 ºC)	Brown trout (*Salmo trutta)*	No change	Beaumont *et al.* (1995); Butler *et al.* (1992)
		Rainbow trout	Increase	Houston *et al.* (1996)

Table 1.1 - Continued

Endpoint	Variable	Species	Response	References
Heart Rate	**Hypoxia**	Toadfish (*Opsanus beta*)	Decrease	Panlilio *et al.* (2016)
		Rainbow trout		Eliason and Farrell (2014); Holeton and Randall (1967a, 1967b); Perry and Desforges (2006); Randall (1982); Sandblom and Axelsson (2005); Wood and Shelton (1980)
		General fish		Randall (1982)
	Warmer temperature (>16 ºC)	Rainbow trout	Increase	Aho and Vornanen (2001); Ekstrom *et al.* (2016); Ekstrom *et al.* (2014); Heath and Hughes (1973); Sandblom and Axelsson (2007); Taylor *et al.* (1996); Wood and Shelton (1980)
		Salmonids		Farrell (2002)
		Brown trout (*Salmo trutta fario)*		Altimiras *et al.* (2002); Vornanen *et al.* (2014)
	Cooler temperature (<6 ºC)	Rainbow trout	Decrease	Aho and Vornanen (2001); Devera and Priede (1991); Tuurala *et al.* (1982); Wood *et al.* (1979)
		Brown trout (*Salmo trutta)*		Beaumont *et al.* (1995); Butler *et al.* (1992)

Table 1.1 - Continued

Endpoint	Variable	Species	Response	References
Oxygen Consumption	**Hypoxia**	Rainbow trout	No change	Eliason and Farrell (2014); Holeton and Randall (1967a)
		General fish		Randall (1982)
	Warmer temperature (>16 ºC)	Steelhead trout (*Oncorhynchus mykiss*)	Increase	Keen and Gamperl (2012)
		Rainbow trout		Heath and Hughes (1973)
		Brown trout (*Salmo trutta*)		Altimiras *et al.* (2002)
	Cooler temperature (<6 ºC)	Brown trout (*Salmo trutta*)	Decrease	Beaumont *et al.* (1995); Butler *et al.* (1992)
Ventilation	**Hypoxia**	Rainbow trout	Increase	Holeton and Randall (1967a, 1967b)
		General fish		Randall (1982)
	Warmer temperature (>16 ºC)	Rainbow trout	Increase	Ekstrom *et al.* (2014); Heath and Hughes (1973)
		Steelhead trout (*Oncorhynchus mykiss*)		Keen and Gamperl (2012)
		Brown trout (*Salmo trutta*)		Altimiras *et al.* (2002)
	Cooler temperature (<6 ºC)	Salmonids	N/A	N/A*

* - No references were found on the effects of low water temperature (starting at 10ºC - 12ºC and reducing to 6ºC or below) on salmonids in Web of Science and PubMed databases.

The blood, now rich in O_2, flows through the efferent lamellar artery to the efferent filamental artery to irrigate the vital organs and peripheral tissues, including the gills themselves. Under hypoxia, the efferent lamellar artery constricts allowing for an increased blood pressure in the gills secondary lamella, thus increasing contact surface area (lamellar recruitment) and gas exchange, allowing fish to maintain oxygen uptake rate, without spending extra energy on hyperventilating or increasing heart rate (Pettersson & Johansen, 1982). Sundin *et al.* (1995) demonstrated that trout may do so by selectively activating the serotonergic system to respond accordingly to the hypoxia severity. Even though one can determine hypoxia thresholds guidelines for fish (e.g., rainbow trout) in the laboratory and *in situ*, hypoxia thresholds have to be taken into account as fish rely on invertebrates that fish often feed on (e.g., such as flies' larva) to thrive.

Since the acute lethal DO concentration in water for cool-water species (e.g., trout) is at 3 mg/L or below, the US-EPA established the minimum dissolved oxygen (DO) concentration at 4 mg/L to protect insects that salmonids often feed on (US EPA – Quality Criteria for Water, 1986). Alberta Environment & Sustainable Resource Development (ESRD) (2014) established a more conservative minimum of 5 mg/L of DO concentration in water for short-term exposures. The guidelines are established to protect the aquatic environment as a whole, meaning that even at the minimum DO established, the aquatic environment is still safe.

1.1.4 Multiple Stressors

WWTP effluents, important carriers of nutrients into the environment, may unintentionally release an increased volume of nutrients into surface waters by overflow or bypassing in adverse circumstances (e.g., snow melts and large storms) (Holeton *et al.*, 2011). Hypoxia generated from eutrophication and organic pollution is amongst one of the most pressing and relevant environmental problems in the world (Goldberg, 1995). Seasonal hypoxic zones or persistent hypoxic zones are often results of nutrient-rich water associated with sewage effluent (McElroy *et al.*, 2012). The nutrient-rich water in the river together with the warm summer temperatures may create favourable conditions for phytoplankton growth. Since phytoplankton do not photosynthesize after sunset, they can deplete dissolved oxygen from the water, which can promote a hypoxic or anoxic condition (McBryan *et al.*, 2013). Favourable concentrations of dissolved oxygen and ambient temperature regimes are among the key factors essential for the survival of aquatic organisms (Smale & Rabeni, 1995). High water temperatures, low water flow and poor surface movement – among other factors – may result in low concentrations of dissolved oxygen in surface waters. Local events of hypoxia or anoxia are likely to co-occur with high temperatures because oxygen is less soluble in warmer water than in colder water. The increased metabolic activity of the phytoplankton and bacteria at higher temperatures also contributes to hypoxic events (McBryan *et al.*, 2013) since the oxygen demand of phytoplankton and bacteria rises, depleting the concentration of dissolved oxygen available to higher vertebrates, such as fish (McBryan *et al.*, 2013).

Until recently, researchers were experimenting with single-stressor models in tightly controlled laboratory environments when assessing the effects of pollutants. A single-stressor model is relevant for determining the potential toxicity of a pollutant by itself on aquatic organisms. However, a large variety of organisms, with different sensitivities, are acutely and chronically exposed to multiple stressors, exerting their effects, often times combined, in the natural aquatic systems. Our understanding of the effects of multiple stressors in fish, specifically as they relate to changes in river flow regimes, remains limited.

In the climate change scenario, low-flow regimes may lead to higher water temperature which influences standard metabolic rate (SMR) in fish. The SMR is defined as the amount of oxygen that a fish consumes while not active, under a standardized feeding regime. The SMR is usually represented by the basal intake of oxygen for the fish to survive. The higher the temperature in an aquatic environment, the higher the SMR value will be. In higher water temperature, the oxygen solubility in water is reduced, potentially leading to a hypoxic or even anoxic environment. It is important to determine how higher temperatures and hypoxia modulate the effects of DTZ in the ecosystem and the community within. The individual effects of hypoxia and hyperthermia in fish are well known (Table 1.1); however, their effects combined with DTZ have not been explored together in a multiple-stressor environment. Also, there are limited data available on the effects of DTZ in fish. The toxicology community has been urging for studies involving multiple stressors in the aquatic environment (Breitburg *et al.*, 2009; Pollock *et al.*, 2007; Schulte, 2007). Chronic exposures to multiple stressors at environmentally relevant concentrations are warranted, in order to better assess their

impacts in the natural environment (Li, 2013). However, effects of acute exposures to multiple stressors must be assessed in initial exploratory studies in order to mimic real-life, yet controlled conditions.

1.2 Objectives and hypothesis

1.2.1 Approach

To understand and predict the physiological tolerance threshold for key species, it is relevant to study key aspects (such as toxicity and physiological effects) using single stressor models, and thereafter, study the combined effect of multiple stressors. The evident knowledge gap concerning the effects of DTZ in the aquatic environment motivated the development of the research project described in this thesis. This thesis project investigated the cardiovascular effects of DTZ in rainbow trout and determined how temperature and hypoxia modulated the physiological response. Rainbow trout was chosen as the test species, as a known sensitive fish model, ease of laboratory rearing, and availability in hatcheries. Fish were exposed to DTZ (waterborne) for 96 hours in the laboratory. In an effort to mimic natural events, a winter experiment (4ºC nominal water temperature) and summer experiment (18ºC nominal water temperature) were carried out to determine the effects of DTZ under different water temperatures. With climate change, likely to increase ambient temperatures of local freshwater systems and lower oxygen content of water, the effects of DTZ on the cardiovascular function of rainbow trout exposed to hypoxic conditions – 4 mg/L (~35% air sat.) – were also tested. The cardiovascular function of rainbow trout was assessed, including hematology screening

(hematocrit, hemoglobin and red blood cell counts), heart rate, oxygen consumption, and ventilation rate.

1.2.2 Objectives

- To characterize the cardiovascular effects of DTZ in rainbow trout under optimal temperature (10ºC) and oxygen saturation (>85% air saturation);
- To determine if temperature (winter or summer regimes) modulates the cardiovascular effects of DTZ in rainbow trout;
- To determine if hypoxia modulates the cardiovascular effects of DTZ in rainbow trout;
- To determine if the combined effects of temperature and hypoxia modulate cardiovascular effects of DTZ in rainbow trout.

1.2.3 Hypotheses

i. DTZ will impair the cardiovascular function in rainbow trout exposed under optimal conditions (i.e. 10ºC water temperature and dissolved oxygen concentrations above 85%);

ii. DTZ will impair the response of the cardiovascular system of rainbow trout exposed to hypoxia (e.g., reducing heart rate.)

iii. DTZ will have similar effects as in humans on the cardiovascular function (i.e. reduction in heart rate) of rainbow trout exposed under summer and winter mimicking conditions (e.g. 18ºC and 4ºC water temperature);

Chapter Two: **The Effects of Multiple Stressors (Diltiazem, Temperature, and Hypoxia) on the Cardiovascular Function of Rainbow Trout**

2.1 Introduction

From early 19th century, the aquatic environment has been affected by constant anthropogenic threats such as the disposal of pharmaceuticals in freshwater bodies – most commonly rivers (Quinn *et al*., 2010; Holeton *et al*., 2011; Garcia *et al*., 2012). The WWTP effluents are main sources of human pharmaceuticals (e.g. diltiazem) and introduction of contaminants into surface waters (Bartram *et al.*, 2012; Garcia *et al.*, 2012). Even though the fate and effects of numerous pharmaceuticals have been extensively studied, it is important to understand the combined effects of ecologically relevant stressors, such as pharmaceuticals, hypoxia and seasonal temperatures, at values that occur in the natural environment. Predicting the effects of these stressors in the aquatic environment and setting protective guidelines is important to avoid losses of aquatic species populations due to toxicity and impairment in invertebrates, fish and riparian vegetation.

The Oldman River, one of the key water bodies in southern Alberta, is an important river for the human population surrounding it to source water. Lethbridge, like several cities and towns close to the Oldman River drainage, relies on the river for water supply, sewage treatment and recreational activities. However, numerous pollutants, including pharmaceuticals such as DTZ, are released from the wastewater treatment plant effluent potentially posing risks to aquatic organisms, such as fish, and the populations who use the river to source water.

Diltiazem (2S,3S)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-

methoxy-phenyl)-1,5-benzothiazepin-4(5H)-one is a calcium channel blocker used in the treatment of heart disease in humans (Dumont *et al.*, 1991). Calcium channel blockers restrain ions of calcium from crossing cell membranes by L-type calcium channel blockade. One of the effects of the calcium channel blocking by DTZ, for instance, is the relaxation of the target tissue, including reducing the heart rate and the force of contraction; therefore, it is prescribed as a heart medication, including treatment of angina pectoris. Calcium channel blockers are also reported to cause vasodilation (Grossman & Messerli, 2004; Hernandez-Hernandez *et al.*, 2002). The effects of DTZ in dogs include peripheral and coronary vasodilation, and decreased heart rate (Dumont *et al.*, 1991). Even though DTZ has been used as a heart medication in human for a long time, little is known about the effect of DTZ in the aquatic environment, including fish.

The bioconcentration factor (BCF), the concentration ratio of a given chemical in an organism and in water at a steady state, has been reported for DTZ in rainbow trout tissues including kidney, liver, muscle and plasma, at BCFs ranging from 0.5 to 194 (Steinbach, Grabic, *et al.*, 2016). It has been suggested that DTZ can also bioconcentrate in osprey birds from feeding on fish exposed to DTZ (Lazarus *et al.*, 2015). DTZ has shown to have antithrombotic properties in zebrafish (Zhu *et al.*, 2016), however, at high concentration DTZ can be lethal. Exposure to DTZ at 8.2 mg/L for 96-h and 14 mg/L for 48-h showed to be lethal to 50% of the *Daphnia magna* population (Kim *et al.*, 2007). The LC_{50} of DTZ to Japanese medaka (*Oryzias latipes*) ranges from 15 to 25.6 mg/L for 96-h and 48-h, respectively (Kim *et al.*, 2007). Even though it is known that DTZ has lethal effects in fish at high concentrations (Kim et al., 2007), there are knowledge gaps on how it affects fish populations at ecologically relevant concentrations. A study carried

out by Steinbach, Burkina, *et al.* (2016), in a subacute setting (21 – 42 days), tested hematological parameters, including hematocrit (Ht), hemoglobin (Hb), red blood cell count, white blood cell count and differential blood cell counts in rainbow trout, and demonstrated that hematocrit was increased by 11% in fish exposed to 30 µg/L of DTZ under normoxic 15ºC water for 21 days and mature neutrophil granulocytes increased by 400% in fish exposed for 42 days at 3 and 30 µg/L of DTZ.

Even though DTZ has a short photo-degradation half-life (less than 48-h) (Li, 2013), relatively high concentrations of DTZ (ranging from 210 to 250 ng/L) were detected in WWTP effluent in Lethbridge, AB, Canada (collaboration with Dr. Bryan Brooks, Baylor University). The concentration detected in the Oldman River was above the average detected in streams in the USA (20 ng/L) (Kolpin *et al.*, 2002) but below the predicted concentration in English rivers (670 ng/L) (Jones *et al.*, 2002). Scott *et al.* (2016) used a predictive model and suggested that fish are perhaps being exposed to DTZ in the aquatic environment resulting in the equivalent of a human therapeutic dose; however, the effects of DTZ under various climate scenarios have not been investigated yet.

Fish frequently experience hypoxia in nature and in aquaculture (Eliason and Farrell, 2014). In response to a hypoxic challenge, fish increase their ventilation rate while maintaining the heart rate and stroke volume proportional to the metabolic demand (Randall, 1982). Invertebrates such as *Daphnia magna*, may cope differently than fish by progressively increasing their heart rate in proportion to the severity of hypoxia (Pirow & Buchen, 2004). In contrast to daphniids, fish can also induce bradycardia when challenged by hypoxia (Sandblom & Axelsson, 2005). Another mechanism activated in

fish under hypoxic conditions is increasing the production of erythropoietin, the hormone that controls erythropoiesis (red blood cells production) (Zhu *et al.*, 2013). An increase in hematocrit may also be seen in some circumstances under hypoxia (Holeton & Randall, 1967b); however, the increase has also been associated with the swelling of the red blood cells due to changes in the pH of the blood (Holeton & Randall, 1967b). Hypoxia triggers vasoconstriction in the gills of fish thus increasing blood pressure; it is suggested that it is likely a response to increase surface area (lamellar recruitment), thus increasing oxygen uptake (Pettersson & Johansen, 1982). Sundin *et al.* (1995) demonstrated that trout can also remodel the gill structure. The secondary lamellae length (SLL) and basal epithelial thickness (BET) represent the most relevant measurements of the gill that can influence the diffusion distance in fish (Hughes & Perry, 1976) and are correlated with the total surface area of the gills available for gas exchange (PAGE) (Stevens, 1992). Some of the main behavioural responses of fish to cope with hypoxia are hyperventilation and surface breathing. Even though some fish species are highly vulnerable to hypoxia, and exhibit low thresholds of tolerance, there are occurrences of hypoxia tolerant fish species and hypoxia tolerant populations.

Alberta surface water quality guideline (2014) established a minimum of 5 mg/L for short-term exposures of dissolved oxygen (DO) concentration in water to protect the aquatic environment as a whole, while US EPA (Quality Criteria for Water, 1986) established the minimum acceptable DO in the US at 4 mg/L to protect insects that salmonids often feed on, even though the acute lethal DO concentration in water for cool-water species (e.g. trout) is at 3 mg/L or below. Seasonal hypoxic zones or persistent hypoxic zones are often the result of nutrient-rich water associated with sewage effluent

(McElroy *et al.*, 2012). The nutrient-rich water in the river together with the warm summer temperatures may create favourable conditions for phytoplankton growth. Since phytoplankton do not photosynthesize after sunset, they can deplete the oxygen concentration in the water, which can promote a hypoxic condition. When the phytoplankton die, they provide biomass for bacterial decomposition and this activity may reduce even more the oxygen concentration dissolved in the water body, which may lead even to an anoxic environment (McBryan *et al.*, 2013). The individual effects of hypoxia and hyperthermia in fish are widely studied and well known. However, their effects combined with DTZ have not been explored together in a multiple-stressor environment.

Therefore, the current study will investigate the effects of DTZ (0, 1, 10, 100 and 1000 µg/L, as a range finding experiment) on the cardiovascular function of rainbow trout at a wide range of water temperature (4ºC, 10ºC and 18ºC), normoxia (8 mg/L) and hypoxia (4 mg/L - 35% air sat.) for 96 hours in the laboratory. Specifically, the modulation potential of hypoxia on the effects of DTZ in the cardiovascular function of rainbow trout will be assessed.

2.2 Materials and Methods

2.2.1 Experimental Fish

Juvenile rainbow trout (RBT) (n = 144, fork length 179.9 ± 2.3 mm [mean ± SE], wet body mass 66.78 ± 2.57g were acquired from Sam Livingston Fish Hatchery and Rearing Station (Calgary, Alberta, Canada).

Fish were held in holding tanks (750 L) for at least two weeks to acclimate to laboratory conditions before being transferred to experimental tanks. The holding tank water quality parameters were monitored twice a day and maintained at 10 ± 1ºC and DO above 8.5 mg/L (>85% air sat.). The water chemistry parameters (pH, NH_3, NO_2 and NO_3) were measured daily (Table 2.1). The fish were fed 1% of wet body mass with EWOS commercial trout chow pellets (Micro Complete Feed for Salmonids, product 501666, EWOS Canada, Surrey, British Columbia).

Table 2.1 – Water quality parameters (mean ± S.E.) in range finding, summer and winter experiments.

Range finding experiment[a]	DO (mg/L)	NH_3 (mg/L)	NO_2 (mg/L)	NO_3 (mg/L)	pH median[b] (range)	Temperature (ºC)
0 µg/L DTZ (10 ºC)	9.97 ± 0.07	0.17 ± 0.01	0.03 ± 0.009	0.91 ± 0.14	7.73 (7.58 – 7.87)	10.0 ± 0.02
1 µg/L (10 ºC)	9.01 ± 0.16	0.19 ± 0.01	0.01 ± 0.002	1.18 ± 0.29	7.79 (7.66 – 7.91)	10.0 ± 0.03
10 µg/L (10 ºC)	9.34 ± 0.11	0.17 ± 0.02	0.01 ± 0.002	1.19 ± 0.29	7.81 (7.67 – 7.9)	10.1 ± 0.02
100 µg/L (10 ºC)	9.21 ± 0.22	0.19 ± 0.02	0.03 ± 0.008	0.75 ± 0.12	7.71 (7.65 – 7.79)	10.1 ± 0.06
1000 µg/L (10 ºC)	9.40 ± 0.06	0.27 ± 0.01	0.03 ± 0.013	0.79 ± 0.11	7.71 (7.65 – 7.81)	10.1 ± 0.05
Summer experiment						
0 µg/L DTZ (10 ºC)	9.93 ± 0.02	0.31 ± 0.04	0.04 ± 0.007	1.23 ± 0.15	7.66 (7.15 – 7.84)	10.2 ± 0.00
0 µg/L DTZ (18 ºC)	8.38 ± 0.01	0.21 ± 0.06	0.25 ± 0.032	1.41 ± 0.16	7.62 (7.15 – 7.71)	18.0 ± 0.07
1 µg/L DTZ (18 ºC)	7.30 ± 0.15	0.12 ± 0.04	0.12 ± 0.018	1.28 ± 0.09	7.66 (7.25 – 7.8)	17.9 ± 0.05
10 µg/L DTZ (18 ºC)	8.30 ± 0.33	0.18 ± 0.06	0.16 ± 0.028	1.38 ± 0.08	7.74 (7.48 – 7.83)	18.0 ± 0.10
Winter experiment						
0 µg/L DTZ (10 ºC)	10.14 ± 0.05	0.02 ± 0.010	0.16 ± 0.005	0.95 ± 0.14	7.98 (7.91 – 8.1)	10.0 ± 0.02
0 µg/L DTZ (4 ºC)	11.53 ± 0.08	0.02 ± 0.005	0.04 ± 0.004	1.00 ± 0.17	8.04 (7.94 – 8.14)	4.3 ± 0.11
1 µg/L DTZ (4 ºC)	10.78 ± 0.30	0.03 ± 0.008	0.04 ± 0.007	0.55 ± 0.19	7.98 (7.91 – 8.08)	4.2 ± 0.09
10 µg/L DTZ (4 ºC)	11.73 ± 0.18	0.03 ± 0.010	0.01 ± 0.002	0.41 ± 0.12	7.98 (7.9 – 8.08)	4.4 ± 0.11

Hypoxia experiment

0 µg/L DTZ (normoxia)	9.75 ± 0.11	0.16 ± 0.051	0.039 ± 0.011	0.84 ± 0.22	8.11 (8.04 – 8.2)	10.0 ± 0.05
0 µg/L DTZ (hypoxia)	3.95 ± 0.07	0.24 ± 0.025	0.009 ± 0.002	0.23 ± 0.13	8.17 (8.04 – 8.29)	10.1 ± 0.06
1 µg/L DTZ (hypoxia)	4.28 ± 0.23	0.09 ± 0.022	0.073 ± 0.011	0.69 ± 0.15	8.23 (8.13 – 8.34)	10.2 ± 0.05
10 µg/L DTZ (hypoxia)	3.83 ± 0.10	0.02 ± 0.006	0.037 ± 0.008	0.36 ± 0.10	8.22 (8.09 – 8.36)	10.0 ± 0.06

[a] n=8 for all groups, except n=16 in 0 µg/L DTZ (10 ºC), [b] median used for pH values.

2.2.2 Exposures to DTZ

Stock Solution Preparation

DTZ stock solutions were prepared with diltiazem hydrochloride (Toronto Research Canada, Catalogue # D291605**)** and double deionized water. Stock solutions were prepared fresh every day before water changes. Water changes were performed daily (50%). Water samples were collected after water changes for water quality control (Table 2.1) and DTZ concentration validation (analysis in progress).

Pilot Study (Range Finding Experiment) – Figure 2.1A

A range finding pilot study was conducted with RBT to select the relevant waterborne concentrations of DTZ hydrochloride for the main experiments (winter, summer and hypoxia experiment) and for validation of the protocols and techniques that would be further applied. The fish were not fed 24 hours before the beginning of the experiment and fasted throughout the duration of the experiment (96 hours). The range of concentrations of DTZ used in the pilot study was 0, 1, 10, 100 and 1000 µg/L. After the 96 hours of exposure to DTZ, respiration, ventilation and electrocardiogram (ECG) data were collected and the fish were euthanized with MS-222 (Aqua Life TMS, Catalogue # 02168510) buffered with sodium bicarbonate (Amresco, Catalogue # 0865). The fork length and wet body mass of the euthanized fish were measured, blood samples were

taken via caudal vessel puncture, livers were extracted and stored at -80ºC, and maturity of gonads was assessed.

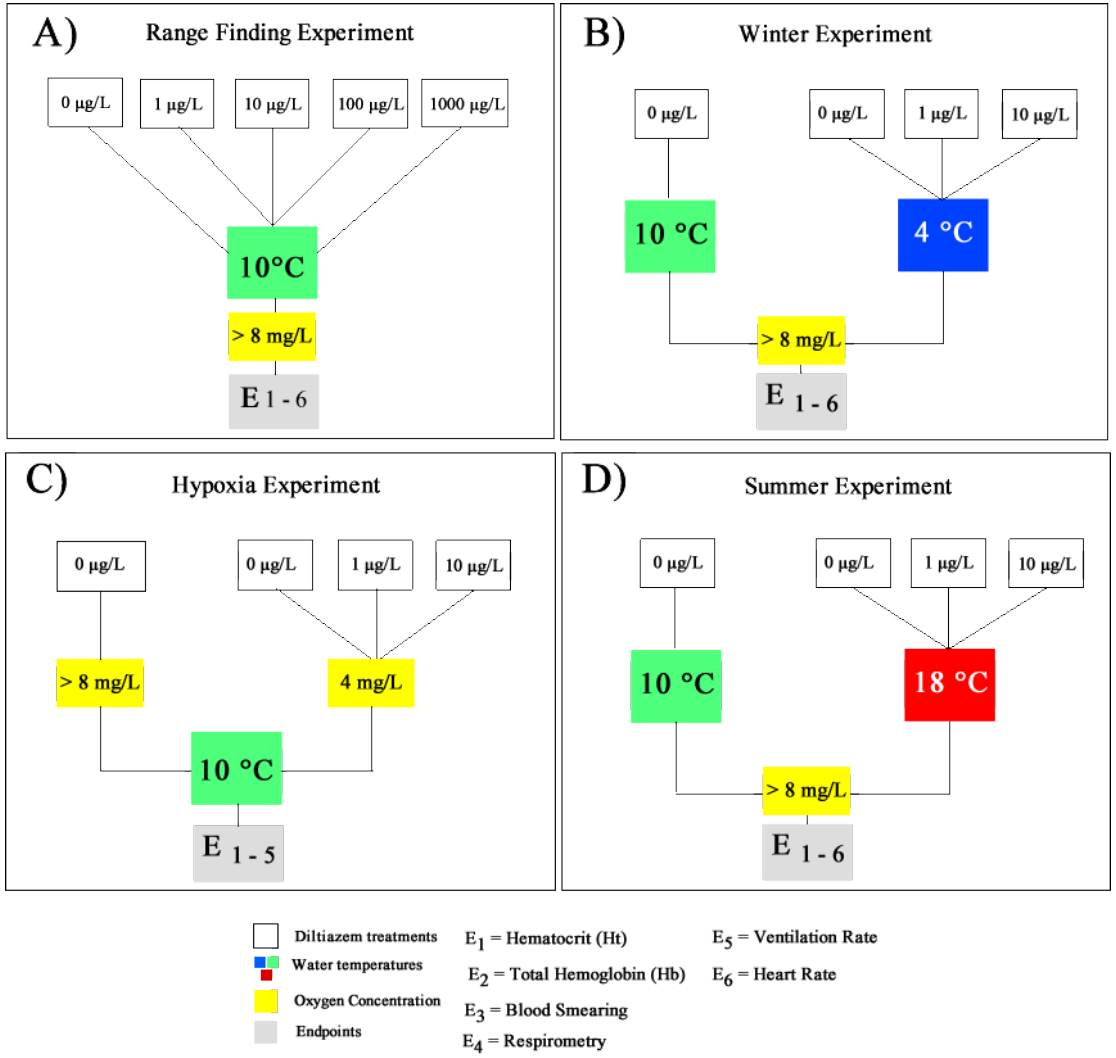

Figure 2.1 – Flow chart of the experimental design.

Diltiazem Exposure: Winter Experiment – Figure 2.1B

At normoxic oxygen saturation (> 85% air sat.), RBT were acclimated to 4ºC from 10ºC by lowering the water temperature 2ºC per day prior to start of the exposure to DTZ. Fish were fed 1% body mass of EWOS commercial trout chow pellets during the 2-day acclimation period and fasted 24 hours before the beginning of the exposures and throughout the experiment. Fish were exposed to 0 µg/L (n = 8), 1 µg/L (n = 8) and 10 µg/L (n = 8) of DTZ at 4ºC for 96 hours. A temperature control group was also used (n = 8; 0 µg/L DTZ at 10 ºC). At the end of the 96 hours of exposure, oxygen consumption, ventilation rate and ECG data were collected (see Section 2.2.3 Endpoints), the fish were euthanized, and fork length and wet body mass were obtained. Blood samples were taken via caudal vessel puncture, livers were extracted and stored at -80ºC, and gonadal maturity assessed.

Diltiazem Exposure: Hypoxia Experiment – Figure 2.1C

For the hypoxia experiment, RBT were acclimated to 4 mg/L dissolved oxygen (35% air sat.) by bubbling nitrogen gas in the water for 2 days, reducing the air saturation no more than 20% per day (2 mg/L/day). Fish were fed 1% body mass of EWOS commercial trout chow pellets during the 2-day acclimation period and fasted 24 hours before the beginning of the DTZ exposures and throughout the experiment. Fish were exposed to 0 µg/L (n = 5), 1 µg/L (n = 6) and 10 µg/L (n = 6) of DTZ (waterborne) at 10ºC and 35% air sat. for 96 hours. A hypoxia control group was also used (n = 6; 0 µg/L DTZ at 10ºC and normoxia [>85% air sat.]). At the end of the 96 hours of exposure,

oxygen consumption and ventilation rate data were collected, the fish were euthanized and the fork length and wet body mass were obtained. Blood samples were taken via caudal vessel puncture, livers were extracted and stored at -80ºC, and gonadal maturity assessed.

Diltiazem Exposure: Summer Experiment – Figure 2.1D

RBT were acclimated to 18ºC by increasing the water temperature 2ºC per day prior to start of the DTZ exposure. Fish were fed 1% body mass of EWOS commercial trout chow pellets during the 4-day acclimation period and fasted 24 hours before the beginning of the exposures and throughout the experiment. Fish were exposed to 0 µg/L (n = 8), 1 µg/L (n = 8) and 10 µg/L (n = 8) of DTZ at 18ºC for 96 hours. A temperature control group was used (n = 8; 0 µg/L DTZ at 10ºC). At the end of the 96 hours of exposure, oxygen consumption, ventilation rate and ECG data were collected, the fish were euthanized and, fork length and wet body mass were obtained. Blood samples were taken via caudal vessel puncture, livers were extracted and stored at -80ºC, and gonadal maturity assessed.

2.2.3 Endpoints

Hematology

Blood samples, obtained through caudal blood vessel puncture, were transferred into Eppendorf vials containing 40 µL of heparin (1000 IU/mL), and stored at 4ºC. The

Eppendorf vials were always refrigerated during all process as they were maintained on ice until stored in the fridge (4ºC).

1. Hematocrit

Hematocrit was measured using a standard protocol (Hesser, 1960); the blood sample was collected from the heparinized Eppendorf vials with hematocrit capillary tubes (Fisher Scientific, catalogue # 22-362574), centrifuged at 13 000 rpm for 5 minutes and hematocrit (%) was determined using the standard scale.

2. Hemoglobin

The blood Hb was determined following a standard protocol (Drabkin's method) (Shiver, 2006) for measuring cyanmethemoglobin. The Hb reference was prepared using human Hb (Sigma Aldrich, Catalogue #H7379) to plot a standard curve (R^2 = 0.99). Drabkin's reagent (Sigma Aldrich, Catalogue #D5941) was mixed with blood samples and incubated for 20 minutes. Aliquots of 200 µg/L from the solution were pipetted, in triplicates, in a 96-well microplate (Corning, catalogue # 3596). Absorbance was measured at 540 nm in a Varioscan Flash microplate reader (Thermo scientific, catalogue # 5250040). The Hb concentration was determined by multiplying the absorbance value of the samples by the standard curve formula (y = 801.74).

3. Red Blood Cell Count

Blood smears were prepared, in triplicate, by smearing one drop of blood onto a microscope slide (Fisher Scientific, Catalogue # 125447) using a standard procedure and allowed to air dry. The slides were fixed in methanol and stored until staining. Slides

were then re-hydrated in double deionized water phosphate-buffer (PO_4) at pH 7.2 for 2 minutes, then immersed for 3 minutes in Wright's stain solution and 5 minutes in Giemsa stain. Images of the blood smears were taken with a microscope camera (Lumenera, model Infinity 1) in two fields under 400x magnification and the blood cells were counted. The software used for the analysis was ImageJ v1.50f with Cell Counter plugin.

Cardiovascular Monitoring

Cardiovascular data were obtained using a custom-built system respiration chamber (Appendix 1) to hold the fish during the oxygen consumption monitoring, an oxygen measuring solution and a temperature sensor (see section "2. Oxygen Consumption"). The heart rate was determined from the electrocardiogram tracings obtained through a custom setup consisting of needle electrodes, signal amplifier, and an interface (see section "1. Heart Rate (Electrocardiogram, ECG)"). The oxygen consumption was obtained by measuring the difference of initial DO concentration and final DO concentration in the respiration chamber and ventilation rates were obtained by video-recording and counting the opercula abductions.

1. Heart Rate (Electrocardiogram, ECG)

The heart rate data acquisition apparatus consisted of a damp chamber to accommodate the anesthetized fish (gills were constantly irrigated with aerated MS-222 solution maintained at the experimental temperature of 4ºC, 10ºC or 18ºC), needle electrodes (ADIstruments, Catalogue code MLA1203), a signal amplifier (Warner

Instruments, Catalogue DP-311), and a PowerLab interface (ADInstruments, PowerLab 4/26). The MS-222 solution was prepared with 60 mg/L of MS-222, buffered to pH 7.4 with 120 mg/L of sodium bicarbonate, and the temperature of the solution (4ºC, 10ºC or 18ºC) was maintained using a wine cooler. The ECG tracing was obtained by inserting needle electrodes surrounding the heart cavity and processed using PowerLab Chart 5 (ADInstruments, version 5.1). All equipment was accommodated in a Faraday cage, except the signal amplifier and the interface. The heart rate was determined by counting the QRS peaks, each peak considered as one heart beat.

2. Oxygen Consumption

The oxygen consumption of fish held in the respiration chamber (Appendix 1) was determined by measuring the difference between the initial DO and final DO in a 30-minute time span. The custom-built respiration chamber, based on the commercially available Loligo systems, was constructed using plexiglass, nylon nuts and bolts, nylon spouts, acrylic glue, PVC caps and tubes and silicon tubing. The system had a closed-loop design, utilizing a 300 L/h pump (Eheim, Catalogue # 1046219). The main chamber (25.5 x 8.9 x 8.9 cm, length x height x depth) where the fish was held, had a containment volume of 2L. The data acquisition of the DO values was performed in a non-invasive setup using a Fibox 3 interface (PreSens, Catalogue # 200000269), an optical fiber (PreSens, Catalogue # 200000241), and sensor spots (PreSens, Catalogue # 200000023) in a remote chamber to minimize visual contact and disturbance of the subject.

3. Ventilation Rate

The ventilation rate was measured by counting opercula abductions for 1 minute, every 5 minutes over a 30-minute time span. A video recording camera was positioned above the main oxygen consumption chamber, oriented in a 90-degree angle in relation to the centre of chamber. All images were captured using Debut Video Capture Software (version 2.16).

Gill Histology

Gill filaments were collected and fixed in Bouin's solution. Histological examination was carried out using a standard histological protocol, including preparation of paraffin embedded sections and staining (hematoxylin and eosin) the second gill arch (5 µm sections) of fish to quantify key aspects of the gill structure under bright field microscopy. The basal epithelial thickness (BET) and secondary lamellae length (SLL) were measured at 3 different sections within a lamella (base, middle and tip) of 3 lamellae per fish. The Proportion Available for Gas Exchange (PAGE) of the secondary lamellae was calculated for each filament of each fish using the following equation: PAGE (%) = 100 x [SLL/(BET + SLL)] (Nero *et al.*, 2006).

2.2.4 Statistical Analyses

Data were analyzed using IBM SPSS (version 22 for Windows). Since a full factorial design was not possible due to logistical limitations, a t-test was used to determine temperature or hypoxia effects alone and a one-way ANOVA (analysis of variance) was employed to determine the effects of DTZ on the cardiovascular function of fish.

Following a determination of statistical significance from the ANOVA, a Dunnett's test was applied for the post-hoc analysis. The significance threshold (α) was set to 0.05 *a priori*; however, due to the exploratory and preliminary nature of this study which necessitated small samples sizes for multiple endpoints a significance threshold of up to 0.1 was used to detect biological trends (Wasserstein & Lazar, 2016). Biological trends (P-values ranging from 0.051 to 0.1) will be referred to as *statistical trend* (*st*) throughout.

2.3 Results

2.3.1 Range Finding Experiment

Hematocrit, oxygen consumption and heart rate were measured in rainbow trout exposed to DTZ at 0, 1, 10, 100, and 1000 µg/L in the range finding experiment (Table 2.2). No statistically significant changes were detected; however, a declining trend was observed in Hb and heart rate between groups exposed to 0 µg/L (control), 1 µg/L and 10 µg/L. The selection of concentrations of DTZ used in the main experiments (i.e. winter, hypoxia and summer experiment) was based on the trend observed in the range finding experiment.

Table 2.2 – Range finding experiment: cardiovascular endpoints (mean ± S.E. [n]) in rainbow trout exposed to DTZ for 96 hours in the laboratory.

Endpoint	0 µg/L DTZ	1 µg/L DTZ	10 µg/L DTZ	100 µg/L DTZ	1000 µg/L DTZ
Hematocrit (%)	50 ± 2 [15]	54 ± 2 [8]	51 ± 2 [8]	49 ± 5 [7]	49 ± 5 [7]
Hemoglobin (mg/ml)	110.40 ± 4.43 [15]	109.67 ± 2.59 [8]	98.91 ± 6.27 [8]	98.44 ± 4.93 [7]	97.72 ± 5.11 [7]
Heart rate (BPM)	41 ± 6 [8]	44 ± 7 [4]	42 ± 7 [4]	41 ± 5 [4]	45 ± 3 [4]
Oxygen consumption (mg/L)	0.14 ± 0.02 [7]	0.16 ± 0.01 [4]	0.12 ± 0.04 [4]	0.13 ± 0.02 [3]	0.14 ± 0.02 [3]

2.3.2 Hematology – Winter, Hypoxia and Summer Experiments

Hematocrit

Hematocrit values of fish exposed to DTZ in the winter experiment at 4ºC and at 10ºC (control 0 µg/L DTZ) are presented in Figure 2.2A. Neither DTZ ($F_{(2,21)}$ = 0.023, p = 0.97) nor temperature ($T_{(14)}$ = 0.84, p = 0.41) had a significant effect. Hypoxia experiment hematocrit values are presented in Figure 2.2B. Neither DTZ ($F_{(2,14)}$ = 2.78, p = 0.09) nor the DO ($T_{(9)}$ = -1.08, p = 0.30) had a significant effect; however, a significant statistical trend for a decrease ($F_{(2,14)}$ = 2.78, p = 0.06) was observed at 1 µg/L. Summer experiment hematocrit values are presented in Figure 2.2C. Neither DTZ ($F_{(2,18)}$ = 0.144, p = 0.86) nor temperature ($T_{(14)}$ = -1.18, p = 0.25) had a significant effect.

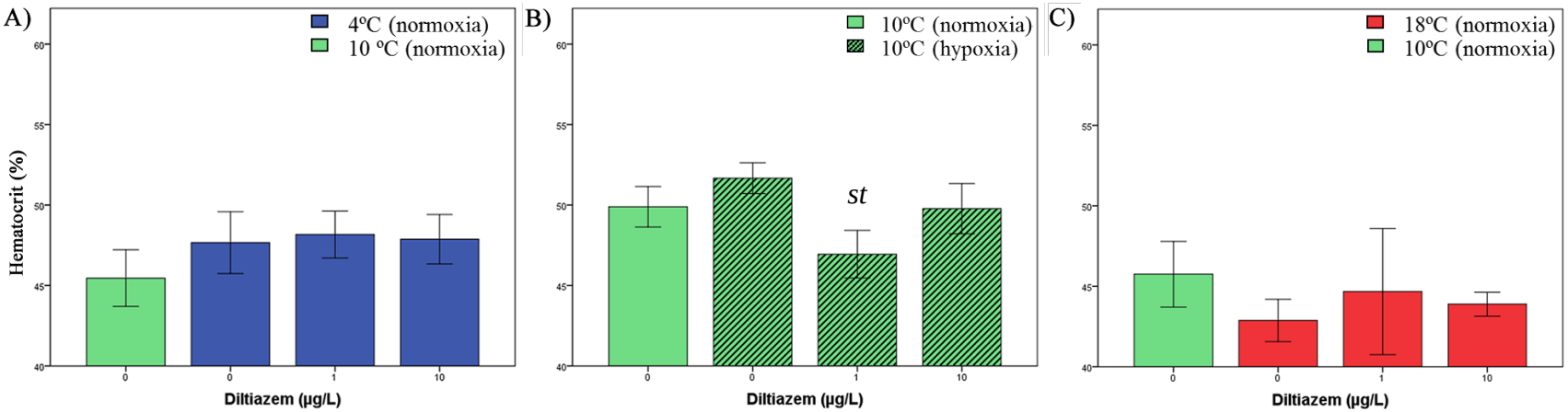

Figure 2.2 – Hematocrit (mean ± S.E.) of rainbow trout exposed to diltiazem (DTZ) under different temperatures and DO regimes for 96 hours. **A)** Winter experiment: water temperature 10ºC and 4ºC, normoxia (> 85% air sat.), n = 6 - 8. **B)** Hypoxia experiment: temperature 10ºC, normoxia and hypoxia (35% air sat.), n = 5 – 6. **C)** Summer experiment: 10ºC and 18ºC, normoxia, n = 6 – 8. *st* – statistical trend (0.051 > P-value < 0.1)

Hemoglobin

Winter experiment blood Hb values of fish exposed to DTZ at 4ºC and at 10ºC (control 0 µg/L DTZ) are presented in Figure 2.3A. Neither DTZ ($F_{(2,21)}$ = 1.242, p = 0.30) nor temperature ($T_{(14)}$ = 0.575, p = 0.57) had a significant effect. Hypoxia experiment blood Hb values are presented in Figure 2.3B. There was a statistically significant effect of DTZ on Hb concentration ($F_{(2,14)}$ = 12.82, p = 0.001) comparing the 0, 1 and 10 µg/L treatment groups in hypoxic water. A post-hoc analysis further determined the statistical difference was between 0 µg/L (control) and 1 µg/L hypoxia groups (p = 0.003) (fish exposed to 1 µg/L had 24% less Hb than 0 µg/L group), and 0 µg/L (control) and 10 µg/L hypoxia groups (p = 0.001) (fish exposed to 10 µg/L had a 30% less Hb than 0 µg/L group.) When testing for the effect of hypoxia alone, there was a statistically significant effect of hypoxia ($T_{(9)}$ = -3.54, p = 0.006) between 0 µg/L (normoxia) and 0 µg/L (hypoxia); fish exposed to 0 µg/L (normoxia) had a 23% increase in Hb than 0 µg/L group (hypoxia.) Summer experiment blood Hb values are presented in Figure 2.3C. Neither DTZ ($F_{(2,20)}$ = 0.472, p = 0.63) nor temperature ($T_{(14)}$ = -1.87, p = 0.85) had a significant effect.

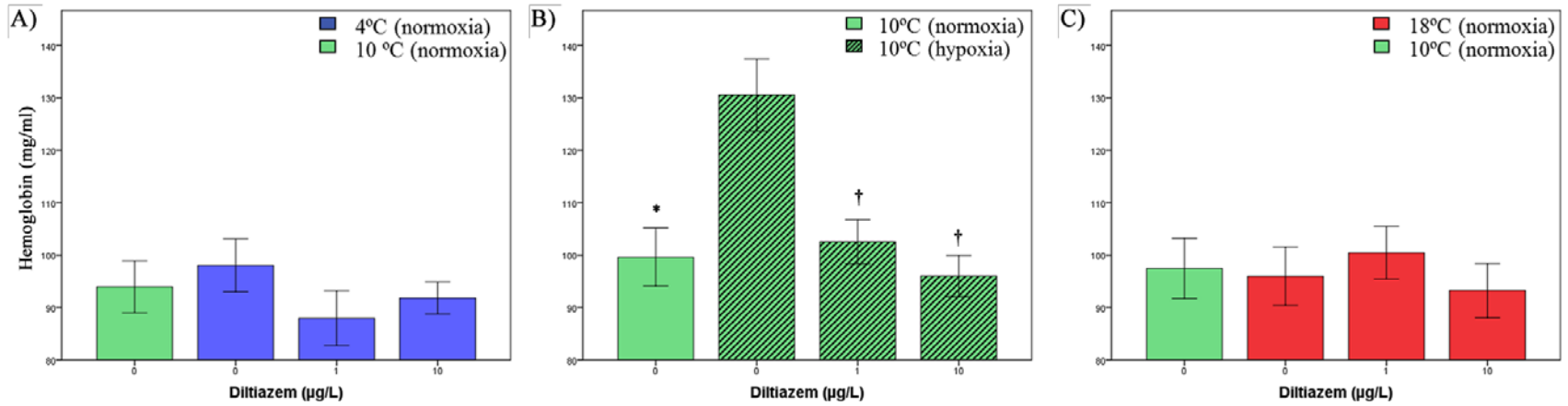

Figure 2.3 – Hb (mean ± S.E.) of rainbow trout exposed to DTZ under different temperatures and DO regimes for 96 hours. **A)** Winter experiment: water temperature 10ºC and 4ºC, normoxia (> 85% air sat.), n = 8. **B)** Hypoxia experiment: temperature 10ºC, normoxia and hypoxia (35% air sat.), n = 5 - 6; * - significance ($P < 0.05$) between controls (T test). † - significance ($P < 0.05$) between treatments (one-way ANOVA). **C)** Summer experiment: 10ºC and 18ºC, normoxia, n = 7 – 8.

Red Blood Cell Count (in hypoxia experiment)

Blood cell count in blood smears of fish exposed to DTZ for 96 hours in normoxic water (control >85% air sat.) and hypoxic water (35% air sat.) at 10ºC are presented in Table 2.3, represented in Figure 2.4, and described below.

IMMATURE RED BLOOD CELL TO MATURE BLOOD CELL RATIO

Immature red blood cell count to mature red blood cell count ratio in blood smears of fish exposed are presented in Figure 2.5. There was a statistically significant effect of DTZ on the ratio between immature red blood cell count over mature red blood cell count ($F_{(2,14)}$ = 5.825, p = 0.014) comparing the 0, 1 and 10 µg/L treatment groups in hypoxic water. A Dunnett (2-tailed) post-hoc test further determined that the statistically significant effect was between the 0 µg/L (control) and 10 µg/L hypoxia groups; 10 µg/L group had 35% higher ratio immature RBC:mature RBC than 0 µg/L (p = 0.032). When testing for the effect of hypoxia alone, there was no statistically significant effect of hypoxia ($T_{(9)}$ = 1.664, p = 0.13).

Table 2.3 – Hypoxia experiment: blood cell counts (mean ± S.E. [n]) in rainbow trout exposed to DTZ and hypoxia for 96 hours in the laboratory.

	Normoxia	Hypoxia		
Endpoint	**0 µg/L DTZ**	**0 µg/L DTZ**	**1 µg/L DTZ**	**10 µg/L DTZ**
Mature RBC count (per field)	175 ± 7 [6]	185 ± 10 [5]	190 ± 10 [6]	188 ± 7 [6]
Leukocytes count (per field)	5 ± 1 [6]	6 ± 1 [5]	5 ± 1 [6]	4 ± 0.5 [6]
Mean RBC surface area (µm^3)	187 ± 6 [6]	199 ± 8 [5]	199 ± 3 [6]	199 ± 4 [6]
Leukocytes/total RBC	0.025 ± 0.005 [6]	0.029 ± 0.007 [5]	0.025 ± 0.005 [6]	0.020 ± 0.002 [6]

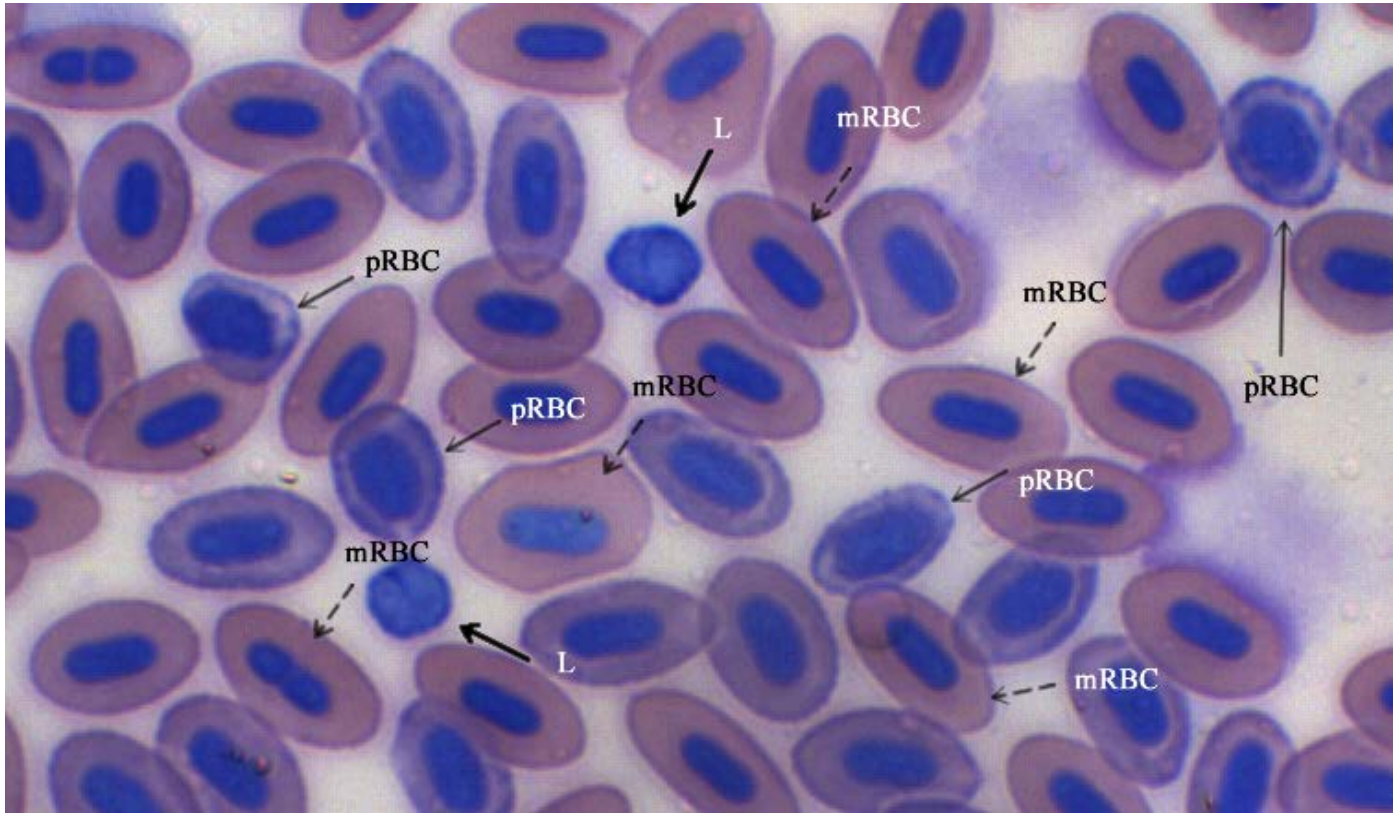

Figure 2.4 – Photomicrograph of rainbow trout blood smear. Dashed-line arrows point to mature red blood cells (mRBC); solid-line arrows point to immature red blood cells (pRBC – polychromatic RBC); solid-line thick arrows point to lymphocytes (L).

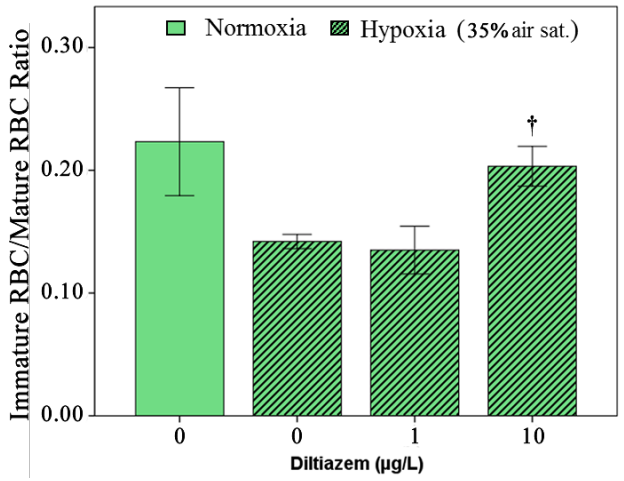

Figure 2.5 – Red blood cell count (mean ± S.E.) of rainbow trout exposed to DTZ under different DO regimes for 96 hours (n = 5 – 6). Immature RBC to mature RBC ratio † - significance ($P < 0.05$) between treatments (one-way ANOVA).

2.3.3 Cardiovascular Monitoring – Winter, Hypoxia and Summer Experiments

Electrocardiogram

Winter experiment heart rate values of fish exposed to DTZ for 96 hours at 4ºC and at 10ºC (control 0 µg/L DTZ) are presented in Figure 2.6A. Neither DTZ ($F_{(2,9)} = 0.280$, $p = 0.76$) nor temperature ($T_{(6)} = -1.634$, $p = 0.153$) had a significant effect. Summer experiment heart rate values are presented in Figure 2.6B. Neither DTZ ($F_{(2,9)} = 0.549$, $p = 0.59$) nor temperature ($T_{(5)} = 0.057$, $p = 0.95$) had a significant effect.

Oxygen Consumption

Winter experiment oxygen consumption values of fish exposed to DTZ for 96 hours at 4ºC and at 10ºC (control 0 µg/L DTZ) are presented in Figure 2.7A. Neither DTZ ($F_{(2,9)} = 2.454$, $p = 0.14$) nor temperature ($T_{(6)} = -1.657$, $p = 0.149$) had a significant effect. Hypoxia experiment oxygen consumption values are presented in Figure 2.7B. Neither DTZ ($F_{(3,19)} = 1.728$, $p = 0.19$) nor the DO ($T_{(9)} = 1.565$, $p = 0.15$) had a significant effect. Summer experiment oxygen consumption values are presented in Figure 2.7C. Neither DTZ ($F_{(2,9)} = 0.241$, $p = 0.79$) nor temperature ($T_{(6)} = 1.265$, $p = 0.25$) had a significant effect.

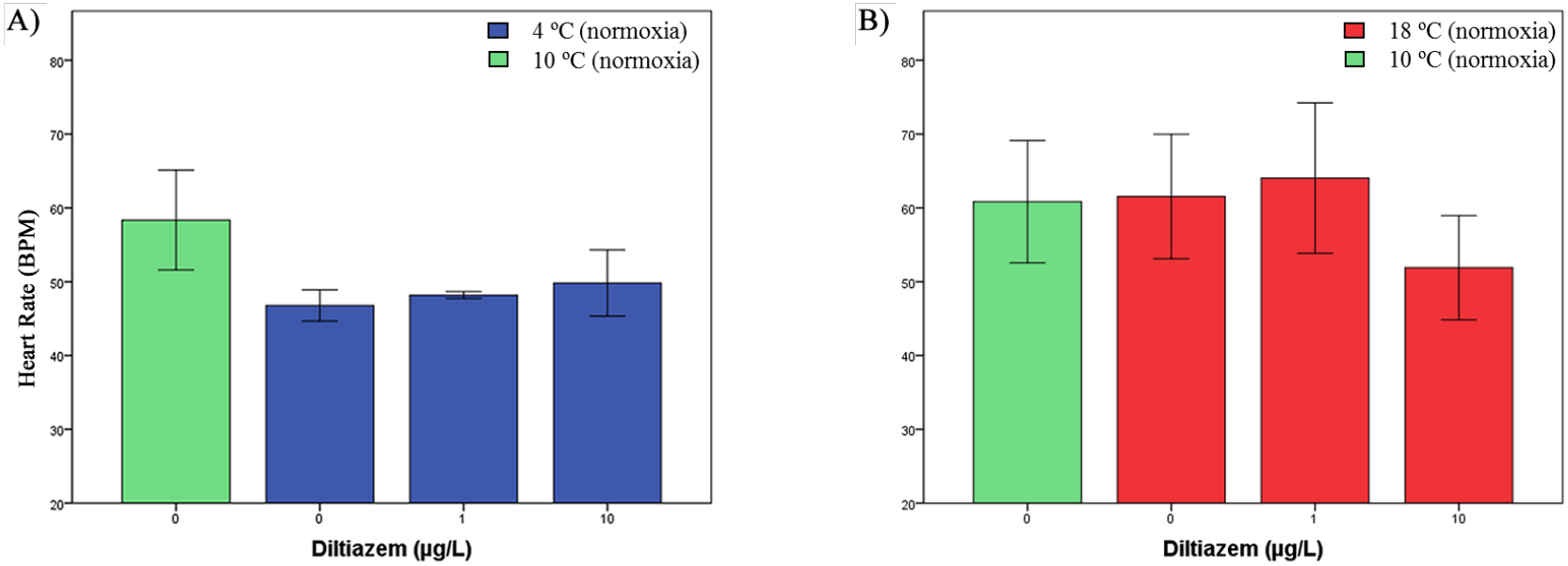

Figure 2.6 – Heart rate (mean ± S.E.) of rainbow trout exposed to DTZ under different temperatures regimes for 96 hours. **A)** Winter experiment: water temperature 10ºC and 4ºC, normoxia (> 85% air sat.), n = 3 – 4. **B)** Summer experiment: 10ºC and 18ºC, normoxia, n = 3 – 4.

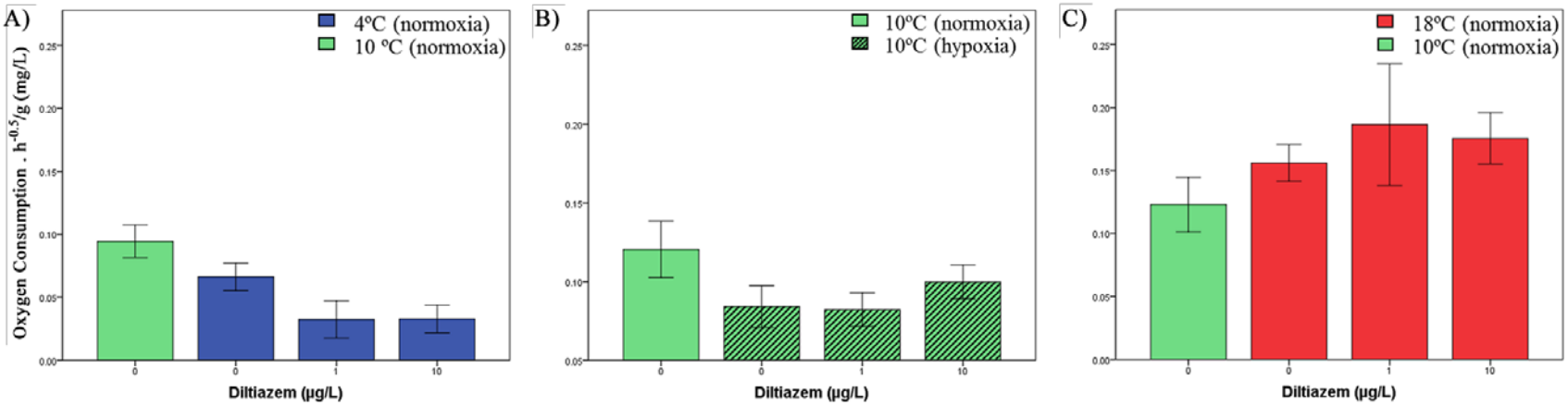

Figure 2.7 – Oxygen consumption (mean ± S.E.) of rainbow trout exposed to DTZ under different temperatures and DO regimes for 96 hours. **A)** Winter experiment: water temperature 10ºC and 4ºC, normoxia (> 85% air sat.), n = 4. **B)** Hypoxia experiment: temperature 10ºC, normoxia and hypoxia (35% air sat.), n = 5 – 6. **C)** Summer experiment: 10ºC and 18ºC, normoxia, n = 4.

Ventilation Rate

Winter experiment ventilation rate of fish exposed to DTZ for 96 hours at 4ºC and 10ºC (control 0 µg/L DTZ) are presented in Figure 2.8A. Temperature ($T_{(6)}$ = -1.512, p = 0.181) had no significant effect; unfortunately DTZ analysis was not possible due to small sample size (n < 3). Hypoxia experiment ventilation rate values are presented in Figure 2.8B. There was a statistically significant effect of DTZ on the ventilation rate ($F_{(2,13)}$ = 3.577, p = 0.05) comparing 0, 1 and 10 µg/L treatment groups in hypoxic water. A Dunnett (2-tailed) post-hoc test further determined that the statistically significant difference was between the 0 µg/L (control) and 1 µg/L hypoxia groups; 1 µg/L group decreased the ventilation rate by 14% compared to 0 µg/L group (p = 0.035). When testing for the effect of hypoxia alone, there was also a statistically significant effect of hypoxia ($T_{(9)}$ = -2.282, p = 0.04) between 0 µg/L (normoxia) and 0 µg/L (hypoxia); 0 µg/L group (hypoxia) increased the ventilation rate by 13% compared to 0 µg/L (normoxia) group. Summer experiment ventilation rate values are presented in Figure 2.8C. Neither DTZ ($F_{(2,6)}$ = 0.131, p = 0.87) nor temperature ($T_{(5)}$ = 1.724, p = 0.14) had a significant effect.

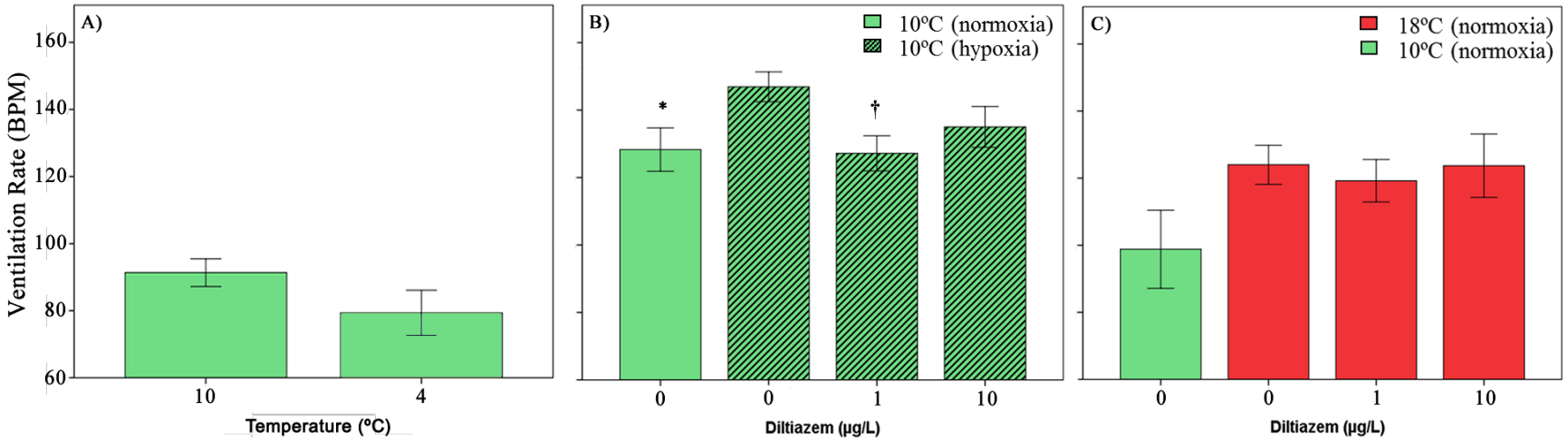

Figure 2.8 – Ventilation rate (mean ± S.E.) of rainbow trout exposed to DTZ under different DO regimes for 96 hours. **A)** Winter experiment: 4ºC and 10ºC, normoxia, n = 4. **B)** Hypoxia experiment: temperature 10ºC, normoxia and hypoxia (35% air sat.), n = 5 – 6; * - significance ($P < 0.05$) between controls (T test). † - significance ($P < 0.05$) between treatments (one-way ANOVA). **C)** Summer experiment: 10ºC and 18ºC, normoxia; n = 3 – 4.

2.3.4 Gills Histology – Hypoxia Experiment

Results from the gill histology analysis of fish exposed to DTZ for 96 hours at 10ºC and hypoxia (4 mg/L) are presented in Figure 2.9. Neither DTZ ($F_{(2,14)}$ = 2.61, p = 0.10) nor hypoxia ($T_{(9)}$ = 0.912, p = 0.38) had a significant effect on BET although a statistical trend for decreasing BET in exposure to DTZ was noted at 10 µg/L ($F_{(2,14)}$ = 2.61, p = 0.09) Figure 2.9A. Neither DTZ ($F_{(2, 14)}$ = 2.646, p = 0.10) nor hypoxia ($T_{(9)}$ = 0.538, p = 0.60) had a significant effect on SLL although again, a statistical trend for decreasing SLL at 1 µg/L DTZ was noted ($F_{(2, 14)}$ = 2.646, p = 0.06) Figure 2.9B. Neither DTZ ($F_{(2,14)}$ = 2.938, p = 0.08) nor hypoxia ($T_{(9)}$ = -0.498, p = 0.63) had a significant effect on PAGE (Figure 2.9C).

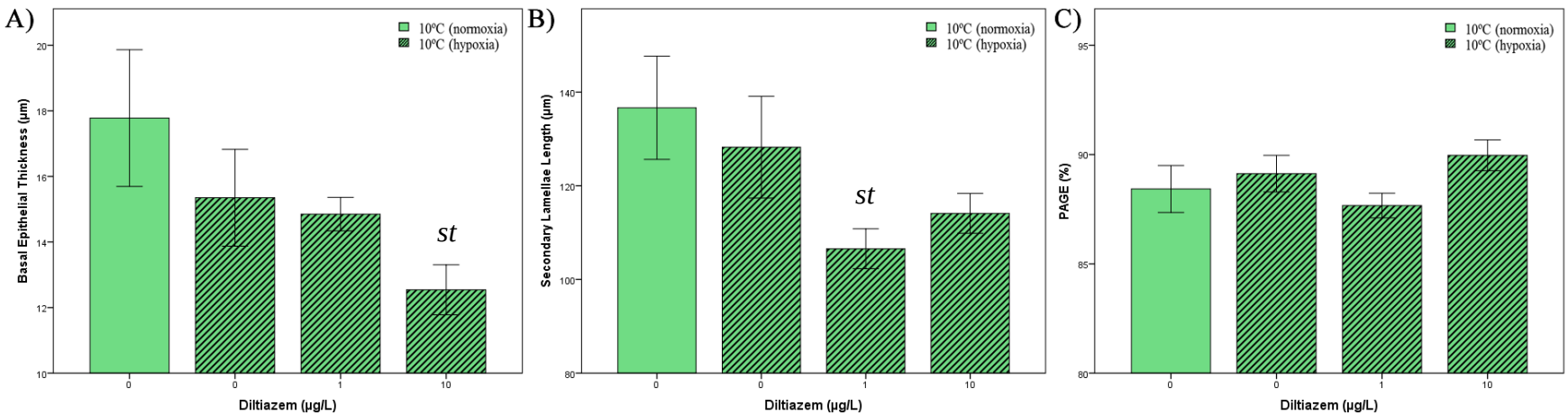

Figure 2.9 – Gill histology measurements (mean ± S.E.) of rainbow trout exposed to DTZ under different DO regimes for 96 hours (n = 5 – 6). A) Basal epithelial thickness (BET); B) Secondary lamellae length (SLL); C) Proportion available gas exchange (PAGE). s*t* – statistical trend (0.051 > P-value < 0.1)

2.4 Discussion

The present study was designed to investigate and test the hypotheses that DTZ impairs the cardiovascular function of rainbow trout (i) under optimal exposure conditions of water temperature (10ºC) and dissolved oxygen concentrations (above 85%). The experiments also tested the hypothesis that (ii) DTZ impairs the response of the cardiovascular function of rainbow trout exposed to hypoxia. Furthermore, experiments were also carried out to test the hypothesis that (iii) DTZ will have similar effects on the cardiovascular function of rainbow trout as those reported in humans (including a reduction of heart rate) when rainbow trout is exposed to DTZ and challenged with summer and winter conditions (18ºC and 4ºC water temperature). This study provided new data on multiple stressors mimicking a climate change scenario (warm temperature, hypoxia), to complement the available data on the effects of DTZ in fish, including the LC_{50} values for DTZ (Kim *et al.*, 2007) and the effects of DTZ on the cardiovascular status of rainbow trout (Steinbach *et al.*, 2016ab).

Even though Steinbach *et al.* (2016ab) have contributed towards filling gaps of knowledge regarding the effects of DTZ in fish, the current study investigated the effects of DTZ (0, 1, 10, 100 and 1000 µg/L) on the cardiovascular function of rainbow trout at a wider range of water temperatures (4ºC, 10ºC and 18ºC) for 96 hours. Steinbach *et al.* (2016a) exposed rainbow trout to 0.03, 3 and 30 µg/L of DTZ at one temperature, 15.4 ± 1.4ºC for 21 – 42 days. Moreover, for the first time, the modulation potential of hypoxia (4 mg/L – 35% air sat.) on the effects of DTZ on the cardiovascular function of rainbow trout was assessed in the current study. DTZ had no significant effect on hematocrit in

rainbow trout exposed to DTZ for 96-hours due to different temperatures (4ºC, 10ºC and 18ºC) or DO concentration (4 mg/L and >8 mg/L); which corroborates previous studies as similar values of hematocrit were detected in RBT under similar conditions of temperature and DO (McCarthy *et al.*, 1973; Rehulka *et al.*, 2004), but Steinbach, Burkina, *et al.* (2016) determined an 11% increase in hematocrit in rainbow trout exposed to 30 µg/L DTZ for 21 days.

Another physiologically important characteristic of blood, in addition to hematocrit, is hemoglobin (Hb) content of blood. It is known that although temperature can affect Hb (Houston *et al.*, 1996; Lewis *et al.*, 2012), it is not the most powerful stimulus to influence the Hb content of blood, including Hb synthesis. The temperature-linked change in dissolved oxygen content of water seems to be the key stimulus for modifying Hb concentration of blood (Tun and Houston, 1986). The increase in temperature *per se* does not modulate the Hb changes in blood, but rather the hypoxia that follows it (decreased oxygen solubility at higher temperatures). The Hb concentration, as expected, did not vary due to temperature *per se* in this study, but hypoxia alone might have triggered a Hb synthesis in RBT, as the blood Hb concentration increased significantly at 0 DTZ 10ºC hypoxia, as reported previously by Tun and Houston (1986). However, DTZ may impair the capacity of rainbow trout to trigger this response, as the Hb synthesis impairment is proportional to the concentration of DTZ, so there may be a negative relationship between diltiazem concentration and Hb synthesis impairment (i.e., the higher concentration of DTZ, the more severe the Hb synthesis impairment).

Hematocrit values and RBC counts are closely related; respectively, hematocrit reflects the packed cell volume in percentage and the RBC counts reflect actual numbers of cells (Handy & Depledge, 1999). Increased immature:mature RBC ratio was observed in animals exposed to 10 µg/L DTZ and 4 mg/L DO at 10 ºC. This result suggests an increased influx of immature RBC into the blood stream, possibly as an emergency measure to cope with hypoxia under exposure to DTZ, a pharmaceutical that seems to impair the Hb synthesis in rainbow trout. The increase of RBC influx in the blood stream of rainbow trout exposed to hypoxia was also reported by Swift and Lloyd (1974).

The increased number of immature RBC in the blood stream due to hypoxia and DTZ (10 µg/L) exposure leads us to speculate that the reducing trend of DTZ observed in the oxygen consumption during winter-mimicking conditions (4ºC nominal water temperature) may be related to the reduced capacity of immature RBC to carry oxygen as efficiently as mature RBC since the ventilation rate did not vary under hypoxia at 10 µg/L.

The ventilation rate analysis is an important tool for monitoring the cardiovascular status of RBT exposed to climate change scenarios. The present study demonstrated that the ventilation rate of RBT increases significantly when fish experience hypoxia, as expected, in order to maintain the oxygen consumption rate. However, when exposed to 1 µg/L DTZ and hypoxia, the ventilation rate was significantly decreased. The temperature *per se* in the winter experiment (4ºC) did not decrease the ventilation rate, possibly due to small sample size, since previous studies (rainbow trout exposed to 8ºC), including Black *et al.* (1991) demonstrated that low temperatures reduce the ventilation rate in rainbow trout. The summer experiment (18ºC)

did not increase the ventilation rate at the higher temperature, again possibly due to small sample size, since previous studies, including Ekstrom *et al.* (2014), demonstrated that high temperatures increase the ventilation rate in rainbow trout; however, DTZ had no significant effect on the ventilation. It must be noted that the sample sizes for some of the ventilation measurements were small (n=3 – 4) because of the experimental design used in the current study where multiple endpoints were measured.

The gills are one of the tissues of fish most exposed to pollutants in the aquatic environment. It is known that fish may increase surface area of the gills through lamellar recruitment (Pettersson & Johansen, 1982) to cope with hypoxia and this may be directly related to the oxygen consumption rate. It is important to evaluate if DTZ has an effect on this response (lamellar recruitment) in fish. A statistical trend for lower BET was observed at 10 µg/L and in SLL at 1 µg/L and no change was observed in PAGE. Based on the trend observed in the SLL graph (Figure 2.9B), DTZ vasodilation effects may overrule the serotonergic system which is responsible for the vasoconstriction regulation in gills (Sundin *et al.*, 1995), which could help fish cope when exposed to hypoxia. The sustained oxygen consumption rate observed under hypoxia when exposed to diltiazem is perhaps related to the remodeling of the gills structure as well, since DTZ seem to impair the Hb synthesis thus reducing the oxygen-carrying capacity of RBCs.

The oxygen consumption rate is also an important endpoint to analyze in the assessment of cardiovascular performance of RBT exposed to climate change scenarios and DTZ exposure. Temperature *per se* did not reduce RBT oxygen consumption at 4ºC. No statistical significance was detected in the hypoxia experiment on oxygen consumption, which corroborates previous studies, including Eliason and Farrell (2014)

and Holeton and Randall (1967b). There was no statistical significance on the effect of DTZ in the summer experiment, but an increasing trend in the oxygen consumption can be observed corroborating previous studies, including Keen and Gamperl (2012).

It is known that DTZ lowers the oxygen demand of the heart through reduction of blood pressure (via vasodilation), cardiac contractility and heart rate (Padial *et al.*, 2016). However, the present study has not detected a reduction in heart rate due to DTZ across the experiments. A heart rate reduction trend in the winter experiment was observed due to the low temperature as expected and demonstrated in previous studies, including Aho and Vornanen (2001). Even though previous studies have demonstrated that the heart rate of RBT increases at higher temperatures (Aho & Vornanen, 2001; Ekstrom *et al.*, 2016; Ekstrom *et al.*, 2014), the present study has not detected it in the summer experiment likely due to the smalll sample size of the current study (n = 3 – 4). Unfortunately the heart rate endpoint measurement was not possible at the hypoxia experiment due to fish availability limitation.

This study demonstrates the effects of DTZ on RBT cardiovascular funcion such as the impairment of the hemoglobin response, ventilation rate, and immature RBC count in a hypoxia challenge. All of these effects were observed in an acute exposure at relatively low concentrations, therefore, a chronic exposure exploration with increased sample size is warranted as the long term exposure may demonstrate other effects that were not statistically significant in an acute setting.

Chapter Three: **Summary, Conclusions, Final Considerations and Future Directions**

3.1 Summary and Conclusions

The present study did not detect DTZ effects in rainbow trout similar to what is seen in humans (such as reduction of heart rate and vasodilation). However, DTZ impaired the hemoglobin synthesis response at both concentrations (1 and 10 µg/L) reducing the concentration of hemoglobin in whole blood. DTZ may also indirectly cause an increased number of immature RBC in the blood stream. The impairment of hemoglobin synthesis may have led to the emergency release of immature RBC in the blood stream under a hypoxic challenge, which was triggered at 10 µg/L. The immature:mature RBC ratio was also reduced in the presence of DTZ at 10 µg/L and under hypoxic conditions. DTZ also reduced the ventilation rate of rainbow trout at 1 µg/L but not at 10 µg/L. This observation could be due to hemoglobin synthesis (greater impairment at 10 µg/L) and influx of RBC in the blood stream (triggered at 10 µg/L) as a compensatory response. Possibly, at 1 µg/L the ventilatory response was not observed because the hemoglobin synthesis impairment did not reach unsustainable metabolic levels (i.e., the reduction in hemoglobin production did not jeopardize the RBC oxygen-carrying capacity sufficiently to not meet the metabolic demand). Oxygen consumption did not vary with DTZ exposure; however, a trend was observed in the hypoxia challenge experiment during which there was a slight increase in the oxygen consumption at 10 µg/L of DTZ, which could corroborate the explanation of the ventilation rate response observed in this study.

This exploratory study was designed to be a broad survey of the effects of DTZ in fish cardiovascular performance. DTZ may not elicit effects on fish similar to what is seen in humans, but it absolutely causes non-lethal detrimental effects at low concentrations in rainbow trout which may or may not compromise the physiological fitness response of rainbow trout.

3.2 Final Considerations and Future Directions

This project yielded relevant new data regarding the effects of DTZ in non-target organisms such as fish. It generated new data to understand how hypoxia and fluctuations of temperature, by themselves and concomitantly, modulate the effects of DTZ in a fish species. Therefore, this study elucidates and helps to understand the effects of the environmental challenges faced by fish, as previously described. However, this study was also a first attempt to investigate the cardiovascular function in fish in the University of Lethbridge. Hurdles were overcome by crafting custom systems that would generate cardiovascular data and provide a first exploratory survey of the physiological effects of DTZ in fish. The respirometry chamber (Appendix 1) was built from acrylic sheets, PVC pipes, vinyl tubing, vinyl spouts, sealed with silicone gaskets and nylon screws, bolts and washers. We were able to replicate a well reputed equipment from Loligo systems that costs about $10 000 on the budget; fully functional – it is my state-of-the-art $1.99 joy. Another excitement is the heart rate monitoring custom setup integrating an electro-olfactogram (EOG) equipment with needle probes to get the readings while testing new protocols never used before in our laboratories – it was something that have never been

performed in any of the laboratories that I worked in nor even in my department; so, it was truly designed from ground up.

Future research should focus on cardiovascular responses, large sample sizes, broad water temperatures and chronic exposures, principally if testing under multiple stressors, since the organisms in the aquatic environment are exposed to a much more complex environment than just one or two variables. It is known that carbamazepine, amongst others, interacts with diltiazem, for instance (Wishart *et al.*, 2006). Mimicking 'real life' exposures and conditions is a goal when researching aquatic ecosystems.

Appendix 1 – The Respirometry Chamber

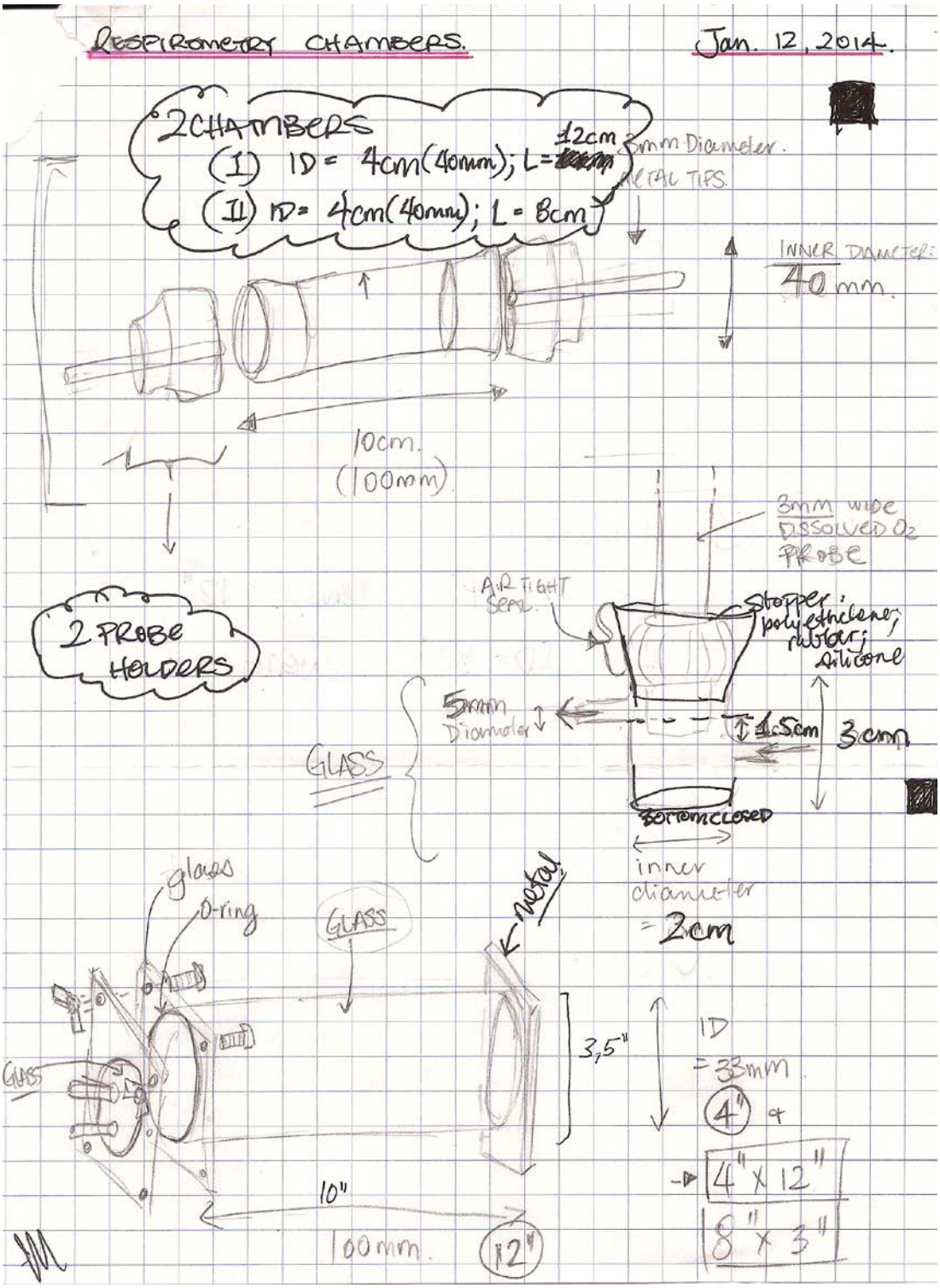

Figure A.1 – Sketch of the respirometry chamber.

Appendix 1 – The Respirometry Chamber (continued)

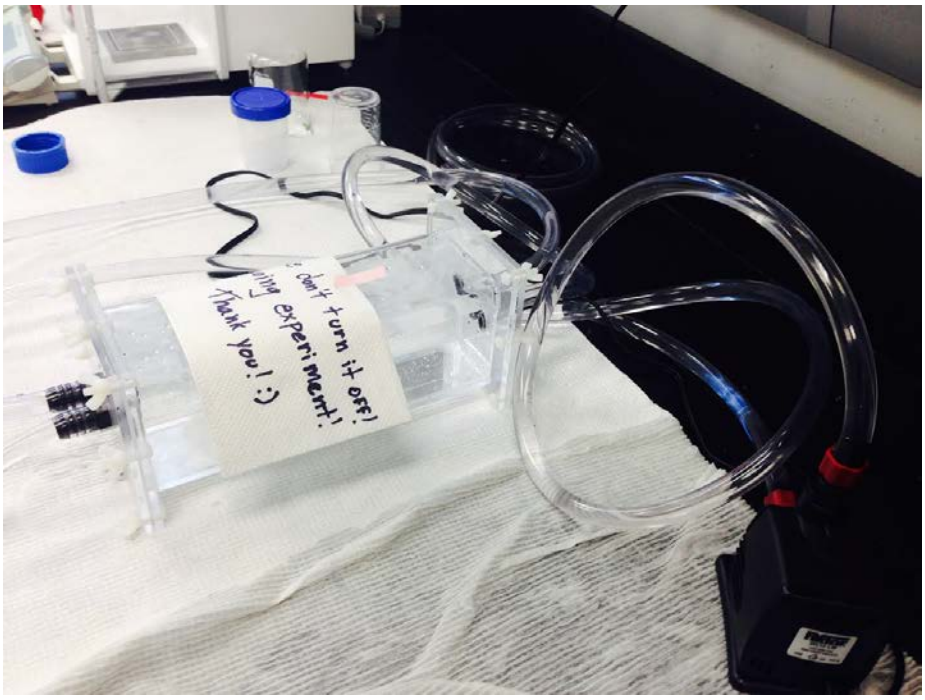

Figure A.2 – Main chamber of the Respirometry prototype setup.

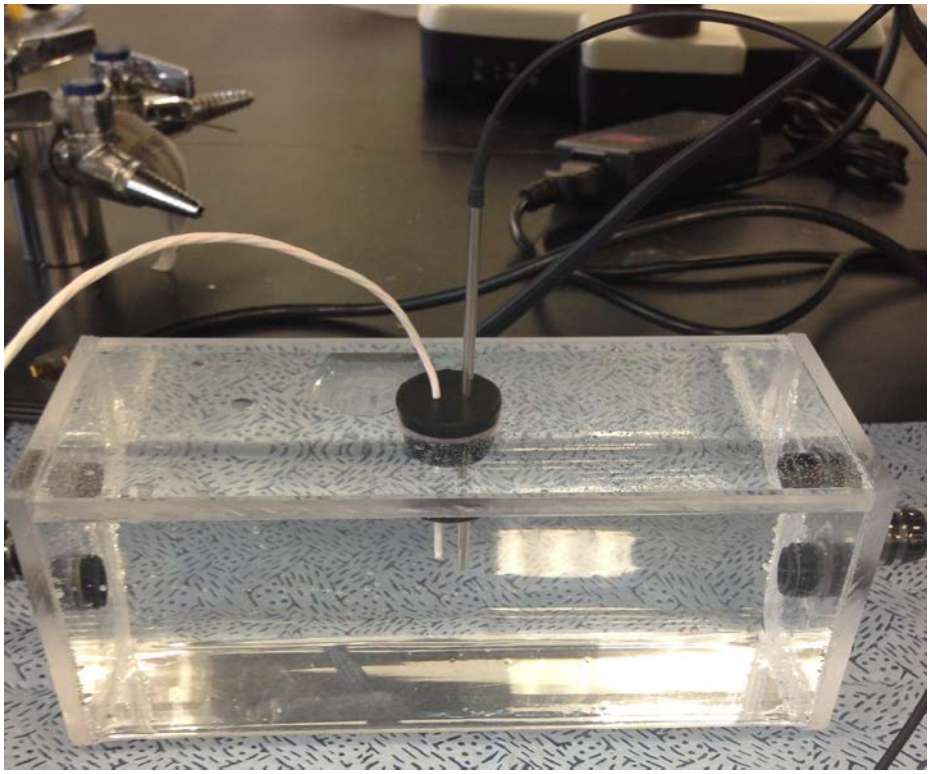

Figure A.3 – Remote chamber of the Respirometry prototype setup.

Appendix 1 – The Respirometry Chamber (continued)

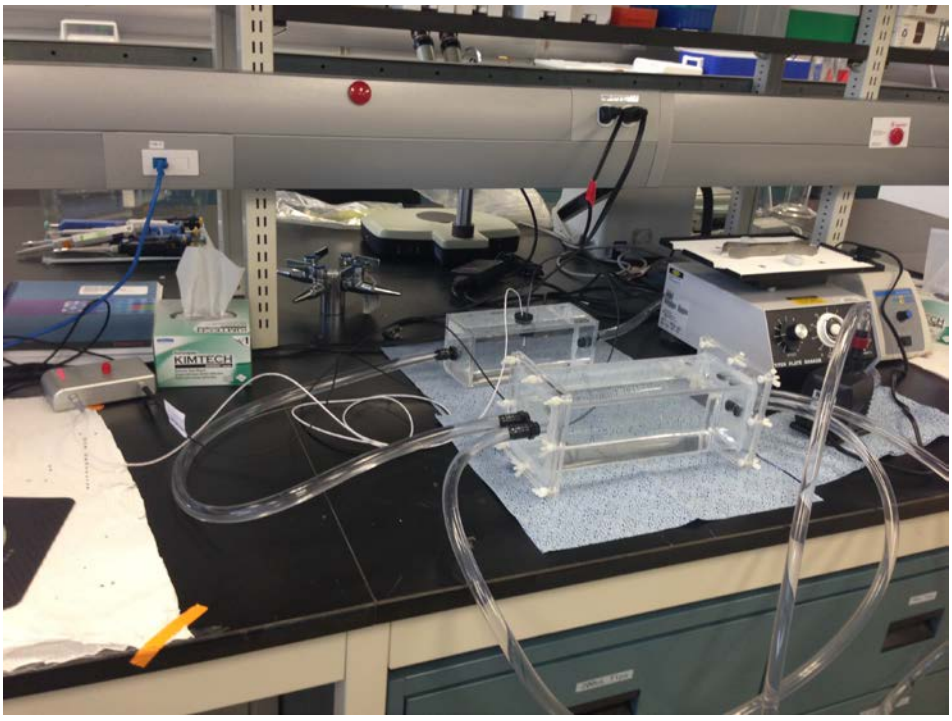

Figure A.4 – Functional Respirometry prototype setup.

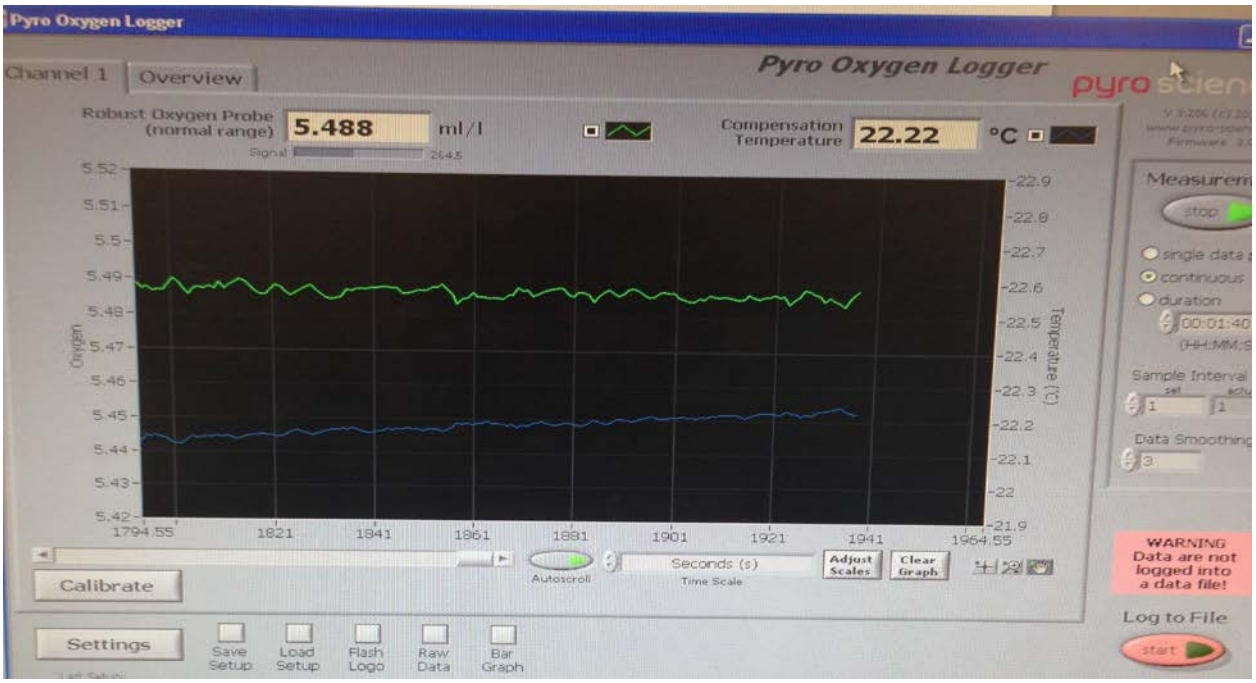

Figure A.5 – Functional Respirometry prototype setup passed airtight test. Green tracing represents oxygen concentration of dissolved oxygen in the system; blue tracing represents the water temperature in the system. No fish was used for this test, hence the linearity of the dissolved oxygen content.

Appendix 2 – The Electrocardiogram Setup

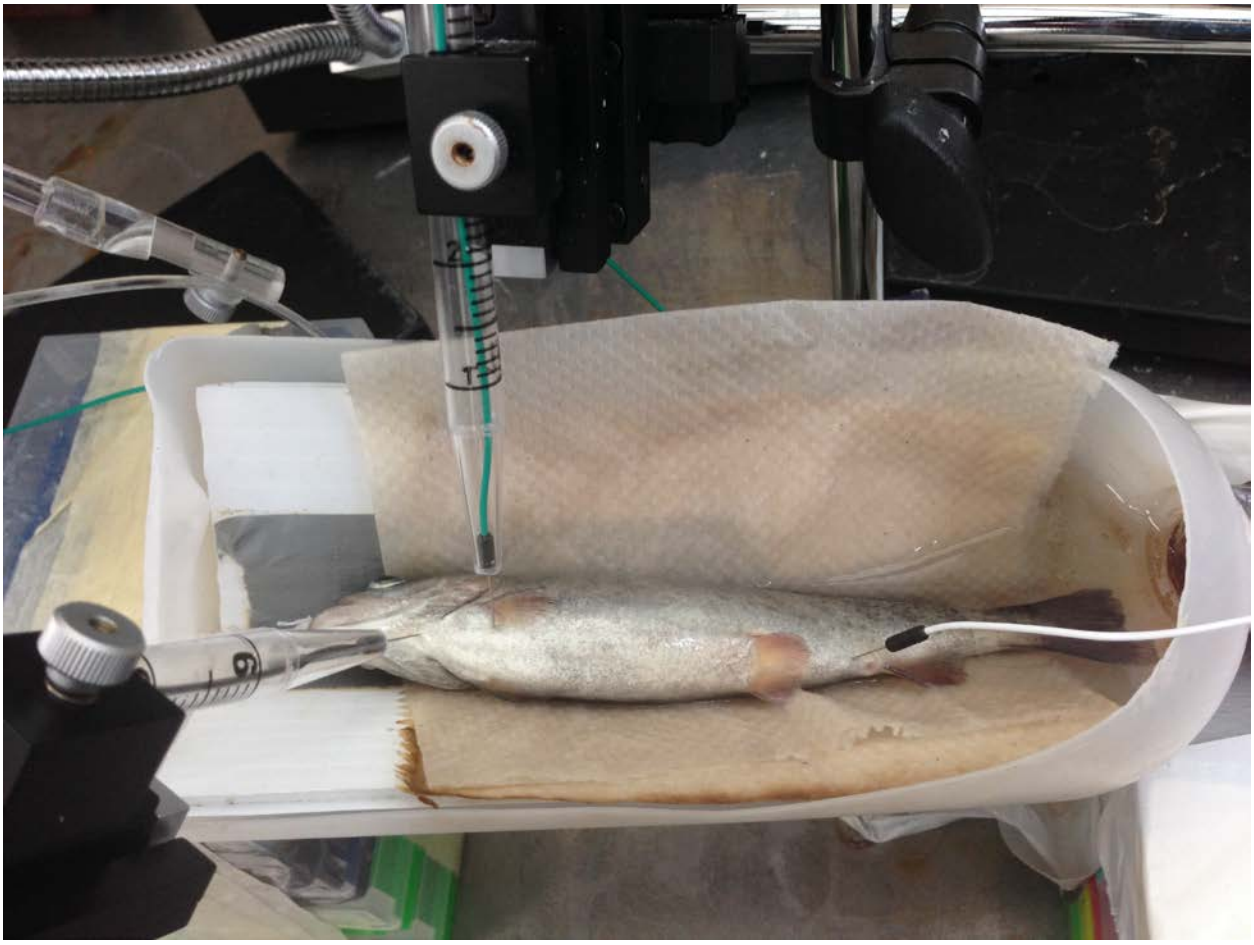

Figure A.1 – Heart rate custom setup test. The green and black needle probes are the positive and negative electrodes; the white needle probe is the ground electrode. An anesthetized juvenile rainbow trout was used for this test.

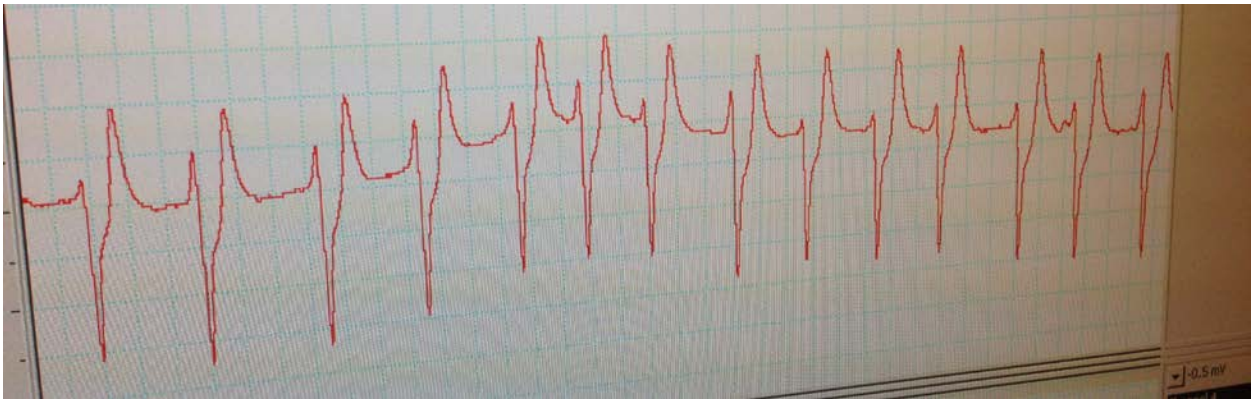

Figure A.2 – Tracing generated from the heart rate custom setup test. Special thanks to Ebi Lari for sharing his expertise with the rig making this reading possible.

Appendix 3 – Aquatic Research Facility

Figure A.1 – State of the art Aquatic Research Facility at the University of Lethbridge. These tanks were used to perform all the experiments, including the hypoxia experiment. This system provides the ability to regulate the dissolved oxygen concentration in each tank individually.

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